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Protective Vaccine Efficacy of the Complete Form of PPE39 Protein from *Mycobacterium tuberculosis* Beijing/K Strain in Mice

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ABSTRACT The aim of this study was to evaluate the protective efficacy of MTBK_24820, a complete form of PPE39 protein derived from a predominant Beijing/K strain of *Mycobacterium tuberculosis* in South Korea. Mice were immunized with MTKB_24820, *M. bovis* Bacilli Calmette-Guérin (BCG), or adjuvant prior to a high-dosed Beijing/K strain aerosol infection. After 4 and 9 weeks, bacterial loads were determined and histopathologic and immunologic features in the lungs and spleens of the *M. tuberculosis*-infected mice were analyzed. Putative immunogenic T-cell epitopes were examined using synthetic overlapping peptides. Successful immunization of MTBK_24820 in mice was confirmed by increased IgG responses ($P < 0.05$) and recalled gamma interferon (IFN-γ), interleukin-2 (IL-2), IL-6, and IL-17 responses ($P < 0.05$ or $P < 0.01$) to MTBK_24820. After challenge with the Beijing/K strain, an approximately 0.5 to 1.0 $\log_{10}$ reduction in CFU in lungs and fewer lung inflammation lesions were observed in MTBK_24820-immunized mice compared to those for control mice. Moreover, MTBK_24820 immunization elicited significantly higher numbers of CD4$^+$ T cells producing protective cytokines, such as IFN-γ and IL-17, in lungs and spleens ($P < 0.01$) and CD4$^+$ multifunctional T cells producing IFN-γ, tumor necrosis factor alpha (TNF-α), and/or IL-17 ($P < 0.01$) than in control mice, suggesting protection comparable to that of BCG against the hypervirulent Beijing/K strain. The dominant immunogenic T-cell epitopes that induced IFN-γ production were at the N terminus (amino acids 85 to 102 and 217 to 234). Its vaccine potential, along with protective immune responses *in vivo*, may be informative for vaccine development, particularly in regions where the *M. tuberculosis* Beijing/K-strain is frequently isolated from TB patients.

KEYWORDS *Mycobacterium tuberculosis*, TB, Beijing/K strain, PPE family, MTBK_24820, vaccine, IFN-γ, IL-17

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Tuberculosis (TB) is caused by infection with *Mycobacterium tuberculosis*. TB is a major global health problem, with approximately 10.4 million new cases and 1.4 million deaths worldwide in 2015 (1). Approximately 58% of new cases were reported in Southeast Asia and Western Pacific regions, including China and South Korea (1). The Beijing strain is prevalent in China and surrounding countries, including South Korea (2). The Beijing strain also has been associated with TB outbreaks in North America (3) and multidrug resistance in New York City in the United States (4, 5).

Bacilli Calmette-Guérin (BCG), the most widely used vaccine against TB, has variable protective efficacy, from 0 to 80% (6, 7), and shows low levels of protection against the modern Beijing lineage strain (8). The Beijing strain induces extensive pneumonia in mice and results in earlier mortality compared to *M. tuberculosis* H37Rv infection in spite of prior BCG vaccination (8). In a human study, the Beijing genotype occurs more frequently in Vietnamese patients with a BCG scar than in those without it (9).

Therefore, the vaccine candidates should be able to protect from prevalent and hypervirulent *M. tuberculosis* strains in preclinical testing.

In South Korea, TB remains a major public health concern, with an incidence of 80 cases per 100,000 people (10). More than 80% of clinical isolates from Korean pulmonary TB patients belong to the Beijing genotype (11, 12, 13). Beijing/K strains were identified as the major cause of pulmonary TB outbreaks (14) and were associated with drug resistance (13) in South Korea. The Beijing/K strain has rapid replication with more severe pathologies at early times after infection compared to *M. tuberculosis* H37Rv in mice (15, 16).

From a whole-genome sequence analysis of the Beijing/K strain, we identified MTBK_24820 (GenBank accession no. AIB49026.1) within the 5.7-kb insertion region compared with the genome of H37Rv strain (17). In the inserted region, ESAT-6 like (*esx*) proteins, such as InsB (MTBK_24790) and InsC (MTBK_24800) (18, 19), and PPE proteins, including InsD (MTBK_24810; partial form of PPE38) and MTBK_24820 (N-terminally added PPE39 protein), are inserted in a row. Unlike other PE-PPE proteins located in a row in the ESX region (20), MTBK_24820 exists independently, without PE proteins in the insertion region. MTBK_24820 is a PPE-MTPR subfamily with repeats of NxGxGNxG in the C terminus (17, 21) and is orthologous to the *M. tuberculosis* H37Rv PPE39 protein (annotated Rv2353c) (22). PPE39 has a number of highly genetic variables among several *M. tuberculosis* isolates, caused by IS*6110* integration and the addition of single-nucleotide polymorphisms (SNPs) (23). PPE39 of H37Rv strain is truncated at the N terminus, whereas MTBK_24820 of Beijing/K strain includes 259 additional amino acids at the N terminus, which is defined as a complete form of PPE39 in this study (see Fig. S1 in the supplemental material). There is also a PPE39 homologue in *M. bovis* BCG Pasteur 1173P2 strain with genetic variation, including SNPs in the N-terminal region, and about 150 amino acids in the C terminus are fused with part of PPE40 (23).

Our previous microarray experiments showed an approximately 8.1-fold overexpression of MTBK_24820 in the Beijing/K strain compared with PPE39 in the H37Rv strain. Sequence analysis of MTBK_24820 showed six transmembrane helices with no signal peptide and the N terminus oriented towards the inside of the cell (TMpred software [http://embnet.vital-it.ch/software/TMPRED_form.html] and SignalP 4.1 server [http://www.cbs.dtu.dk/services/SignalP/]). However, the function of PPE39 has not yet been proved.

Some PE/PPE proteins play a role in mycobacterial pathogenesis linked to bacterial growth in host macrophages or macrophage maturation processes. For example, PE_PGRS33 and PPE38 inhibited phagocytosis of *M. tuberculosis* (24, 25), and deletion of PPE25 in *M. avium* induces inhibition of phagolysosomal fusion (26). PE4-expressing *M. smegmatis* showed improved survival in murine macrophages (27). *ppe18* knockout *M. tuberculosis*-infected mice decreased bacterial burden and showed less tissue damage, suggesting that PPE18 plays a role in survival of *M. tuberculosis* (28). In addition, PE/PPE family proteins have highly immunogenic T-cell epitopes that induce secretion of gamma interferon (IFN-γ) (29, 30). A multiepitope DNA vaccine, including peptides derived from PE19 and PPE25, induces potent IFN-γ responses (31).

