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An Unbiased Genome-Wide Mycobacterium tuberculosis Gene Expression Approach To Discover Antigens Targeted by Human T Cells Expressed during Pulmonary Infection

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Mycobacterium tuberculosis is responsible for almost 2 million deaths annually. Mycobacterium bovis bacillus Calmette-Guérin, the only vaccine available against tuberculosis (TB), induces highly variable protection against TB, and better TB vaccines are urgently needed. A prerequisite for candidate vaccine Ags is that they are immunogenic and expressed by M. tuberculosis during infection of the primary target organ, that is, the lungs of susceptible individuals. In search of new TB vaccine candidate Ags, we have used a genome-wide, unbiased Ag discovery approach to investigate the in vivo expression of 2170 M. tuberculosis genes during M. tuberculosis infection in the lungs of mice. Four genetically related but distinct mouse strains were studied, representing a spectrum of TB susceptibility controlled by the supersusceptibility to TB 1 locus. We used stringent selection approaches to select in vivo–expressed M. tuberculosis (IVE-TB) genes and analyzed their expression patterns in distinct disease phenotypes such as necrosis and granuloma formation. To study the vaccine potential of these proteins, we analyzed their immunogenicity. Several M. tuberculosis proteins were recognized by immune cells from tuberculin skin test-positive, ESAT6/CFP10-responsive individuals, indicating that these Ags are presented during natural M. tuberculosis infection. Furthermore, TB patients also showed responses toward IVE-TB Ags, albeit lower than tuberculin skin test-positive, ESAT6/CFP10-responsive individuals. Finally, IVE-TB Ags induced strong IFN-γ+/TNF-α+ CD8+ and TNF-α+/IL-2+ CD154+/CD4+ T cell responses in PBMC from long-term latently M. tuberculosis–infected individuals. In conclusion, these IVE-TB Ags are expressed during pulmonary infection in vivo, are immunogenic, induce strong T cell responses in long-term latently M. tuberculosis–infected individuals, and may therefore represent attractive Ags for new TB vaccines.

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Tuberculosis (TB) remains a leading cause of death, particularly in low and middle income countries (1). One third of the world population is estimated to be latently infected with Mycobacterium tuberculosis, and 3–10% of these will develop active TB during their lifetime. In HIV-infected individuals this proportion increases to 7–10% per life year. The emergence of multidrug-resistant, extensively drug-resistant, and more recently also totally drug-resistant M. tuberculosis strains is further aggravating the TB epidemic. Currently, Mycobacterium bovis bacillus Calmette-Guérin (BCG) is the only available vaccine against TB. Although BCG vaccination can prevent severe childhood TB (2), it induces highly variable and inconsistent protection against pulmonary TB, the contagious form of TB in adults (3). A more recently identified drawback of live BCG vaccination is the occurrence of disseminating BCG infections in HIV-infected children (4), similar to severe BCG infections in individuals with genetic defects in the IL-12/IL-23/IFN-γ axis (5, 6). Thus, new TB vaccines are needed that are more effective and safer than BCG.

Understanding the intracellular behavior of M. tuberculosis during in vivo infection is important not only for understanding its

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Abbreviations used in this article: B6, C57BL/6J; BCG, Mycobacterium bovis bacillus Calmette-Guérin; C3H, C3HeB/FvJ; E/C, ESAT6/CFP10 hybrid; EHR, enduring hypoxic response; HC, healthy control (donor not exposed to Mycobacterium tuberculosis); iMFI, integrated median fluorescence intensity; INH, isoniazid; Ipr1, intracellular pathogen resistance 1; IVE-TB, in vivo–expressed Mycobacterium tuberculosis; LTBI, latent tuberculosis infection; LT-LTBI, long-term latent tuberculosis infection; QFT-GIT, QuantiFERON-TB Gold In-Tube test; RGCN, relative gene copy number; RT, reverse transcriptase; ss1, supersusceptibility to tuberculosis 1; TB, tuberculosis; TST, tuberculin skin test; WBA, whole blood assay.

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infection biology, but it is also essential for the identification of possible novel TB vaccine candidate Ags. Infection stage and site-related differences in in vivo *M. tuberculosis* gene expression patterns can clearly affect the repertoire of potential *M. tuberculosis* Ags that is available for immune recognition in the primary infected organ, the lung. Ags expressed in the lungs of *M. tuberculosis*–infected, susceptible individuals could represent interesting new candidate Ags for TB vaccination, because they would induce responses capable of recognizing in situ *M. tuberculosis*–infected cells.

*M. tuberculosis* has a remarkable ability to adapt to environmental changes by altering its metabolic state. A major environmental stress factor that *M. tuberculosis* is thought to encounter during host infection is the deprivation of oxygen and nutrients. In vitro hypoxia induces the expression of the *M. tuberculosis* dormancy regulon (7), which is controlled by the master regulator DosR (*Rv3133c*). The expression of the *M. tuberculosis* DosR regulon is also induced by low-dose NO, carbon monoxide exposure, and during infection in IFN-γ–activated macrophages (7, 8). Previously, we have reported broad human T cell responses to *M. tuberculosis* DosR regulon–encoded Ags and showed that responses to these Ags were prominent and associated with latent TB infection (LTBI) in ethnically and geographically distinct populations (9–13). Other work has shown that nutrient limitation can induce the expression of specific *M. tuberculosis* genes such as *Rv2660c* (14). This gene was found to encode a “starvation” Ag with promising long-term vaccine efficacy in preclinical TB infection models, both in mice (15) and in nonhuman primates (16). The more recently described enduring hypoxic response (EHR) genes represent an alternative hypoxia-induced response model, which includes most of the DosR regulon–encoded genes complemented with an additional number (>200) of *M. tuberculosis* stress response genes (17). This model has also helped to identify new *M. tuberculosis* Ags (18).

A limitation of the models discussed above is that the identification of differentially regulated *M. tuberculosis* genes relies on in vitro models supposed to recapitulate relevant environmental stress conditions that *M. tuberculosis* encounters upon host infection. First, however, many of these environmental stress factors may not be known as yet, limiting the value of such hypothesis-driven studies. Second, there may be additive or synergistic effects between multiple stress factors in vivo that may easily be missed when studied in isolation in vitro. Third, and perhaps more importantly, certain key features of host response–induced stress cannot readily be recapitulated in vitro, including granuloma formation and TB necrosis, both being cardinal features of TB. To overcome these limitations several laboratories have started to analyze the gene expression profiles of intracellular *M. tuberculosis*, either in infected human or murine macrophages (8, 19), in the infected tissue of different mouse strains (C3H, B6) (20), or in artificial granuloma mouse models (21). However, none of these mouse models developed granulomatous necrotic TB lesions (22). We therefore have studied *M. tuberculosis* genome-wide gene expression patterns in mice strains carrying different genotypes of the supersusceptibility to TB 1 (*sst1*) locus. This genetic locus is located on chromosome 1 and controls the progression of *M. tuberculosis* infection to severe and necrotic lesions in a lung-specific manner: C3HeB/FeJ (C3H) mice carrying the susceptible *sst1* allele develop TB pneumonia with strong inflammatory responses with exudation throughout the lung and early onset of massive necrosis, whereas C57BL/6J (B6) mice carrying the resistant *sst1* allele develop smaller, interstitial granulomas without necrotic lesions that control bacterial multiplication. C3H.B6-sst1 congenic mice carrying the (B6-derived) resistant *sst1* locus on the C3H background showed increased survival after *M. tuberculosis* infection compared with the susceptible C3H mice, but less prominently than did the resistant B6 mice. Finally, *M. tuberculosis*–infected B6.C3H-sst1 mice, carrying the susceptible C3H-sst1 locus on the B6 background, develop robust granulomas that are fenced from the healthy tissue where lesions contain foamy macrophages and develop late-onset necrosis, resembling pulmonary TB in human adults. In contrast, the B6 strain does not display this phenotype, confirming the specific role for *sst1* in the control of cell death (23).

