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Protective vaccine efficacy of MTBK_24820, the complete form of PPE39 protein from *Mycobacterium tuberculosis* Beijing/K strain in mice

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Directed by Professor Sang-Nae Cho

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학위과정을 시작하여 논문을 작성할 때까지 여러 우여곡절이 있었지만 무사히 마침표를 찍을 수 있도록 도움을 주신 분들께 감사인사를 드리고자 합니다.

특히, 석사과정을 시작할 때의 20대 초반에 처음 인연을 시작하여, 회사생활을 하던 중 다시 학문의 길을 걷기로 결심했을 때 흔쾌히 손을 내밀어주신 조상래교수님께 감사의 인사를 드립니다. 긴 학위 과정 동안 많은 기회를 주시고 따뜻한 시선으로 끝까지 지도해주셔서 감사합니다. 교수님의 마지막 제자로서 제가 있는 자리에서 항상 최선을 다하겠습니다.

또한 바쁘신 일정 가운데 논문작성을 꼼꼼하게 지도해주시고 심사해주신 이경원교수님, 장준교수님, 최인홍교수님, 신성재교수님께도 진심으로 감사 드립니다.

그리고 미생물학교실의 연구자들에게 훌륭한 본보기가 되어주시고 학생들에게 많은 가르침을 주시는 박전한교수님, 신전수교수님, 김종선교수님, 이재면교수님, 윤상선교수님, 유제욱교수님께도 감사 말씀 드립니다.

대학원 생활의 첫 시작부터 지금까지 하루의 대부분을 같은 공간에서 생활하면서 많은 도움을 주신 같은 연구실의 박사님들, 선생님들께도 감사 드립니다. 특히 연구주제를 선정할 때 도움을 주신 한미영박사님, 폭넓은 시야를 가질 수 있도록 옆에서 조언을 많이 해주시는 허윤경박사님, 10여 년의 세월을 함께 해 온 김영미선생님, 이은희선생님, 강미진선생님, 선화선생님에게 감사 인사 드립니다.

더불어 미생물학교실의 일원으로 생활하면서 소중한 인연을 맺었던 여러 강사조교선생님들, 고시환선생님을 비롯한 모든 학생, 연구원 분들께도 감사 인사 드립니다.

마지막으로 제가 결정하는 모든 일들에 대해 믿음과 사랑의 눈으로 기도해주시는 사랑하는 부모님, 멋진 준순이 가족, 많은 응원을 해주시는 시부모님과 형님내외분께도 감사 드립니다. 앞으로 성숙한 성인으로써 더욱 노력하는 딸, 며느리가 되겠습니다.

무엇보다도 학위 과정 중에 부부의 인연이 되어 저의 모든 일상을 함께 공유하며, 사랑하는 서진이의 아버지로서도 항상 최선을 다해주고 있는 남편에게 더욱 깊은 사랑의 인사를 남기고 싶습니다.

이외에도 제가 아는 모든 분들께 감사드리며
김 아 름 올림

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ABBREVIATION

TB, Tuberculosis

M. tb, Mycobacterium tuberculosis

BCG, Bacilli Calmette-Guérin

DDA, Dimethyl dioctadecyl ammonium bromide

MPL, Monophospholipid A

CFU, Colony forming unit

qRT-PCR, Quantitative real time RT-PCR

IPTG, Isopropyl-β-d-thiogalactopyranoside

BCA, Bicinchoninic acid assays

OADC, Oleic acid-albumin-dextrose-catalase

H&E, Hematoxylin and eosin

NGS, Normal goat serum

TMB, Tetramethylbenzidine

ConA, Concanavalin A

Esx, ESAT-6

OD, Optical density

ABSTRACT

Protective vaccine efficacy of MTBK_24820, the complete form of PPE39 protein from *Mycobacterium tuberculosis* Beijing/K strain in mice

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(Directed by Professor Sang-Nae Cho)

The aim of this study was to evaluate the protective efficacy of MTBK_24820, the complete form of PPE39 protein derived from a predominant Beijing/K strain of *Mycobacterium tuberculosis*, in tuberculosis outbreaks in South Korea. Mice were immunized with adjuvant of dimethyl dioctadecyl ammonium bromide and monophospholipid A, Bacilli Calmette-Guérin, or recombinant MTKB_24820 with adjuvant prior to a high-dose 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. tb*-infected mice were analyzed. Putative immunogenic T-cell epitopes were examined using synthetic overlapping peptides.

Compared with adjuvant-control mice, MTBK_24820-immunized mice showed increased IgG responses against MTBK_24820 ($p<0.05$) and recalled antigen-specific IFN-γ, IL-2, IL-6, and IL-17 responses in spleens ($p<0.01$). After challenge with the Beijing/K strain, an approximately 0.5-1.0 $\log_{10}$ reduction in lungs in colony forming units and fewer lung inflammation lesions were observed in MTBK_24820-immunized mice compared with 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 the lungs and spleens ($p<0.01$) and $CD4^+$ multifunctional T cells producing IFN-γ, TNF-α, and/or IL-17 ($p<0.01$) than in control mice, results in comparable protection to BCG against the hypervirulent Beijing/K strain. The dominant immunogenic T-cell epitopes that induced IFN- γ production was at the N-terminal (amino acids 85-102 and 217-234).

Its vaccine potential along with protective immune responses *in vivo* may be informative for vaccine development, particularly in regions where the *M. tb* Beijing/K-strain is frequently isolated from TB patients.

Key Words: *Mycobacterium tuberculosis*; TB; Beijing/K strain; PPE family; MTBK_24820; vaccine; IFN-γ; IL-17

I. INTRODUCTION

1. Tuberculosis and Beijing strain

Tuberculosis (TB) is caused by infection with *Mycobacterium tuberculosis* (*M. tb*). 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 South-East 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 has also been associated with TB outbreaks in North America[3] and multidrug-resistance in New York City, NY, USA.[4,5]

2. Limitations of BCG

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 Beijing modern lineage strain.[8] The Beijing strain induces extensive pneumonia in mice and results in earlier mortality compared to *M. tb* H37Rv infection in spite of prior BCG vaccination.[8] In a human study, the Beijing genotype occurs more frequent in Vietnamese patients with a BCG scar than in those without it.[9] Therefore, the vaccine

candidates should be able to protect from prevalent *M. tb* strains type with high virulence in preclinical testing.

3. *M. tuberculosis* Beijing/K strain in South Korea and MTBK_24820 antigen

In South Korea, TB remains a major public health concern with an incidence of 80 cases per 100,000 population.[10] More than 80% of clinical isolates from Korean pulmonary TB patients belong to the Beijing genotype.[11-13] 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. tb* H37Rv in mice.[15, 16]

From a whole-genome sequence analysis of the Beijing/K strain, I identified MTBK_24820 (http://www.ncbi.nlm.nih.gov/protein/646276346 [Genbank accession no. AIB49026.1]), which is a predicted member of the PPE family and is located within the 5.7-kb insertion region compared with the genome of H37Rv strain.[17] Within this region, MTBK_24820 is arranged in a row with the ESAT-6 like-(*esx*) proteins,[18, 19] indicating that the cluster containing MTBK_24820 likely plays an immuno-pathogenic role in hosts infected with the Beijing/K strain.

4. PPE family of *M. tuberculosis*

In the literature, 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. tb*,[20, 21] and deletion of PPE25 in *M. avium* induces inhibition of phagolysosomal fusion.[22] PE4-expressing *M. smegmatis* showed improved survival in murine macrophages.[23] *ppe18* knock-out *M. tb*-infected mice showed reduced colony forming unit (CFU) burdens and less tissue damage, suggesting that PPE18 could play a role in survival of *M. tb*.[24] In addition, PE/PPE family proteins have highly immunogenic T-cell epitopes that induce secretion of IFN-γ as determined by immunoinformatic analyses.[25, 26] A multi-epitope DNA vaccine including peptides derived from PE19 and PPE25 induces potent IFN-γ responses.[27]

5. Purpose of this study

In this study, I tried to identify characterization of MTBK_24820 antigen, a member of PPE family derived from *M. tb* Beijing/K strains. To evaluate the protective efficacy of the MTBK_24820 antigen, I assessed the performance of immunization with MTBK_24820 in comparison with BCG following challenge with the hypervirulent clinical isolate Beijing/K in mice. The bacterial load, histopathology, and cytokine signatures in

lungs and spleens of the mice were examined at 4- and 9-wks post infection. In addition, the immunogenic T-cell epitopes of MTBK_24820 necessary to elicit IFN-γ production were determined.

II. MATERIAL AND METHODS

1. RNA extraction of *M. tb* strains

M. tb Beijing/K strain and *M. tb* H37Rv cultures were centrifuged at 2,500 × *g* for 20 min. After removing the supernatant, the pellets were stored at -80℃ until use. Total RNA extraction was performed using TRIzol® (Invitrogen, Carlsbad, CA, USA) and RNeasy® mini kit (Qiagen Inc., Valencia, CA, USA) according to the manufacturer's instructions. The cell pellet from 50 ㎖ cultures was resuspended with 800 ㎕ TRIzol® solution and transferred to Lysing matrix B (MP Biomedicals, Santa Ana, CA, USA) containing 0.1 mm silica spheres. Disruption of cells was conducted using Thermo Savant FastPrep® 120 cell homogenizer (Speed: 6 m/sec, time: 40 sec, four times repeats) (Thermo Fisher Scientific, Waltham, MA, USA). 300 ㎕ chroloform was added and centrifuged at 10,000 × *g* for 10 min. The yellowish supernatant was transferred to new tube and equal volume of isoprophanol (Merck, Darmstadt, Germany) was added.

After inverting several times, precipitate were obtained after centrifugation at 10,000 ×g for 10 min. After removal of supernatant, precipitate was washed with 70% ethanol and dried. The extracted RNA was purified using spin column of RNeasy® mini kit (Qiagen Inc.) and dissolved in RNase free water. The concentration of total RNA was

measured using Nanodrop ND-100 (Biocompare Inc., San Francisco, CA, USA) and stored at -80℃ until use.

2. Quantitative real time RT-PCR (qRT-PCR)

RNA was converted to first-strand cDNA with reverse transcriptase Superscript III (Invitrogen) according to manufacturer's instructions using 50 ng/㎕ random hexamer. The synthesized 2 ㎕ cDNA and primers (Table 1) were mixed with 12.5 ㎕ DyNAmo™ HS SYBR® Green Master Mix (Thermo Fisher Scientific) for a final volumn of 25 ㎕. Fluorescence-monitored PCR was carried out with an Applied Biosystems StepOnePlus™ Real-Time PCR system (Thermo Fisher Scientific) using SYBR® Green as an indicator. The major housekeeping sigma factor gene sigA was used as the normalization control. Change of mRNA levels of MTBK_24820 in *M. tb* Beijing/K strain and H37Rv strain was calculated and statistically analyzed by comparing mRNA expression level of sigA and MTBK_24820.

3. Animals

All mouse experiments were in accordance with guidelines and used animal protocols (perrmit number: 2013-0089) approved by the Institutional Animal Care and Use Committee, Yonsei University College of Medicine (Seoul, South Korea). C57BL/6N (female, 5-6 wks of age) mice were

purchased from SLC, Inc. (Shijuoka, Japan) and maintained in the Animal Bio Safety Level 3 facility at the Yonsei University College of Medicine.