Based on sequence analyses and the overexpression of MTBK_24820 in the Beijing/K strain, we hypothesized that the complete form of PPE39 has protective efficacy against *M. tuberculosis* infection, particularly in mice infected with the hypervirulent clinical isolate Beijing/K. We assessed the performance of immunization with MTBK_24820 compared to that with BCG following challenge with the Beijing/K strain in mice. The bacterial loads, histopathology, and cytokine signatures in lungs and spleens of the mice were examined at 4 and 9 weeks postinfection. In addition, the immunogenic T-cell epitopes of MTBK_24820 necessary to elicit IFN-γ production were determined.

RESULTS

MTBK_24820-induced immune responses in mice. Successful immunization of mice with MTBK_24820 was confirmed by MTBK_24820-specific IgG responses. Mice
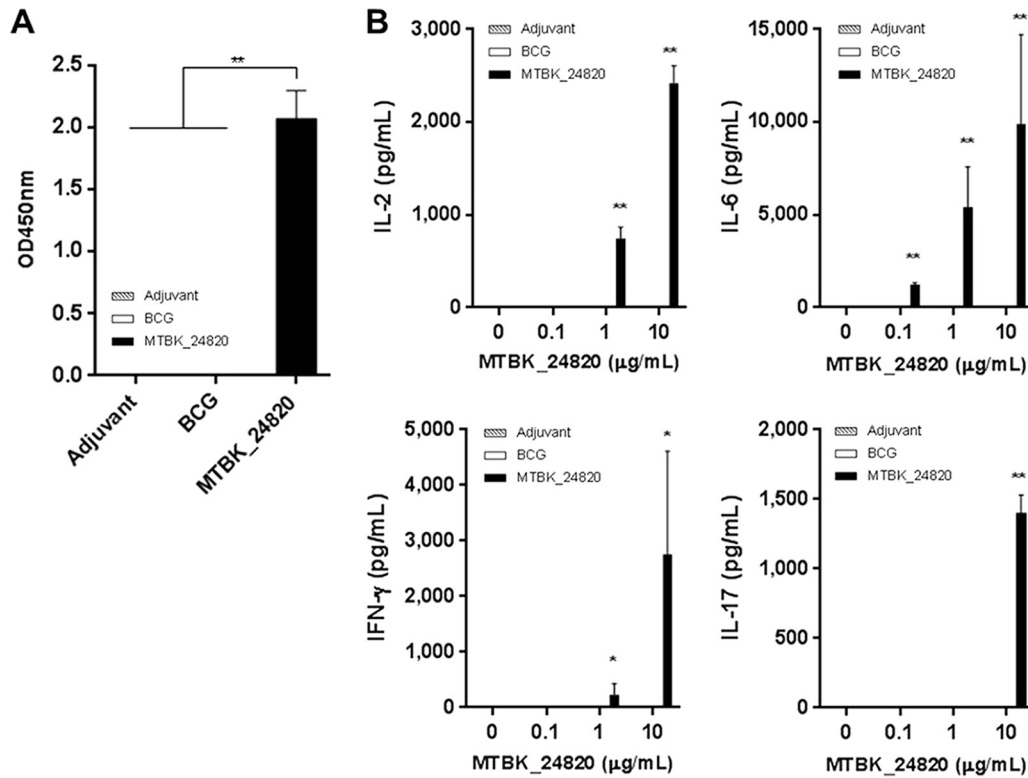
FIG 1 MTBK_24820-specific immune responses in C56BL/6 mice immunized with adjuvant, *M. bovis* BCG, or MTBK_24820. (A) Three weeks after the last immunization with adjuvant alone, BCG, or MTBK_24820, the IgG antibodies specific to MTBK_24820 were measured in sera. OD, optical density; adjuvant, DDA and MPL. (B) MTBK_24820-specific recall responses induced by immunization were measured in spleen cell culture supernatants after stimulation with 0, 0.1, 1, or 10 μg/ml of MTBK_24820 prior to *M. tuberculosis* Beijing/K strain aerosol infection. Concentrations of IL-2, IL-6, IFN-γ, and IL-17 were determined using multiplex bead assays. Data are presented as means ± SD for duplicate determinations from three mice. Significant differences between groups were analyzed by one-way ANOVA (*, $P < 0.05$; **, $P < 0.01$).

immunized with MTBK_24820 had significantly higher MTBK_24820-specific IgG responses than mice immunized with adjuvant or BCG ($P < 0.01$) (Fig. 1A).

To determine the immunogenicity of MTBK_24820, expression of 10 cytokines, including Th1 and Th2 cytokines, was examined in the lungs and spleens of mice immunized with MTBK_24820. Among the cytokines tested, IFN-γ, interleukin-2 (IL-2), IL-6, and IL-17 production was significantly increased in a dose-dependent manner in mice immunized with MTBK_24820 ($P < 0.05$ for IFN-γ and $P < 0.01$ for IL-2, IL-6, and IL-17) (Fig. 1B). Despite the presence of PPE39 homologue in *M. bovis* BCG, none of the cytokines were detected in the mice immunized with BCG. Th2 cytokines, such as IL-4 and IL-5, were not detected in any groups (data not shown).

MTBK_24820-induced protective efficacy against TB. To evaluate the protective properties of MTBK_24820, the immunized mice were challenged with the Beijing/K strain of *M. tuberculosis* and bacterial counts were assessed in the lungs and spleens. The MTBK_24820-immunized group showed an approximately 0.5-log reduction in CFU in the lungs at 4 weeks postinfection ($P < 0.05$), and it was nearly the same level of protection as that seen in the BCG-immunized group ($P < 0.01$) (Fig. 2A). At 9 weeks postinfection, the CFU reduction in MTBK_24820-immunized mice was still significant compared with the level for the control group ($P < 0.01$) (Fig. 2A), although the CFU reduction had fallen to 0.2 log. In spleens, MTBK_24820-immunized mice showed superior protection over the control group ($P < 0.05$), whereas BCG did not significantly lower the bacterial loads at 9 weeks postinfection (Fig. 2B).

