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Efficacy and safety of live attenuated persistent and rapidly cleared *Mycobacterium tuberculosis* vaccine candidates in non-human primates

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Abstract

Tuberculosis (TB) remains a global health burden for which safe vaccines are needed. BCG has limitations as a TB vaccine so we have focused on live attenuated *Mycobacterium tuberculosis* mutants as vaccine candidates. Prior to human studies, however, it is necessary to demonstrate safety in non-human primates (NHP). In this study, we evaluate the safety and efficacy of two live attenuated *M. tuberculosis* double deletion vaccine strains mc²6020 (Δ *lysA* Δ *panCD*) and mc²6030 (Δ *RD1* Δ *panCD*) in cynomolgus macaques. In murine models, mc²6020 is rapidly cleared while mc²6030 persists. Both mc²6020 and mc²6030 were safe and well tolerated in cynomolgus macaques. Following a high-dose intrabronchial challenge with virulent *M. tuberculosis*, mc²6020-vaccinates were afforded a level of protection intermediate between that elicited by BCG vaccination and no vaccination. BCG vaccinates had reduced tuberculosis-associated pathology and improved clinical scores as compared to saline and mc²6030 vaccinates, but survival did not differ among the groups.

Keywords

Vaccine; Mycobacteria; Mycobacterium; Tuberculosis; Non-human primate; BCG; Safety

1. Introduction

Worldwide one-third of the population is infected with *Mycobacterium tuberculosis* resulting in 1.6 million deaths annually. The vaccine Bacille Calmette Guérin (BCG) affords

protection against pediatric disseminated tuberculosis [1,2], but can cause disease in HIV-positive neonates. The WHO has advised against BCG vaccination in HIV-infected children even if they are asymptomatic [3] as the risk of disseminated disease in HIV-infected infants [4] clearly outweighed the potential benefits [5]. Furthermore, BCG has variable (0–80%) protection against adult pulmonary TB [5]. Innovative approaches are needed to make safe and effective TB vaccines [6] and this work focuses on evaluation of live attenuated *M. tuberculosis* vaccines in a non-human primate model. A working group sponsored by WHO, TB-VAC, and Aeras Global TB Vaccine Foundation put forth guidelines for developing live attenuated mycobacterial vaccines [7]. This pre-clinical pathway includes testing safety and efficacy in mouse and guinea pig models and when possible, evaluation of safety in non-human primates prior to phase I studies.

Live attenuated vaccines were derived from virulent *M. tuberculosis* with the reasoning that such *M. tuberculosis* strains are adapted for humans and might be more immunogenic than an *M. bovis* BCG strain which is bovine adapted. We had previously established the vaccine safety and efficacy of mc²6020 (*M. tuberculosis* H37Rv Δ *lysA* Δ *panCD*) and mc²6030 (*M. tuberculosis* H37Rv Δ *RD1* Δ *panCD*) in mice and guinea pigs. In C57Bl/6 mice, mc²6020 was cleared in less than 30 days [8], while mc²6030 persisted for over 200 days [9]. Vaccine efficacy studies in the murine model with these vaccine candidates demonstrated protection from a challenge of virulent *M. tuberculosis* up to 8 months after vaccination [8,9]. This study tested the safety and efficacy of the live attenuated *M. tuberculosis* vaccine strains mc²6020 and mc²6030 in non-human primates at a dose 50-fold higher than the recommended human dose for BCG.