4. Preparation of recombinant MTBK_24820 antigen

MTBK_24820 from the Beijing/K strain was cloned into pYUB1062 with the *Nde*I and *Hin*dIII (New England BioLabs, Ipswich, MA, USA) digestion.[28] The MTBK_24820 gene was amplified using the primers (Table 2) from *M. tb* Beijing/K strain genomic DNA. The constructed plasmid was transformed into *E. coli* BL21(DE3) and the strain containing MTBK_24820 was cultured in LB media containing 150 μg/㎖ hygromycin (A.G. Scientific, Inc., San Diego, CA, USA) at 37℃ until the OD_{600} reached 0.6-0.7. 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 Ni-NTA agarose resin (Qiagen, Venlo, Netherlands). Further purification was conducted using MonoQ anion exchange columns on an ÄKTA-FPLC system (GE Healthcare Biosciences, Pittsburgh, PA, USA). The purified recombinant MTBK_24820 protein was refolded and confirmed by SDS-PAGE analysis. Bicinchoninic acid assays (BCA, Thermo Fisher Scientific, Inc., Rockford, IL, USA) were used to measure protein concentrations. Samples were sterilized by gamma radiation and stored at -80℃ until use.

Table 1. Primer sequences for quantitative real time RT-PCR (qRT-PCR)

Primer	PCR primer sequence
SigA-F	5’-ATGGTCGAGGTGATCAACAA-3’
SigA-R	5’-GGGGTGATGTCCATCTCTTT-3’
PPE39-Rv-F	5'-GCAACTCGGGCTATGTCAAT-3'
PPE39-Rv-R	5'-AAAATCCCGAACTCATCACG-3'
MTBK_24820-N-F	5'-TCGGGCTTGCTAGATAGTGC-3'
MTBK_24820-N-R	5'-AAATTGAGGGACCCGATGTT-3'

Table 2. Primer sequences to amplify *MTBK_24820* derived from *M. tb* Beijing/K strain

Primer	PCR primer sequence	Restriction enzyme site
MTBK_24820-F	5'-TA**CATATG**GTGGTGAATTTTTCGGTGTTG-3'	*Nde*I
MTBK_24820-R	5'-CCA**AAGCTT**TCCGAACAAGTTCTTGAAGA-3'	*Hin*dIII

5. 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 (OACD, Becton Dickinson, Sparks, MD, USA) and 0.02% glycerol for 4 wks at 37℃. 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℃ until use.

6. Immunization and infection

Mice were immunized by subcutaneous injection with a dose of 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-wks intervals. PBS emulsified with DDA and MPL was used for the sham-immunized group.[29] BCG (2×10^5 CFUs/dose), as a control vaccine, was subcutaneously injected into mice once 6-wks before Beijing/K infection. Three weeks after the final

immunization, sera and spleens from three mice of each group were obtained for analysis of MTBK_24820-induced immune responses.

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

7. Determination of bacterial load and histopathological examination

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 ㎖ of PBS. Ten-fold serial dilutions of each homogenate were prepared and plated onto Middlebrook 7H11 agar plates supplemented with OADC containing amphotericin B (Sigma-Aldrich). Plates were incubated for 4-wks at 37℃ and bacterial colonies were then counted.

For histopathological examination 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 examination (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, Maryland, USA). Results are represented as the percentage of area with lesions.

8. Preparation of lung and spleen cells

Lungs and spleens were removed from immunized and/or infected mice. Lung tissue was chopped and incubated in RPMI media (Welgene, Daejeon, Korea) containing collagenase type II (Worthington Biochemical Co., Lakewood, NJ, USA) for 30 min at 37℃ 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_4Cl, 1 mM $KHCO_3$, and 0.1 mM Na_2EDTA). Cells were washed and resuspended in RPMI media containing 10% fetal bovine serum and 1 unit/㎖ Antibiotic-Antimycotic (Invitrogen, Grand Island, NY, USA). Cells were stimulated with 0.1, 1, or 5 μg/㎖ MTBK_24820 for 24 hr at 37℃ for determination of cytokine concentrations. Cells were stimulated with 5 μg/㎖ MTBK_24820 for 24 hr at 37℃ for intracellular cytokine staining.

9. Intracellular cytokine staining

Single lung or spleen cells were stimulated with 5 μg/㎖ MTBK_24820 for 12 hr at 37℃ in the presence of GolgiPlug (BD Biosciences). Cells were then stained with PerCP-Cy5.5-conjugated anti-CD4, APC-Cy7-conju

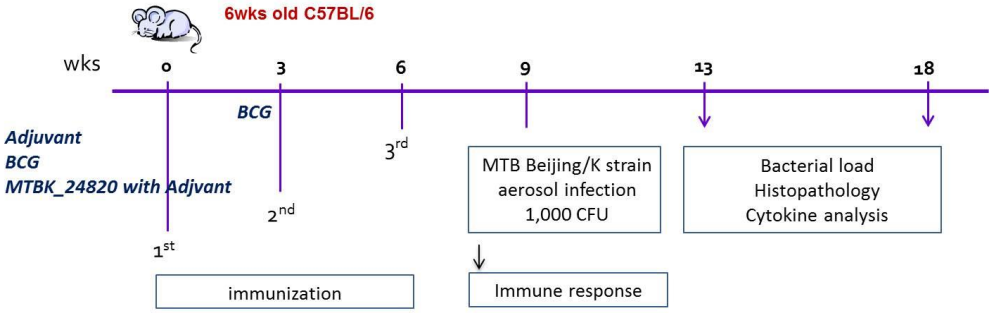

Figure 1. Design of protection against *M. tb* Beijing/K strains conferred by MTBK_24820 immunization. The time points for immunizations, infection, and analysis contents are shown. Mice were immunized subcutaneously with a single dose of BCG (BCG group) or DDA-MPL (adjuvant group) or DDA-MPL-MTBK_24820 (MTBK_24820 group). Sera and spleens were processed to determine immune responses induced by immunization prior to aerosol infection of *M. tb*. Three weeks after immunization, mice were challenged with a hypervirulent strain of *M. tb* Beijing/K strain. At 4- and 9-wks after infection, the lungs were processed to determine bacterial loads by counting the CFUs and hematoxylin-eosin (HE) staining. The lungs and spleens were also obtained for analysis of immune responses.

gated anti-CD8, and FITC-conjugated anti-CD44 antibodies (eBiosciences, Vienna, Austria) for 30 min at 4℃. Cells were fixed and permeabilized using a Cytofix/Cytoperm kit (BD Biosciences) and stained with PE-conjugated anti-IFN-γ, PC-conjugated anti-TNF-α, and PE-Cy7-conjugated anti-IL-17 (eBiosciences). All analyses were performed using a FACS Verse 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 was also determined using FlowJo software (FlowJo, LLC).

10. Quantification of IgG antibodies specific to MTBK_24820

Blood samples were collected from the mice 3 wks after the final immunization. Sera were separated after clotting of whole blood at room temperature followed by centrifugation at 1,500 × *g* for 15 min and stored at -20℃ until use. To determine the level of the anti-IgG antibodies in response to MTBK_24820, anti-IgG ELISAs were performed as previously described.[18] Briefly, 5 μg/㎖ MTBK_24820 was diluted in 0.5 M carbonate-bicarbonate buffer and coated onto 96-well plates (Corning Inc., Oneonta, NY, USA) for 16 hr at 4℃. 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 hr at 37℃, peroxidase-conjugated anti-mouse IgG antibody (1:10,000 dilution; Merck, Darmstadt, Germany)

was added and incubated for 1 hr at 37℃. Reactions within the plates were visualized using tetramethylbenzidine (TMB) substrate (KPL, Gaithersburg, MD, USA), and the reactions were stopped with 2.5 N H_2SO_4. Absorbencies at 450 nm were read using a VersaMax ELISA reader (Molecular Devices, Sunnyvale, CA, USA).

11. Multiplex bead array

Th1 and Th2 type cytokines including IFN-γ, IL-1α, IL-2, IL-4, IL-5, IL-6, IL-10, GM-CSF, TNF-α and IL-17 concentrations in supernatants from lung- and spleen-cell culture supernatants stimulated with MTBK_24820 were simultaneously measured using FlowCytomix (catalog no: 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 of the standard curve were adjusted by setting the minimum and maximum values.

12. Design of synthetic MTBK_24820 peptides and IFN-γ ELISAs

The 18-mer synthetic peptides overlapped by 6-mer that span 259 amino acids at the N-terminus of MTBK_24820 were designed for determination of potential epitope sites (GenScript, Piscataway, NJ, USA) (Figure 2). Peptides were diluted in RPMI medium at 1 mg/㎖ and stored at -20℃ until use. Spleen cells from infected mice were stimulated with 10 μg/㎖

peptide or 1 μg/㎖ concanavalin A (ConA) (Sigma-Aldrich). After incubation for 24 hr at 37℃, cell culture supernatants were harvested and IFN-γ responses were detected using ELISAs (eBioscience) according to the manufacturer's protocol.

13. Statistical analysis

Data were analyzed using Prism 6.0 software (Graph Pad, La Jolla, CA, USA). Mean values and standard deviations were calculated for each experimental group. In qRT-PCR, differences of gene expression of PPE39 or MTBK_24820 between of *M. tb* Beijing/K strains and H37Rv strains were compared using two-tailed unpaired *t*-tests. In animal experiments, differences among the adjuvant-alone, BCG, and MTBK_24820 groups were compared using one-way ANOVA followed by Dunn's multiple comparison tests or two-tailed unpaired *t*-tests. Values of $*p<0.05$, $**p<0.01$, and $***p<0.001$ were considered significant.

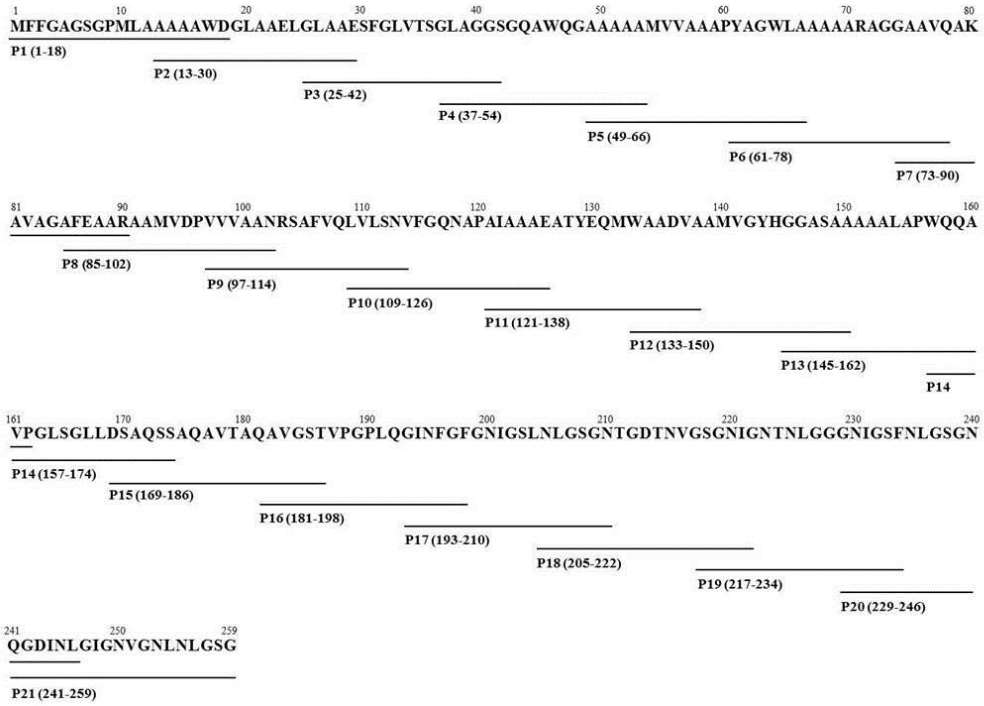

Figure 2. Synthetic peptides with overlapping 6-mer spanning the N-terminus of MTBK_24820. The peptides that covered 259 amino acids of N-terminus of MTBK_24820 were designed for determination of potential T cell epitope sites. Peptides were diluted in RPMI medium at 1 mg/㎖ stored at -20℃ until use. Spleen cells from infected mice were stimulated with 10 μg/㎖ peptide diluted in RPMI media.