The *sst1* locus carries 22 genes, 1 of which was highly expressed in *M. tuberculosis*–infected lungs of C3H.B6-sst1 but not of hypersusceptible C3H mice. Interestingly, the expression of this gene, termed intracellular pathogen resistance 1 (*Ipr1*), decreased *M. tuberculosis* multiplication in susceptible macrophages and induced a switch from necrotic to apoptotic cell death (24). The lack of *Ipr1* expression in C3H-susceptible *sst1* locus is therefore responsible for the development of lung-specific necrosis upon *M. tuberculosis* infection (25). The closest human homolog of *Ipr1* is SP110b. The expression of both *Ipr1* and SP110b is regulated by IFNs, indicating a role in immunity (26–28). Genetic association studies performed in West Africa identified three polymorphisms in the SP110b gene that were associated with genetic susceptibility to TB (29). However, a number of other studies performed in Ghana, Russia, South Africa, and Indonesia did not replicate this finding (30–33). A SP110b homolog was also identified in cattle, which correlated to susceptibility to *Mycobacterium avium* ssp. *paratuberculosis* (34).

These four (congenic) mouse models we have used in this study show a spectrum of TB susceptibility that ranges from highly susceptible (C3H) to resistant (B6) mice, with the development of necrotic lesions depending on the *sst1* locus and the modifying genetic background in which the locus is expressed. This mouse model replicates key features of human *M. tuberculosis* infection. In this study, we have taken advantage of this disease spectrum and 1) analyzed quantitative real-time expression patterns of all *M. tuberculosis* genes predicted to be the first gene in each operon, in the lungs of *M. tuberculosis*–infected mice, aiming to identify the *M. tuberculosis* genes that are highly or differentially expressed in the lung during in vivo infection (in vivo–expressed *Mycobacterium tuberculosis* [IVE-TB] genes); 2) compared these *M. tuberculosis* gene expression patterns between susceptible (B6.C3H-sst1 and C3H) and resistant (C3H.B6-sst1 and B6) mouse strains in an attempt to correlate expression patterns to infection phenotype; and 3) selected a set of the most consistently expressed *M. tuberculosis* genes, produced these as recombinant proteins, and analyzed their immunogenicity in tuberculin skin test (TST) healthy, TB–affected individuals as well as long-term LTBI (HILTBI) as a first step toward their validation as new TB vaccine candidate Ags.

**Materials and Methods**

**Mouse strains**

C3H and B6 mice were purchased from The Jackson Laboratory (Bar Harbor, ME). Congenic C3H.B6-sst1 and B6.C3H-sst1 mouse strains carrying the resistant and susceptible alleles of the *sst1* locus, respectively, were generated as previously described (24, 34). Briefly, an ~20-cM segment of chromosome 1, containing the *sst1* locus, was introgressed in the opposite background strain via ≥10 backcrosses. Mice were bred and housed under specific pathogen-free conditions at the Harvard Medical School of Public Health.

**Bacterial strains**

*M. tuberculosis* suspensions were used as previously described (36). In short, *M. tuberculosis* (Erdman strain; Trudeau Institute, Saranac Lake,
NY) cultures were grown to midlog phase in Middlebrook 7H9 medium (BD Biosciences, Franklin Lakes, NJ) (10% oleic acid/albumin/dextrose/catalase [OADC; Difco], 0.05% Tween 80 [Sigma-Aldrich], and 0.5% glycerol [Sigma-Aldrich]). Bacteria were washed and stored at ~80°C. Prior to infection, bacteria were thawed, sonicated, and diluted in PBS to 10^8 CFU/ml.

**Infection of mice**

Mice were infected by aerosol with 25–50 CFU M. tuberculosis using a Madison chamber (College of Engineering shops at the University of Wisconsin, Madison, WI) with n = 2 per time point (24). B6 and C3H mice were sacrificed both 6 and 9 wk postinfection, whereas B6.C3H-sstl and C3H.B6-sstl mice were sacrificed 9 and 6 wk postinfection, respectively. For the reactivation model, B6 and B6.C3H-sstl mice were infected i.v. via the tail vein with 5 × 10^6 CFU M. tuberculosis per mouse as previously described (23). Twelve weeks after challenge the mice were given isonit- azid (INH) supplied via the drinking water (10 mg/100 ml) for 90 d. Mice were sacrificed 8 wk after INH treatment withdrawal.

**Genome-wide M. tuberculosis transcription profiling via a two-step multiplex real-time RT-PCR**

Quantification of M. tuberculosis mRNA gene expression was performed as previously described (15, 37, 38). The protocol is based on first-strand cDNA synthesis and controlled multiplex amplification of cDNAs, which is followed by individual real-time PCR (TaqMan) quantification of amplified cDNAs in a 384-well format using a LightCycler 480.

Total M. tuberculosis RNA was isolated from the infected mouse lung tissue by homogenization in TRIZol and bacillary disruption by bead beating (MP Biomedicals, Solon, OH). Total RNA was isolated using RNeasy columns (Qiagen, Valencia, CA). RNA was precipitated, cleaned with two consecutive off-column RQI DNase digestions (Promega, Madison, WI), and resuspended in 50 μl RNase-free water (Applied Biosystems/ Ambion, Austin, TX).

cDNA synthesis was performed using 50 ng total RNA, which was separated in reverse transcriptase (RT*) and RT reactions to control for DNA contamination. Exo-resistant random primer (0.5 μl, 1 μl 10 mM dNTPs, and nuclease-free water was added and incubated for 3 min at 70°C in a thermal cycler. Subsequently, 4 μl 5× Maxima RT buffer, 0.5 μl RiboLock RNase inhibitor, 0.5 μl Maxima RT enzyme (replaced by water for RT control samples) (all Fermentas, Glen Burnie, MD), and nuclease-free water were added and incubated at 50°C for 1 h, 95°C for 2 min to inactivate, and then kept at 4°C.