2. Materials and methods

2.1. Experimental animals

24 cynomolgus macaques (*Macaca fascicularis*), 2–7.8 kg and 3.8–7.6 years of age were randomized by weight, sex, and age into groups. Prior to the initiation of the study all animals received physical exams including complete blood counts (CBC), serum chemistry and chest radiographs. In addition the animals were confirmed to be negative for immunosuppressive retroviruses (type D retrovirus, simian immunodeficiency virus) and simian T cell leukemia virus by both serology and PCR. The animals also had negative intradermal palpbral tuberculin skin tests (TST using 0.1 mL mammalian old tuberculin, Synbiotics, San Diego, CA) and Primagam® (Prionics AG, Schlieren, Switzerland) assays. Primagam® is an IFN γ response assay that has been validated for both rhesus and cynomolgus macaques and is similar to the human QuantiferonGold® assay except that *M. bovis* and *M. avium* spp. *paratuberculosis* PPD are the test antigens. During the study animal monitoring and sample collection included blood collection, bronchoalveolar lavage (BAL), tuberculin skin tests, radiographs and complete physicals including body weight and temperature. Samples of blood and BAL fluid were utilized as described below. Animals were housed in a biosafety level 3 facility (BSL-3) at the Tulane National Primate Research Center (TNPRC), an AAALAC International-accredited facility. Housing and handling of animals were in accordance with the Guide for the Care and Use of Laboratory Animals (U.S. Public Health Service) and the Animal Welfare Act. All protocols and procedures were reviewed and approved by the Tulane University Institutional Animal Care and Use Committee.

2.2. Vaccination

Animals were vaccinated with either saline, BCG Danish, mc²6020 (*M. tuberculosis* H37Rv Δ *lysA* Δ *panCD*) [8], or mc²6030 (*M. tuberculosis* H37Rv Δ *RD1* Δ *panCD*) [9]. Consistent with a previous safety study in guinea pigs [9], the non-human primates were vaccinated

with the recommended single dose of 50 times the equivalent dose of BCG. For BCG Danish the recommended single human dose is 1.8×10^5 CFU so for each animal in the BCG group, five vials of lyophilized BCG Danish (Statens Serum Institute, Copenhagen, Denmark) were resuspended in a total volume of 1.0 mL PBS (9×10^6 CFU/animal) and was administered intradermally between the scapulae. Frozen stocks of mc²6020 and mc²6030 grown in supplemented Middlebrook 7H9 media as previously described [8,9] were diluted to a final concentration of 9×10^6 CFU/mL and administered in 1.0 mL PBS intradermally between the scapulae. Following vaccination the animals were monitored for 8 months. During this time blood was collected weekly for the first month and then twice per month. Bronchoalveolar lavage was performed monthly, radiographs every other month, and tuberculin skin tests at 2, 3 and 4 months post-vaccination. In addition, a biopsy of the axillary lymph node that drains the vaccination site was performed 7 months post-vaccination.

2.3. Experimental infection

Animals were sedated with ketamine HCL (10 mg/kg) prior to bronchoscope instillation of the challenge strain *M. tuberculosis* Erdman (Mycos Research, Loveland, CO). At day 228, the *M. tuberculosis* Erdman challenge strain thawed from frozen stock was diluted to 500 CFU/mL in PBS, briefly sonicated, and 2 mL was administered by bronchoscope to the lung for a final challenge dose of 1000 CFU. Animals were monitored for 6 months following challenge and during this time blood was collected weekly for the first month and then twice per month. Bronchoalveolar lavage was performed twice in the first month post-challenge and monthly thereafter, radiographs and skin tests were performed monthly.

2.4. Immunological assays

To enumerate specific interferon (IFN γ) production capacity of mycobacteria-specific T cells, peripheral-blood lymphocytes (PBLs) were assessed for their specific recognition of purified protein derivative (PPD) and ESAT6 antigens by ELISPOT assay, as described previously [10,11]. The ESAT6 used for immune assays was the pooled synthetic 15-mer overlapping by 11-mer peptides that span the entire ESAT6 protein (GenScript Corporation, Piscataway, NJ). The working concentration was 1 μ g/mL for each ESAT6 peptide.