III. RESULTS

1. Identification of MTBK_24820 in *M. tb* Beijing/K strain

After whole genome sequencing of *M. tb* Beijing/K strains,[30] several insertion regions were found in *M. tb* Beijing/K strain compared with *M. tb* H37Rv strain. In this study, the 5.7 kb insertion region containing ESAT-6 (*esx*) like proteins and MTBK_24820 cluster was found by comparative sequence analysis (Figure 3). I focused on MTBK_24820 predicted as a member of PPE family, which is orthologous of PPE39 protein in H37Rv strain. In sequence alignment, the 259 amino acid of N-terminus of MTBK_24820 was absent in H37Rv genome (Figure 4). In previous study, MTBK_24820 was overexpressed in Beijing/K strain about 8.8 fold compared with H37Rv strain in microarray experiments (Data not shown). In qRT-PCR experiments, the consensus sequence of MTBK_24820 and PPE39 was significantly expressed in Beijing/K strains compared with H37Rv strain ($p<0.001$) (Figure 5), which was consistent with microarray results. The N-terminus of MTBK_24820 absent from H37Rv genome was expressed only in Beijing/K strains ($p<0.001$), and this result corresponded to sequence alignment analysis (Figure 5).

2. Preparation of MTBK_24820 antigen

After detection of expression of MTBK_24820 in Beijing/K strains, I prepared recombinant MTBK_24820 protein. The MTBK_24820 was cloned into pYUB1062 vector using *Nde*I/*Bam*HI restriction enzyme site (Figure 6A). This recombinant plasmid was expressed in *E. coli* by T7 expression system and purified using histidine affinity chromatography followed by anion exchange purification method. About 60 kDa of recombinant MTBK_24820 protein was confirmed by SDS-PAGE analysis (Figure 6B). The purified MTBK_24820 protein was emulsified with MPL, DDA adjuvants in mouse experiments.

3. MTBK_24820 -induced immune responses in mice

Immunization of mice with MTBK_24820 was confirmed by MTBK_24820-specific IgG responses. Mice immunized with MTBK_24820 had significantly higher MTBK_24820-specific IgG responses compared with mice immunized with adjuvant ($p<0.01$) (Figure 7A).

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, IL-2, IL-6, and IL-17 production was significantly increased in a dose-dependent manner in mice immunized with MTBK_24820 ($p<0.05$ in IFN–γ and $p<0.01$ in IL-2, IL-6, IL-17) (Figure 7B). Despite the presence of MTBK_24820 homologs in BCG, none of the cytokines were

