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Characterization of a Novel Antigen of *Mycobacterium tuberculosis* K strain and Its Use in Immunodiagnosis of Tuberculosis

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***Mycobacterium tuberculosis*-specific antigens would be of great value in developing immunodiagnostic tests for tuberculosis (TB), but regional differences in molecular types of the organism may result in antigenic variation, which in turn affects the outcome of the tests. For example, the Beijing strains of *M. tuberculosis* are prevalent in East Asia, and in particular, the K strain and related strains of the Beijing family, are most frequently isolated during school outbreaks of TB in South Korea. From comparison of genome sequences between *M. tuberculosis* K strain and the H37Rv strain, a non-Beijing type, we identified a K strain-specific gene, *InsB*, which has substantial homology with the ESAT-6-like proteins. This study was, therefore, initiated to characterize the InsB protein for its immunogenicity in mice and to confirm its expression in TB patients by detecting antibodies to the protein. The *InsB* gene was cloned from *M. tuberculosis* K strain and expressed in *Escherichia coli*. The recombinant InsB protein was used for immunization of mice. All mice showed strong antibody responses to the InsB protein, and splenocytes stimulated with InsB showed strong IFN-γ and IL-17 responses and a weak IL-2 response, all of which have been implicated in disease expression and used for the immunodiagnosis of TB. Serum samples from TB patients also showed significant antibody responses to the InsB protein as compared to healthy control samples. These results indicate that the InsB protein is an *M. tuberculosis* K-strain-specific antigen that could further improve the current immunodiagnostic methods, especially for the South Korean population.**

Keywords: *Mycobacterium tuberculosis*, K strain, ESAT-6-like protein, InsB, immunodiagnosis

Introduction

Tuberculosis (TB) is a major infectious disease in the world, and its causative agent, *Mycobacterium tuberculosis,* has infected about one third of the world's population. According to the World Health Organization, there were approximately 8.8 million new cases of TB with nearly 1.1 million deaths due to the disease in 2010 (WHO, 2011). Though a majority of those infected with *M. tuberculosis* do not progress to active disease, the organisms remain in a latent phase and can re-emerge if the immune system becomes weakened (Kaufmann, 2001). In order to control such a disease effectively, rapid and accurate diagnosis of latent *M. tuberculosis* infection (LTBI) is needed for preventive therapy before reactivation.

The tuberculin skin test (TST), which has been used to detect LTBI for nearly the past century, has much room for improvement in terms of sensitivity and specificity. Over the last several decades, new developments have brought new methods for detecting of LTBI. First, assays that measure the antibody titer against *M. tuberculosis*-specific antigens have been extensively explored but are still being refined in order to increase their diagnostic sensitivity and specificity (Steingart *et al*., 2009). Secondly, assays that measure the immune response of patient peripheral blood mononuclear cells (PBMC) to *M. tuberculosis* specific antigens *ex vivo* have already been widely adopted in detecting LTBI. Measuring the IFN-γ levels in response to stimulation with *M. tuberculosis*-specific antigens has proven to be a more accurate and sensitive method to detect LTBI than TST (Walzl *et al*., 2011).

The current QuantiFERON-TB Gold kit is one of the IFN-γ release assays (IGRA). This kit requires incubation of whole blood with *M. tuberculosis*-specific antigens followed by measuring the IFN-γ concentration by ELISA. The antigens used to stimulate lymphocytes are two proteins secreted by *M. tuberculosis* known as ESAT-6 and CFP-10 (Mori *et al*., 2004; Diel *et al*., 2009). The ESAT-6 and CFP-10 proteins are parts of the ESX-1 secretion pathway, coded in the RD1 region of the *M. tuberculosis* genome. It has been shown that strains lacking these genes have a significant reduction in virulence (Simeone *et al*., 2009). In particular, the *M. tuberculosis* K strain (Kim *et al*., 2001), which belongs to the Beijing family, shows a significant up-regulation of these two proteins when compared to non-Beijing strains such as H37Rv (Shin *et al*., 2008). These findings indicate that these two well-characterized antigens could be useful in identifying K strain infections as well. Considering that the *M. tuberculosis* K strain, and its related strains (as determined by restriction fragment length polymorphism using IS6110, forming the K family), have been found more frequently than others during school outbreaks of TB in South Korea, the K family strains are likely more efficient in transmission and in fact,

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were more virulent in a mouse model of TB (Jeon *et al.*, 2012).