2.5. Necropsy procedures and analysis of gross and histopathologic lesions

Animals were sacrificed by intravenous barbiturate overdose, and immediately necropsied in a biological safety cabinet within a BSL-3 facility. Multiple specimens from all tissues with gross lesions and remaining major organs were harvested. Specifically, each lobe of lung, bronchial, mesenteric, axillary and inguinal lymph nodes, tonsils, thymus, bone marrow and other major organs were collected and labeled. Gross observations including but not limited to the presence, location, size, number and distribution of lesions were recorded. The percent of total organ involvement was estimated based on consolidation and discoloration as viewed from organ exterior and cut surfaces. The estimate of the percent of total organ involvement was used to score each organ from 0 to 4 (normal, minimal, mild, moderate, and severe). The maximum score for an organ would be 4 and the maximum score for a group of animals would be 24. Adjacent blocks of tissues were collected and flash frozen for bacterial culture, placed in RPMI with 5% fetal bovine serum for immunologic assays, fixed in buffered 10% formalin with ionized zinc (Z-FixTM; Anatech, LTD, Battle Creek, MI), frozen with and without Optimal Cutting Temperature (OCT) compound (Sakura Finetek USA Inc., Torrance, CA), or placed in RNA-laterTM (Ambion, Austin, TX) for additional study. Histologic specimens were embedded in paraffin and sectioned at 5 μ m for routine staining with hematoxylin and eosin (H&E) and selected staining with Ziehl-Neelsen acid-fast stain. The pathologist doing the necropsy was blinded to the group of animals and the assessment of tissue sections was also blinded. The severity of lung lesions was scored from

0 to 4 (0 = normal, 1 = focal granulomas, 2 = few granulomas, 3 = numerous granulomas and 4 = grossly visible granulomas). The extent of lung involvement for each lung lobe was determined using digital scans of each lobe of lung to record total pixel counts on H&E stained material and specimen area measured in square cm using Image-Pro Plus software (MediaCybernetics, Silver Spring, MD). Together this provides a measure of both the extent and severity of TB lesions.

2.6. Bacterial burden measurements

Quantification of mycobacterial burden was accomplished by measuring bacterial colony counts of tissue homogenates, blood, and bronchoalveolar lavage fluid. Tissue homogenates were made using a stomacher (Seward, UK; Stomacher 80) and diluting the homogenate in sterile PBS + 0.05% Tween-80. Bronchoalveolar lavage fluid was decontaminated with the MycoPrep solutions (Becton Dickinson Cat. 240862). Serial dilutions of samples were plated on Middlebrook 7H11 supplemented with OADC (10%), glycerol (0.5%), Casamino acids (0.2%; Becton Dickinson 223120), Lysine (80 mg/L; Sigma L8662), Pantothenate (24 mg/L; Sigma P5710), Polymyxin B (50,000 units/L; Sigma P1004), AmphotericinB (5 mg/L; Sigma A2942), Nalidixic acid (20 mg/L; Sigma N4382), Trimethoprim (5 mg/L; Sigma T7883) and Azlocillin (5 mg/L; Sigma A7926).