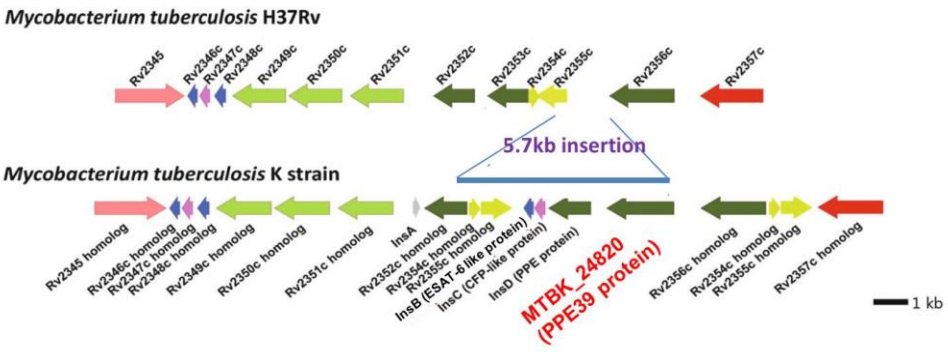

Figure 3. Genetic map of the region containing the MTBK_24820 in the *M. tb* H37Rv and Beijing/K strains. The 5.7 kb insertion region found in Beijing/K strains is indicated by the blue bar. MTBK_24820 (orthologous of PPE39 in H37Rv) is located in this insertion region, arranged in a row with the ESAT-6-like (*esx*) proteins.

```
K_2353c        MFFGAGSGPMLAAAAAWDGLAAELGLAAESFGLVTSGLAGGSGQAWQGAAAAAMVVAAAP 60
H37Rv_Rv2353   ------------------------------------------------------------

K_2353c        VAGWLAAAAARAGGAAVQAKAVAGAFEAARAAMVDPVVVAANRSAFVQLVLSNVFGQNAP 120
H37Rv_Rv2353   ------------------------------------------------------------

K_2353c        AIAAAEATVEQMWAADVAAMVGVHGGASAAAAALAPWQQAVPGLSGLLDSAQSSAQAVTA 180
H37Rv_Rv2353   ------------------------------------------------------------

K_2353c        QAVGSTVPGPLQGINFGFGNIGSLNLGSGNTGDTNVGSGNIGNTNLGGGNIGSFNLGSGN 240
H37Rv_Rv2353   ------MPGRFR-------------------------------------------------- 6
                     :** ::

K_2353c        QGDINLGIGNVGNLNLGSGNFGSQNLGSGNIGSTNVGSGNIGSTNVGSGNIGDTNFGNGN 300
H37Rv_Rv2353   -------------------NFGSQNLGSGNIGSTNVGSGNIGSTNVGSGNIGDTNFGNGN 47
                                  ****************************************

K_2353c        NGNFNFGSGNTGSNNIGFGNTGSGNFGFGNTGNNNIGIGLTGDGQIGIGGLNSGSGNIGF 360
H37Rv_Rv2353   NGNFNFGSGNTGSNNIGFGNTGSGNFGFGNTGNNNIGIGLTGDGQIGIGGLNSGSGNIGF 107
               ************************************************************

K_2353c        GNSGTGNVGLFNSGTGNVGFGNSGTANTGFGNAGNVNTGFWNGGSTNTGLANAGAGNTGF 420
H37Rv_Rv2353   GNSGTGNVGLFNSGTGNVGFGNSGTANTGFGNAGNVNTGFWNGGSTNTGLANAGAGNTGF 167
               ************************************************************

K_2353c        FDAGNYNFGSLNAGNINSSFGNSGDGNSGFLNAGDVNSGVGNAGDVNTGLGNSGNINTGG 480
H37Rv_Rv2353   FDAGNYNFGSLNAGNINSSFGNSGDGNSGFLNAGDVNSGVGNAGDVNTGLGNSGNINTGG 227
               ************************************************************

K_2353c        FNPGTLNTGFFSAMTQAGPNSGFFNAGTGNSGFGHNDPAGSGNSGIQNSGFGNSGYVNTS 540
H37Rv_Rv2353   FNPGTLNTGFFSAMTQAGPNSGFFNAGTGNSGFGHNDPAGSGNSGIQNSGFGNSGYVNTS 287
               ************************************************************

K_2353c        TTSMFGGNSGVLNTGVGNSGFYNAAVNNTGIFVTGVMSSGFFNFGTGNSGLLVSGNGLSG 600
H37Rv_Rv2353   TTSMFGGNSGVLNTGVGNSGFYNAAVNNTGIFVTGVMSSGFFNFGTGNSGLLVSGNGLSG 347
               ************************************************************

K_2353c        FFKNLFG 607
H37Rv_Rv2353   FFKNLFG 354
               *******
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Figure 4. Sequence alignment of MTBK_24820 and PPE39. The amino acid sequence of MTBK_24820 of Beijing/K strain (designated as K_2353) was aligned with sequence of PPE39 of H37Rv strain (designated as H37Rv_Rv2353). The 259 amino acid of N-terminus of MTBK_24820 was absent in H37Rv genome. MTBK_24820, the complete form of PPE39 was prepared of recombinant protein for this study.

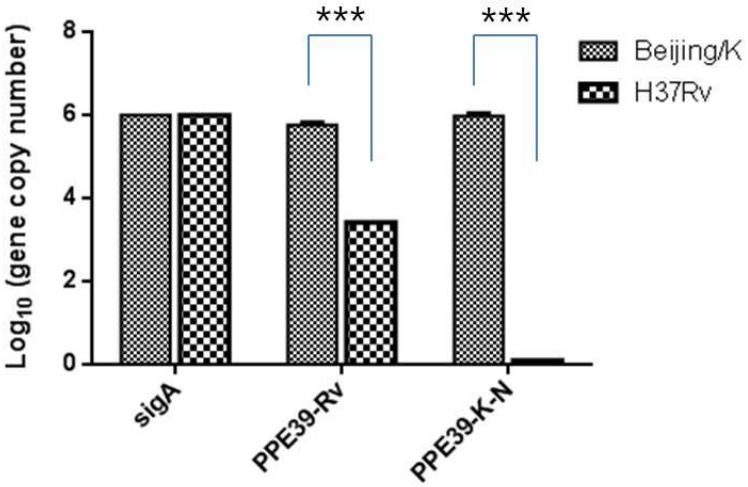

Figure 5. Quantitative real-time PCR analysis of mRNA of the MTBK_24820 in *M. tb* H37Rv and Beijing/K strains. The calculated gene copy number of PPE39 gene (designated as PPE39-Rv) and MTBK_24820 N-terminal region absent in H37Rv genome (designated as PPE39-K-N) in *M. tb* H37Rv and Beijing/K strain were shown at Y-axis. *sigA* was used as the normalization control. Data are presented as means ± S.D. of duplicate determination. Significant differences between strains were confirmed by unpaired t test (***$p < 0.001$).

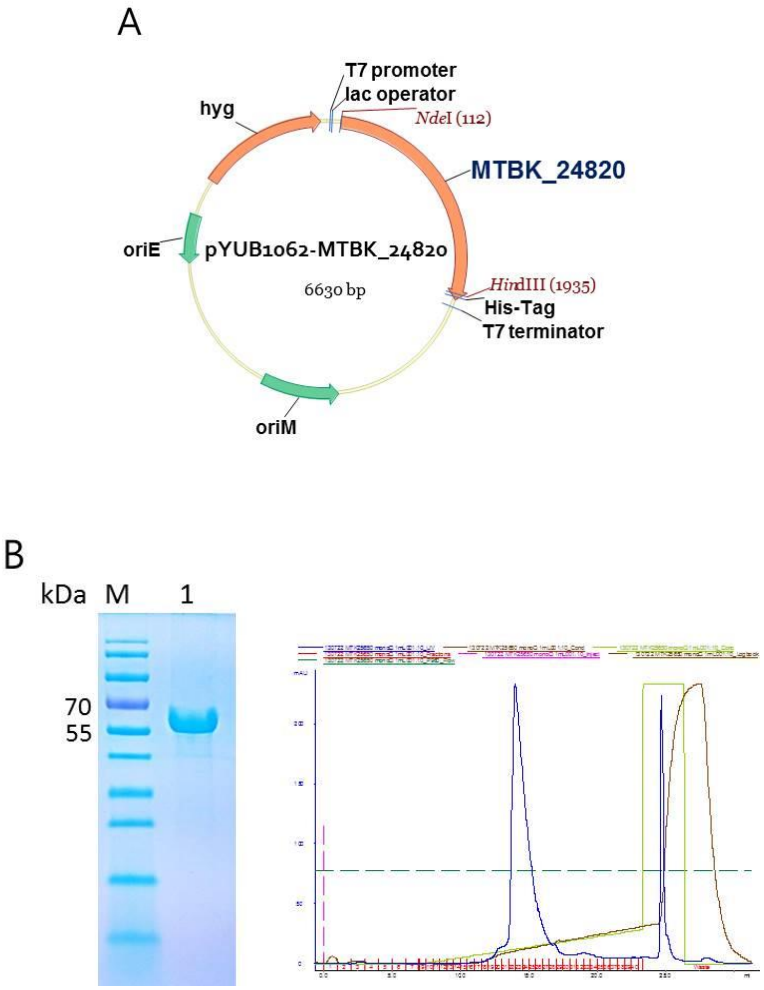

Figure 6. Preparation of recombinant MTBK_24820 antigen. (A) pYUB1062 plasmid containing a T7 promoter was used to cloning of MTBK_24820 gene. The amplified *MTBK_24820* was inserted into this plasmid using *Nde*I and *Bam*HI restriction enzymes. (**B)** A recombinant MTBK_24820 was overexpressed and purified in *E. coli* using histidine affinity chromatography followed by a FPLC purification method. Recombinant MTBK_24820 (approximately 60 kDa) was confirmed by SDS-PAGE. Lanes: M; marker, 1: 10 μg of MTBK_24820.

detected in the mice immunized with BCG. Th2 cytokines, such as IL-4 and IL-5, were not detected in all groups (Data not shown).

4. MTBK_24820-induced protective efficacy against TB

Although MTBK_24820 immunization-induced immune responses were observed in mice, it is not sufficient to prove the protective ability of this protein. To evaluate its protective property, mice were immunized and then challenged with Beijing/K strain of *M. tb* (Figure 1).

Bacterial counts were assessed in the lungs and spleens of Beijing/K strain infected mice. The MTBK_24820-immunized group showed approximately a 0.5-log reduction in CFUs in lungs at 4-wks post infection ($p<0.05$), and it was nearly the same level of protection as that seen in the BCG-immunized group ($p<0.01$) (Figure 8A). At 9-wks post infection, the CFU reduction in MTBK_24820-immunized mice was still significant compared with the control group ($p<0.01$) (Figure 8A), 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 reduce the bacterial loads at 9-wks post infection (Figure 8B).

The protective efficacy of MTBK_24820 against TB infection was also examined by histopathology. At 4-wks post infection, the calculated percent of inflammation lesions was higher in control mice immunized

with adjuvant (19.4-27.5%) than in mice immunized with BCG (5.4-12.9%) (p<0.01) or MTBK_24820 (9.1-16.4%) (p<0.01) (Figure 8C). At 9-wks post challenge, the inflammation of the lesions was more severe than at 4 wks, but the BCG- (20.0-24.3%) (p<0.01) and MTBK_24820-immunized mice (17.5-28.2%) (p<0.01) still showed fewer inflammation lesions than the adjuvant control mice (35.1-39.4%) (Figure 8D). There was no significant difference in inflammation lesion areas between BCG- and MTBK_24820-immunized mice (Figure 8D). These results indicate that MTBK_24820 had protective efficacy against the virulent Beijing/K strain comparable to the BCG vaccine.

5. MTBK_24820-induced cellular immune responses in mice infected with the Beijing/K strain of *M. tb*

To assess whether the Beijing/K strain of *M. tb* 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 *M. tb* infection (Figure 9).

MTBK_24820-immunized mice showed significantly higher concentrations of IL-2, IL-6, IFN-γ, and IL-17 production in both lungs and spleens at 4- and 9-wks post infection compared with the adjuvant-alone control group (p<0.01 in all cases) (Figure 9). IFN-γ production in

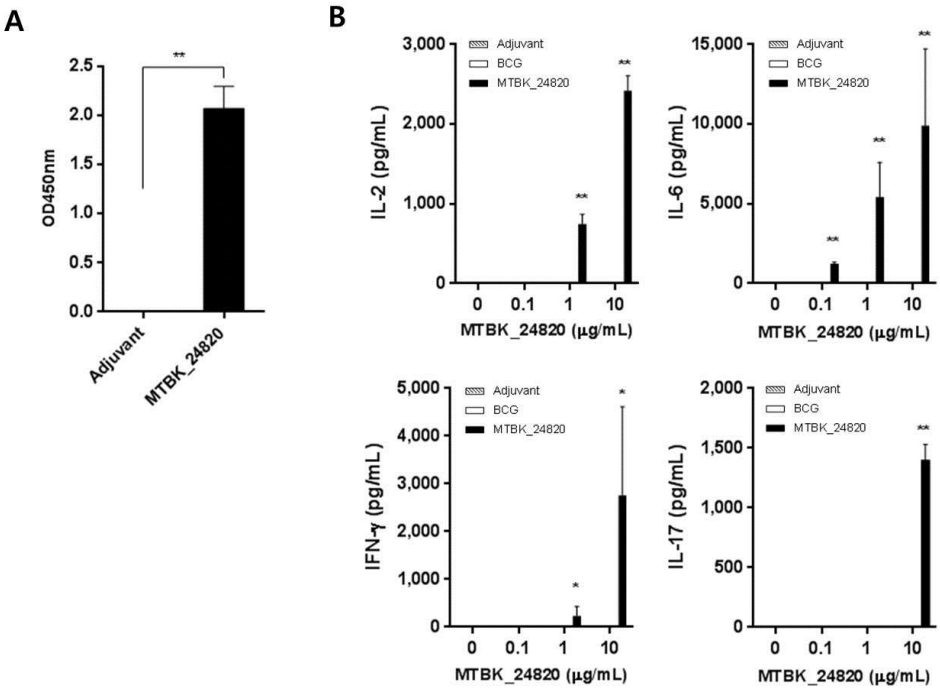

Figure 7. MTBK_24820-specific immune responses in C56BL/6 mice immunized with adjuvant, BCG, or MTBK_24820. (A) Three weeks after the last immunization with adjuvant alone, MTBK_24820 with adjuvant, anti-MTBK_24820-specific IgG was measured in sera. OD: optical density; Adjuvant: dimethyl dioctadecyl ammonium bromide (DDA) and monophospholipid A (MPL); (**B**) MTBK_24820-specific recall responses induced by immunization were measured in spleen cell culture supernatants from each immunized mice after stimulation with 0, 0.1, 1, or 10 μg/㎖ of MTBK_24820 prior to *M. tb* Beijing/K strain aerosol infection. IL-2, IL-6, IFN-γ and IL-17 concentrations were determined using multiplex bead assays. Data are presented as means ± S.D. of duplicate determinations from three mice. Significant differences between groups were confirmed by one-way ANOVA tests followed by Dunn's multiple comparison tests (*p<0.05; **p<0.01).

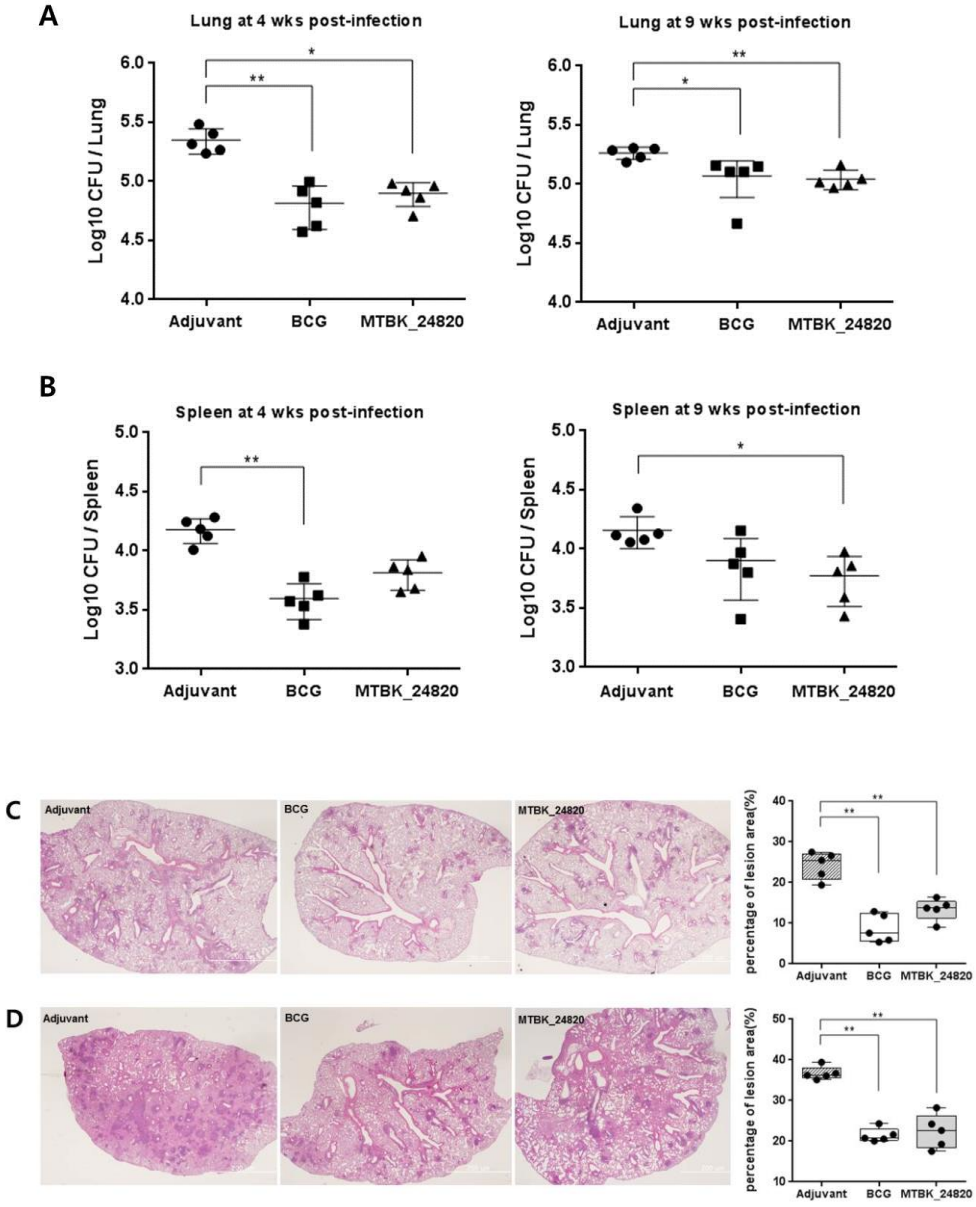