The protective efficacy of MTBK_24820 against TB infection was also examined by histopathology. At 4 weeks postinfection, the calculated percentage of inflammation lesions was higher in control mice immunized with adjuvant (19.4 to 27.5%) than in
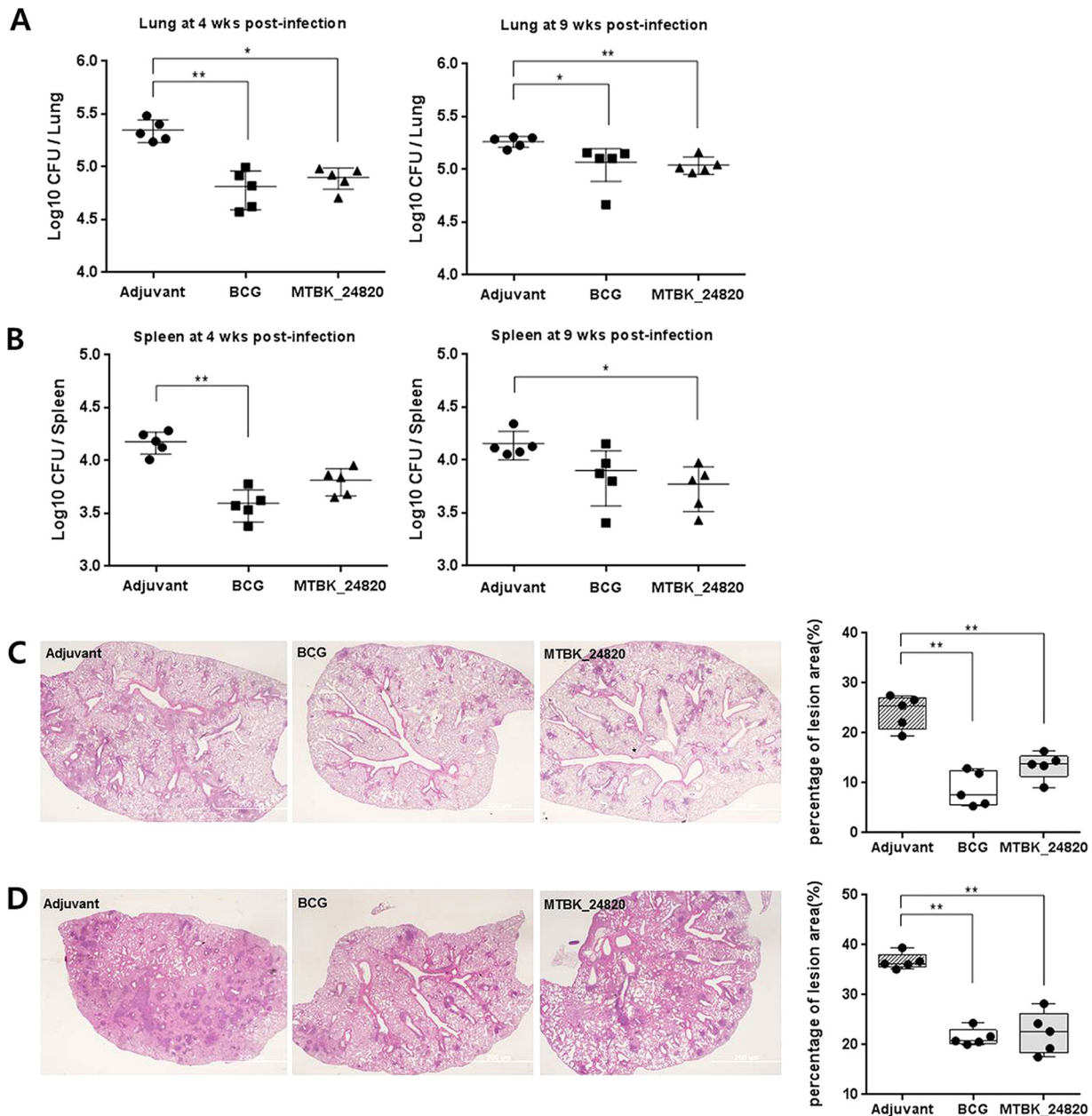
FIG 2 Protective efficacy of immunization with MTBK_24820 in mice against the *M. tuberculosis* Beijing/K strain. Three weeks after the final immunization, mice were challenged with 1,000 CFU of virulent Beijing/K strain. At 4 and 9 weeks postinfection, all mice were sacrificed and bacterial burden (CFU) was measured from homogenized lungs (A) and spleens (B). Dots represent the mean $\log_{10}$ values obtained for individually tested animals, and lines represent the means ± SD from five mice. For histopathologic analysis of the lungs from immunized mice, mice were sacrificed at 4 (C) and 9 (D) weeks postinfection. The lung sections were stained with H&E (×20 magnification; bar, 200 μm). The percentage of inflammation lesions was calculated using ×10 magnification fields and ImageJ software. Significant differences between multiple groups were confirmed by one-way ANOVA (*, $P < 0.05$; **, $P < 0.01$).