The generated cDNAs were further amplified via controlled multiplex preamplification with a mix of 2179 M. tuberculosis gene-specific primers (23.8 μl primer mix: ~50 μM per amplification reaction). 2 μl cDNA, 3 μl 10X Advantage 2 buffer (Clontech, Mountain View, CA), 0.6 μl 1× Advantage 2 polymerase mix (Clontech, CA), and 0.6 μl 10 mM dNTPs (Fermentas) to a volume of 30 μl (ftp://smd-ftp.stanford.edu/tbdb/rtpcr/taqman oligos.fa). (15). Sequences and design of PCR primer/probe sets are available at: http://genes.stanford.edu/technology.php and http://www. tdbb.org/rtpcrData.shtml. A comparative control of 100 pg (2 × 10^6 gene copy number) genomic H37Rv DNA was also included. As an additional control, 25 M. tuberculosis reference genes were used to control for variation across amplification mixes. The gene primer sets were designed using Primer Express software (PerkinElmer, Foster City, CA) to cover at least one gene of each predicted M. tuberculosis operon. Each reaction was heated at 95°C for 5 min, followed by 15 cycles at 95°C for 30 s, 60°C for 20 s, and 68°C for 1 min. Previously we have validated conditions for multiplex PCR preamplification via linearity of amplification assay using all of the genes used in the assay. We also validated all individual TaqMan assays from our collection for sensitivity and linearity before we started using them in gene expression profiling in this study.

Individual gene transcript quantification was carried out using TaqMan primer/probe sets (Biosearch Technologies). Quantitative real-time PCR mix contained 0.07 μl preamplified cDNA, 2 μl TaqMan primer/probe mix, 5 μl 2× LightCycler 480 Probes Master Mix, and 2.93 μl Probes Master PCR-grade water (Roche) to a final volume of 10 μl. Reactions were heated at 95°C for 5 min, followed by 40 cycles at 95°C for 30 s and 60°C for 20 s. A cool down step of 40°C for 30 s was run for one cycle. Cycle threshold values were converted to relative gene copy numbers (RGCN) based on logarithmic transformation/linear regression equations devised from calibration curves. The data set is available at: http://www. tdbb.org/pubdata/tdbb/publications/Raw-Data-Harvard-Mice.xls.

Correction for some biological heterogeneity between the different mice and mouse strains such as differences in bacterial load was not possible because these were inherent to the extensive differences in genetic TB susceptibility.

**IVE-TB gene selection procedure**

First, genes were selected that were expressed in one data set (>1000 RGCN) but not in the other data set (+/-), thus selecting M. tuberculosis genes that are differentially expressed owing to genetic host susceptibility and/or infection phenotype variations.

The second approach was to select genes that were highly expressed in both data sets from two different mouse strains in the chosen comparison (+/+), selecting for M. tuberculosis genes that are expressed independent of the genetic makeup of the host. For this selection, the RGCN data were ranked from the highest to the lowest value and overlapping genes were selected from the top 100 highest expressed genes of both data sets. This number of 100 genes was arbitrarily chosen to limit the number of candidates to be analyzed further.

The third and last approach included genes that were expressed in data set 2 (>1000 RGCN) but not in data set 1 (-/+), following the same rationale as approach 1. Hence, approaches 1 and 3 included differentially expressed genes. There was no number restriction limit (because fewer genes were identified compared with the second approach [+/-]). (Figs. 1, 2).

**Recombinant proteins**

Recombinant proteins were produced from the selected M. tuberculosis genes as described previously (39). Briefly, M. tuberculosis genes were amplified by PCR from genomic H37Rv DNA and cloned by Gateway technology (Invitrogen, Carlsbad, CA) in a bacterial expression vector containing a histidine tag at the N terminus. Vectors were overexpressed in Escherichia coli BL21 (DE3) and purified. Size and purity of recombinant proteins were analyzed by gel electrophoresis and Western blotting with an anti-His Ab (Invitrogen) and an anti-E. coli polyconal Ab (gift of Statens Serum Institute, Copenhagen, Denmark). RV2380c, RV2453c, and RV2737c proteins were prepared as two or three recombinant protein fragments owing to their large sizes (C, middle [M], and N termini). Endotoxin contents were <50 EU/μg as tested using a Limulus amebocyte lysate assay (Cambrex, East Rutherford, NJ). All recombinant proteins were tested in lymphocyte stimulation assays to exclude Ag nonspecific T cell stimulation and cellular toxicity using PBMC of in vitro–purified protein derivative of M. tuberculosis−negative healthy Dutch donors (Sanquin Blood Bank, Leiden, The Netherlands) (12, 40−42). Purified protein derivative of M. tuberculosis was purchased from Statens Serum Institute.

**FIGURE 1.** Overview of IVE-TB gene selection procedure. (A) The M. tuberculosis RGCN profiles of each mouse model were independently compared with each other. (B) Three gene selection procedures were used to select genes for each comparison: genes that were expressed in data set 1 (>1000 RGCN) but not in data set 2 (+/-); genes highly expressed in both data sets (top 100 highest expressed genes in both models and select overlapping genes) (+/+); and genes not expressed in data set 1 but expressed in data set 2 (>1000 RGCN) (-/+). (see also Materials and Methods).
Lymphocyte stimulation test
PBMC (1.5 × 10^6/well) were cultured in triplicate in 96-well round-bottom plates (Nunc) and incubated with or without protein (10 μg/ml) in IMDM (Life Technologies/Invitrogen) containing 10% pooled human serum (Invitrogen) at 37°C in 5% CO2. After 6 d, supernatants were harvested, pooled, and stored at −20°C for future use in IFN-γ ELISAs.

IFN-γ ELISAs
IFN-γ concentrations in supernatants were measured by a standard ELISA technique (U-CyTech, Utrecht, The Netherlands). ELISA samples were tested in duplicate and the assay was performed according to the manufacturer’s guidelines. Detection limit of the assay was set arbitrarily at 20 pg/ml for whole blood assay (WBA) and 100 pg/ml for a lymphocyte stimulation test.

Flow cytometric analysis
PBMC (1–2 × 10^6/tube) were thawed and rested overnight and subsequently stimulated for 16 h with protein (10 μg/ml) in the presence of costimulatory Abs anti-CD28 and anti-CD49d (Sanquin and BD Biosciences, respectively). Brefeldin A (3 μg/ml; Sigma-Aldrich) was added after the first 4–6 h. Cells were stained with Live/Dead fixable violet dead cell stain (VIVID; Invitrogen) to discriminate between live and dead cells according to manufacturer’s instructions. Cells were stained for 1 h at 4°C with the following surface markers: anti-CD3 PE-Cy5 (BD Biosciences), anti-CD4 Texas Red (Caltag), and anti-CD19 Pacific Blue (BD Biosciences). Additionally, anti-CD14 Pacific Blue and anti-CD19 Pacific Blue (both Invitrogen) were included to select for CD14+ and CD19+ live cells. Intracellular staining was performed with anti–IFN-γ Alexa 700 (BD Pharmingen), anti–TNF-α PE-Cy7 (BD Biosciences), anti–IL-2 PE (BD Pharmingen), and CD154 allophycocyanin-Alexa 780 (eBioscience) using the ADG Fix&Perm kit (An Der Grub Bio Research, Vienna, Austria).