Figure 8. **Protective efficacy of immunization with MTBK_24820 in mice against the *M. tb* Beijing/K strain.** Three weeks after the final immunization, mice were challenged with 1,000 CFUs of virulent Beijing/K strain. At 4- and 9-wks post infection, all mice were sacrificed and bacterial burden (CFUs) was measured from homogenized lungs **(A)** and spleens **(B)**. Dots represent the mean log10 values obtained for individually tested animals and lines represent the means ± S.D from five mice. For histopathological examination of the lungs from immunized mice, at 4- **(C)** and 9-wks **(D)** post infection, mice were sacrificed, and lung sections were stained with H&E (20× magnification, bar = 200 μm). The lesion area percentage was obtained using 10× magnification fields and Image J software. Significant differences between multiple groups were confirmed by one-way ANOVA tests followed by Dunn's multiple comparison tests (*p<0.05; **p<0.01).

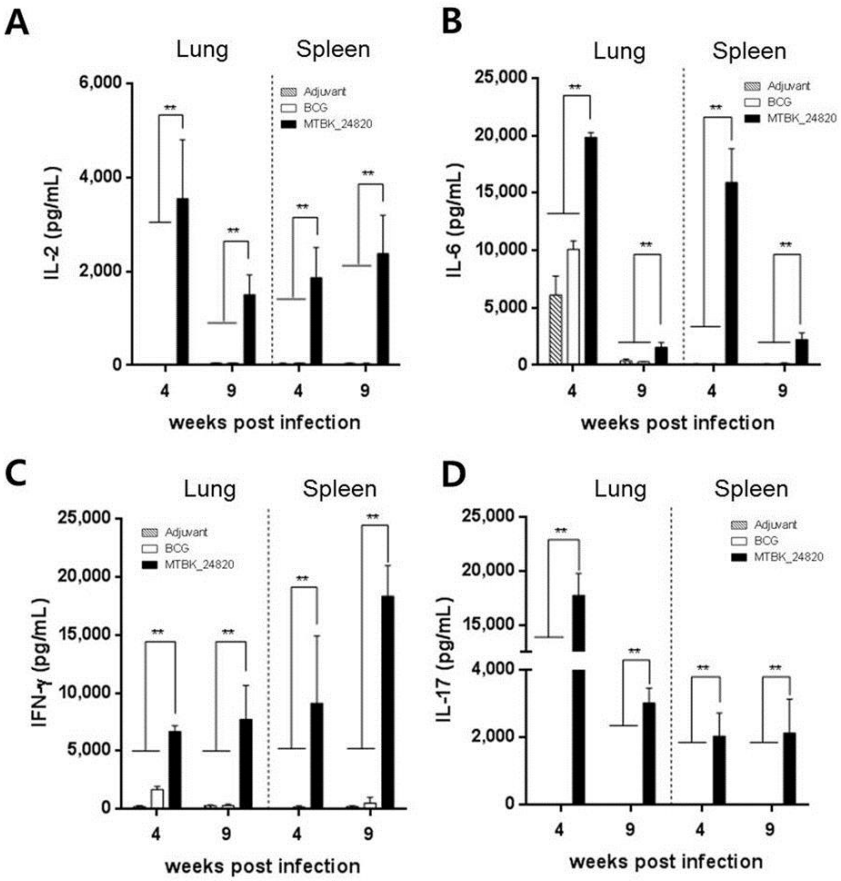

Figure 9. Cytokine production in the lungs and spleens from *M. tb* Beijing/K-infected mice following *ex vivo* stimulation with MTBK_24820. Concentrations of protection-related cytokines to MTBK_24820 (5 μg/㎖) were determined *ex vivo* at 4- and 9-wks post infection. IL-2 **(A),** IL-6 **(B),** IFN-γ **(C),** and IL-17 **(D)** concentrations in cell culture supernatants were also measured. Data are presented as means ± S.D from five mice. Significant differences between multiple groups were confirmed by unpaired t tests (**p<0.01).

MTBK_24820-immunized mice was elevated even more at 9-wks post infection in the spleen compared to 4-wks post infection ($p<0.01$) (Figure 9C). On the contrary, the IL-2, IL-6, and IL-17 responses of the MTBK_24820-immunized mice were significantly higher at 4-wks post infection than at 9-wks post infection in the lungs ($p<0.01$) (Figure 9A, C, D).

Next, I focused on 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 both in the lung and spleen than the adjuvant-alone control group ($p<0.01$) (Figure 10A, B). Similarly, the percentage of $CD4^+$ T cells producing IL-17 in MTBK_24820-immunized mice was higher than in the adjuvant-alone control group ($p<0.01$) (Figure 10C, D). The proportion of $CD8^+$ T cells producing IFN-γ in response to MTBK_24820 was also significantly higher in MTBK_24820-immunized mice than the adjuvant-alone control group ($p<0.01$) (Figure 11).

These results suggest that MTBK_24820 may generate MTBK_24820-specific Th1- and Th17-cytokine responses that could be maintained during chronic infections, which may play a role in protection against TB.

6. MTBK_24820 immunization induced antigen-specific multifunctional T cells in Beijing/K-infected mice

Based on reports showing an association between multifunctional T cells and protection against TB in *M. tb*-infected mice,[31, 32] MTBK_24820-specific multifunctional $CD4^+$ T cells were examined. 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. The gating strategy for multifunctional T cell populations is represented (Figure 12). 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-wks post infection (Figure 13). Although the proportion of triple-positive $CD4^+$ T cells producing IFN-γ, TNF-α, and IL-17 were lower than the proportion of double-positive $CD4^+$ T cells, immunization with MTBK_24820 induced triple positive $CD4^+$ T cells following *M. tb* infection ($p<0.05$ for Figure 13D or $p<0.01$ for Figure 13A, B, C). BCG-immunized mice did not produce IL-17, but were limited to IFN-γ and TNF-α production (Figure 13A, 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. tb*, and these responses may be involved in protection against TB.

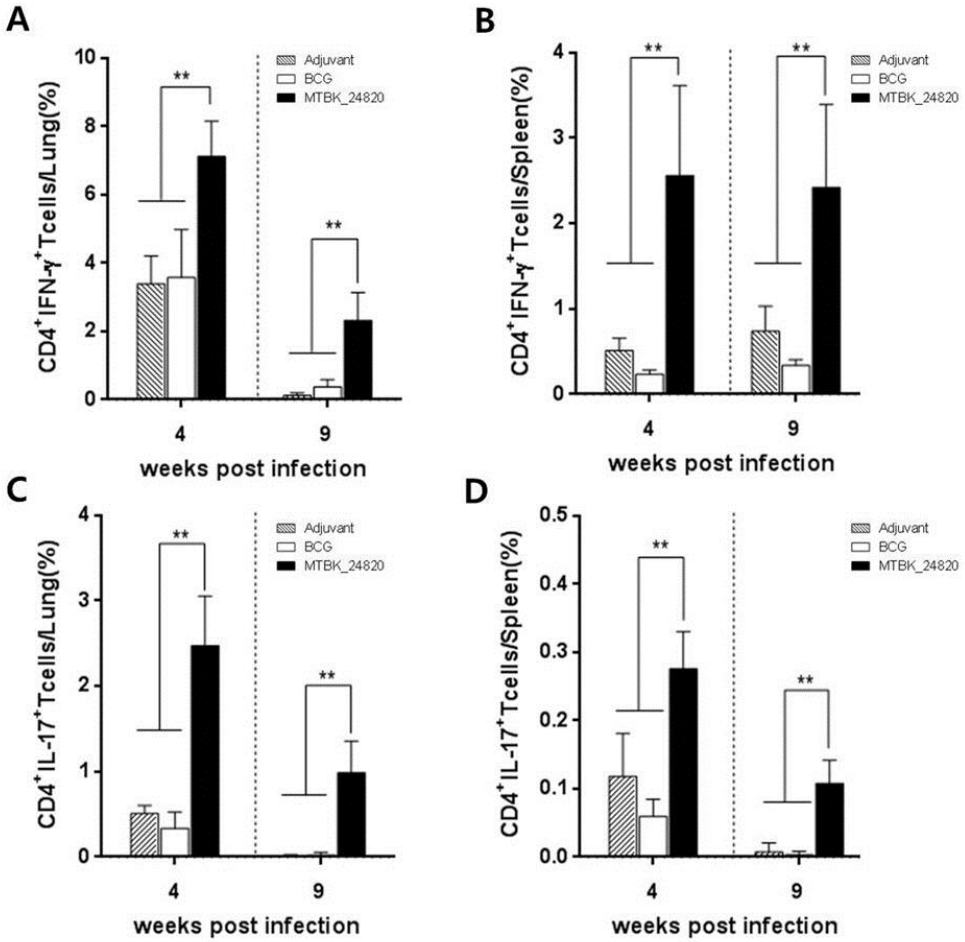

Figure 10. Proportion of CD4$^+$ T cells producing IFN-γ or IL-17 in the lungs and spleens of *M. tb* Beijing/K-infected mice in response to MTBK_24820. At 4- and 9-wks post infection, cells from lungs and spleens were stimulated with MTBK_24820 (5 μg/㎖) for 24 hr, and the percentage of IFN-γ-positive CD4$^+$ T cells in lungs **(A)** and spleens **(B)**, and IL-17-positive CD4$^+$ T cells in lungs **(C)** and spleens **(D)** were determined. Data are presented as means ± S.D. of two independent experiments with five mice. Significant differences between multiple groups were confirmed by unpaired *t* tests (**p<0.01).

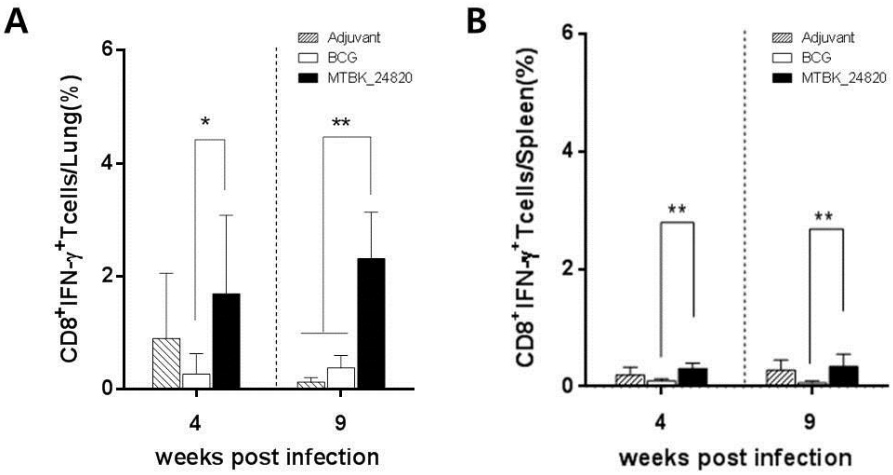

Figure 11. Proportion of CD8$^+$ T cells producing IFN-γ in the lungs and spleens of *M. tb* Beijing/K-infected mice in response to MTBK_24820. At 4- and 9-wks post infection, cells from lungs and spleens were stimulated with MTBK_24820 (5 μg/㎖) for 24 hr, and the percentage of IFN-γ-positive CD8$^+$ T cells in lungs **(A)** and spleens **(B)** were determined. Data are presented as means ± S.D. of two independent experiments with five mice. Significant differences between multiple groups were confirmed by unpaired *t* tests (**p<0.01).

7. The dominant epitope of MTBK_24820 in T cells of Beijing/K-infected mice

To determine potential epitopes in the N-terminus 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-wks post infection (Figure 2). Two peptides (amino acids 85-102 and 217-234) induced IFN-γ production in spleen cells from mice infected with the Beijing/K strain. The dominant epitope was at amino acids 85-102 (AFEAARAAMVDPVVVAAN) and amino acids 217-234 (GSGNIGNTNLGGGNIGSF) (Figure 14). Amino acids 85-102 stimulation showed more IFN-γ secretion at early infection time point, whereas amino acids 217-234 stimulation induced superior IFN-γ secretion at late infection time point, respectively (Figure 14).

However, IFN-γ secretion in response to the peptides was not observed in naïve C57BL/6 mice and *M. tb* H37Rv infected mice (Data not shown).

These data indicate that the N-terminus of MTBK_24820 may have potent T-cell epitopes that contribute to protection against TB specifically induced by the Beijing/K strain.

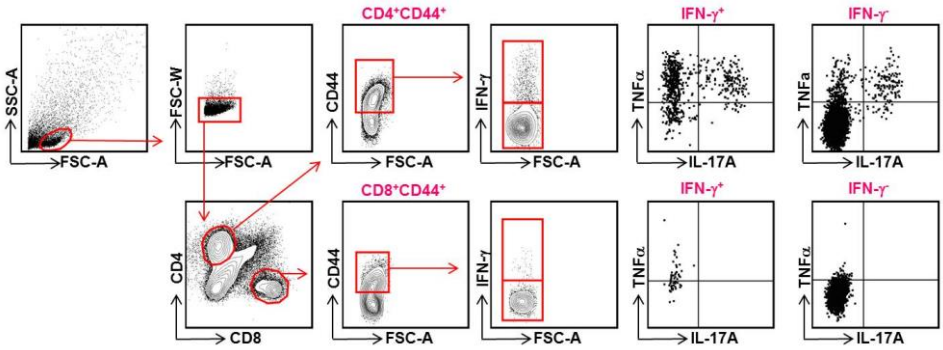

Figure 12. The gating strategy using Flowjo program. The gating strategy was used to determine the frequency of the seven $CD4^+$ or $CD8^+$ T cell subsets, each expressing one of the possible combinations of IFN-γ, TNF-α and IL-17A. Splenocytes stimulated with MTBK_24820 were firstly gated for lymphocytes (SSC-A vs. FSC-A). The lymphocytes were further analyzed for $CD4^+/CD44^+$ and $CD8^+/CD44^+$ T cells. The gated $CD4^+/CD44^+$ or $CD8^+/CD44^+$ T cells expressing INF-γ were categorized into cells expressing TNF–α and IL-17A.

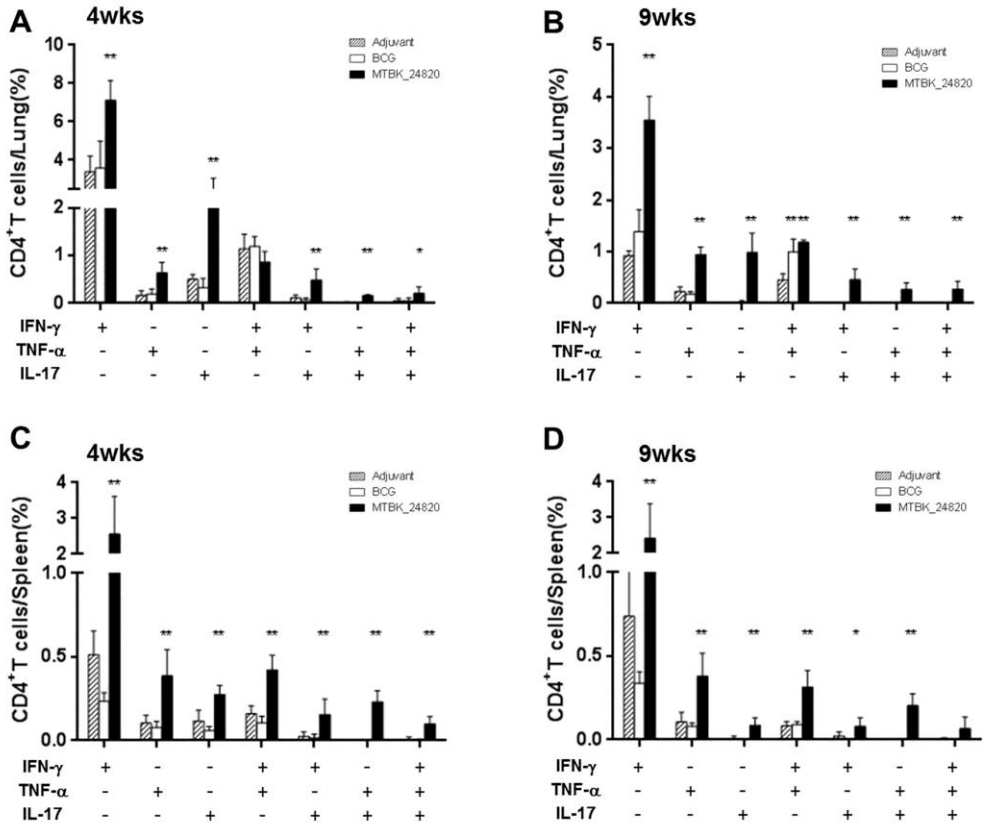

Figure 13. Functional profiles of MTBK_24820-specific CD4$^+$ T cells based on IFN-γ, TNF–α and IL-17 production. Multifunctional CD4$^+$ T cells in lungs and spleens from each group of immunized mice were analyzed by flow cytometry. At 4- and 9-wks post infection, cells were stimulated with MTBK_24820 (5 μg/㎖) for 12 hr in the presence of GolgiPlug. The percentage of IFN-γ, TNF-α and/or IL-17-producing CD4$^+$ T cells in lungs **(A, B)** and spleens **(C, D)** are presented as means ± S.D. of two independent experiments with five mice. Significant differences compared to the adjuvant control group were confirmed by unpaired *t* tests (*p<0.05 and **p<0.01).

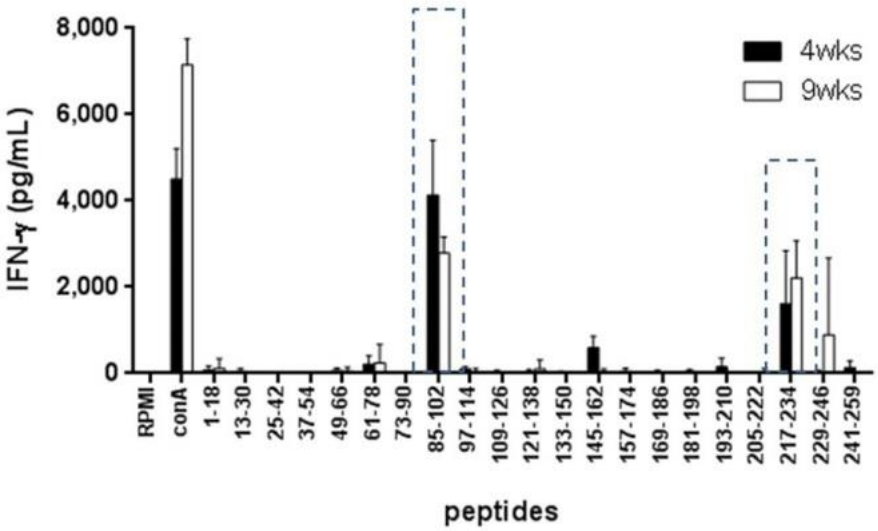

Figure 14. IFN-γ responses induced by overlapping peptides of MTBK_24820 in *M. tb* Beijing/K-infected mice. At 4- and 9-wks post infection, splenocytes were stimulated with control medium (RPMI), ConA (1 μg/㎖), or 10 μg/㎖ of the synthetic peptides covering the N-terminus of MTBK_24820 for 48 hr for measurement of the IFN-γ responses in cell supernatants. Peptides spanning the amino acids of the MTBK_24820 N-terminus are showed on the X-axis and dotted boxes represent peptides that induced IFN-γ responses. Data are presented as means ± S.D. of two independent experiments with four mice.