mice immunized with BCG (5.4 to 12.9%) ($P < 0.01$) or MTBK_24820 (9.1 to 16.4%) ($P < 0.01$) (Fig. 2C). At 9 weeks postchallenge, the inflammation of the lesions was more severe than at 4 weeks, but the BCG-immunized mice (20.0 to 24.3%) ($P < 0.01$) and MTBK_24820-immunized mice (17.5 to 28.2%) ($P < 0.01$) still showed fewer inflammation lesions than the control mice (35.1 to 39.4%) (Fig. 2D). There was no significant difference in inflammation lesion areas between BCG- and MTBK_24820-immunized mice (Fig. 2D). These results indicate that MTBK_24820 had protective efficacy against the virulent Beijing/K strain.
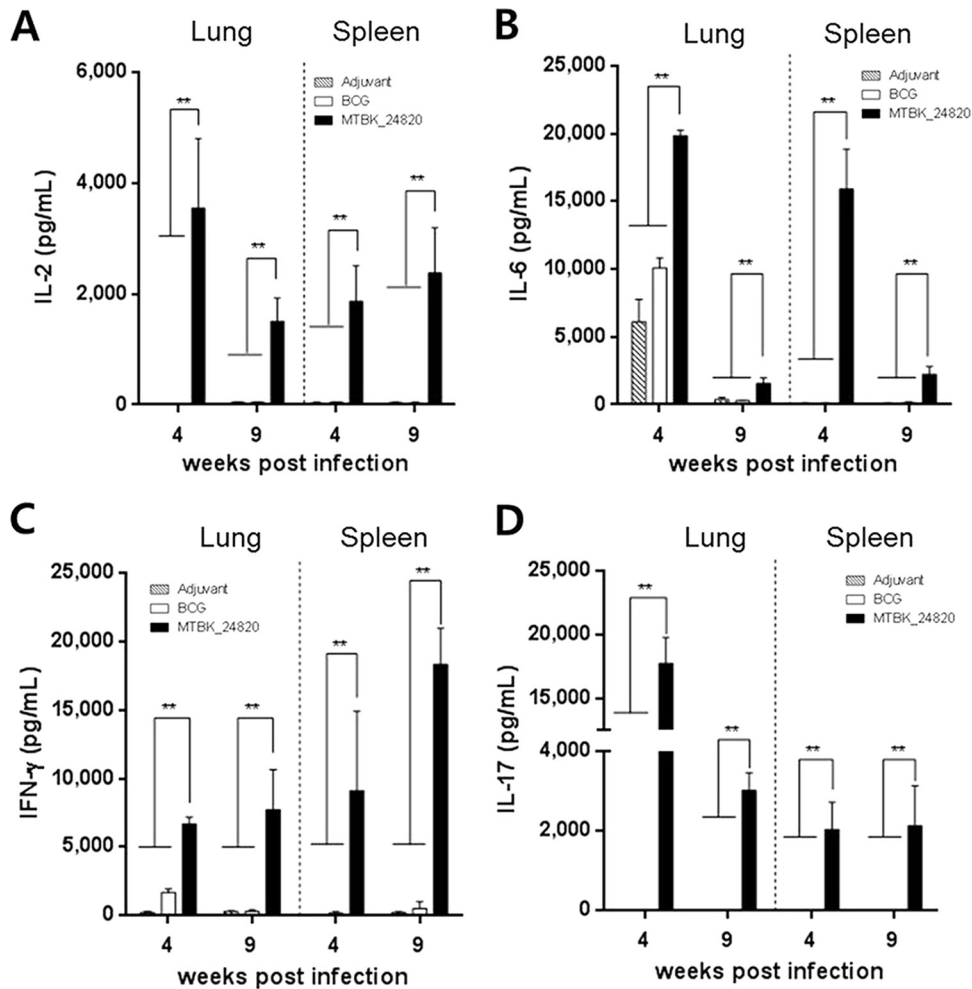
FIG 3 Cytokine production in the lungs and spleens from *M. tuberculosis* Beijing/K-infected mice following *ex vivo* stimulation with MTBK_24820. Concentrations of protection-related cytokines to MTBK_24820 (5 μg/ml) were determined *ex vivo* at 4 and 9 weeks postinfection. Concentrations of IL-2 (A), IL-6 (B), IFN-γ (C), and IL-17 (D) in cell culture supernatants were measured. Data are presented as means ± SD from five mice. Significant differences between multiple groups were analyzed by unpaired *t* tests (**, $P < 0.01$).

MTBK_24820-induced cellular immune responses in mice infected with the Beijing/K strain of *M. tuberculosis*. To assess whether the Beijing/K strain of *M. tuberculosis* could recall immune responses that were induced by previous immunization with MTBK_24820, cytokine responses were measured in the lungs and spleens. The production of IL-2, IL-6, IFN-γ, and IL-17 in response to MTBK_24820 immunization was maintained after the infection with Beijing/K strain (Fig. 3). MTBK_24820-immunized mice showed significantly higher concentrations of IL-2, IL-6, IFN-γ, and IL-17 production in both the lungs and spleens at 4 and 9 weeks postinfection compared to those of the adjuvant group ($P < 0.01$ in all cases) (Fig. 3). IFN-γ production in MTBK_24820-immunized mice was elevated even more at 9 weeks postinfection than at 4 weeks postinfection ($P < 0.01$) (Fig. 3C). On the contrary, the IL-2, IL-6, and IL-17 responses of the MTBK_24820-immunized mice were decreased at 9 weeks postinfection in the lungs ($P < 0.01$) (Fig. 3A, B, and D).

We next examined T cells producing IFN-γ and IL-17 in response to MTBK_24820. Mice immunized with MTBK_24820 had a higher proportion of CD4^{+} T cells producing IFN-γ than did the mice with adjuvant alone ($P < 0.01$) (Fig. 4A and B). Similarly, the percentage of CD4^{+} T cells producing IL-17 in MTBK_24820-immunized mice was higher than that in the adjuvant group ($P < 0.01$) (Fig. 4C and D). The proportion of CD8^{+} T cells producing IFN-γ or IL-17 in response to MTBK_24820 was also significantly
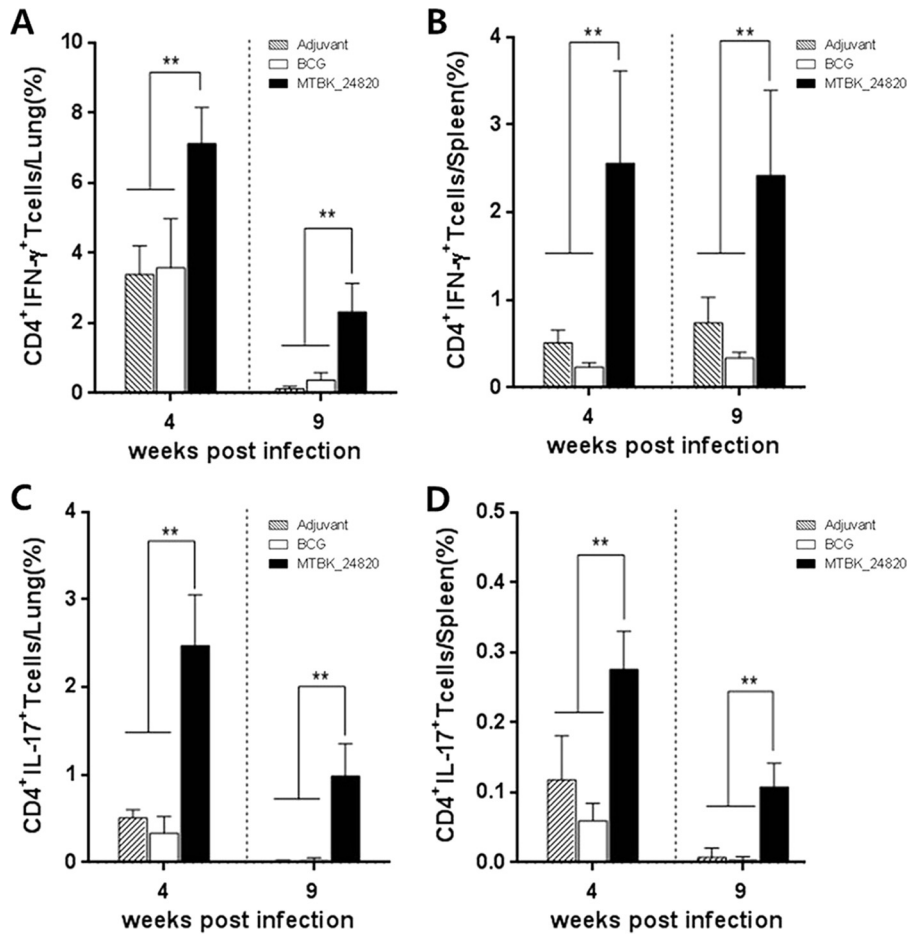
FIG 4 CD4$^+$ T cells producing protection-related cytokines in the lungs and spleens of *M. tuberculosis* Beijing/K-infected mice in response to MTBK_24820. At 4 and 9 weeks postinfection, cells from the lungs and spleens were stimulated with MTBK_24820 (5 μg/ml) for 24 h, and the percentage of IFN-γ-positive CD4$^+$ T cells in the lungs (A) and spleens (B) and IL-17-positive CD4$^+$ T cells in the lungs (C) and spleens (D) were determined. Data are presented as means $\pm$ SD from two independent experiments with five mice. Significant differences between multiple groups were analyzed by unpaired *t* tests (**, $P < 0.01$).

