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Protection by novel vaccine candidates, *Mycobacterium tuberculosis* *mosR* and *echA7*, against challenge with a *Mycobacterium tuberculosis* Beijing strain

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Abstract

Mycobacterium tuberculosis, the etiological agent of tuberculosis (TB), infects over two billion people, claiming around 1.5 million lives annually. The only vaccine approved for clinical use against this disease is the Bacillus Calmette-Guérin (BCG) vaccine. Unfortunately, BCG has limited efficacy against the adult, pulmonary form of tuberculosis. This vaccine was developed from *M. bovis* with antigen expression and host specificity that differ from *M. tuberculosis*. To address these problems, we have designed two novel, live attenuated vaccine (LAV) candidates on an *M. tuberculosis* background: *mosR* and *echA7*. These targeted genes are important to *M. tuberculosis* pathogenicity during infection. To examine the efficacy of these strains, C57BL/6 mice were vaccinated subcutaneously with either LAV, BCG, or PBS. Both LAV strains persisted up to 16 weeks in the spleens or lungs of vaccinated mice, while eliciting minimal pathology prior to challenge. Following challenge with a selected, high virulence *M. tuberculosis* Beijing strain, protection was notably greater for both groups of LAV vaccinated animals as compared to BCG at both 30 and 60 days post-challenge. Additionally, vaccination with either *mosR* or *echA7* elicited an immune response similar to BCG. Although these strains require further development to meet safety standards, this first evidence of protection by these two new, live attenuated vaccine candidates shows promise.

Keywords

Mycobacterium tuberculosis; live attenuated vaccine; tuberculosis; BCG; Beijing strain

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Introduction

Despite 90% global vaccination coverage against tuberculosis (TB) [1], the disease continues to claim a million and a half lives annually [2]. While the current *M. bovis* Bacillus-Calmette Guérin (BCG) vaccine provides some protection, particularly against disseminated tuberculosis in childhood [3], *M. tuberculosis* still manages to infect over one-third of the world population [2]. The causes behind the vaccine's poor performance are unclear, but may include exposure to environmental mycobacteria [4], variable and reduced efficacy of different BCG strains [5,6], or variation in the infecting *M. tuberculosis* strain. In particular, the BCG vaccine has been found to be less effective against the widespread *M. tuberculosis* Beijing family [7,8], members of which have been associated with increased levels of virulence and drug resistance [9]. These troubling statistics highlight the urgent need for new, more effective vaccines against TB.

The search for a better tuberculosis vaccine has intensified over the past two decades upon sequencing of the *M. tuberculosis* genome, allowing for the development of more rationally designed vaccines [10]. In that time many possible solutions for replacing or boosting the BCG vaccine have been presented, reaching so far as phase II clinical trials, however many have not yielded results as promising as hoped, as reviewed in [11]. One area of focus for developing a vaccine to replace BCG has been on live attenuated vaccines (LAVs), like BCG itself, using BCG or *M. tuberculosis* as the parent strain [12,13]. Both genetic backgrounds have yielded promising results. Vaccine VPM1002, *M. bovis* BCG *ureC::hly* Hm^R, recently demonstrated safety and immunogenicity during phase I clinical trials [14]. Additionally, MTBVAC, consisting of *Mycobacterium tuberculosis* *phoP* and *fadD26* and representing the first vaccine on an *M. tuberculosis* background to reach clinical trials, is currently being evaluated in phase I [15]. While BCG based vaccines present an advantage in safety which must be carefully addressed with *M. tuberculosis* LAVs [12,13], use of an *M. tuberculosis* parent strain may prove superior in protection. Unlike *M. bovis* BCG, an *M. tuberculosis* background would provide specificity to the human host and antigens from numerous regions of difference that have been lost in BCG [16]. Given these advantages, we chose to pursue this latter strategy for developing novel vaccine candidates.