Data were acquired on a BD LSRFortessa (BD Biosciences) and analyzed using FlowJo version 7.6.5 (Tree Star, Ashland, OR). Single CD14+/CD19−/CD3+ donors: A and B. Nonsusceptible donors: A, B, C, and D. Control donors: 1) the average of donors with ≥10 resistant donors and/or travel to high endemic countries (n = 76). TST− individuals entered a follow-up study at recruitment. Blood samples were collected by venipuncture. M. tuberculosis–unexposed donors were included as healthy control (HC) donors (n = 11). PBMC from TST− donors and treated TB patients (n = 7) were used in lymphocyte stimulation assays. PBMC from IltLTBI (41, 42) (n = 6) were used for polychromatic flow cytometry assays. Informed consent was obtained prior to venipuncture. The study protocols (P07.048 and P027/99) were approved by the Institutional Review Board of the Leiden University Medical Center and the Regional Committees for Medical and Health Research Ethics in Norway.

Whole blood assay
Blood was diluted 1:10 in AIM-V medium (Invitrogen, Breda, The Netherlands), incubated in 48-wells plates (Nunc, Roskilde, Denmark), and cultured with or without recombinant protein (10 μg/ml), PHA (2 μg/ml), or M. tuberculosis lysate (5 μg/ml) at 37°C, 5% CO2. After 6 d supernatant was harvested and stored at −20°C for use at a later stage.

Study subjects
One hundred thirty-three donors were selected that responded to M. tuberculosis–purified protein derivative by TST (weak positive, 5–11 mm; average, 16 mm) and that had documented exposure to a TB index case (n = 76). TST− individuals entered a follow-up study at recruitment. A QuantiFERON-T B Gold In-Tube test (QFT-GIT) was performed (Cellestis, Carnegie, VIC, Australia). The test was considered positive when there were $>$ 0.3 IU/ml. Blood samples were collected by venipuncture. M. tuberculosis–unexposed donors were included as healthy control (HC) donors (n = 11). PBMC from TST− donors and treated TB patients (n = 7) were used in lymphocyte stimulation assays. PBMC from IltLTBI (41, 42) (n = 6) were used for polychromatic flow cytometry assays. Informed consent was obtained prior to venipuncture. The study protocols (P07.048 and P027/99) were approved by the Institutional Review Board of the Leiden University Medical Center and the Regional Committees for Medical and Health Research Ethics in Norway.

M. tuberculosis lysate
M. tuberculosis H37Rv organisms were grown to a late log phase in a culture flask at 37°C and collected in a V-bottom tube. The pellet was washed twice with PBS and heat killed for 30 min at 80°C. The cell suspension was subsequently collected in a BioSpec tube containing 0.1-mm glass beads. The bacteria were disrupted using a MiniBeadBeater (BioSpec Products). Concentrations of lysates were measured using the BCA assay (Thermo Scientific Pierce).

Results
Identification of IVE-TB genes in the lungs of genetically resistant and/or susceptible mice
To start identifying novel candidate M. tuberculosis Ags in an unbiased and M. tuberculosis genome-wide fashion, we analyzed the gene expression patterns of 2170 M. tuberculosis genes, most of which represent the first gene of each predicted M. tuberculosis operon, in the lungs of four different M. tuberculosis–infected mouse strains (B6, C3H, B6.C3H-sst1, and B6.C3H-sst1) that show a spectrum of TB susceptibility (Table I). The RGCN were determined using quantitative PCR (15). This allows for absolute quantification of the level of transcripts per sample, because the data are normalized against a standard reference gene number copy (as described in Materials and Methods).

In this IVE-TB gene selection screen we used the following criteria to select candidate genes for further analysis. First, we used the most strongly upregulated M. tuberculosis genes in all four analyzed mouse models. This group of genes includes genes that are expressed independently of the host susceptibility background. Second, we used M. tuberculosis genes specifically upregulated in either B6, C3H, B6.C3H-sst1, or C3H.B6-sst1. The expression of these genes is influenced by the host genetic background and may therefore include genes whose expression is associated to particular TB disease characteristics such as granuloma formation and...
necrosis. The obtained RGCN of all four mouse models were independently compared with each other as visualized in Fig. 1A. All four mouse models were infected with a low-dose aerosol \textit{M. tuberculosis} inoculum. Additionally, a subset of B6.C3H-sstl was infected using a previously described TB relapse model to study features of gene expression during \textit{M. tuberculosis} reactivation (23).

Every individual \textit{M. tuberculosis} gene’s expression data (e.g., B6 versus C3H, B6 versus B6.C3H-sstl) was subjected to three different selection approaches as indicated in Fig. 1B and described in detail in \textit{Materials and Methods}. The gene selection results for each comparison are visualized in Fig. 2 and Supplemental Table I. The results shown in Fig. 2 were then used to select IVE-TB genes whose expression was associated with particular disease characteristics as indicated in Fig. 3. These included 1) \textit{M. tuberculosis} genes highly expressed independently of host genetic background (expressed in all four mouse models; these \textit{M. tuberculosis} genes included esxA encoding ESAT6 and other esx genes); 2) \textit{M. tuberculosis} genes expressed in association with necrosis (expressed in both C3H and B6.C3H-sstl, but not in B6 or C3H.B6-sstl); 3) \textit{M. tuberculosis} genes expressed in association with severe necrotic infection or susceptibility (expressed in C3H, but not in B6, C3H.B6-sstl, or B6.C3H-sstl); 4) \textit{M. tuberculosis} genes expressed in association with dense granuloma development (only expressed in B6.C3H-sstl but not in C3H, B6, or C3H.B6-sstl); 5) \textit{M. tuberculosis} genes expressed in association with diffuse granuloma development (expressed in C3H but not in B6, C3H-sstl, B6, or C3H.B6-sstl); 6) \textit{M. tuberculosis} genes expressed in association with resistance (expressed in every more resistant mouse strain per comparison); 7) \textit{M. tuberculosis} genes expressed in association with low inflammation (expressed in B6, but not in C3H.B6-sstl); 8) inflammation (expressed in C3H.B6-sstl, but not in B6); and 9) relapse (expressed in i.v. \textit{M. tuberculosis}-infected, INH-treated B6.C3H-sstl, but not low-dose aerosol–infected B6. C3H-sstl). An overview of the resulting IVE-TB genes is presented in Supplemental Table II.