higher in MTBK_24820-immunized mice than in the adjuvant group ($P < 0.01$) (data not shown). These results suggest that MTBK_24820 generates MTBK_24820-specific Th1 and Th17 cytokine responses that could be maintained during delayed infections, which may play a role in protection against TB.

MTBK_24820 immunization induced antigen-specific multifunctional T cells in Beijing/K-infected mice. Based on reports showing the association between multifunctional T cells and protection against TB in mice (32, 33), the existence of multifunctional CD4$^+$ T cells was examined in mice immunized with MTBK_24820. In response to MTBK_24820 immunization, CD4$^+$ T cells producing IFN-γ, TNF-α, and IL-17 were observed in lung and spleen cells from MTBK_24820-immunized mice (Fig. 5). Double-positive CD4$^+$ T cells producing IFN-γ and TNF-α, IFN-γ and IL-17, or TNF-α and IL-17 were identified at 4 and 9 weeks postinfection (Fig. 5). MTBK_24820 immunization also induced triple-positive CD4$^+$ T cells, although the proportion of triple-positive CD4$^+$ T cells producing IFN-γ, TNF-α, and IL-17 was lower than that of double-positive CD4$^+$ T cells ($P < 0.01$ for Fig. 5A, B, and C and $P < 0.05$ for Fig. 5D). BCG-immunized mice did not produce IL-17 but were limited to IFN-γ and TNF-α production in the lungs (Fig. 5A and B). IFN-γ-producing CD8$^+$ T cells were observed in all groups, whereas none of the mice produced multifunctional CD8$^+$ T cells (data not shown). These results indicate that MTBK_24820 induces multifunctional T-cell responses to the Beijing/K strain of *M. tuberculosis*, and these responses may be involved in protection against TB.
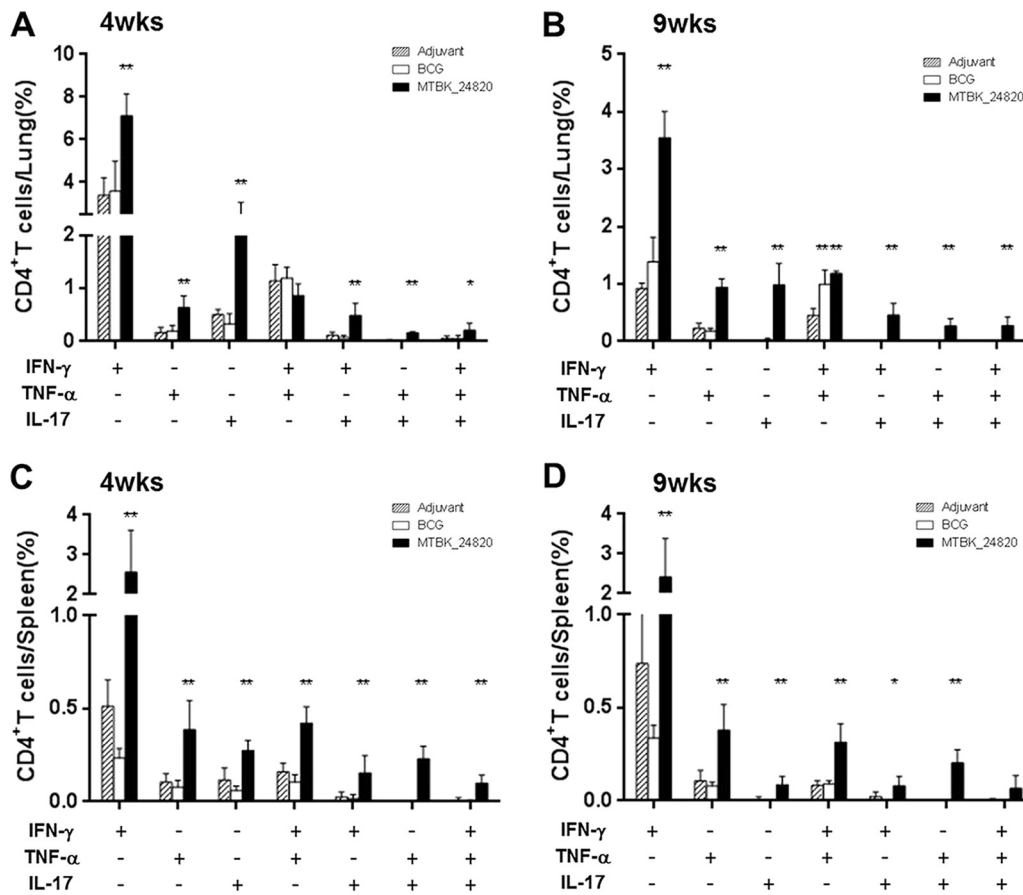
FIG 5 Functional profiles of MTBK_24820-specific CD4+ T cells based on IFN-γ, TNF-α, and IL-17 production. Multifunctional CD4+ T cells in the lungs and spleens from each group of immunized mice were analyzed by flow cytometry. At 4 and 9 weeks postinfection, cells were stimulated with MTBK_24820 (5 μg/ml) for 12 h in the presence of GolgiPlug. The percentage of IFN-γ-, TNF-α-, and/or IL-17-producing CD4+ T cells in the lungs (A and B) and spleens (C and D) are presented as means $\pm$ SD from two independent experiments with five mice. Significant differences compared to the adjuvant control group were analyzed by unpaired t tests (*, $P < 0.05$; **, $P < 0.01$).