Recently, we completed partial genome sequence analysis of the *M. tuberculosis* K strain and identified a K strain-specific ESAT-6-like protein, InsB, within the PPE protein region, which is not found in the genome of the H37Rv strain. This study was, therefore, initiated to explore its potential use in improving current immunodiagnostic methods. The *InsB* gene was cloned into *Escherichia coli*, and its recombinant protein was prepared and used to immunize BALB/C mice along with the ESAT-6 protein. Serum samples and splenocytes from immunized mice were analyzed for their immunogenicity. In addition, the antigen candidate was used for antibody detection in serum samples from TB patients. The results indicate that InsB, specific to the *M. tuberculosis* K strain, can induce both humoral and cell-mediated immune responses and may give an additive effect to ESAT-6 in the detection of antibodies in TB patients, thus improving sensitivity of immunodiagnosis of TB.

Materials and Methods

Selection of antigen candidate

Comparison of the partial genome sequences between the K and H37Rv strains revealed a 5.7 kb insertion found only in the genome of the K strain (Fig. 1). This insertion was found in the PPE protein region. PE/PPE proteins induce a strong immune response in the host and are known to have a role in *M. tuberculosis* virulence and pathogenesis (Delogu *et al.*, 2008; Sampson, 2011). The approximately 5.7 kb insertion, called the insertion region, codes for several proteins of interest. These protein sequences were then compared to those of known immunogenic antigen markers such as ESAT-6 and CFP-10.

Cloning and expression of the InsB gene

M. tuberculosis K strain was grown in Sauton media at 37°C and its genomic DNA was prepared by using *N*-acetyl-*N*,*N*,*N*-trimethyl ammonium bromide (CTAB) buffer as described by Somerville (Somerville *et al.*, 2005). Polymerase chain reaction (PCR) was performed to amplify the InsB sequence with a primer set (InsB-F: 5′-TTGCATATGAC GATCAATT-ATCAGTTCGG-3′ including the *Nde*I site; InsB-R: 5′-GCGGATCCAGCCCAGCT-GGAACCCACT-3′ including the *Bam*HI site). The PCR product was confirmed on a 1.5% agarose gel, and sequences containing a 6× histidine tag were inserted into the pET11a_KB by incubating with *Bam*H1 and *Nde*I enzymes (NEB, USA) for 1.5 h at 37°C. After the addition of calf intestinal alkaline phosphatase, the DNA was incubated for an additional 30 min at 37°C. The DNA was run on a 1.5% agarose gel and the band was excised and extracted with an Expin Gel SV extraction kit (GeneAll, Korea). The vector and DNA inserts were combined in a 1:5 ratio, then incubated at 60°C for 5 min. Next, 1 µl 10× T4 DNA ligase buffer (NEB), 1 µl T4 DNA ligase (NEB) and distilled water were added for a final volume of 10 µl and incubated for an hour at 25°C to complete ligation. The ligated DNA was transferred to 100 µl of *E. coli* DH5α, and plasmids were then extracted using an Expin Plasmid purification kit (GeneAll).

The recombinant protein was expressed in *E. coli* BL21 (DE3) and purified with Ni-NTA resin (Qiagen, Netherlands) for histidine affinity purification. The recombinant proteins were further purified by fast protein liquid chromatography (FPLC) using an ÄKTA FPLC (GE, USA) with a MonoQ anion exchange column, in which the NaCl concentration in the Tris buffer was slowly increased to separate individual proteins. Following separation by FPLC, proteins were confirmed with SDS-PAGE for purity. Samples then underwent dialysis for refolding overnight at 4°C in a buffer containing 20 mM Tris (pH 8.0), 20 mM 2-mercaptoethanol (ME) and 5% glycerol, then transferred into a similar buffer without 2-ME and again placed at 4°C overnight while stirring. A bicinchoninic acid assay was performed to quantify the recombinant proteins. The assay was performed according to the manufacturer's instructions (Pierce, USA). Finally, proteins underwent gamma irradiation for sterilization before use in splenocyte stimulation.