IV. DISCUSSION

In this study, it was demonstrated that MTBK_24820, a complete form of PPE39 derived from the Beijing/K strain of *M. tb* provided protective efficacy against *M. tb* infection in mice. This finding was supported by the strong immunogenicity of MTBK_24820, which included an increase in multifunctional $CD4^+$ T cells that may confer protection against *M. tb* infection.

MTBK_24820 is a member of the PPE family and is orthologous to the *M. tb* H37Rv PPE39 protein (annotated Rv2353c).[33] Sequence analysis showed six transmembrane helices with no signal peptide and the N-terminus oriented to the inside of the cell (TMpred software; http://embnet.vital-it.ch/software/TMPRED_form.html, SignalP 4.1 server; http:// www.cbs.dtu.dk/services/SingalP/). As determined by a comparative proteomic approach, PPE39 has high genetic variation between several *M. tb* isolates caused by IS6110 integration and SNPs[34] In the H37Rv genome, the function of PPE39 seems to be abolished due to IS6110 insertion.[35] Sequences homologous to MTBK_24820 by BLAST analyses were found in other clinical isolates, including the *M. tb* Haarlem family that has a higher frequency of MDR-TB [34] and CDC1551, which is highly transmissible and virulent in humans.[36]

The function of the PPE family depends on subcellular localization. The PPE41-PE25 dimer is secreted into culture filtrates via ESX-5 secretion systems[37] and induces TNF-α production and necrosis following *ex vivo* stimulation in mouse

macrophages. This suggests that the PPE41-PE25 dimer may enhance bacterial spread through necrosis, resulting in TB reactivation.[38] Another PPE family member, PPE36, is a TB immunogen that is localized in the membrane[39] and induces Th1-cytokine responses in PPE36-immunized mice.[38] In a human study, PPE36 showed significantly higher IgA antibody responses compared with healthy control subjects in TB patients' sera.[40]

Here, I examined the immunogenicity of the MTBK_24820 protein by immunizing mice with the MTBK_24820 protein that was emulsified in DDA and MPL as adjuvants to strengthen cellular accumulation in the lungs.[29] Robust cytokine production in response to MTBK_24820 immunization was indicated by high levels of IFN-γ, IL-2, IL-6, and IL-17 in spleen cells; however, there were relatively low levels of TNF-α in spleen cells (Data not shown). The immunogenicity of MTBK_24820 was prolonged following *M. tb* challenge as seen by continued elevated cytokine production at 9-wks post infection. MTBK_24820-immunized mice showed antigen-specific $CD4^{+}IFN\text{-}\gamma^{+}$ and $CD4^{+}IL\text{-}17^{+}$ T-cell responses.

Several reports have suggested that IFN-γ and IL-17 production by $CD4^{+}$ T cells is targeted in vaccine-induced immunity to *M. tb* infection in mice models.[42, 43] IL-17 produced by Th17 cells is a pro-inflammatory cytokine that induces neutrophil recruitment, which may result in protection against TB.[44] 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. tb* infection.[45] Moreover, the IL-17 requirement for host protection against TB depends on the *M. tb* 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. tb* HN878 infection, while IL-17 does not impact protective immunity against laboratory-adapted *M. tb* H37Rv infection.[46] Our data demonstrated that high levels of IL-17 production were induced in the lungs of MTBK_24820-immunized mice at the early phase of infection, which may function in the control of bacterial burden during the course of *M. tb* Beijing/K infection.

The high frequency of multifunctional $CD4^+$ T cells producing IFN-γ, IL-2, and/or TNF-α is also strongly correlated with vaccine-induced protection against TB infection in mice; therefore, multifunctional $CD4^+$ T cells are considered an important determinant of protective vaccination. A subunit vaccine in which Ag85A and ESAT-6 are fused causes $CD4^+$ multifunctional T cells expressing IFN-γ, IL-2, and TNF-α to be maintained 1-year post vaccination.[31, 32] In my study, immunization with MTBK_24820 induced a higher frequency of $CD4^+$ T cells expressing IFN-γ, TNF-α, and IL-17 in response to MTBK_24820 compared with mice vaccinated with BCG. Further study will evaluate the persistence of MTBK_24820-specific multifunctional T cells in a term longer than 1-year post infection in mice.

A report suggested that immunogenic, IFN-γ generating T-cell epitopes in PE/PPE family proteins could be potential TB vaccines[27] and diagnostic

biomarkers.[47] Therefore, I determined the dominant epitopes of MTBK_24820 using synthetic peptides that covered the 259 amino acids at the N-terminus of MTBK_24820 that are absent in *M. tb* H37Rv. The dominant epitope site of MTBK_24820 was at amino acids 85-102. Three potential epitope sites at C-terminus of the Beijing/K, H37Rv and the truncated region in BCG showed weak IFN- γ responses compared to the dominant N-terminus peptide (Data not shown).

There are now TB vaccine candidates containing PPE genes in clinical trial phase II; the ID93 sponsored by IDRI has PPE42.[48] It decreased bacterial loads and elicited $CD4^+$ and $CD8^+$ multifunctional T cell responses in mice infected with multidrug resistant TN5904 as well as H37Rv.[49] The Mtb72F developed by GSK contains PPE18 which was produced from H37Rv strain.[48, 50] Several genetic variations including SNPs in PPE18 were observed in clinical isolates resulting in alteration of amino acid sequences even in T cell epitopes.[51] It means that the Mtb72F may have limitation as a vaccine to recognize some *M. tb* strains due to the antigenic variation. However, a vaccine composed of PPE genes may be valuable due to the cross-reactivity with many PPE homologues throughout the *M. tb* genome.[52]

Although protective efficacy of MTBK_24820 determined by a CFU counting and histological pathology was not significantly different from that of BCG in mice, the efficacy by the MTBK_24820 seems to be longer lasted than BCG based on the CFU counting. Meanwhile, the protective immune responses were significantly higher in the mice immunized with MTBK_24820 compared with those with BCG.

Considering the high prevalence of the Beijing/K strain of *M. tb* and relatively poor protection of BCG against the W-Beijing genotype of *M. tb*,[8, 46] it is worth testing of the MTBK_24820 derived from the strain as a vaccine candidate, particularly in the endemic area. The dominant epitope sites of the T cells observed in the MTBK-24820 also exists in *M. bovis* BCG, but the sub-dominant epitope sites were not found in *M. bovis* BCG. Therefore, it is necessary to evaluate the protective efficacy of the MTBK_24820 using the potential epitopes in mice infected with the Beijing/K strain. These further studies may give the clue to the scarce immune responses in mice immunized with BCG in this study. Persistence of protective efficacy and multifunctional T cells in mice immunized with the MTBK_24820 should be also confirmed in a term longer than at least 9 weeks post infection.

Taken together, the 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 the MTBK_24820 may give the helpful information for development of new vaccines in endemic areas. The MTBK_24820 could be used as an antigen for improved future vaccines against the highly transmissible and virulent *M. tb* including Beijing/K.

V. CONCLUSION

In this study, I tried to characterize MTBK_24820, PPE39 protein, within the 5.7 kb insertion region in *M. tb* compared to H37Rv strain. The MTBK_24820 was evaluated as a TB vaccine candidate against hypervirulent Beijing/K strain in mice aerosol infection model.