The selection of targets for LAV development came after *in vivo* transcriptional studies revealed induction of *mosR* and *echA7*, among many other genes, in mice following a low dose aerosol infection as compared to a standard Middlebrook 7H9 culture [17,18]. Notably, *mosR*, a transcriptional repressor of its own operon, was highly induced during the late chronic stage of TB, 20 weeks post-infection. To better understand the role *mosR* plays during infection, a mutant strain was studied revealing a drastic reduction in virulence [19]. Furthermore, transcriptional analysis of *mosR* revealed the induction of genes coding for at least 8 antigenic proteins, including *hspX*. The potential superior immunogenicity of this strain combined with its attenuation made it a prime candidate for this LAV study. The other gene of interest, *echA7*, was induced earlier at 3-4 weeks as part of the 20 gene *in vivo* expressed genomic island (iVEGI). It encodes one of 21 probable enoyl-CoA hydratases annotated in the *M. tuberculosis* genome [10]. Among several other iVEGI knockout strains tested, only *echA7* did not cause lethal infection [20].

Here we set out to determine how these novel LAV candidates perform against the gold standard of TB vaccination, BCG. To better characterize the protective immunity elicited by these LAVs, we compared the performance of *mosR*, *echA7*, BCG, or PBS following challenge with the high virulence clinical isolate, *M. tuberculosis* Beijing 4619, characterized previously [21,22]. Prior to challenge, it was noted that both LAV strains persisted while causing minimal pathology, emphasizing the safety of the strains in an immunocompetent host. Challenged mice were examined at 30 and 60 days when samples were taken to determine bacterial load, histopathology, and the immune response. Both LAVs protected mice from *M. tuberculosis* challenge superior to BCG, showing nearly a log reduction in colonization of the lungs at both time points, and a reduction in lung pathology. Immunological assays revealed an *M. tuberculosis* specific T-cell response similar to, and in some cases exceeding that primed by BCG.

Materials and Methods

Bacterial strains and growth media

M. bovis BCG (Pasteur TMC 1011) was used in this study as a positive vaccine control. *M. tuberculosis* CDC1551 *mosR* and *M. tuberculosis* H37Rv *echA7* were constructed in this lab and have been characterized in previous studies [19,20].

Briefly ~800bp fragments flanking the open reading frames of *mosR* or *echA7* were cloned into vector pYUB854 which was then introduced into a specialized transducing mycobacteriophage [23] prior to transduction of wild type *M. tuberculosis* strains CDC1551 and H37Rv, respectively. Hygromycin was used to select for successful knockout strains which were confirmed by PCR and sequencing. Clinical isolate, *M. tuberculosis* Beijing strain 4619 was a generous gift from Dr. Ian Orme.

All strains were grown in Middlebrook 7H9 medium (Remel™, Lenexa, KS) supplemented with 10% ADC and 0.05% TWEEN 80 as well as 30µg/ml hygromycin where appropriate. For vaccine and challenge stocks, strains were grown to an approximate OD₆₀₀ of 1.0 prior to aliquoting and storage at -80°C until use. Where required for enumeration of colony forming units (CFU), strains were plated on solid Middlebrook 7H10 medium (BD, Sparks, MD) supplemented with 10% ADC and, where appropriate, 30µg/ml hygromycin (Invitrogen, Carlsbad, CA) or 2 µg/ml 2-thiophenecarboxylic acid (TCH) (Sigma-Aldrich, St. Louis, MO) to select for LAV strains or against BCG, respectively. Strains were allowed to grow on solid media 2-4 weeks before collection for CFU counts. Negative plates were monitored for 6 weeks to confirm absence of growth.