The dominant epitope of MTBK_24820 in T cells of Beijing/K-infected mice. To determine potential epitopes of MTBK_24820, the IFN-γ responses to synthetic peptides that overlapped within the N terminus of MTBK_24820 were examined in spleen cells from the Beijing/K strain-challenged mice at 4 and 9 weeks postinfection. The dominant epitopes were at amino acids 85 to 102 (AFEAARAAMVDPVVVAAN) at the early stage of infection and amino acids 217 to 234 (GSGNIGNTNLGGGNIGSF), with increased IFN-γ, at 9 weeks postinfection (Fig. 6). However, IFN-γ secretion in response to the peptides was not observed in naive C57BL/6- and *M. tuberculosis* H37Rv-infected mice (data not shown). These data indicate that the N terminus of MTBK_24820 has potent T-cell epitopes that contribute to protection against TB specifically caused by the Beijing/K strain.

DISCUSSION

Evidence so far indicates that MTBK_24820 has protective efficacy and induces strong Th1 and IL-17 responses against *M. tuberculosis* Beijing/K infection in mice immunized with MTBK_24820. Several reports have suggested that IFN-γ and IL-17 production by CD4+ T cells is targeted in vaccine-induced immunity to *M. tuberculosis* infection in mouse models (34, 35). The immunogenic IFN-γ-generating T-cell epitopes in PE/PPE family proteins are potential TB vaccines (30) and diagnostic biomarkers (36). In this study, two predominant epitope sites were demonstrated by strong IFN-γ-inducing T cells at amino acids 85 to 102 and 217 to 234.
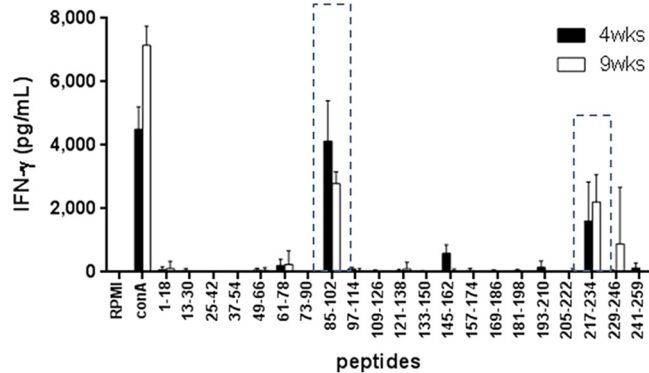
FIG 6 IFN-γ responses induced by MTBK_24820 overlapping peptides in *M. tuberculosis* Beijing/K-infected mice. At 4 and 9 weeks postinfection, splenocytes were stimulated with control medium (RPMI), ConA (1 µg/ml), or 10 µg/ml of the synthetic peptides covering the N terminus of MTBK_24820 for 48 h for measurement of the IFN-γ responses in cell culture supernatants. Peptides spanning the amino acids of MTBK_24820 at the N terminus are shown on the *x* axis, and dotted boxes represent peptides that induced IFN-γ responses. Data are presented as means ± SD from two independent experiments with four mice.

An additional 3 potential epitope sites with weak IFN-γ responses were also observed at the C terminus of MTBK_24820 (data not shown) that were absent from *M. bovis* BCG. Therefore, the insufficient immune responses to MTBK_24820 in mice immunized with BCG likely are due to the genetic variation at the C terminus between the two strains.

IL-17 is a proinflammatory cytokine that induces neutrophil recruitment, which may result in protection against TB (37). Mice with genetically inactivated IL-17A receptor failed to control bacterial burden, resulting in accelerated mortality in long-term infections even though the bacterial burden was controlled during the acute phase of *M. tuberculosis* infection (38). Moreover, the IL-17 requirement for host protection against TB depends on the *M. tuberculosis* strain in mice. IL-17 contributes to early protection by inducing CXCL-13, which is required for T-cell localization within lung lymphoid follicles in response to hypervirulent *M. tuberculosis* HN878 infection, while IL-17 does not impact protective immunity against laboratory-adapted *M. tuberculosis* H37Rv infection (39). Our data demonstrated that high levels of IL-17 production were induced in the lungs of MTBK_24820-immunized mice at early phases of infection and may function in the control of bacterial burden during the course of *M. tuberculosis* Beijing/K infection.

There are now TB vaccine candidates containing PPE genes in clinical trial phase II trials. The ID93 vaccine candidate, sponsored by IDRI, has PPE42 (40). This vaccine has decreased bacterial loads and elicited CD4+ and CD8+ multifunctional T-cell responses in mice infected with multidrug-resistant TN5904 as well as H37Rv (41). Mtb72F, developed by GSK, contains PPE18, which was produced from the H37Rv strain (40, 42). Several genetic variations, including SNPs in PPE18, have been observed in clinical isolates and result in alteration of amino acid sequences even in T-cell epitopes (43). This means that Mtb72F may have limitations as a vaccine to recognize some *M. tuberculosis* strains attributable to antigenic variation. A vaccine composed of PPE genes, conversely, may be valuable due to the cross-reactivity with many PPE homologues throughout the *M. tuberculosis* genome (44). In contrast to the genetic variations of the PPE genes, according to BLAST analyses, better than 99% homology for MTBK_24820 was found in other clinical isolates, namely, the *M. tuberculosis* Haarlem family, which caused a higher frequency of multidrug-resistant TB (45), and CDC1551, which was highly transmissible and virulent in humans (46). This suggests that MTBK_24820 is a vaccine candidate that can protect against other *M. tuberculosis* strains as well as Beijing/K.