2.7. Identity testing of mycobacteria

Identity testing was performed to confirm that the mycobacteria recovered from the samples at necropsy were the *M. tuberculosis* Erdman challenge strain and not the BCG, mc²6020 or mc²6030 vaccine strains. Two types of PCR reactions were performed. Amplification with the *lysA*-A and *lysA*-B primers as described below yield a 1607 bp product (*M. tuberculosis* Erdman, BCG Danish, or *M. tuberculosis* mc²6030) or a 1412 bp product for *M. tuberculosis* mc²6020. For the *RD1* locus, amplification with the RD1-B and RD1-C primers yields no product (BCG Danish or *M. tuberculosis* mc²6030) or a 146 bp product (*M. tuberculosis* Erdman or mc²6020). In contrast, amplification with RD1-A and RD1-C yields a 200 bp fragments for BCG Danish, a 1200 bp fragment for *M. tuberculosis* mc²6030 and no product for *M. tuberculosis* Erdman. DNA was prepared from representative colonies isolated from necropsy tissue samples by transferring single colonies into 2 mL screwcap tubes containing 100 µl H₂O and boiling for 30 min. PCR reactions for the *lysA* locus used primers *lysA*-A (5' -TCGATGATGCGGACAACCTC-3') and *lysA*-B (5' -GCCGCTACCTGGAAGACCTG-3'). The PCR mix contained 10 mM Tris-HCl (pH 8.3), 50 mM KCl, 0.001% (w/v) gelatin, 2 mM MgCl₂, 200 µM of each deoxynucleoside triphosphate, 1.25 U of Taq DNA-polymerase (AmpliTaq®, Applied Biosystems, Foster City, CA), and 20 pmol each of primers *lysA*-A and *lysA*-B. For each colony three PCR mixtures containing 1 µl, 5 µl and 10 µl colony lysate (preparation see above) were prepared. Thereactions were denatured for 5 min at 95 °C and then 25 times subjected to the following temperature program: 94 °C for 30 s, 60 °C for 30 s and 72 °C for 1 min. A final extension for 10 min at 72 °C followed the last cycle. PCR reactions for the *RD1* locus used primers RD1-A (5' -AAGCGGTTGCCGCGGACCG ACC-3'), RD1-B (5' -CTGGCTATATTCTGGGCCCCGG-3') and RD1-C (5' -GAGGCGATC TGGCGGTTTGGGG-3') as described by Talbot et al. [12]. The BD Advantage™ 2 Polymerase Mix (Cat. Nos. 639201 and 639202) was used in two separate PCR reactions. For each colony three PCR reactions containing 1 µl, 5 µl and 10 µl colony lysate (preparation see above) were prepared for each PCR mix. One PCR mix contained 5 µl of the 10× BD Advantage™ 2 PCR buffer, 200 µM of each deoxynucleoside triphosphate, 1 µl of the 50× BD Advantage™ 2PCR Polymerase Mix, 25 pmol of primer RD-A and 5 pmol of primer RD-C in a total volume of 50 µl. The second PCR mix contained 5 µl of the 10× BD Advantage™ 2 SA PCR Buffer, 200 µM of each deoxynucleoside triphosphate, 1 µl of the 50 BD Advantage™ 2 PCR Polymerase Mix, 25 pmol of primer RD-B × and 25 pmol of

primer RD-C in a total volume of 50 μ l. Both PCR mixtures were cycled together. After an initial denaturation for 5 min at 95 °C the samples were subjected 35 times to the following temperature program: 95 °C for 30 s, 65 °C for 10 s and 72 °C for 30 s. A final extension for 7 min at 72 °C followed the last cycle. In addition to PCR identity analysis, representative colonies recovered from the plates were acid-fast stained (Kinyoun method) and examined by light microscopy.

2.8. Statistical analysis

Survival was compared by the Fisher Exact Test. Survival times and the numbers of antigen-specific IFN γ -producing cells were compared by the Wilcoxon rank sum test [13]. An unadjusted *p*-value <0.05 was considered statistically significant. All tests were two-tailed.

3. Results

3.1. Clinical response to vaccination

The primary objective of this study was to show safety of live attenuated *M. tuberculosis* strains mc²6020 (*M. tuberculosis* H37Rv Δ lysA Δ panCD), or mc²6030 (*M. tuberculosis* H37Rv Δ RD1 Δ panCD) as vaccine candidates. Six healthy PPD-negative cynomolgus macaques in each group were vaccinated (saline, BCG, mc²6020 and mc²6030) intradermally. Weekly examination following vaccination revealed no signs of local reactions at the injection sites in any animal. Monitoring of body temperature at weekly physical exams showed no elevated temperatures for any of the animals during the 8 months following vaccination. For all animals in all groups CBC, serum concentrations of electrolytes, blood urea nitrogen, creatinine, transaminases, total protein, and albumin were in the normal range for each of the 8 months following vaccination (data not shown) with the exception of one animal in the saline control group (FG16) and one animal in the BCG group (FG11) that each exhibited mild transient anemia. Chest radiographs at days 54, 123, and 224 post-vaccination were performed and were normal for all animals in all groups. All cynomolgus macaques remained clinically healthy and 23 of the 24 gained weight following vaccination (Fig. 1) (FB37 in the mc²6020 vaccinated group had an adverse reaction to the bronchial procedures). There was no evidence of local or systemic reaction to any of the vaccination regimens. As this study was primarily designed as a safety study, we patterned our parameters from the Guinea Pig Freedom From Virulence Assay (GPFVA) described previously [9]. In the GPFVA, a 50 $\times$ human dose of vaccine is administered to guinea pigs. Guinea pigs are monitored for 6 weeks for weight loss and at necropsy, a gross pathology exam including of spleen is measured. Guinea pigs are extremely sensitive to infection with virulent mycobacteria and a 6-week interval is sufficient to detect disease in response to infection with virulent mycobacteria. Since cynomolgus macaques are probably not as sensitive as guinea pigs to infection with virulent mycobacteria, a shorter interval could have masked seeing adverse event for attenuated strains (i.e. reversion, etc.) so we chose a longer timeframe to monitor safety.