Mouse vaccination

All animal experiments were approved of by the Institutional Animal Care and Use Committee, University of Wisconsin-Madison. Groups of 10-13 mice, age 5-6 weeks, were vaccinated subcutaneously at the base of the tail with 10⁶ CFU vaccine strain in 100µl PBS, or 100µl PBS alone. At 8 weeks post-vaccination, 7-10 mice from each group were challenged using the *M. tuberculosis* Beijing strain 4619. Approximately 40 CFU were administered by aerosol using the Glas-Col inhalation system (Glas-Col, LLC, Terre Haute,

IN). The dose was confirmed by plating lungs of an infected mouse 1 day post-challenge. Mice were monitored regularly for adverse reactions to the vaccination and for progression of infection. At 8 weeks post-vaccination and 4 and 8 weeks post-challenge 3-5 mice were sacrificed from each group. Lungs, spleen, and liver were collected to determine bacterial load by CFU counts. Briefly samples were homogenized in PBS brought up to a total volume of 2ml before plating undiluted and 10 fold serial dilutions of samples onto antibiotic free and selective media to differentiate between challenge and vaccine strains. For histopathological analysis, sections were cut from embedded samples of lung, spleen, and liver and stained with H&E. Slides were scored by a trained pathologist blinded to the samples. Samples were scored on a severity scale of 0 to 5 as follows: 1, minimal; 2, mild; 3, moderate; 4, severe; and 5, massive. Images of lungs at 40× magnification were also analyzed in Photoshop CS2 (Adobe, San Jose, CA) to determine the percent area of inflamed lung as compared to the total area of the lung for each animal post challenge. Lung and spleen were also used for lymphocyte isolation and immunological analysis.

Lymphocyte isolation and stimulation

Lung and spleen for lymphocyte isolation were placed in RPMI (Corning, Manassas, VA) supplemented with 1% FBS (Atlanta biological, Lawrenceville, GA), 1% L-glutamine (Gibco, Grand Island, NY), 1% penicillin-streptomycin (Mediatech, Inc., Manassas, VA) and 1% nonessential amino acids (Gibco). Splenocytes were isolated by passing the spleen through wire mesh screens. Lymphocytes were isolated from the lung after digesting the tissue in 2mg/ml collagenase B (Roche, Mannheim, Germany) and 28U/ml DNase I (Roche) and passing through a 70µ cell strainer. Lung lymphocytes were further purified using a Percoll (GE Healthcare, Uppsala, Sweden) gradient. Red blood cells were lysed in samples from both organs by ammonium chloride treatment. Cells were washed and resuspended in fresh media containing 10% FBS, counted, and seeded in a flat bottom 96 well plate at a density of 10⁶ cells per well. Cells were stimulated in the presence of 100U/ml IL-2 (BD Biosciences, San Jose, CA) for 24 hours with media alone or with 10µg/ml whole cell lysate prepared from *M. tuberculosis* culture grown to OD 1.0 and lysed in protein lysis buffer using 2 45s pulses in a bead beater. Nonsoluble material was removed by centrifugation and protein content measured by BCA assay (Thermo Fisher Scientific, Rockford, IL). Aliquots were stored at -80°C until use.

Immunological Assays

Intracellular cytokine staining followed by flow cytometry was performed on splenocytes and lymphocytes isolated from mouse lungs as described above. All reagents were obtained from BD Pharmingen (San Diego, CA). Following 18hrs of stimulation, GolgiPlug was added to all wells. At 24hrs cells were collected for staining. Following CD16/CD32 blocking with Fc Block, cell surfaces were stained with PE conjugated α-mouse CD4 antibody, clone RM4-5 and PerCP conjugated α-mouse CD8 antibody, clone 53-6.7. Cells were fixed and permeabilized with Cytotfix/Cytoperm and stained intracellularly with APC conjugated α-mouse IFN-γ antibody, clone XMG1.2 and FITC conjugated α-mouse IL-17a, clone eBiol7B7 (eBioscience, San Diego, CA). Cells were analyzed using a BD FACSCalibur and data was analyzed using FlowJo (FlowJo, LLC, Ashland, OR). The supernatant of the same samples was analyzed by enzyme-linked immunosorbent assay

(ELISA) for IFN- γ levels using the Mouse IFN- γ ELISA MAX™ Deluxe kit following the manufacturer's instructions (Biolegend, San Diego, CA). Readings were acquired and analyzed with SoftMax Pro software (Molecular Devices, Sunnyvale, CA).