Although the protective efficacy of MTBK_24820, determined by CFU counting and

histological pathology, was not significantly different from that of BCG in mice, the efficacy of MTBK_24820 seems to be longer lasting than that of BCG based on the CFU counts. Meanwhile, the protective immune responses were significantly higher in mice immunized with MTBK_24820 than in those immunized with BCG, which suggests that BCG has methods other than the pathways releasing the cytokines in response to MTBK_24820 for protection against *M. tuberculosis* Beijing/K infection. We also determined bacterial loads and histopathological features in mice challenged with the Erdman strain. We observed an approximately 0.5 to 1.0 $\log_{10}$ reduction of CFU in lungs and fewer lung inflammation lesions in MTBK_24820-immunized mice at 4 and 10 weeks postinfection compared with the control mice (adjuvant only or BCG vaccinated). Compared with mice with the Beijing/K strain, Erdman-challenged mice showed higher reduction of bacterial loads by MTBK_24820 immunization, and the protective efficacy of MTBK_24820 was better than that of BCG at 10 weeks postinfection (see Fig. S2 in the supplemental material). Considering the high prevalence of the Beijing/K strain of *M. tuberculosis* and the relatively poor protection of BCG against the W-Beijing genotype of *M. tuberculosis* (8, 47), it would be worthwhile to test MTBK_24820 derived from the strain as a vaccine candidate, particularly in an area where TB is endemic. The dominant epitope sites of the T cells observed in MTBK_24820 also exist in *M. bovis* BCG, but the subdominant epitope sites were not found in *M. bovis* BCG. Therefore, it is necessary to evaluate the protective efficacy of MTBK_24820 using the potential epitopes in mice infected with the Beijing/K strain. These further studies may give a clue to the scarce immune responses observed in mice immunized with BCG in this study. The persistence of protective efficacy and the response of multifunctional T cells in mice immunized with MTBK_24820 also should be confirmed at least 9 weeks postinfection or later.

Taken together, MTBK_24820, a complete form of PPE39, showed protective efficacy against infection with the Beijing/K strain. The protective efficacy and strong immune responses of MTBK_24820 may give helpful information for development of new vaccines in areas where TB is endemic. MTBK_24820 could be used as an antigen for improved future vaccines against the highly transmissible and virulent *M. tuberculosis* strains, including Beijing/K.

MATERIALS AND METHODS

Animals. All mouse experiments were in accordance with guidelines and used animal protocols (permit number 2013-0089) approved by the Institutional Animal Care and Use Committee, Yonsei University College of Medicine (Seoul, South Korea). C57BL/6N (female, 5 to 6 weeks of age) mice were purchased from SLC, Inc. (Shijuoka, Japan), and maintained in the animal biosafety level 3 facility at the Yonsei University College of Medicine.

Preparation and purification of recombinant MTBK_24820 antigen. *MTBK_24820* from the Beijing/K strain was cloned into pYUB1062, which contains six histidine tags at the C terminus, with NdeI and HindIII (New England BioLabs, Ipswich, MA, USA) digestion (48). The MTBK_24820 gene was amplified using the following primers from *M. tuberculosis* Beijing/K strain genomic DNA: MTBK_24820F, 5′-TACATATGGTGGTGAATTTTTCGGTGTTG-3′, including the underlined NdeI site, and MTBK_24820R, 5′-CCAAAGCTTTCCGAACAAGTTCTTGAAGA-3′, including the underlined HindIII site. The constructed plasmid was transformed into *Escherichia coli* BL21(DE3), and the strain containing MTBK_24820 was cultured in LB medium containing 150 μg/ml hygromycin (A.G. Scientific, Inc., San Diego, CA, USA) at 37°C until the optical density at 600 nm (OD_{600}) reached 0.6 to 0.7. Overexpression of MTBK_24820 was carried out by addition of 1 mM IPTG (isopropyl-β-D-thiogalactopyranoside; Bio-World, Dublin, OH, USA) and purified using nickel-nitrilotriacetic acid (Ni-NTA) agarose resin (Qiagen, Venlo, Netherlands). Further purification was conducted using MonoQ anion exchange columns on an ÄKTA fast protein liquid chromatography system (GE Healthcare Biosciences, Pittsburgh, PA, USA) (see Fig. S3A in the supplemental material). The final purified product was confirmed by SDS-PAGE analysis (Fig. S3A). Bicinchoninic acid (BCA) assays (Thermo Fisher Scientific, Inc., Rockford, IL, USA) were used to measure protein concentrations. Samples were sterilized by gamma radiation and stored at −80°C until use.

Mycobacterial strains. The *M. bovis* BCG Pasteur 1173P2 strain was kindly provided by the Pasteur Institute (Paris, France). The *M. tuberculosis* Beijing/K strain was obtained from the Korean Institute of Tuberculosis (KIT; Osong, Chungchungbukdo, South Korea). All strains were grown in Middlebrook 7H9 broth (Difco Laboratories, Detroit, MI, USA) supplemented with 10% oleic acid-albumin-dextrose-catalase (OADC; Becton Dickinson, Sparks, MD, USA) and 0.02% glycerol for 4 weeks at 37°C. Single-cell suspensions of each strain were prepared as previously described (16). The concentrations of each lot of both strains were determined by plating serial dilutions on Middlebrook 7H11 agar (Difco Laboratories) supplemented with OADC (Becton Dickinson). Aliquots of each strain were stored at −80°C until use.

Immunization and infection. Mice were immunized by subcutaneous injection with 20 μg of MTBK_24820 protein. The protein was emulsified in dimethyl dioctadecyl ammonium bromide (DDA; 250 μg/dose; Sigma-Aldrich, St. Louis, MO, USA) and monophospholipid A (MPL; 25 μg/dose; Sigma-Aldrich). Injections were given three times at 3-week intervals. Phosphate-buffered saline (PBS) emulsified with DDA and MPL was used for the sham-immunized group (49). BCG (2×10^5 CFU/dose), as a control vaccine, was subcutaneously injected into mice once 6 weeks before the Beijing/K infection. Three weeks after the final immunization, sera and spleens from three mice of each group were obtained for MTBK_24820-induced immune response analysis.

Mice were challenged with approximately 1,000 CFU of the Beijing/K strain using an aerosol apparatus (Glas-Col, Terre Haute, IN, USA) 3 weeks after the final immunization. The initial dose was confirmed the following day. The protective efficacy was evaluated using CFU counts at 4 and 9 weeks postinfection.