After *M. tb* Beijing/K challenge, CFU reduction and fewer lung inflammation lesions were observed in mice immunized with MTBK_24820. The immune parameters induced by MTBK_24820 immunization were significantly higher $CD4^+$ T cells producing inflammatory cytokines such as IFN-γ and IL-17, and $CD4^+$ multifunctional T cells producing IFN-γ, TNF-α and/or IL-17. Moreover, there were two dominant T cell epitope sites in the N-terminal of MTBK_24820 to generate IFN-γ responses.

In conclusion, MTBK_24820, the complete form of PPE39 antigen, derived from the *M. tb* Beijing/K strain showed protective efficacy against infection with the hypervirulent Beijing/K strain. The protective efficacy and strong immune responses induced *in vivo* in response to immunization with MTBK_24820 may be informative for vaccine development, particularly in regions where the *M. tb* Beijing/K strain is frequently isolated from TB patients.

REFERENCES

1. World Health Organization. Global tuberculosis report 2016. Available at: http://apps.who.int/iris/bitstream/10665/250441/1/9789241565394-eng.pdf?ua=1 Accessed 1 April 2017.
2. Van Soolingen D, Qian L, de Haas PE, Douglas JT, Traore H, Portaels F. Predominance of a single genotype of *Mycobacterium tuberculosis* in countries of East Asia. J Clin Microbiol 1995;33:3234–38.
3. Bifani PJ, Mathema B, Kurepina NE, Kreiswirth BN. Global dissemination of the *Mycobacterium tuberculosis* W-Beijing family strains. Trends Microbiol 2002;10:45-52.
4. Frieden TR, Sherman LF, Maw KL, Fujiwara PI, Crawford JT, Nivin B. A multi-institutional outbreak of highly drug-resistant tuberculosis: epidemiology and clinical outcomes. JAMA 1996; 276:1229–35.
5. Agerton TB, Valway SE, Blinkhorn RJ, Shilkret KL, Reves R, Schluter WW, et al. Spread of strain W, a highly drug-resistant strain of *Mycobacterium tuberculosis*, across the United States. Clin Infect Dis 1999;29:85-92.
6. Fine PE. Variation in protection by BCG: implications of and for heterologous immunity. Lancet 1995;346:1339-45.

7. Andersen P, Doherty TM. The success and failure of BCG-implications for a novel tuberculosis vaccine. Nat Rev Microbiol 2005;3:656-62.

8. López B, Aguilar D, Orozco H, Burger M, Espitia C, Ritacco V, et al. A marked difference in pathogenesis and immune response induced by different *Mycobacterium tuberculosis* genotypes. Clin Exp Immunol 2003;133:30-7.

9. Anh DD, Borgdorff MW, Van LN, Lan NT, van Gorkom T, Kremer K, et al. *Mycobacterium tuberculosis* Beijing genotype emerging in Vietnam. Emerg Infect Dis 2000;6:302-5.

10. World Health Organization. Tuberculosis country profiles; Republic of Korea. Available at: http://www.who.int/tb/country/data/profiles/en/ Accessed 20 August 2016.

11. Kang HY, Wada T, Iwamoto T, Maeda S, Murase Y, Kato S, et al. Phylogeographical particularity of the *Mycobacterium tuberculosis* Beijing family in South Korea based on international comparison with surrounding countries. J Med Microbiol 2010;59:1191-97.

12. Choi GE, Jang MH, Song EJ, Jeong SH, Kim JS, Lee WG, et al. IS*6110*-restriction fragment length polymorphism and spoligotyping analysis of *Mycobacterium tuberculosis* clinical isolates for investigating epidemiologic distribution in Korea. J Korean Med Sci 2010;25:1716-21.

13. Shamputa IC, Lee J, Allix-Béguec C, Cho EJ, Lee JI, Rajan V, et al. Genetic diversity of *Mycobacterium tuberculosis* isolates from a tertiary care tuberculosis hospital in South Korea. J Clin Microbiol 2010;48:387-94.

14. Kim SJ, Bai GH, Lee H, Kim HJ, Lew WJ, Park YK, et al. Transmission of *Mycobacterium tuberculosis* among high school students in Korea. Int J Tuberc Lung Dis 2001;5:824–30.

15. Jeon BY, Kwak J, Hahn MY, Eum SY, Yang J, Kim SC, et al. *In vivo* characteristics of Korean Beijing *Mycobacterium tuberculosis* strain K1 in an aerosol challenge model and in the Cornell latent tuberculosis model. J Med Microbiol 2012;61:1373–79.

16. Kim WS, Kim JS, Cha SB, Han SJ, Kim H, Kwon KW, et al. Virulence-dependent alterations in the kinetics of immune cells during pulmonary infection by *Mycobacterium tuberculosis*. PLoS One 2015;10:e0145234.

17. Han SJ, Song T, Cho YJ, Kim JS, Choi SY, Bang HE, et al. Complete genome sequence of *Mycobacterium tuberculosis* K from a Korean high school outbreak, belonging to the Beijing family. Stand Genomic Sci 2015;10:78.

18. Park PJ, Kim AR, Salch YP, Song T, Shin SJ, Han SJ, et al. Characterization of a novel antigen of *Mycobacterium tuberculosis* K

strain and its use in immunodiagnosis of tuberculosis. J Microbiol 2014;52:871-8.

19. Hur YG, Chung WY, Kim A, Kim YS, Kim HS, Jang SH, et al. Host immune responses to antigens derived from a predominant strain of Mycobacterium tuberculosis. J Infect 2016;73:54-62.

20. Basu S, Pathak SK, Banerjee A, Pathak S, Bhattacharyya A, Yang Z, et al. Execution of macrophage apoptosis by PE_PGRS33 of *Mycobacterium tuberculosis* is mediated by Toll-like receptor 2-dependent release of tumor necrosis factor-alpha. J Biol Chem 2007;282:1039-50.

21. Dong D, Wang D, Li M, Wang H, Yu J, Wang C, et al. PPE38 modulates the innate immune response and is required for *Mycobacterium marinum* virulence. Infect Immun 2012;80:43-54.

22. Jha SS, Danelishvili L, Wagner D, Maser J, Li YJ, Moric I, et al. Virulence-related *Mycobacterium avium* subsp *hominissuis* MAV_2928 gene is associated with vacuole remodeling in macrophages. BMC Microbiol 2010;10:100.

23. Singh SK, Tripathi DK, Singh PK, Sharma S, Srivastava KK. Protective and survival efficacies of Rv0160c protein in murine model of

Mycobacterium tuberculosis. Appl Microbiol Biotechnol 2013;97:5825-37.

24. Bhat KH, Ahmed A, Kumar S, Sharma P, Mukhopadhyay S. Role of PPE18 protein in intracellular survival and pathogenicity of *Mycobacterium tuberculosis* in mice. PLoS One 2012;7:e52601.

25. Chaitra MG, Hariharaputran S, Chandra NR, Shaila MS, Nayak R. Defining putative T cell epitopes from PE and PPE families of proteins of *Mycobacterium tuberculosis* with vaccine potential. Vaccine 2005;23:1265-72.

26. Chaitra MG, Shaila MS, Nayak R. Characterization of T-cell immunogenicity of two PE/PPE proteins of *Mycobacterium tuberculosis*. J Med Microbiol 2008;57:1079-86.

27. Wu M, Li M, Yue Y, Xu W. DNA vaccine with discontinuous T-cell epitope insertions into HSP65 scaffold as a potential means to improve immunogenicity of multi-epitope *Mycobacterium tuberculosis* vaccine. Microbiol Immunol 2016;60:634-45.

28. Wang F, Jain P, Gulten G, Liu Z, Feng Y, Ganesula K, et al. *Mycobacterium tuberculosis* dihydrofolate reductase is not a target relevant to the antitubercular activity of isoniazid. Antimicrob Agents Chemother 2010;54:3776-82.

29. Sable SB, Cheruvu M, Nandakumar S, Sharma S, Bandyopadhyay K, Kellar KL, et al. Cellular immune responses to nine *Mycobacterium tuberculosis* vaccine candidates following intranasal vaccination. PLoS One 2011;6:e22718.

30. Han SJ, Song T, Cho YJ, Kim JS, Choi SY, Bang HE, et al. Complete genome sequence of *Mycobacterium tuberculosis* K from a Korean high school outbreak, belonging to the Beijing family. Stand Genomic Sci. 2015;14;10:78.

31. Lindenstrøm T, Agger EM, Korsholm KS, Darrah PA, Aagaard C, Seder RA, et al. Tuberculosis subunit vaccination provides long-term protective immunity characterized by multifunctional $CD4^+$ memory T cells. J Immunol 2009;182:8047-55.

32. Derrick SC, Yabe IM, Yang A, Morris SL. Vaccine-induced anti-tuberculosis protective immunity in mice correlates with the magnitude and quality of multifunctional $CD4^+$ T cells. Vaccine 2011;29:2902-09.