Statistical Analysis

Statistical analyses were done using GraphPad Prism (La Jolla, CA). Data were analyzed using a Student's t-test or the Kruskal-Wallis test followed by Dunn's multiple comparisons test. *P*-values less than 0.05 were considered significant.

Results

Safety of LAV candidates

To evaluate the safety of the two live attenuated vaccine candidates, *mosR* and *echA7*, mice were immunized with approximately 10⁶ CFU of either LAV strain, or *M. bovis* BCG Pasteur TMC 1011 as a positive control. After 8 weeks post-vaccination, mice were sacrificed and a gross pathological analysis was done of the vaccine site. All mice appeared healthy, and no lesions were observed in any of the vaccine groups; however 2 of 3 mice in both *mosR* and *echA7* showed enlarged inguinal lymph nodes (data not shown). Colonization of BCG was not observed in any of the organs examined while *mosR* was detected in the spleen of one mouse, but not in the lungs or liver. On the other hand, *echA7* colonies were isolated from the spleen and liver of all mice in the group, and from the lungs of most mice (Table 1). Using selective media, persistence of the LAV strains was followed for another 8 weeks post-challenge, for a total of 16 weeks post-vaccination. At 4 and 8 weeks post-challenge persistence was most notable in the spleens where colonies were found in at least half of the mice of each group at each time point. Low levels of persistence were found in the lungs and liver at 4 weeks and were absent by 8 weeks post-challenge (Table 1).

Upon histological examination, a small amount of granulomatous inflammation was observed in the lungs of a single mouse vaccinated with *echA7*. No inflammation was seen in the lungs of any of the other mice. The spleen also remained free of inflammation in all groups. Interestingly, the organ observed to have the most inflammation was the liver, particularly in mice vaccinated with BCG (Figure S1). LAV vaccinated mice had a more mild level of inflammation; none was seen in the PBS control group.

Vaccination primed immune response

To analyze the immune response primed by each vaccine, spleen and lung tissue were collected at 8 weeks post-vaccination. Upon sacrifice, lymphocytes were isolated from the lung and spleen and stimulated with *M. tuberculosis* whole cell lysate, or media alone to establish background levels. At 24 hours post-stimulation, cells were collected for intracellular cytokine staining to detect markers of immunity. The percentage of *M. tuberculosis* specific CD4⁺ and CD8⁺ IFN- γ ⁺ cells isolated from the spleen, was similar in BCG and *echA7* vaccinated mice, while *mosR* vaccination did not elicit quite as strong a response. The CD4⁺ IL17a⁺ response differed in that *mosR* primed the greatest response followed by BCG with no response from *echA7* above background levels. Nevertheless, no

significant difference was detected between the percentages of responsive, mycobacterial antigen specific T-cell populations in the spleen (Figure 1A). Thus the LAVs showed a general immune response on par with the standard BCG vaccine. In the case of cells isolated from the lung, however, both *mosR* and *echA7* primed a stronger localized CD4⁺ IFN- γ ⁺ immune response than BCG (Figure 1B). This difference was significant between *echA7* and BCG ($P = 0.01$), and may be attributed to the early dissemination of *echA7* to the lungs. Levels of CD4⁺ IL17a⁺, and CD8⁺ IFN- γ ⁺ T-cell populations in the lungs were not detected above background.