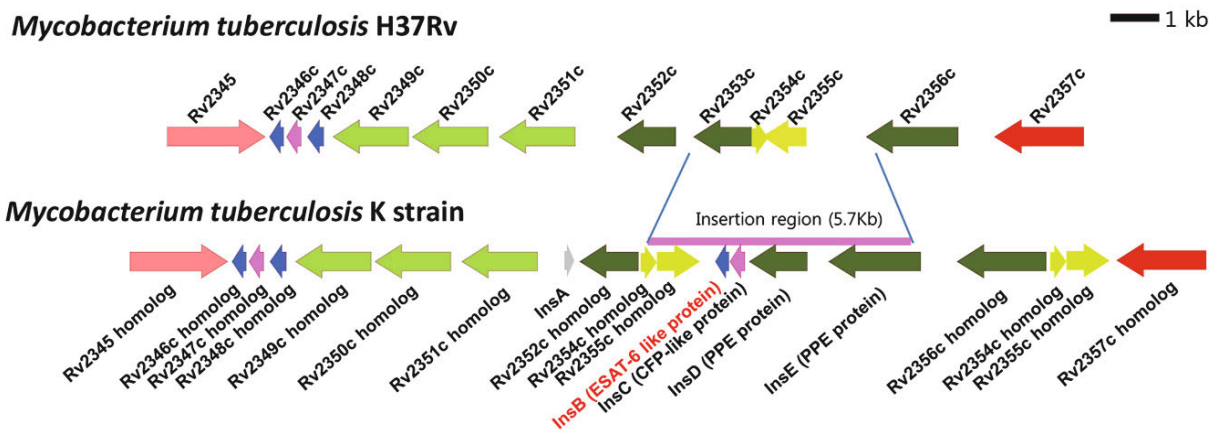

Fig. 1. Genetic map of a region containing the ESAT-6- and CFP-10-like proteins in the *M. tuberculosis* H37Rv and K strains. The 5.7 kb insertion region found only in the K strain is indicated with a purple bar. InsB is found in this insertion, while the K strain ESAT-6 protein is found outside the inserted sequence (InsB).

Immunization of mice

All mouse experiments were in accordance with IACUC guidelines at Yonsei University Health System. BALB/C mice, between 5–6 weeks old, were immunized with a dose of 20 μg of target antigen emulsified with 250 μg dimethyl dioctadecylammonium bromide (Sigma, USA) and adjuvanted with 25 μg monophospholipid A (Sigma; Guinn *et al*., 2004). Five mice were in each immunization group. Control groups, containing four mice each, were injected with either saline or the adjuvant mix only. The injections (0.2 ml/mice) were given subcutaneously in the back three times, at two-week intervals. Serum samples were collected retro-orbitally 3–4 days prior to the initial injection and two weeks after the last injection and stored at -20°C until use.

Cytokine analysis

Spleens were removed from the mice two weeks after the last injection and passed through a 40 μm cell strainer (BD Biosciences, USA). Cells were suspended in RPMI, which contained 10% fetal bovine serum and 1 unit/ml of antibiotic-antimycotic solution (Invitrogen, USA). The cells were spun down at 1,200 rpm for 5 min and the supernatant was discarded. A volume of 2 ml ACK lysis buffer (0.15 M NH_4Cl, 1 mM $KHCO_3$, 0.1 mM Na_2EDTA) was then added. After 5 min, 8 ml RPMI were added and again spun down at 1,200 rpm for 5 min. The supernatant was discarded, 5 ml RPMI were added, and again spun down. The supernatant was discarded and the cells were re-suspended in a final volume of 1 ml RPMI. A volume of 100 μl containing 1×10^6 cells was added to the wells of a 96-well plate and stimulated with either RPMI with 10% FBS or the recombinant proteins at a concentration of 5 μg/μl. A positive control group of naïve mouse splenocytes were stimulated with concanavalin A. Cells were then incubated for 48 h and the supernatant was collected and stored at -75°C. Flow cytometry was performed to quantify cytokine levels using a kit from eBioscience (USA) and a BD LSRII flow cytometer (USA).

ELISA for detection of antibodies

Plates were coated overnight at 4°C with the InsB protein at 5 μg/μl (ESAT-6 was coated at 2 μg/μl) in coating buffer (0.5 M carbonate-bicarbonate buffer). After washing with PBST, plates were blocked with PBST containing 5% NGS (PBST-NGS) and incubated at 37°C for 1 h. The plates were then emptied and incubated with mouse serum samples diluted (1:2,000) in PBST-NGS for 2 h at 37°C. The plates were washed with PBST, and peroxidase-conjugated anti-mouse IgG enzyme-labeled antibody diluted (1:10,000) in PBST-NGS was incubated for 1 h at room temperature. After several more washes, the plates were visualized using tetramethylbenzidine (KPL, USA) and the reaction was stopped with 2.5 N H_2SO_4. The absorbance was read at OD_{450}.

Serum samples, which were obtained from 86 TB patients and 40 healthy controls after obtaining informed consent based on an IRB-approved protocol, were used in this study. Serum was diluted to 1:300 for detection of antibodies, and a peroxidase-conjugated anti-human IgG enzyme-labeled antibody (Calbiochem, USA) was diluted to 1:10,000 for this study. The rest of the procedures were the same as described above.

Statistical analyses

Statistical calculations were performed using Graphpad Prism 5. Differences in B-cell response for non-immunized and immunized mice were analyzed using the paired Student's t-test. Differences between patients with active tuberculosis and healthy control patients were calculated with the Mann-Whitney test. Differences were considered significant if $P < 0.05$. The diagnostic accuracy of each of the candidates was evaluated using receiver operating characteristic (ROC) curve analysis, which correlates true and false-positive rates [sensitivity and (1 - specificity)]. In addition, the differences in the area under the curve (AUC) values were determined.