3.2. Immunological response to vaccination

All animals were TB skin test and Primagam®-negative prior to vaccination. TB skin tests with mammalian old tuberculin were performed at days 54, 82, 118, and 221. None of the animals in any of the groups had a positive TB skin test (described in Ref. [14]). The median ELISPOT PPD IFN γ responses for all groups (Fig. 2) show that all three vaccinated groups had median ELISPOT PPD responses exceeding the saline group at most time points and these differences were statistically significant at some time points as shown. Fig. 3 shows the ELISPOT PPD or ESAT6 IFN γ responses for individual animals. There was substantial variation in these responses within groups and over time. ELISPOT responses to ESAT6 in the three vaccinated groups did not differ significantly from the saline group.

3.3. Determination of vaccination load in nearest lymph node, BAL and blood

To evaluate the persistence of vaccine strains, the axillary lymph node near the vaccination site was biopsied, and BAL and blood were collected for all 24 animals at 7 months. Blood and BAL samples as well as homogenates of the lymph node biopsies were plated on supplemented media. No mycobacteria were recovered for any of the lymph node, BAL, or blood samples from any of the 24 animals.

3.4. Clinical response to live *M. tuberculosis* challenge

A dose of 1000 CFU of *M. tuberculosis* Erdman was administered by bronchoscope. Thoracic radiographs showed progressive abnormalities over time. At 5 weeks post-challenge four of six animals immunized with mc²6030 and four of six saline controls had radiographic abnormalities while only one of six animals immunized with BCG or mc²6020 had such abnormalities. By 4 months post-challenge, all animals remaining alive had radiographic abnormalities and the pattern of the changes had progressed from a mild interstitial pattern to one with nodular infiltrates and enlargement of the mediastinum. Animals were monitored weekly for body temperature and weight loss, which is reported as percent body weight change (Fig. 1E–H). Animals with a greater than 20% loss of body weight were euthanized. All of the groups except the mc²6020-vaccinates had at least one animal with a percent weight change greater than 10%. Chemistry blood panels as well as C-reactive protein were determined at weeks 1, 5, 8, and 14 post-challenge (Fig. 4). Survival after challenge is shown in Fig. 5. Two animals were terminated for reasons unrelated to the *M. tuberculosis* challenge. Overall survival and survival time did not differ among the groups.

3.5. Immunological response to challenge

Patterns of antigen-specific cell responses following *M. tuberculosis* challenge were different among the vaccinated and control groups. Three monkeys in the mc²6020 group developed remarkable recall immune responses of ESAT6-specific IFN γ -producing cells 2 and 4 weeks after *M. tuberculosis* challenge although the response to this early-secreted antigen was not stable in the blood (Fig. 4). Interestingly, at the end point of studies, PPD-specific IFN γ -producing cells in the blood, lung, bronchial lymph node, spleen, liver and kidney in the BCG and mc²6020 groups were significantly lower than the saline and mc²6030 groups (Fig. 6). A low level of PPD-specific IFN γ -positive T effector cells at the later stage of *M. tuberculosis* challenge appeared to be consistent with lower TB burdens (for the BCG group) or longer survivals of these two groups of vaccinated animals. Thus, although the development of early recall responses of vaccine-elicited cell responses was inconclusive for prediction of differences in protection against TB among three vaccinated groups, the end point studies of antigen-specific IFN γ -positive T effector cells in the whole immune system provided data suggesting correlation between TB-driven responses of IFN γ -positive T effector cells and favorable clinical status of tuberculous disease in the BCG and mc²6020 groups.