Protection against colonization with *M. tuberculosis* Beijing strain

In order to assess the efficacy of the LAV vaccines, 8 weeks post-vaccination, mice vaccinated with *mosR*, *echA7*, BCG, or PBS were challenged by a low dose aerosol infection of *M. tuberculosis* Beijing strain 4619 and assessed 30 or 60 days later. Examination of the bacterial load at day 30 showed that colonization of the lungs in both *mosR* and *echA7* groups was, significantly, over two logs lower than that of the PBS group ($P = 0.04$ and 0.02 respectively) (Figure 2A). Average *M. tuberculosis* colonization of BCG vaccinated mice was intermediate, approximately one log lower than the PBS control. As such, the LAV groups showed less colonization than BCG at day 30, amounting to superior protection over the standard vaccine strain. At day 60 this trend continued, however colonization levels dropped from day 30 for all groups. Markedly, colonization levels in all *mosR* vaccinated mice fell below the limit of detection (20 CFU/lung), significantly over 3 logs lower than the PBS control ($P = 0.01$) and approximately 2 logs lower than that detected in BCG vaccinated mice. The *echA7* vaccinated mice showed a more intermediate phenotype, with colonization between BCG and *mosR* levels.

The disparity in challenge strain colonization levels between BCG and the LAVs was not as pronounced when the total bacterial load found in the spleen was examined. At day 30, *mosR* vaccinated mice had nearly the same level of challenge strain colonization as BCG, while *echA7* performed better than BCG, showing significant reduction in *M. tuberculosis* Beijing levels as compared to control mice ($P = 0.01$) (Figure 2B). By day 60 post-vaccination, the difference between vaccinated and PBS treated mice was greater than at day 30. Similar to the lungs, no colonies from the challenge strain were found in the spleens of the *mosR* vaccinated mice, significantly less than the PBS group ($P = 0.01$). Likewise, only one of the *echA7* vaccinated mice showed challenge strain colonization in the spleen, while BCG mice showed an intermediate level of colonization. Furthermore, we examined the *M. tuberculosis* Beijing dissemination to the liver. At day 30, both LAV vaccinated groups showed a similar 2 log reduction in colonization of the liver as compared to the PBS control (Figure 2C). BCG vaccinated mice, on the other hand only showed a reduction in half of the mice examined. By day 60, no *M. tuberculosis* could be isolated from either LAV-vaccinated group. To the contrary, *M. tuberculosis* was isolated from PBS and BCG vaccinated mice, the latter near the limit of detection.

Histopathology following *M. tuberculosis* challenge

The extent of *M. tuberculosis*-induced lesions in each vaccine group was also examined, using histopathology. At 30 days post-challenge lesions were larger and more numerous in

PBS control vaccinated mice as compared to BCG or LAV vaccinated mice (Figure 3A). The calculated percent area of inflammation in *mosR* and *echA7* vaccinated mice was found to be significantly lower than PBS treated mice ($P = 0.004$ and 0.05 , respectively). In particular, granulomatous inflammation was severe in lungs examined from most PBS control mice, while BCG vaccinated mice showed mild inflammation, and in some lungs examined in *mosR* and *echA7* vaccinated mice inflammation was totally absent, with severity scores differing significantly from PBS groups ($P = 0.02$ and $P = 0.01$, respectively) (Figure S2A). On the other hand, lymphocytic inflammation was absent from PBS vaccinated mice, but significantly detected in BCG vaccinated mice at a mild to moderate level ($P = 0.02$) (Figure S2B). *mosR* and *echA7* vaccinated mice showed a more intermediate phenotype with only a few mice showing a lymphocytic inflammatory response. By day 60, all groups showed more severe pathology (Figure 3B, Figure S2), with a higher percent area of inflammation, however *mosR* vaccinated mice still showed significantly less overall inflammation than PBS ($P = 0.05$). Granulomatous inflammation was detected in all vaccinated samples, in addition to PBS controls, and lymphocytic inflammation was detected in PBS as well as vaccinated strains. Notably, both of the LAV vaccinated groups showed less pathology than BCG or PBS. At both 30 and 60 days, lesions were seen in the liver, however no significant difference was seen between groups (Figure S1). Interestingly, the moderate amount of inflammation seen in the livers of BCG vaccinated mice pre-challenge had decreased by the post-challenge time points. No lesions were observed within the spleen at any time point for any groups.