Results

Comparison of sequences of ESAT-like proteins

As described above, comparison of the genome sequences of the *M. tuberculosis* K and H37Rv strains revealed several insertions found only in the K strain. Examination of the 5.7 kb insertion found in the PPE region revealed a protein with conserved amino acid sequence compared to the ESAT6 (Rv3875) protein of the H37Rv strain (Figs. 1 and 2). The *InsB* gene was chosen for further study because of its similarity in sequence with the well-characterized ESAT-6 antigen and of its presence only in the K strain.

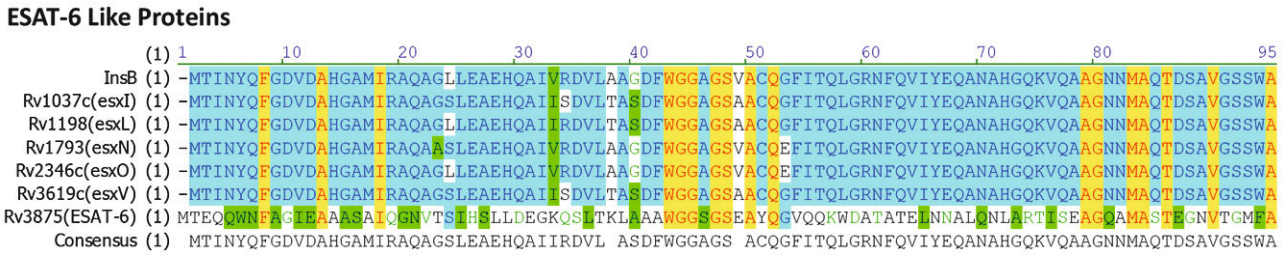

Fig. 2. Alignment of InsB compared with related proteins in the family and ESAT-6 of *M. tuberculosis* H37Rv. Sequence analysis of the K-specific InsB protein shows differences only in several amino acids when compared to other related ESAT-6-like proteins, except the ESAT-6 sequence found in the H37Rv genome (Rv3875). The alignment result of amino acid sequences between ESAT-6 and InsB showed only consensus amino acid sequence conservation in each antigen.