3.6. Confirmation of challenge strain

Tissue homogenates from necropsy samples were plated on supplemented Middlebrook media. Plates were examined for growth and random colonies were selected for further characterization. Approximately 300 representative colonies were chosen for both acid-fast staining and PCR analysis to determine if the mycobacterial colonies recovered were the challenge strain *M. tuberculosis* Erdman or any of the vaccine strains. All mycobacterial colonies recovered were *M. tuberculosis* Erdman based on PCR analysis (data not shown).

3.7. Pathology

Granulomatous lesions were grossly apparent in all TB-challenged animals characterized by miliary to confluent to solitary grayish-white nodules (1–3 mm diameter) in most lobes of lung and bronchial lymph nodes (Fig. 7A). Lung involvement was most extensive and severe in the right posterior lobe where the inoculum was deposited (Fig. 7B). For the other lung lobes, the BCG vaccinated group had the lowest total lesion intensity, the saline-vaccinated group had the most severe lesions, and the mc²6020 and mc²6030-vaccinated animals were intermediate between these two groups (Fig. 7B). The rank order for gross lesion intensity in lung and bronchial lymph node was BCG < mc²6020 < mc²6030 < saline (Fig. 7B). Single and occasionally multiple granulomas distributed most often to the liver, spleen, and kidney but no significant differences were detected among the groups in spread of lesions outside the chest (Fig. 7C).

Histologically, granulomas in all groups were characterized by dense aggregates of epithelioid macrophages with a thin peripheral lymphocytic infiltrate and a few multinucleated giant cells surrounding a central zone of necrotic debris and degenerating polymorphonuclear leukocytes. Fibrosis was notably absent. No qualitative differences in the character of the lesions were observed among organs within an animal, or among the different groups of animals (Fig. 7D). The only measurement for which there was a statistical difference among groups was the extent of lung involvement reflected in digitized measurements of individual lung lobes. The treatment group average (SD) reflects a decrease in lesion area for the BCG group (16.2 cm² + 11.5) compared to saline (46.8 cm² + 32.7) and non-significant reductions for the mc²6030 group (32.5 cm² + 39.7) and the mc²6020 group (33.0 cm² + 23.3).

4. Discussion

Non-human primates are an important model system to evaluate safety and efficacy of tuberculosis vaccines [14–21] and have been recommended for pre-clinical evaluation of new vaccine candidates [7]. BCG vaccination can protect non-human primates from *M. tuberculosis* challenge, although the degree of protection varies widely [15–17,20] and the 8-month interval between vaccination and challenge of the monkeys in this study is significantly longer than previously reported 1–4-month intervals between vaccination and challenge [15–17].

The primary objective of this study was to determine the safety of two live attenuated *M. tuberculosis* vaccine candidates in non-human primates. The mc²6030 strain is most similar to BCG in that the *RDI* region is deleted in both strains and both mc²6030 and BCG persist in murine models following vaccination [8,9,22]. In contrast, mc²6020 has an intact *RDI* region and undergoes limited replication in the mouse model following vaccination. In this study, we demonstrated that both vaccine candidates mc²6020 and mc²6030 were well tolerated in cynomolgus macaques and had safety profiles indistinguishable from BCG Danish. In all safety parameters including physical exams, local vaccination site reaction, blood work, body temperature, chest radiographs, and body weight, all three vaccination regimens were safe and well tolerated. Although safety differences have been observed between BCG and mc²6020 or mc²6030 in immunocompromised mice, we did not expect to observe differences in safety in adult immunocompetent macaques with these three vaccination regimens.