Post-challenge immune response

To evaluate the *M. tuberculosis* specific immune response post-challenge, ELISA was used to analyze IFN- γ production by lymphocytes isolated from the lung or spleen and stimulated with *M. tuberculosis* whole cell lysate. For all samples at each time point, a clear response could be seen when compared to unstimulated cells. At day 30 post-challenge cells from the lungs of mice vaccinated with *mosR* showed the strongest response, significantly greater than BCG ($P < 0.05$) (Figure 4A). Cells from both BCG and *echA7* vaccinated mice on the other hand produced less IFN- γ than the unvaccinated PBS control. By day 60 however, the IFN- γ response of PBS treated mice dropped while the response of the vaccinated mice remained steady, with *mosR* treated mice more closely matching the levels of the other two vaccine strains. Splenocytes were also analyzed from mice at day 60. Overall there was no significant difference between the *M. tuberculosis* specific IFN- γ responses of these cells, though *echA7* showed the greatest level of response, and *mosR* the lowest (Figure 4B). Flow cytometry results of the same samples showed the presence of both CD4⁺ and CD8⁺ T-cells producing IFN- γ in all samples, but no significant differences between LAV, BCG, or PBS vaccinated mice were detected (data not shown).

Discussion

Given the poor efficacy of BCG against adult pulmonary tuberculosis [3], in particular against *M. tuberculosis* Beijing sublineages [7,8], research into new alternatives is of paramount importance. The results presented above - showing that both novel, live attenuated vaccine candidates, *M. tuberculosis mosR* and *echA7*, provide protection

superior than that provided by BCG - demonstrate a promising strategy for addressing this problem. Most remarkably, mice vaccinated with *mosR* and several vaccinated with *echA7* were able to clear the lungs of viable *M. tuberculosis* within 60 days post-challenge while bacterial loads in the BCG and PBS vaccinated mice remained one and two logs higher, respectively. Additionally, the new LAVs persisted longer than BCG in the vaccinated mice and reached the lung, which may be advantageous, giving the LAV candidates a longer period to stimulate the immune system at the primary site of infection and hence, provide better overall protection than BCG [24].

More experiments are needed to understand the mechanisms responsible for the improved protection of *M. tuberculosis mosR* and *echA7* compared to the BCG vaccine. Among LAVs currently in the pipeline, two strategies, one based on use of a BCG background and those utilizing attenuated *M. tuberculosis* prevail. Both approaches have yielded encouraging results [14,15]. While BCG provides the advantage of a safe genetic background, already approved for use and effective against some forms of tuberculosis, it still has its disadvantages. Unlike vaccines of an *M. tuberculosis* background, BCG has lost regions containing known antigens, such as ESAT-6 and CFP-10 [6] and BCG strains have lost over 100 T-cell epitopes through passaging, compromising the efficacy of their protection [25]. While attempts to replace or overexpress a few of these antigens in BCG have been made [26], they still cannot compensate for all of the genome wide differences between the two species. However, whether including these epitopes in a vaccine is helpful or harmful is still up for debate [27].

In addition to the advantages provided by an *M. tuberculosis* background, these rationally designed LAV strains, may be an improvement over BCG, and potentially other *M. tuberculosis* based vaccines, due to the transcriptional changes induced by the loss of *mosR* or *echA7*. It has been shown that even infection with wild type *M. tuberculosis* does not elicit protection any stronger than BCG vaccination [28]. Therefore, our goal cannot be the development of an *M. tuberculosis* LAV that merely mimics the wild type *M. tuberculosis* antigen expression seen during natural infection, but to enhance expression of these antigens in a targeted manner. Transcriptional analysis of *M. tuberculosis* H37Rv *mosR* was completed in a previous study and the data mined for potential signatures of antigenic expression that may be contributing to the vaccine's efficacy [19]. This search showed the induction of several antigens, some of which have already proven their role in protection. Six dormancy induced proteins, previously shown to be immunogenic, were induced in the *mosR* strain as much as 15 fold: *hspX*, Rv1733c, Rv2032, Rv2626c, Rv2627c, and Rv2628 [29,30]. Notably, *hspX* (2.5 fold) is found to enhance the protection afforded by BCG when overexpressed in a recombinant BCG strain [25]. Similarly, *fbpC2*, encoding Antigen 85C, was induced 2.7 fold in the mutant strain. As with *hspX*, an rBCG strain overexpressing Ag85C improved upon the protective effect of BCG vaccination [31]. Not only is Ag85C antigenic, but it is important for monocyte uptake [32]. In fact, an rBCG strain overexpressing both antigens, HspX and Ag85C, has been studied, again improving the efficacy of BCG [33]. We have achieved similar results here without sacrificing the advantages of using an *M. tuberculosis* background, and plan to assess the specific role these