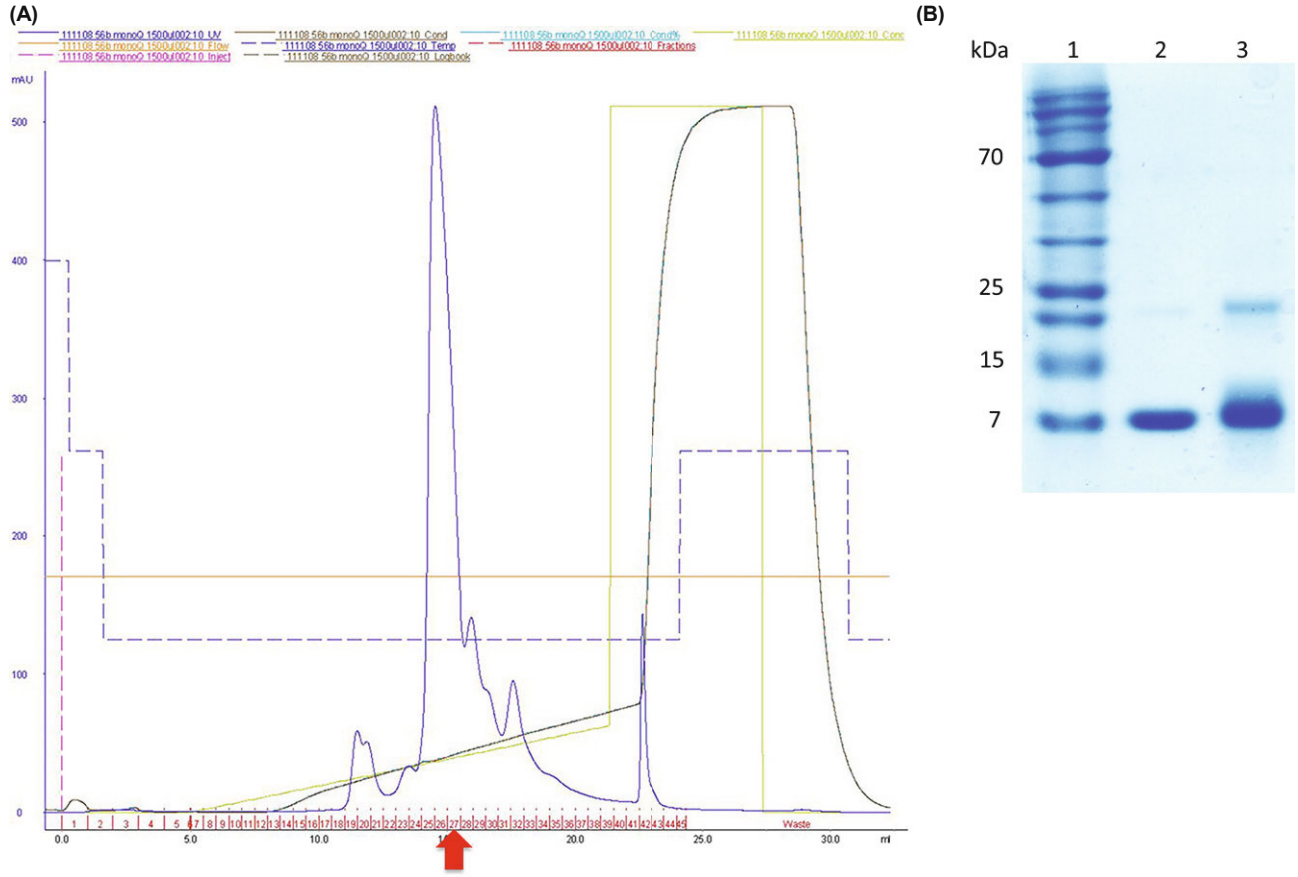

Fig. 3. Purification of recombinant InsB expressed in *E. coli* BL21. (A) Purification of antigen candidate using AKTA-FPLC with a MonoQ anion exchange column. (B) SDS-PAGE of purified recombinant InsB fractions after ÄKTA-FPLC purification. Lanes: 1, Broad-range protein marker; 2, Fraction 27 (Red arrow); 3, Fraction 28.

Characterization of the recombinant InsB protein and antibody responses in mice

The *InsB* gene was cloned into the pET11a_KB vector, and the gene in the plasmid was successfully expressed in *E. coli* BL21 (DE3) with a 6× histidine tag. The protein was then affinity purified using a Ni-NTA column followed by further purification using FPLC (Fig. 3A). SDS-PAGE analysis of the protein confirmed that the recombinant protein was approximately 6 kDa in size, similar to ESAT-6 (Fig. 3B). In order to examine immunogenicity of the recombinant proteins, two groups of mice were immunized with the InsB and ESAT-6 proteins, respectively. After three immunization

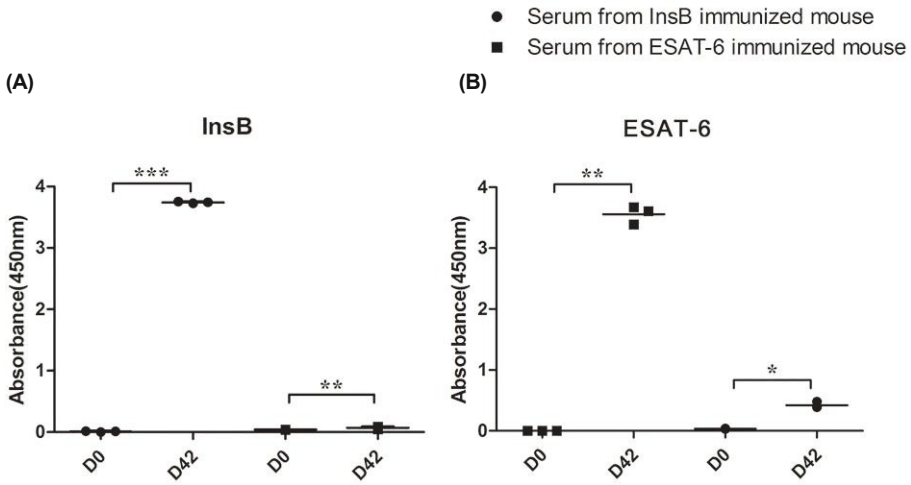

Fig. 4. IgG antibody responses determined by ELISA in BALB/c mice immunized with ESAT-6 or InsB protein. Blood was collected retro-orbitally from mice prior to immunization and two weeks after the final immunization. An enzyme-labeled anti-mouse IgG antibody was used to detect antibody levels. (** $P< 0.001$; *** $P<0.0001$).