In addition to safety, we were able to assess the efficacy of both persistent and rapidly cleared vaccine strains following an intrabronchial challenge 8 months after vaccination with a high dose (1000 CFU) of virulent *M. tuberculosis* Erdman. The ELISPOT responses to PPD in blood and tissues at necropsy showed statistical significance between the BCG

and mc²6020 vaccinated animals compared to the mc²6030 or unvaccinated controls. In this study, the higher ELISPOT responses of mc²6030 and unvaccinated animals could be interpreted as an index for inability to control disease. Our data would suggest that IFN γ production is not a reliable correlate of immune protection for vaccination protocols and may be more relevant for active disease. IFN γ -positive cells appear to represent potential effector cells of an *M. tuberculosis*-specific T-cell population. It is likely that numbers of effector cells are driven by tuberculosis burdens, whereas vaccine-induced protection is coincident more with central memory phenotype without robust IFN γ production. Loss of body weight was correlated with disease progression and increases in C-reactive protein in 50% of the controls but only 30% of the vaccinated animals, suggesting a more controlled response. For many measurements of efficacy, this study was not powered sufficiently to show statistical significance, however, an overall qualitative trend emerged: for weight loss, C-reactive protein, survival, gross lesions, and histopathology, in most cases BCG was better by some degree than mc²6020 which was quite a bit better than mc²6030 and certainly saline.

BCG and mc²6020 differ in that BCG is lacking the *RDI* region and BCG persists after vaccination compared to the rapidly cleared mc²6020. Presumably mc²6020 is unable to scavenge sufficient amounts of lysine and pantothenate and consequently undergoes limited replication. The limited replication of the mc²6020 strain could provide a safety advantage over BCG. The mc²6020 vaccine primed a strong response to ESAT6 after challenge with *M. tuberculosis* compared to the responses of the BCG and mc²6030 vaccinates, suggesting *RDI* could be important for protection even though it is missing from BCG. The lack of protection in mc²6030 vaccinates raises the possibility that *RDI* deletions of *M. tuberculosis* might be less capable of eliciting a protective immune response in comparison to *RDI* deletions of *M. bovis*. The mc²6030 vaccine strain was tested in the neonatal bovine model and although it was safe and well tolerated, it did not protect against virulent *M. bovis* aerosol challenge while BCG vaccination did provide protection [23]. Although both mc²6020 and mc²6030 were safe and well tolerated, the study was not statistically powered to demonstrate differences in efficacy.

While the vaccine candidate mc²6030 is safe in four animal models (mouse [8,9], guinea pig [9], neonatal calf [23], and non-human primates), the discordant protective efficacy between models highlights the utility of non-rodent animal models to refine TB vaccine development. Because of their safety profile, the mc²6020 and mc²6030 strains have been reclassified for work in BSL2 containment (the same containment level for *M. bovis* BCG or *M. tuberculosis* H37Ra) by the Albert Einstein College of Medicine Institutional Biosafety Committee. Moreover, Institutional Biosafety Committees from 15 research organizations and universities have granted permission for the mc²6020 and mc²6030 to be used in BSL2 containment at their facilities.

The primary goal of this study was to evaluate the safety of attenuated *M. tuberculosis* vaccine strains in a non-human primate model. Towards a class as a whole, concerns have been raised about how well attenuated *M. tuberculosis* strains could be accepted as TB vaccine candidates in humans. Through studies in immunocompromised mice we have shown that the attenuated *M. tuberculosis* vaccine candidates mc²6020 and mc²6030 are unequivocally safer than BCG. This study shows clearly that mc²6020 and mc²6030 vaccines are as safe as BCG in non-human primates. The size and scope of this study (small group numbers) did not show any statistical differences in survival for any vaccine. In other measures both BCG and mc²6020 were comparable while mc²6030 vaccinates were more like the saline group. Our conclusion from this study is that the mc²6020 and mc²6030 strains are safe as TB vaccine candidates and should be considered as a platform for Phase I studies in humans. Recombinant mycobacteria including BCG and attenuated *M.*