antigens may play in priming the immune response in future studies. The transcriptional basis of protection conferred by *echA7* will also be probed.

Overall, this study presents two promising live attenuated vaccine candidates: *mosR* and *echA7*. As such, our next step will be to test the safety of each candidate more rigorously in guinea pig and immunocompromised mouse models. Given the safety profiles of these strains observed thus far, we expect that they will serve as acceptable candidates for further development where a second, attenuating mutation will be added in accordance with safety requirements outlined in the Geneva consensus [12,13]. Although there may be concern that these strains are less attenuated than BCG in their current state, there is precedent for incorporating mutations that do not render *M. tuberculosis* completely avirulent into final *M. tuberculosis* LAVs. Such is the case with MTBVAC which uses *fadD26* as a second attenuating mutation to fulfill the requirements of the Geneva consensus [15]. Although MT103 *fadD26* is attenuated in the mouse model, it does not reach the level of avirulence that is observed with BCG [34,35]. While the data presented here represent the efficacy of only our early stage vaccine candidates, there is also precedent for using data from studies of a prototype vaccine, *M. tuberculosis* MT103 *phoP* also known as SO2, to support the efficacy and safety of the final vaccine, MTBVAC, as it heads towards clinical trials [15]. We expect that a carefully selected secondary mutation will leave the antigenic profiles of the current strains intact while further improving their safety. The continued study of the LAVs presented here will contribute to the next generation of *M. tuberculosis* live attenuated vaccines in the hope of replacing the current BCG vaccine with one providing high levels of lifelong protection against all forms of TB.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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- The currently used BCG vaccine does not protect against pulmonary TB.
- Genes induced during mouse infection were targeted yielding LAVs *mosR* and *echA7*.
- Both LAV strains provide protection against an *M. tb* Beijing strain challenge.
- Both provided improved protection over BCG in terms of bacterial load and pathology.
- Both gave an *M. tb* specific immune response and persisted within the host.

Figure 1. T-cell response to *M. tuberculosis* specific stimulation post-vaccination

Lymphocytes were isolated from **A)** spleen or **B)** lung of C57BL/6 mice vaccinated with BCG (diagonal striped), *mosR* (white), or *echA7* (vertical striped) 8 weeks prior. Cells were stimulated with *M. tuberculosis* whole cell lysate or media alone for standardization. Intracellular cytokine staining was used to assess the populations of CD4⁺ and CD8⁺ T-cells positive for IFN- γ and CD4⁺ T-cells positive for IL-17a. Error bars show the standard error of the mean. * $P < 0.05$.

Figure 2. Bacterial load of vaccinated mice after *M. tuberculosis* Beijing challenge. 8 weeks post-vaccination, C57BL/6 mice vaccinated with *mosR* (squares), *echA7* (triangles), BCG (circles), or PBS control (diamonds) mice were challenged with *M. tuberculosis* Beijing. At days 30 and 60 post-challenge, tissues were collected for enumeration the bacterial load of the challenge strain in the **A)** lungs **B)** spleen and **C)** liver. Shown are total counts for each individual. Solid lines indicate mean CFU counts and dashed lines show limits of detection. * $P < 0.05$; ** $P < 0.01$.