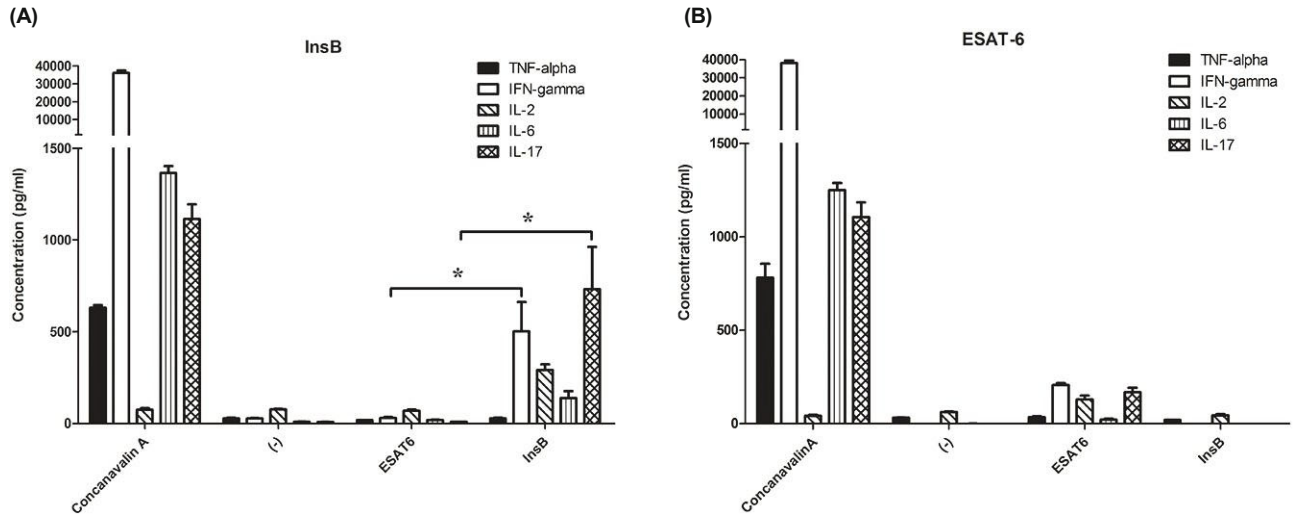

Fig. 5. Cytokine analysis of mice immunized with InsB or ESAT-6 protein. Splenocytes from mice immunized with InsB (A) or ESAT-6 (B) were stimulated with either medium (RPMI with 10% FBS), InsB, or ESAT-6, and their supernatants were analyzed by flow cytometry.

treatments over a six-week period, serum samples were obtained from mice prior to the initial immunization and approximately two weeks after the last immunization. As shown in Fig. 4, a significant level of antibodies was detected in mice immunized with ESAT-6 and with the K-specific InsB protein (P<0.01). ELISA with ESAT-6 protein and sera from InsB immunized mice, and vice versa, showed no reactivity. These result indicate that both InsB and ESAT-6 proteins are immunogenic in terms of eliciting humoral antibody responses and that they have no cross reactivity.

Cell-mediated immune responses in mice

Splenocytes from mice were prepared two weeks after the third and final immunization. Cells were stimulated with the InsB and ESAT-6 proteins for 48 h, and the supernatant was stored at -70°C until use. Supernatants were then analyzed using a multiplex cytokine analysis kit from eBioscience and a BD LSRII flow cytometer (BD Biosciences). A high level of IFN-γ and IL-17 production in response to the InsB protein was noted in mice immunized with the protein (Fig. 5A). However, there was little response to the ESAT-6 protein. Likewise, there were a moderate levels of