\textbf{Immunogenicity of newly identified IVE-TB Ags}

\textit{Further down selection of IVE-TB genes}. The goal of the above selection of IVE-TB genes was to identify potentially interesting new vaccine candidate Ags. Thus, we next determined their immunogenicity. To this end, a number of \textit{M. tuberculosis} genes were further selected that were either present in more than one group or were among the top number of genes in the IVE-TB selections performed (Table II). Some \textit{M. tuberculosis} genes identified using our unbiased genome-wide approach were also identified in previous studies as environmental stress induced proteins (7, 14, 17).

A subsequent literature search revealed that almost all further selected IVE-TB proteins (14 of the 16) have been identified previously in \textit{M. tuberculosis} proteomic studies, confirming the protein expression of the \textit{M. tuberculosis} genes identified in our study (44–57) (Table III). Indeed, we observed that \textit{M. tuberculosis}-

\begin{table}[h]
\centering
\begin{tabular}{|c|c|c|c|c|c|}
\hline
Mouse Strain$^a$ & Genetic Background & \textit{Sst} Allele & Inflammation & Lung Necrosis & Clinical TB Correlate \\
\hline
C3H & C3H & Susceptible & +++ & + (Early) & Caseous pneumonia \\
C3H.B6-sstl & C3H & Resistant & +++ & – & Progressive interstitial granuloma without necrosis \\
B6.C3H-sstl & B6 & Susceptible & ++ & + (Late) & Causative granuloma without necrosis \\
B6 & B6 & Resistant & + & – & Chronic persistent interstitial granuloma without necrosis \\
\hline
\end{tabular}
\caption{Mouse strains, genetic background, and TB infection phenotypes}
\end{table}

\footnotesize
$^a$Pichugin et al. (23) and Pan et al. (24).

\begin{figure}[h]
\centering
\includegraphics[width=\textwidth]{figure3}
\caption{Selection of IVE-TB genes associated with particular TB disease characteristics. Flowchart of analysis to identify IVE-TB genes related to TB disease phenotypes is shown. Letters in graphs refer to the specific selections indicated in Fig. 2. \textit{M. tuberculosis} genes highly expressed independent of host genetic background are presented in Fig. 2A (a), 2B (e), 2C (h), 2D (k), 2E (n), and 2F (q). \textit{M. tuberculosis} genes highly expressed in association with necrosis are presented in Fig. 2A (c), 2B (f), 2D (j), and/or 2F (p). \textit{M. tuberculosis} genes highly expressed in association with severe necrotic infection or susceptibility are presented in Fig. 2A (c), 2D (j), and/or 2C (i). \textit{M. tuberculosis} genes highly expressed in association with dense granuloma are presented in Fig. 2C (g), 2B (f), and/or 2F (p). \textit{M. tuberculosis} genes highly expressed in association with diffuse granuloma are presented in Fig. 2C (i), but also Fig. 2A (c) and 2D (j). \textit{M. tuberculosis} genes highly expressed in association with resistance are presented in one or more of the following selections: Fig. 2A (a), 2B (d), 2D (l), and/or 2F (m). \textit{M. tuberculosis} genes highly expressed in association with relapse are presented in Fig. 2E (o). \textit{M. tuberculosis} genes highly expressed in association with relapse-associated genes are presented in Fig. 2G (u).}
\end{figure}
infected C3H mice recognized most of the selected IVE-TB Ags as measured by Ag-specific IFN-γ production by splenocytes (Supplemental Fig. 1). Furthermore, we analyzed the conservation of these IVE-TB proteins using protein BLAST searches on different *M. tuberculosis* strains as well as other mycobacterial species. This showed that the IVE-TB protein sequences are strongly conserved among all tested *M. tuberculosis*, *M. bovis*, and *Mycobacterium africanum* strains. Strong conservation was observed also for other mycobacterial strains (Table IV).

### Table II. Predicted function and classification of selected IVE-TB genes

<table>
<thead>
<tr>
<th>Gene</th>
<th>Name</th>
<th>IVE-TB Selection</th>
<th>Selections</th>
<th>Function</th>
<th>Category</th>
<th>Classification</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>Rv1284</td>
<td>Rv1284</td>
<td>High expression</td>
<td>b + c + h + k + n + q + t</td>
<td>Conserved hypothetical protein (carbonic anhydrase)</td>
<td>7</td>
<td>EHR/starvation</td>
<td>14, 17, 43</td>
</tr>
<tr>
<td>Rv2380c</td>
<td>mbtE</td>
<td>High expression</td>
<td>b + c + h + k + n + q + t</td>
<td>Peptide synthetase mbtE</td>
<td>1</td>
<td></td>
<td>21</td>
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<tr>
<td>Rv3515c</td>
<td>fadD19</td>
<td>High expressed ssl1</td>
<td>h + q + t</td>
<td>Probable fatty acid-CoA ligase fadD19 (involved in lipid degradation)</td>
<td>1</td>
<td>EHR</td>
<td>17, 21</td>
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<tr>
<td>Rv0079</td>
<td>Rv0079</td>
<td>Necrosis and sni</td>
<td>c + f + j + p + c + j + u</td>
<td>Hypothetical protein</td>
<td>10</td>
<td></td>
<td>7, 9, 12, 20, 21</td>
</tr>
<tr>
<td>Rv2324</td>
<td>Rv2324</td>
<td>sni and relapse</td>
<td>c + j + u</td>
<td>Probable transcriptional regulator, asnC family</td>
<td>9</td>
<td>EHR</td>
<td>17</td>
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<tr>
<td>Rv2737c</td>
<td>recA</td>
<td>Necrosis and sni</td>
<td>c + f + j + p</td>
<td>Recombination protein recombinase A (recA; <em>M. tuberculosis</em> recA intein)</td>
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<td></td>
</tr>
<tr>
<td>Rv2838c</td>
<td>rfbA</td>
<td>sni, diffuse granuloma, and relapse</td>
<td>c + i + j + u</td>
<td>Probable ribosome-binding factor A (P15B protein)</td>
<td>2</td>
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</tr>
<tr>
<td>Rv3420c</td>
<td>rimI</td>
<td>sni, diffuse granuloma, and relapse</td>
<td>c + i + j + u</td>
<td>Ribosomal-protein-alanine acetyltransferase rimI arsR type repressor protein</td>
<td>2</td>
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<td></td>
</tr>
<tr>
<td>Rv2034</td>
<td>Rv2034</td>
<td>Resistance, diffuse granuloma, low inflammation, and relapse</td>
<td>o + i + j + m + u</td>
<td>Transcriptional regulator (possible antitoxin; TA operon with Rv1955)</td>
<td>0</td>
<td>EHR/starvation</td>
<td>14, 17, 20</td>
</tr>
<tr>
<td>Rv1956</td>
<td>Rv1956</td>
<td>Resistance, diffuse granuloma, low inflammation, and relapse</td>
<td>o + i + j + m + u</td>
<td>Transcriptional regulator (possible antitoxin; TA operon with Rv1955)</td>
<td>0</td>
<td>EHR/starvation</td>
<td>14, 17, 21</td>
</tr>
<tr>
<td>Rv2225</td>
<td>panB</td>
<td>Dense granuloma</td>
<td>f + g + p</td>
<td>Probable 3-methyl-2-oxobutanoate hydroxymethyltransferase (panB)</td>
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<td>21</td>
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<tr>
<td>Rv2465c</td>
<td>rpiB</td>
<td>Dense granuloma</td>
<td>f + g + p</td>
<td>Isomerase (ribose 5-phosphate isomerase)</td>
<td>7</td>
<td>EHR</td>
<td>17, 20</td>
</tr>
<tr>
<td>Rv2982c</td>
<td>gpsA</td>
<td>Resistance, inflammation, and relapse</td>
<td>l + o + r + u</td>
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<td>Rv3353c</td>
<td>Rv3353c</td>
<td>Relapse</td>
<td>u</td>
<td>Conserved hypothetical protein</td>
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<td>Rv1363c</td>
<td>Resistance</td>
<td>s</td>
<td>Possible membrane protein</td>
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<td>Rv2435c</td>
<td>Rv2435c</td>
<td>Resistance and inflammation</td>
<td>l + o</td>
<td>Probable cyclase (adenylate or guanylate cyclase)</td>
<td>7</td>
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<td></td>
</tr>
</tbody>
</table>