Figure 3. Lung histopathology of vaccinated mice post-challenge

Lungs were taken for histopathological analysis from C57BL/6 mice vaccinated with *mosR*, *echA7*, BCG, or PBS control 30 and 60 days post-challenge. 40× magnification of H&E stained sections of lung from mice vaccinated with PBS, BCG, *mosR*, or *echA7*, from top left to bottom right, **A)** 30 days or **B)** 60 days post-challenge. The mean and standard error of the mean percent inflammation for each group are shown in the bottom left corner of each panel. Scale bar is equivalent to 500μm.

Figure 4. IFN- γ response to *M. tuberculosis* specific stimulation post-challenge

Lymphocytes were isolated from **A)** lung or **B)** spleen of C57BL/6 mice vaccinated with BCG (diagonal striped), *mosR* (white), or *echA7* (vertical striped) or PBS control (black) 30 or 60 days post-challenge with *M. tuberculosis* Beijing. Cells were stimulated with *M. tuberculosis* whole cell lysate or media alone for standardization. ELISA was used to detect IFN- γ levels produced by the lymphocytes in the supernatant collected 24hrs after stimulation. Error bars show the standard error of the mean. * $P < 0.05$.

Table 1

Tissue colonization and persistence of LAV candidates

Collection time	Vaccine	Lung		Spleen		Liver	
		Animals colonized	Avg (CFU/lung $\pm$ SEM)	Animals colonized	Avg (CFU/g $\pm$ SEM)	Animals colonized	Avg (CFU/g $\pm$ SEM)
Pre-challenge;	BCG	0 of 0	< 2.00 $\times$ 10	0 of 0	< 1.00 $\times$ 10 ³	0 of 0	< 2.00 $\times$ 10 ²
8 weeks	<i>masR</i>	0 of 3	< 2.00 $\times$ 10	1 of 3	2.93 $\times$ 10 ³ $\pm$ 1.93 $\times$ 10 ³	0 of 3	< 2.00 $\times$ 10 ²
Post-vaccination	<i>echA7</i>	2 of 3	1.20 $\times$ 10 ² $\pm$ 8.08 $\times$ 10	3 of 3	1.18 $\times$ 10 ⁴ $\pm$ 4.72 $\times$ 10 ³	3 of 3	5.66 $\times$ 10 ² $\pm$ 2.90 $\times$ 10 ²
30 days	<i>masR</i>	2 of 5	6.20 $\times$ 10 ² $\pm$ 6.00 $\times$ 10 ²	3 of 5	9.61 $\times$ 10 ⁴ $\pm$ 6.63 $\times$ 10 ⁴	1 of 5	3.60 $\times$ 10 ² $\pm$ 1.60 $\times$ 10 ²
Post-challenge	<i>echA7</i>	1 of 4	2.0 $\times$ 10 ² $\pm$ 2.30 $\times$ 10 ²	2 of 4	1.13 $\times$ 10 ³ $\pm$ 3.15 $\times$ 10 ²	0 of 3	< 2.00 $\times$ 10 ²
60 days	<i>masR</i>	0 of 5	< 2.00 $\times$ 10	2 of 4	1.95 $\times$ 10 ⁴ $\pm$ 1.82 $\times$ 10 ⁴	0 of 5	< 2.00 $\times$ 10 ²
Post-challenge	<i>echA7</i>	0 of 3	< 2.00 $\times$ 10	2 of 3	3.00 $\times$ 10 ³ $\pm$ 1.53 $\times$ 10 ³	0 of 3	< 2.00 $\times$ 10 ²

Post-challenge samples were plated on 7H10+ADC with hygromycin to differentiate vaccine strains from the challenge strain. Limit of detection for lungs: 20 CFU/lung, spleen: 1000 CFU/g, and liver: 200 CFU/g. SEM, standard error of the mean.