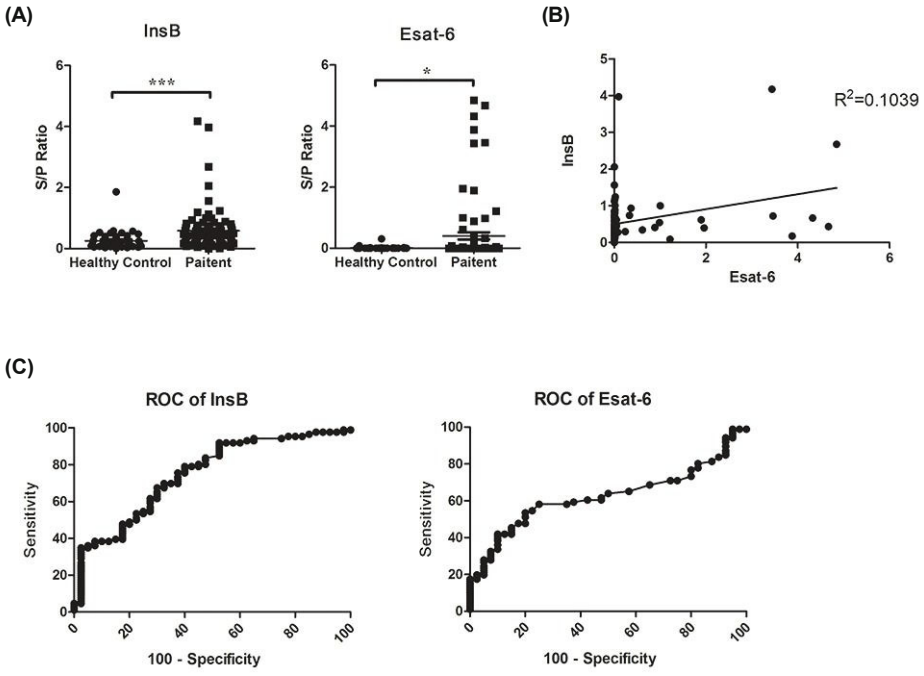

Fig. 6. Antibody responses to ESAT6 and InsB proteins in sera from TB patients and healthy controls determined by ELISA. (A) S/P ratios of IgG antibodies to ESAT-6 and InsB proteins in TB patients and healthy controls (* P < 0.05; *** P < 0.0001). (B) Dispersion diagram of correlation between S/P ratios of IgG antibodies to InsB and to ESAT-6 antigens. (C) Receiver operating characteristic (ROC) curves of ESAT-6 and InsB antigens against TB patient serum for the diagnosis of TB. ROC curves were derived by plotting the relationship between the specificity and sensitivity at various cut-off levels. The diagnostic accuracy of the test is expressed by the area under the curve (AUC). InsB had a larger AUC (0.75) than ESAT-6 AUC (0.62).

IL-17 and IFN-γ responses to the ESAT-6 protein in mice immunized with that protein, while no response was noted in the cells stimulated with InsB (Fig. 5B). Both groups of mice exhibited a low level of IL-2 response to the respective proteins but no or little TNF-α and IL-6 response were noted in the mice.

Antibody responses in TB patients

To obtain evidence of expression of the InsB protein and immune responses to the protein during *M. tuberculosis* infection in humans, serum samples from TB patients were examined for the presence of its antibodies by ELISA. These results were compared with those from healthy controls. As shown in Fig. 6A, a substantial portion of the TB patients had elevated antibodies against both the ESAT-6 and InsB proteins, thus indicating that the InsB protein was expressed and elicited immune responses like ESAT-6. In comparison, there were few healthy controls showing antibody responses to either the ESAT-6 or InsB protein. Interestingly, there was little correlation between antibody responses to the ESAT-6 and InsB proteins (Fig. 6B), indicating a marked difference in B-cell epitopes between the two proteins despite their similarity in amino acid sequences. When the results were analyzed using the Mann-Whitney test, there was a significant response for both ESAT-6 ($P < 0.001$) and InsB ($P < 0.0001$), showing greater significance of InsB than ESAT-6. The ROC curve of each antigen showed that InsB was more sensitive and more specific than ESAT-6 in the serum of a patient with active TB. The AUC value of ESAT-6 and InsB were 0.62 and 0.75, respectively (Fig. 6C).

Discussion

Identifying new antigen candidates useful for detection of *M. tuberculosis* infection is crucial for improving immunodiagnostic methods. The characterization of ESAT-6 and CFP-10 was a major step toward immunodiagnosis of LTBI. ESAT-6 is able to elicit a strong T-cell response in patients with active tuberculosis, resulting in elevated levels of IFN-γ. For both its similarity to ESAT-6 and its specificity to the K strain, the InsB protein was selected for further studies on its diagnostic potential in this study. The immunogenicity of InsB was approached from both a humoral and cellular perspective.

Previous studies differ regarding the reliability of the humoral response for serodiagnosis of *M. tuberculosis* infection. Some studies on ESAT-6 claim that the humoral response to the antigen is not a particularly accurate method of diagnosis, while others believe there is a possible use for antibody measurements as an alternative diagnostic method (Silva *et al.*, 2003; Davidow *et al.*, 2005; Greenaway *et al.*, 2005). Our results showed a significant humoral response ($P < 0.001$) with ESAT-6 in serum samples of patients with active tuberculosis compared to healthy controls. Interestingly, a more significant difference between patients with active TB and healthy controls was seen with InsB ($P < 0.0001$). Results from the ROC curve analysis demonstrated that AUC of InsB is about 1.2 fold higher than that of ESAT-6. Considering the variability in previous experiments, it is difficult to draw any conclusion on the efficacy of InsB antibody titers as a diagnostic measurement. However, the use of InsB with other antigen candidates to establish an antibody profile may prove to be a more accurate method of diagnosis and warrants further study (Davidow *et al.*, 2005). In our mouse immunization experiments, we also found a significant difference in humoral response between serum samples pre- and post-immunization for both ESAT-6 ($P < 0.01$) and InsB ($P < 0.0001$). Though the sample size was small, it seems that the mice used for the cytokine analysis experiment were successfully immunized with the antigen candidates.

The cellular immune response to InsB provides additional support for its use as a diagnostic biomarker. IFN-γ has been established as a key component in controlling *M. tuberculosis*, and up-regulation of the cytokine during infection has been well established in previous studies. Therefore, IFN-γ is the foundation for a new generation of diagnostic tests of LTBI such as the interferon gamma release assay (IGRA) and is an important marker for any new antigen candidate considered for immunodiagnosis (Flynn *et al.*, 1993; Pai *et al.*, 2004). In our experiments, mice immunized with the K strain-specific InsB antigen showed a strong IFN-γ response, even when compared to those mice immunized with ESAT-6, further supporting its potential use as a biomarker for TB.