*a*In reference to Figs. 2 and 3.


*c*TubercuList functional classification codes are available at: http://genolist.pasteur.fr/tuberculist.

sni, Severe necrotic infection.

### Table III. Identification of IVE-TB proteins in *M. tuberculosis*

<table>
<thead>
<tr>
<th>Gene</th>
<th>Protein Name</th>
<th>Protein Location</th>
<th>Membrane/Lipid</th>
<th>Cytosol</th>
<th>CF</th>
<th>WCL</th>
<th>Essential In Vitro</th>
<th>Essential In Vivo</th>
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<td>Rv0079</td>
<td>Rv0079</td>
<td>49, 51, 52</td>
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<td>Rv1284</td>
<td>Rv1284</td>
<td>45–48, 51</td>
<td>X X</td>
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<td>Yes (54)</td>
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<tr>
<td>Rv1363c</td>
<td>Rv1363c</td>
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<td>Rv1956</td>
<td>Rv1956</td>
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<td>X X</td>
<td>No (53, 55)</td>
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<td>Rv2034</td>
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<td>X</td>
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<td>Rv2225</td>
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<td>Rv3515c</td>
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</table>

Note that the locations of the proteins indicated may not be exclusive given limitations and difficulties in annotating exact protein localization (46).

CF, Culture filtrate; NT, not tested; WCL, whole cell lysate.
### Table IV. IVE-TB protein sequence identity among mycobacterial strains

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<thead>
<tr>
<th>Species</th>
<th>Strain</th>
<th>Rv0079</th>
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<th>Rv1956</th>
<th>Rv2034</th>
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<th>Rv2324</th>
<th>Rv2380c</th>
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<th>Rv2465c</th>
<th>Rv2737c</th>
<th>Rv2838c</th>
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</table>


*Note: The Journal of Immunology* 1665

The table shows the sequence identity of IVE-TB proteins among various mycobacterial strains. The data are presented as percentages, indicating the similarity in protein sequence between different mycobacterial species and strains. The table includes columns for different species and strains of *Mycobacterium*, with rows indicating the sequence identity for each protein. The highest identity is 100%, indicating a perfect match, while lower values show decreasing similarity. The table highlights the specific proteins and species that exhibit high or low sequence identity, aiding in the identification and understanding of immune responses to *Mycobacterium* infection.
FIGURE 4. IFN-γ responses to IVE-TB Ags in E/C+ (A), E/C− (B), and HC donors (C). A total of 43 E/C+ donors (A), 90 E/C− donors (B), and 11 HC donors (C) were analyzed for their IFN-γ WBA responses to Ags and controls; the Ags were *M. tuberculosis* Rv1284, Rv1956, Rv2034, Rv2324, Rv3353c, and Rv3420c. Ten E/C+ donors (A), 36 E/C− donors (B), and 9 HC donors (C) were also analyzed for responses to *M. tuberculosis* Ags Rv0079, Rv1363c, Rv2225, Rv2380c, Rv2435c, Rv2465c, Rv2737c, Rv2828, Rv2982, and Rv3515c IFN-γ. The proportion of responders for each Ag is indicated at the top of the graph. For comparative purposes, medium background values were subtracted for each response in each donor. Horizontal bars represent the median IFN-γ responses. The dotted line indicates the cut-off value for positivity, arbitrarily calculated as 3 × medium value.

T cell responses toward IVE-TB Ags in long-term latent *M. tuberculosis*-infected individuals. Because the IVE-TB Ags were strongly recognized by TST+ individuals, we subsequently analyzed the immune responses toward the seven best recognized Ags in more detail using PBMC from donors that had been exposed to *M. tuberculosis* decades ago, but had never developed TB despite the lack of any preventive treatment, designated ltLTBI (41, 42). Of additional importance, the availability of several vials of PBMC also allowed more detailed cell subset analysis.

Interestingly, high frequencies of TNF-α- and IL-2–producing CD4+ T cells were observed after stimulation with the IVE-TB Ags, whereas only intermediate frequencies of IFN-γ–producing CD4+ T cells were detected (Fig. 7A). In contrast, high frequencies of IFN-γ–producing as well as TNF-α+ CD8+ T cells were present in these donors, whereas fewer IL-2+ T cells were detected compared with CD4+ T cells. Besides IFN-γ, TNF-α, and IL-2, also the Ag-induced CD4+ T cell activation marker CD154 (58) was expressed.

More detailed analysis of the multifunctional Th1 responses among CD4+ and CD8+ T cells showed that CD154+CD4+ T cells were mostly TNF-α+/IL-2– and TNF-α– (Fig. 7B). Furthermore, intermediate frequencies of IFN-γ+/TNF-α+/IL-2– CD154+CD4+ T cells were detected. Finally, a CD154– population was detected, producing none of the IFN-γ, TNF-α, and IL-2 cytokines. Intriguingly, the same pattern was observed for every IVE-TB Ag or E/C control Ag. Furthermore, interindividual variation of Ag recognition was observed. Remarkably, few TNF-α+/IL-2–CD8+ T cells were detected compared with TNF-α+/IL-2–CD4+ T cells. IFN-γ+/TNF-α–CD8+ T cells were the most prominent population present, followed by TNF-α– CD8+ T cells. Also, intermediate IFN-γ+/TNF-α+/IL-2– and IFN-γ– CD8+ T cells were observed. Again, as mentioned for CD4+ T cells, the same patterns were observed for every Ag within the CD8+ T cell population as well as interindividual variation of Ag recognition.