Determination of bacterial load and histopathologic analysis. To estimate the numbers of viable bacteria in the lungs and spleens of infected mice, tissues were removed aseptically at designated times and homogenized in 2 ml of PBS. Tenfold serial dilutions of each homogenate were plated onto Middlebrook 7H11 agar plates supplemented with OADC containing amphotericin B (Sigma-Aldrich). Plates were incubated for 28 days at 37°C and then bacterial colonies were counted.

For histopathologic analysis of the lungs, the right posterior lobes were collected and fixed in 10% formaldehyde buffer. Samples were cut into 5-μm-thick slices and stained with hematoxylin and eosin (H&E) for microscopic analysis (Olympus, Tokyo, Japan). Lung inflammation lesions relative to the area of the total visual field were evaluated by ImageJ software (National Institutes of Health, Bethesda, MD, USA). Results are represented as the percentage of area with lesions.

Preparation of lung and spleen cells. The lungs and spleens were removed from immunized and/or infected mice. The lung tissue was chopped and incubated in RPMI medium (Welgene, Daejeon, South Korea) containing collagenase type II (Worthington Biochemical Co., Lakewood, NJ, USA) for 30 min at 37°C and passed through a 40-μm cell strainer (BD Biosciences, San Jose, CA, USA). Spleen cells were isolated by passing through a mesh strainer. Red blood cells were lysed using ACK lysis buffer (0.15 M NH$_4$Cl, 1 mM KHCO$_3$, and 0.1 mM Na$_2$EDTA). Cells were washed and resuspended in RPMI medium containing 10% fetal bovine serum and 1 U/ml antibiotic-antimycotic (Invitrogen, Grand Island, NY, USA). Cells were stimulated with 0.1, 1, or 5 μg/ml of MTBK_24820 for 24 h at 37°C for determination of cytokine responses. For the intracellular cytokine staining, cells were stimulated with 5 μg/ml of MTBK_24820 for 24 h at 37°C.

Intracellular cytokine staining. The isolated lung or spleen cells were stimulated with 5 μg/ml of MTBK_24820 for 12 h at 37°C in the presence of GolgiPlug (BD Biosciences). Cells were stained with peridinin chlorophyll protein (PerCP)-Cy5.5-conjugated anti-CD4, allophycocyanin (APC)-Cy7-conjugated anti-CD8, and fluorescein isothiocyanate (FITC)-conjugated anti-CD44 antibodies (eBioscience, Vienna, Austria) for 30 min at 4°C. Cells were fixed and permeabilized using a Cytofix/Cytoperm kit (BD Biosciences) and stained with phycoerythrin (PE)-conjugated anti-IFN-γ, APC-conjugated anti-TNF-α, and PE-Cy7-conjugated anti-IL-17 (eBioscience). All analyses were performed using a FACSVerse flow cytometer (BD Biosciences). Acquired data were analyzed using FlowJo 10.0 software (FlowJo, LLC, Ashland, OR, USA). The gating strategy for multifunctional T-cell populations is represented in Fig. S4.

Quantification of IgG antibodies specific to MTBK_24820. Blood samples were collected from the mice 3 weeks after the final immunization. Sera were separated after clotting of whole blood at room temperature, followed by centrifugation at 1,500 $\times$ g for 15 min and storage at -20°C until use. To determine the level of the anti-IgG antibodies in response to MTBK_24820, anti-IgG enzyme-linked immunosorbent assays (ELISAs) were performed as previously described (18). Briefly, 5 μg/ml MTBK_24820 was diluted in 0.5 M carbonate-bicarbonate buffer and coated onto 96-well plates (Corning Inc., Oneonta, NY, USA) for 16 h at 4°C. Wells were blocked with PBS containing 5% normal goat serum (NGS). Serum samples were diluted 1:1,000 and added to the wells. After 1 h at 37°C, peroxidase-conjugated anti-mouse IgG antibody (1:10,000 dilution; Merck, Darmstadt, Germany) was added and incubated for 1 h at 37°C. Reactions within the plates were visualized using tetramethylbenzidine substrate (KPL, Gaithersburg, MD, USA) and stopped with 2.5 N H$_2$SO$_4$. Absorbance was read at 450 nm using a VersaMax ELISA reader (Molecular Devices, Sunnyvale, CA, USA).

Multiplex bead array. Concentrations of IL-2, IL-6, IFN-γ, and IL-17 from the lung and spleen cell culture supernatants stimulated with MTBK_24820 were simultaneously measured using FlowCytomix (BMS820FF; eBioscience) according to the manufacturer's protocol. Standard curves for each analyte were obtained by the best fit of the data points using FlowCytomix Pro software (eBioscience), and values outside the standard curve were adjusted by setting the minimum and maximum values.

Design of synthetic MTBK_24820 peptides and IFN-γ ELISAs. A total of 21 peptides which overlap by six amino acids were synthesized for determination of potential epitope sites (GenScript, Piscataway, NJ, USA) (Fig. S3B). The overlapping peptides span 259 amino acids at the N terminus of MTBK_24820. Peptides were diluted in RPMI medium at 1 mg/ml and stored at -20°C until use. Spleen cells from infected mice were stimulated with 10 μg/ml of each peptide or 1 μg/ml of concanavalin A (ConA) (Sigma-Aldrich). After incubation for 24 h at 37°C, cell culture supernatants were harvested and IFN-γ responses were detected using ELISAs (eBioscience) according to the manufacturer's protocol.

Statistical analysis. Data were analyzed using Prism 6.0 software (GraphPad, La Jolla, CA, USA). Mean values and standard deviations (SD) were calculated for each experimental group. Differences among the adjuvant-alone, *M. bovis* BCG, and MTBK_24820 groups were compared using one-way analysis of variance (ANOVA) followed by Dunn's multiple-comparison tests or two-tailed unpaired t tests. P values of <0.05 (*), <0.01 (**), and <0.001 (***) were considered significant.

SUPPLEMENTAL MATERIAL

Supplemental material for this article may be found at https://doi.org/10.1128/CVI .00219-17.

SUPPLEMENTAL FILE 1, PDF file, 0.1 MB.
SUPPLEMENTAL FILE 2, PDF file, 0.1 MB.
SUPPLEMENTAL FILE 3, PDF file, 0.1 MB.
SUPPLEMENTAL FILE 4, PDF file, 0.1 MB.

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