Interestingly, InsB also showed a strong response upon stimulation of another cytokine, IL-17. In response to *M. tuberculosis* infection, a population of distinct IL-17-producing T-cells has been shown to increase. The characteristic expression of the chemokine receptor CCR7 in the absence of CD45RA by these IL-17-secreting cells suggests a possible role as long-lived memory cells (Sallusto *et al.*, 1999; Scriba *et al.*, 2008). In *M. tuberculosis* infection, the release of IL-17 by CD4+ T cells leads to inflammation, as well as the recruitment of neutrophils and granuloma formation. Down-regulation of IL-17 has been shown to decrease proper granuloma formation in mice infected with tuberculosis (Curtis and Way, 2009).

IFN-γ has been shown to have a complicated relationship with IL-17. Studies show that IFN-γ can down-regulate IL-17, thus modulating the inflammation response. It has even been suggested that a decrease in IFN-γ levels leads to increased tissue damage through the overproduction of IL-17 (Cruz *et al.*, 2006). However, IL-17 has been suggested to have a priming effect for IFN-γ secreting T cells as well. The Th1 memory response was shown to be accelerated by IL-17. In an experiment involving mice vaccinated with ESAT-6, IL-17 secreting cell recruitment preceded the recruitment of IFN-γ cells. Mice with down-regulated IL-17 had difficulty mounting an IFN-γ response, while treatment with exogenous IL-17 restored the recall response of IFN-γ cells in these mice (Khader *et al.*, 2007). Our study guesses that in combination with other cytokine markers, levels of IL-17 might be used to distinguish between active and latent TB in diagnosis, while another study indicates that there are varying levels of IL-17 secretion between drug-resistant and drug-susceptible strains (Sutherland *et al.*, 2010; Basile *et al.*, 2011). IL-17 is an important cytokine in *M. tuberculosis* infection, and a protein that can elicit an IL-17 response could contribute to a more detailed diagnosis of *M. tuberculosis* infection.

An IL-2 response was also shown in mice immunized with both the ESAT-6 and InsB proteins in this study. The function of IL-2 is that it strongly induces T cell proliferation, but also moderates the immune response by up-regulation of Treg cells (Malek and Bayer, 2004). Several papers suggest that IL-2 decreases during active disease, while levels increase as treatment progresses or in LTBI (Millington *et al*., 2007; Sargentini *et al*., 2009). Based on the release of IL-2 and IFN-γ, it would seem that the cytokine response is strongly Th1-based. Interestingly, there was little to no response in TNF-α, a Th1 cytokine that regulates type-1 activation (Zganiacz *et al*., 2004). TNF-α deficient mice infected with *M. bovis* BCG were shown to have increased tissue damage due to overproduction of IFN-γ and IL-12, while in human patients, anti-TNF-α therapy led to increased cases of reactivation (NúñezMartínez *et al*., 2001; Zganiacz *et al*., 2004). TNF-α release was higher in PBMC and macrophages from patients with active TB compared to healthy controls upon stimulation (Takashima *et al*., 1990; Ogawa *et al*., 1991). However, the study by Takashima does indicate a higher level of TNF-α release in new TB patients as compared to chronic TB patients, which might explain the decreased production of TNF-α in our mouse experiments, whose cytokine release profile was analyzed over a month after initial injection. Also, considering that the K strain is highly associated with multidrug-resistant (MDR) cases, a study looking at whole blood of patients with either MDR or drug-susceptible TB found a significant decrease in TNF-α production in the drug-resistant cases, while comparable levels of IFN-γ were released in both groups (Eum *et al*., 2008). These factors, along with the fact that our studies were conducted in a mouse model using single antigen immunization should be taken into account when comparing our results to experiments with human patients.

In summary, an ESAT-6 like K-strain-specific protein, InsB, was immunogenic in mice as well as humans. The initial characterization of InsB shows that it has potential as a biomarker for *M. tuberculosis* infection. Cytokines elicited by the protein in mice include IL-17, IFN-γ, and IL-2, which are all involved in the anti-mycobacterial response. However, our studies were conducted in a mouse model, and additional studies with human PBMC would be needed to confirm the relationship of the cytokine profile to the InsB protein in human patients. The ability to elicit a humoral response in human serum samples does illustrate, to an extent, the immunogenicity of InsB, but again further studies would be needed to establish a role for it in TB diagnostics. In conclusion, the initial characterization of InsB merits further exploration of the protein as a candidate diagnostic biomarker for *M. tuberculosis* infection and may lead to a more accurate method of diagnosis, particularly for TB patients in South Korea.

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