The integrated median fluorescence intensity (iMFI) was calculated to determine the quantitative contribution of cytokines produced by the different multiple and single cytokine producing CD154+/CD4+ and CD8+ T cells (Fig. 8). IFN-γ+/TNF-α+/IL-2– CD154+CD4+ T cells had the highest iMFI, which gradually declined for double producing and single IFN-γ+ CD154+CD4+ T cells. IFN-γ+/TNF-α– CD8+ T cells contributed the most to IFN-γ production, directly followed by the IFN-γ+/TNF-α+/IL-2– CD8+ T cells. IFN-γ+/TNF-α– CD8+ T cells were also the main contributors for TNF-α, whereas IFN-γ+/TNF-α+/IL-2– CD8+ T cells showed a higher IL-2 iMFI. TNF-α and IL-2 iMFIs were also the highest for TNF-α+/IL-2– CD154+CD4+ T cells, followed by the IFN-γ+/TNF-α+/IL-2– CD154+CD4+ T cells. Thus the TNF-α+/IL-2– CD4 and IFN-γ+/TNF-α– CD8 T cells contribute strongly to the production of Th1 cytokines, followed by the triple-positive T cells. Single cytokine–producing cells only showed a relatively minor contribution.

In conclusion, seven of the identified IVE-TB Ags are strongly immunogenic, triggering specific and high cellular immune responses in E/C+ TST+ individuals and long-term ltLTBI individuals, but not in E/C− TST+ individuals, healthy mycobacterial naive individuals, and TB patients. The strong IVE-TB responses that were measured in the ltLTBI group were identified as IFN-γ+/TNF-α– CD8+ T cells and TNF-α+/IL-2– CD4+ T cells, which were the most prominent contributors to the produced cytokines, followed by triple-positive T cells.

Discussion

Using quantitative genome-wide *M. tuberculosis* transcriptional profiling, we have identified a series of *M. tuberculosis* genes that
are expressed during in vivo *M. tuberculosis* infection in the lungs of resistant and susceptible mice, which we term IVE-TB. Most of the genes identified have previously been found to be expressed in the *M. tuberculosis* proteome, and thus encode bona fide *M. tuberculosis* proteins. This is further supported by their immunogenicity profiles, as many of these proteins triggered IFN-γ production in human WBA and lymphocyte stimulating assays in *M. tuberculosis* ESAT6/CFP10-responsive patients, but not in ESAT6/CFP10⁰ TST⁰ individuals, HC donors, or TB patients. This is particularly relevant in the case of Rv2435c and Rv3353c, as their protein products have not been identified yet; however, IFN-γ responses were demonstrated in E/C⁰ TST⁰ individuals, indirectly showing that these *M. tuberculosis* proteins are presented to the human immune system during mycobacterial infection.

Many of the IVE-TB genes we identified have been described previously in relationship to the adaptative response of *M. tuberculosis*. This is further supported by their immunogenicity profiles, as many of these proteins triggered IFN-γ production in human WBA and lymphocyte stimulating assays in *M. tuberculosis* ESAT6/CFP10-responsive patients, but not in ESAT6/CFP10⁰ TST⁰ individuals, HC donors, or TB patients. This is particularly relevant in the case of Rv2435c and Rv3353c, as their protein products have not been identified yet; however, IFN-γ responses were demonstrated in E/C⁰ TST⁰ individuals, indirectly showing that these *M. tuberculosis* proteins are presented to the human immune system during mycobacterial infection.

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FIGURE 5. Cumulative IFN-γ responses induced by IVE-TB Ags, calculated per individual in the E/C⁺, E/C⁻, and HC groups. Cumulative IFN-γ responses to all 20 IVE-TB protein and protein fragments in E/C⁺ (n = 43), E/C⁻ (n = 90), and HC donors (n = 11). Squares indicate cumulative IFN-γ response of all 20 IVE-TB Ags, and circles indicate cumulative IFN-γ response of Rv1284, Rv1956, Rv2034, Rv2324, Rv3533c, and Rv3420c Ags. Horizontal bars represent the median cumulative IFN-γ responses.

FIGURE 6. PBMC IFN-γ responses toward IVE-TB Ags in TB patients and WBA E/C⁺ TST⁺ donors. PBMC of WBA E/C⁺ TST⁺ donors (n = 8) (A) and TB patients (n = 7) (B) were stimulated with IVE-TB Ags and control conditions for 6 d. Levels of IFN-γ were measured and medium background values were subtracted for each response in each donor for comparative purposes. The proportion of responders for each Ag is indicated at the top of the graph. Horizontal bars represent the median IFN-γ responses. The dotted line indicates the cut-off value for positivity, arbitrarily set at 100 pg/ml.
berculosis to environmental stress conditions, especially those that *M. tuberculosis* likely encounters during host infection. We identified one *M. tuberculosis* DosR regulon–encoded gene (*Rv0079*) (7) as well as six genes that are part of the EHR regulon (*Rv1284*, *Rv1956*, *Rv2034*, *Rv2324*, *Rv2465*, and *Rv3515*) (17). Three of these have also been described as starvation/nutritional stress genes (*Rv1284*, *Rv1956*, and *Rv2034*) (14). This function of the IVE-TB genes in responding to host-induced stress conditions during in vivo pulmonary infection enhances the biological plausibility of our findings and lends validity to our approach.

Of further interest, nine of the *M. tuberculosis* genes identified in this study have not been described previously in relationship to *M. tuberculosis* host infection, although some of their functions have been linked to possible adaptation to in vitro host-induced stress conditions (Supplemental Table III). Several of these genes have a role in metal transport, metalloregulatory transcriptional regulation, or represent metalloenzymes. Furthermore, genes were identified that play a role in lipid metabolism. This is in agreement with the documented shift toward using fatty acids as an alternative carbon source instead of carbohydrates under nutrient-limiting conditions. Altogether, many of the IVE-TB genes we have identified appear to be related to the adaptation of *M. tuberculosis* to environmental stress conditions encountered in the host. Of additional importance, the identification of these genes in our in vivo model supports previous findings mostly obtained in in vitro models by showing that they are induced during pulmonary *M. tuberculosis* infection in vivo. On a cautionary note, however, our data do not allow us to discriminate whether the observed differential *M. tuberculosis* gene expression patterns are cause or

![FIGURE 7. Polychromatic flow cytometric analysis of IVE-TB–specific T cell responses in long-term latent *M. tuberculosis*–infected individuals. PBMC from long-term LTBI (n = 6) were stimulated for 16 h with the seven best recognized Ags as determined in Fig. 4. Frequencies of IFN-γ, TNF-α, IL-2, and CD154–producing CD4+ and CD8+ T cells were determined (A). Subsequently, “multifunctional” responses were determined by analyzing combinations of IFN-γ, TNF-α, IL-2, and CD154 responses for CD4+ T cells and IFN-γ, TNF-α, and IL-2 responses for CD8+ T cells. Results for two representative IVE-TB Ags are shown (*Rv2034* and *Rv3420c*) (B). For comparative purposes, medium background values were subtracted for each response in each donor. Horizontal bars represent the median frequency of cytokine-producing CD4+ or CD8+ T cells.](http://www.jimmunol.org/doi/fig/1668-NOVEL-M-TUBERCULOSIS-AGS-EXPRESSED-IN-PULMONARY-INFECTION)
consequence of variations in host susceptibility (background and/or \(sst1\) locus).