tuberculosis strains as live attenuated TB vaccines are being developed by a number of research groups: *M. tuberculosis* leucine and pantothenate auxotrophic strains [24], *phoP* deletion strains [25]; *M. bovis* BCG overexpressing Ag85B [26], with listeriolysin of *Listeria monocytogenes* [27] or endosome escape and antigen overexpression [28]. However, a limitation for any recombinant mycobacterial vaccine is the inherent ability of *M. bovis* and *M. tuberculosis* to evade host immune functions. Since the safety of the mc²6020 and mc²6030 backgrounds has been established, future studies will include incorporation of additional mutations to counter immune evasion and thus increase vaccine efficacy. One candidate gene is *secA2* as deletion of *secA2* increases the apoptosis of an *M. tuberculosis*-derived strain and thus enhances cross-presentation of mycobacterial antigens to make a more effective vaccine for tuberculosis [29].

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Fig. 1. Percent body weight change following vaccination and challenge. Animals were weighed weekly for the first 4 months following vaccination then bi-monthly. Weight change relative to weight pre-vaccination is expressed as a percentage for each animal (saline (A), BCG (B), mc²6020 (C), and mc²6030 (D)). Weight change relative to weight pre-challenge is expressed as a percentage for each animal (saline (E), BCG (F), mc²6020 (G), and mc²6030 (H)).

Fig. 2.

PBMC-specific IFN γ responses to PPD during the safety phase following vaccination. An ELISPOT assay for IFN γ production in response to PPD was performed at days 12, 18, 25, 40, 68, 103, 118, and 207 post-vaccination. The median responses of each group (saline (square), BCG (diamond), mc²6020 (triangle) and mc²6030 (circle)) of PPD-specific IFN γ producing cells per 10⁶ PMBC are plotted. Measurements were made in triplicate and the median is shown. *p*-Values were determined by both a paired *t*-test and two-way ANOVA. A *p* < 0.05 is indicated by a single asterisk and a *p* < 0.01 by two asterisks.

Fig. 3. PBMC-specific IFN γ responses to PPD or ESAT6. Responses of individual animals to PPD are plotted after vaccination (saline (A), BCG (B), mc²6020 (C), and mc²6030 (D)) and to ESAT6 after vaccination (saline (E), BCG (F), mc²6020 (G), and mc²6030 (H)). The break in the axis represents challenge with virulent *M. tuberculosis* Erdman was at day 228 and is indicated by an arrow.

Fig. 4. C-reactive protein post-challenge. C-reactive protein (mg/dl) was measured at 0, 1, 5, 8, 14, and 16 weeks post-challenge. Saline (A), BCG (B), mc²6020 (C), and mc²6030 (D) groups are graphed. (E) The highest C-reactive protein value for each animal post-challenge in each group.

Fig. 5.

Survival after challenge. Six animals in each group (saline (square), BCG (diamond), mc²6020 (dashed line marked with triangle), and mc²6030 (circle)) were followed for survival after aerosol challenge. At 45 days post-challenge, an mc²6030-vaccinated animal died from unrelated causes and at 66 days post-challenge an mc²6020 animal was necropsied based on a transcription error of body weight. Both of these animals are noted by small arrows above a circle (mc²6030) and triangle (mc²6020) but these animals have not been censored from any of the post-challenge data including survival.

Fig. 6.

PPD-specific IFN γ -producing cells at necropsy were measured in samples from PMBC (A), lymph nodes (B), lungs (C), spleen (D), kidney (E), and liver (F). Measurements were made in triplicate and the median value is shown. p -Values were determined by both a paired t -test and two-way ANOVA. A $p < 0.05$ is indicated by a single asterisk and a $p < 0.01$ by two asterisks.

Fig. 7.

Gross lesions and histology. (A) Sum of gross lesion intensity score for each group for lung, bronchial lymph node, liver, and spleen derived as described in materials and methods. (B) Sum of histology lesion intensity score for each lung lobe. Note that the right posterior lung lobe where the inoculum was deposited was most severely involved. (C) The number of animals in each group with grossly visible granulomas in extra-thoracic organs. (D) Sum of histology lesion intensity score for bronchial lymph node and extra-thoracic organs.