33. Fishhein S, van Wyk N, Warren RM, Sampson SL. Phylogeny to function: PE/PPE protein evolution and impact on *Mycobacterium tuberculosis* pathogenicity. Mol Microbiol 2015;96:901-16.

34. McEvoy CR, van Helden PD, Warren RM, Gey van Pittius NC. Evidence for a rapid rate of molecular evolution at the hypervariable and

immunogenic *Mycobacterium tuberculosis* PPE38 gene region. BMC Evol Biol 2009;9:237.

35. Marais BJ, Victor TC, Hesseling AC, Barnard M, Jordaan A, Brittle W, et al. Beijing and Haarlem genotypes are overrepresented among children with drug-resistant tuberculosis in the Western Cape Province of South Africa. J Clin Microbiol 2006;44:3539-43.

36. Valway SE, Sanchez MP, Shinnick TF, Orme I, Agerton T, Hoy D, et al. An outbreak involving extensive transmission of virulent strain of *Mycobacterium tuberculosis.* N Engl J Med 1998;338:633-9.

37. Ekiert DC, Cox JS. Structure of a PE-PPE-EspG complex from *Mycobacterium tuberculosis* reveals molecular specificity of ESX protein secretion. Proc Natl Acad Sci USA 2014;111:14758-63.

38. Tundup S, Mohareer K, Hasnain SE. *Mycobacterium tuberculosis* PE25/PPE41 protein complex induces necrosis in macrophages: Role in virulence and disease reactivation? FEBS Open Bio 2014;4:822-8.

39. Le Moigne V, Robreau G, Borot C, Guesdon JL, Mahana W. Expression, immunochemical characterization and localization of the *Mycobacterium tuberculosis* protein p27. Tuberculosis 2005;85:213-9.

40. Le Moigne V, Robreau G, Mahana W. Flagellin as a good carrier and potent adjuvant for Th1 response: study of mice immune response to the

p27 (Rv2108) *Mycobacterium tuberculosis* antigen. Mol Immunol 2008;45:2499-507.

41. Le Moigne V, Le Moigne D, Mahana W. Antibody response to *Mycobacterium tuberculosis* p27-PPE36 antigen in sera of pulmonary tuberculosis patients. Tuberculosis 2013;93:189-91.

42. Cooper AM, Dalton DK, Stewart TA, Griffin JP, Russell DG, Orme IM. Disseminated tuberculosis in interferon gamma gene-disrupted mice. J Exp Med 1993;178:2243-47.

43. Khader SA, Bell GK, Pearl JE, Fountain JJ, Rangel-Moreno J, Cilley GE, et al. IL-23 and IL-17 in the establishment of protective pulmonary $CD4^+$ T cell responses after vaccination and during *Mycobacterium tuberculosis* challenge. Nat Immunol 2007;8:369-77.

44. Monin L, Griffiths KL, Slight S, Lin Y, Rangel-Moreno J, Khader SA. Immune requirements for protective Th17 recall responses to *Mycobacterium tuberculosis* challenge. Mucosal Immunol 2015;8:1099-109.

45. Freches D, Korf H, Denis O, Havaux X, Huygen K, Romano M. Mice genetically inactivated in interleukin-17A receptor are defective in long-term control of *Mycobacterium tuberculosis* infection. Immunology 2013;140:220-31.

46. Gopal R, Monin L, Slight S, Uche U, Blanchard E, Fallert Junecko BA, et al. Unexpected role for IL-17 in protective immunity against hypervirulent *Mycobacterium tuberculosis* HN878 infection. PLoS Pathog 2014;10:e1004099.

47. Mustafa AS, AI-Attiyah R, Hanif SN, Shaban FA. Efficient testing of large pools of *Mycobacterium tuberculosis* RD1 peptides and identification of major antigens and immunodominant peptides recognized by human Th1 cells. Clin Vaccine Immunol 2008; 15:916-24.

48. AERAS. Global clinical portfolio of TB vaccine candidates. 2017. Available at: http://www.aeras.org/pages/global-portfolio Accessed 10 July 2017.

49. Bertholet S, Ireton GC, Ordway DJ, Windish HP, Pine SO, Kahn M, et al. A defined tuberculosis vaccine candidate boosts BCG and protects against multidrug-resistant *Mycobacterium tuberculosis*. Sci Transl Med 2010;2:53ra74.

50. Gillard P, Yang PC, Danilovits M, Su WJ, Cheng SL, Pehme L, et al. Safety and immunogenicity of the M72/AS01E candidate tuberculosis vaccine in adults with tuberculosis: A phase II randomised study. Tuberculosis (Edinb) 2016;100:118-27.

51. Hebert AM, Talarico S, Yang D, Durmaz R, Marrs CF, Zhang L, et al. DNA polymorphisms in the pepA and PPE18 genes among clinical strains of *Mycobacterium tuberculosis*: implications for vaccine efficacy. Infect Immun 2007;75:5798-805.

52. Sayes F, Pawlik A, Frigui W, Gröschel MI, Crommelynck S, Fayolle C, et al. $CD4^+$ T cells recognizing PE/PPE antigens directly or via cross reactivity are protective against pulmonary *Mycobacterium tuberculosis* infection. PLoS Pathog 2016;28:12:e1005770.

ABSTRACT (IN KOREAN)

동물모델에서의 결핵균 Beijing/K 균주 MTBK_24820 항원의 방어면역원성 평가

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김 아 름

본 연구는 국내에서 발생한 결핵집단감염사례에서 높은 빈도로 분리된 결핵균 Beijing/K 균주에 존재하는 PPE39 항원의 방어면역원성을 동물모델에서 평가하고자 하였다. 이를 위해 결핵균 H37Rv 균주와 Beijing/K 균주의 유전체 비교분석을 하여 Beijing/K 균주에서 5.7 kb의 유전자 삽입부분을 확인하였고, 이 부위에 존재하는 여러 개의 ORF들 중에서 PPE39 항원인 MTBK_24820 단백질을 연구대상으로 하여 대장균에서 발현, 정제하였다.

MTBK_24820 재조합단백질의 면역원성을 확인하기 위하여 adjuvant, BCG 균주, 그리고 adjuvant와 섞은 MTBK_24820 단백질을 각각 마우스에 면역시켰다. MTBK_24820 면역마우스에서 분리한 혈청에서 항원 특이적 IgG 반응을 확인하였고($p<0.05$), 특히, 이 면역 마우스

그룹에서 비장세포를 분리 한 후 MTBK_24820 항원을 자극시켰을 때 IFN-γ, IL-2, IL-6 그리고 IL-17 등의 사이토카인이 항원자극농도에 따라 의존적으로 분비되는 면역회상반응을 확인하였다($p<0.01$).

MTBK_24820 단백질의 방어면역원성을 마우스 모델에서 확인하고자 adjuvant, BCG, MTBK_24820의 세 면역그룹에 고용량의 Beijing/K 균주를 공기 감염하였다. 감염 4주, 9주 후에 마우스의 폐를 분리하여 조직 병변의 변화를 분석하고, 폐와 비장을 분리하여 조직 내의 결핵 균 수를 측정, 그리고 각 조직에서 세포를 분리하여 면역학적 특성을 분석하였다.

결핵균 감염 후, MTBK_24820 면역 마우스의 폐에서 adjuvant 면역 마우스와 비교하여 약 0.5~1.0 $\log_{10}$ 정도의 균의 감소가 확인되었고($p<0.05$), 폐조직의 염증성 병변도 adjuvant 면역마우스에 비하여 완화됨을 확인하였으며($p<0.01$), 이러한 양상은 BCG균주로 면역을 준 마우스에서 보이는 방어 정도와 유사함을 알 수 있었다. 또한 이 면역 마우스 그룹으로부터 얻어진 폐와 비장에서 세포를 분리하여 MTBK_24820 항원 자극 주었을 때, IFN-γ, IL-17 등의 결핵 방어면역에 관련된 사이토카인이 분비됨을 확인하였다($p<0.01$). FACS 분석을 통하여 이러한 사이토카인들이 $CD4^+$ T 세포에서 분비되고($p<0.01$), IFN-γ의 경우 $CD8^+$ T 세포도 함께 관여하여 분비됨을 확인하였다($p<0.01$). 결핵 방어면역평가에 이용되고 있는 지표로써 다기능 T 세포(multifunctional T cell)에 대한 분석도 시행하였는데, MTBK_24820 면역 마우스 그룹에서 얻어진 폐와 비장에서 세포를 분리한 후, MTBK_24820 자극을 주면 항원특이적인 IFN-γ, TNF-α, IL-17의 사이토카인이 각각, 또는 함께 분비됨을 확인할 수 있었다($p<0.01$).

본 연구에서는 위의 면역반응을 유도하는 MTBK_24820 단백질의 T세포 항원 결정기를 찾고자, 결핵균 H37Rv균주에서는 결손 되어있으면

서 Beijing/K 균주에만 존재하는 MTBK_24820 서열의 아미노산을 포함할 수 있도록 펩티드들을 합성하였다. Beijing/K 균주를 공기 감염 시킨 후 4주, 9주 후에 비장세포를 분리하여 각 펩티드로 자극을 주었을 때 가장 높은 IFN-γ 반응을 일으킨 2개의 펩티드(85-102, 217-234)가 강력한 T세포 항원 결정기로 제시되었다.

이처럼 국내 결핵 환자들에게서 높은 빈도로 분리되는 결핵균 Beijing/K에서 유래한 항원을 발굴하여 동물모델에서 방어면역원성을 평가하는 방법으로 특히 결핵균 Beijing/K 균주가 집단감염을 일으키는 지역에서 효과적인 결핵 백신을 개발하는데 유용한 정보를 제공할 수 있을 것으로 기대된다.

핵심되는 말: 결핵균 Beijing/K균주, PPE family, MTBK_24820, 백신, IFN-γ, IL-17

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Nam JS, Kim AR, Yoon JC, Byun Y, Kim SA, Kim KR, et al. The humoral immune response to the inactivated influenza A (H1N1) 2009 monovalent vaccine in patients with Type 2 diabetes mellitus in Korea. Diabet Med 2011;28:1541.

Park PJ, Kim AR, Salch YP, Song T, Shin SJ, Han SJ, et al. Characterization of a novel antigen of *Mycobacterium tuberculosis* K strain and its use in immunodiagnosis of tuberculosis. J Microbiol 2014;52:871-8.

Hur YG, Kim A, Kang YA, Kim AS, Kim DY, Kim Y, et al. Evaluation of antigen-specific immunoglobulin G responses in pulmonary tuberculosis patients and contacts. J Clin Microbiol 2015;53:904-9.

Hur YG, Kang YA, Jang SH, Hong JY, Kim A, Lee SH, et al. Adjunctive biomarkers for improving diagnosis of tuberculosis and monitoring therapeutic effects. J Infect 2015;70:346-55.

Hur YG, Chung WY, Kim A, Kim YS, Kim HS, Jang SH, et al. Host immune responses to antigens derived from a predominant strain of *Mycobacterium tuberculosis*. J Infect 2016;73:54-62.

Kim AR, Hur YG, Gu SW, Cho SN. Protective vaccine efficacy of the complete form of PPE39 protein from *Mycobacterium tuberculosis* Beijing/K strain in mice. Submitting to the journal of Clinical and vaccine immunology