As already mentioned, \(M.\) \textit{tuberculosis} gene expression profiling has been performed in the past, mostly focusing on in vitro–cultured bacteria grown under a variety of different conditions. Subsequent work assessed \(M.\) \textit{tuberculosis} gene expression profiles following infection of host cells (8, 19, 43), and some recent studies have analyzed \(M.\) \textit{tuberculosis} gene expression patterns also in vivo (8, 59). Ward et al. (59) showed that there was little overlap in the \(M.\) \textit{tuberculosis} genes reported to be expressed in different studies reporting on \(M.\) \textit{tuberculosis} intracellular infection, likely as a result of methodological differences. Nonetheless, the two studies Ward et al. described (8, 60) indicate that similar functional categories of \(M.\) \textit{tuberculosis} genes are expressed during intracellular infection. In line with this notion, when comparing our data to previous reports there are few overlapping individual \(M.\) \textit{tuberculosis} genes, but we nevertheless do identify genes with previous described functional categories. These differences are probably due to differences in selection criteria, in experimental settings such as infection route, and the specific mouse models we have used, which have not been analyzed previously.

Despite these differences, several of our selected IVE-TB genes do overlap with \(M.\) \textit{tuberculosis} genes identified in other studies as indicated in Table II. The \(M.\) \textit{tuberculosis} gene \(Rv2225\) whose expression was TB granuloma associated was also significantly expressed in the artificial granuloma model of Karakousis et al. (21). This strengthens their association with host granuloma formation. Our in vivo pulmonary TB granuloma-associated \(M.\) \textit{tuberculosis}–expressed genes did not overlap with the granuloma-associated genes or macrophage-associated genes described by Ramakrishnan and colleagues (61, 62) for \(M.\) \textit{marinum}, which might be due to differences between the mycobacterial species studied. Moreover, several of the IVE-TB genes we identified to be highly expressed have also been described previously, including \(Rv0467\) (\textit{icl}), which encodes an enzyme in the glyoxylate pathway, which is important for \(M.\) \textit{tuberculosis} persistence of \(M.\) \textit{tuberculosis} (63, 64), and \(Rv0991c\), which is part of the so-called in vivo–expressed genomic island (20).

The new \(M.\) \textit{tuberculosis} Ags we have identified in this study may represent interesting targets for vaccination, as they are expressed during \(M.\) \textit{tuberculosis} infection in the (genetically susceptible) lung, which we consider a critical parameter for appropriate Ag selection. Moreover, successful vaccine Ags should be conserved between multiple \(M.\) \textit{tuberculosis} strains. All protein sequences examined were conserved among the tested \(M.\) \textit{tuberculosis} strains. Additionally, for almost all IVE-TB genes multiple proteome studies have documented their expression as proteins in \(M.\) \textit{tuberculosis} (Table III). A subset of the analyzed IVE-TB proteins was shown to be strongly immunogenic as judged by Th1 responses in WBA, lymphocyte stimulation assays, and polychromatic flow cytometry. Indeed, the highest IFN-\(\gamma\) responses were identified within the E/C\(^*\) population of our TST\(^+\) cohort.

### FIGURE 8. iMFI of IVE-TB–specific CD154\(^+\)CD4\(^+\) and CD8\(^+\) T cell subsets in long-term latent \(M.\) \textit{tuberculosis}–infected individuals. iMFI values for IFN-\(\gamma\), TNF-\(\alpha\), and/or IL-2 were calculated via multiplication of CD154\(^+\)CD4\(^+\) and CD8\(^+\) T cell subset frequency by their MFI. Six ltLTBI donors were analyzed. For comparative purposes, medium background iMFI values were subtracted for each response in each donor. Light gray boxes represent CD154\(^+\) CD4\(^+\) T cell responses and dark gray boxes CD8\(^+\) T cell responses. Lines within boxes represent the medians. The lower boundary of the box represents the 25th percentile and upper boundary the 75th percentile. Whiskers extend to the lowest and highest values.
whereas no differences in mitogen-induced responses were seen. No responses were seen in M. tuberculosis nonresponder healthy individuals, suggesting that T cell recognition of IVE-TB Ags is indeed Ag specific and is correlated with M. tuberculosis exposure based on TST and QFT-GIT conversions. Interestingly, TB patients showed relatively low recognition of the IVE-TB Ags, suggesting that they did not develop strong Th1 immunity against these Ags. Importantly, IVE-TB Ag-specific responses could be detected in IltLTBI, which have been exposed to M. tuberculosis many years ago and never developed TB symptoms despite not having had preventive treatment. The most prominent T cell subsets with activity against IVE-TB Ags included IFN-γ/TNF-α CD8+ T cells and TNF-α/IL-2 CD154+CD4+ T cells. Thus, CD8+ T cells were the major contributors of IFN-γ production. Interestingly, also a population of Ag-specific–activated CD154+CD4+ T cells was observed that did not produce IFN-γ, TNF-α, or IL-2. These cells may exert alternative functionalities, which could include IL-17 production, immune regulation, or yet other functions, which need further study. Finally, we previously reported multifunctional CD4+ and CD8+ T cell responses toward resuscitation promoting factor and DosR proteins and showed that IFN-γ/ TNF-α CD8+ T cells were also the most prominent subset in the response to these Ags, suggesting that the development of specific differential T cell subsets may be unrelated to the nature of the specific protein Ag involved.

CD8+ T cells are activated upon recognition of epitopes present via MHC class I molecules, indicating that the Ags are present and processed via the canonical cytosolic pathway or via alternative (e.g., cross-priming) pathways (65). Both CD4+ and CD8+ T cells are important in M. tuberculosis control, and CD4+ and CD8+ T cell–deficient mice, for example, have increased susceptibility to M. tuberculosis (66). CD4+ T cells were recently shown to play an important (IFN-γ–independent) role in the indirect activation of IFN-γ–CD8+ T cells (67). In any case, our data obtained in the IltLTBI individuals show that the M. tuberculosis Ag-specific CD4+ and CD8+ T cells recognizing IVE-TB Ags must be long lived.

The immunogenicity of some of the IVE-TB Ags has been analyzed previously. The immunogenicity of the DosR Rv0079 protein was analyzed in TST+ (endemic) individuals as well as all blood donors who participated in this study. We thank Louis Wilson for the production of the DosR protein. We acknowledge T.H.M.O. is coinventor of an M. tuberculosis latency Ag patent, which is owned by Leiden University Medical Center. The other authors have no financial conflicts of interest.

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Disclosures

T.H.M.O. is coinventor of an M. tuberculosis latency Ag patent, which is owned by Leiden University Medical Center. The other authors have no financial conflicts of interest.

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