Exploration of Novel Cellular and Serological Antigen Biomarkers in the ORFeome of Mycobacterium tuberculosis*

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Increasing evidence demonstrates that antigen-specific cellular and humoral immunity plays an indispensable role in protection against Mycobacterium tuberculosis infection. Antigen is a key element in the development of a successful diagnostic method and vaccine. However, few antigens are available, and a systemic study on M. tuberculosis ORFeome-based antigen screening is still lacking. In the current study, a genome-wide examination was conducted on high-throughput M. tuberculosis encoding proteins and novel antigens were identified via a comprehensive investigation of serological and antigen-specific cellular responses. The serological immunoglobulin G level of each protein was detected in pooled sera from 200 pulmonary tuberculosis patients by means of semi-quantitative Western blot. Of the 1,250 detected proteins, 29 were present at a higher level relative to the commercialized 38-kDa protein. Furthermore, the top 12 of the 29 proteins had not been previously reported, and their antigenicity was validated in serum from each individual patient. Results confirmed that the 12 proteins displayed nearly identical immunoglobulin G antibody levels in patients with pulmonary and extrapulmonary tuberculosis. Antigen-specific cellular interferon-γ secretion was also evaluated using a cell-based ELISPOT assay. Thirty-four of the proteins were able to induce positive interferon-γ production by peripheral blood mononuclear cells from pulmonary tuberculosis patients as judged by positive (commercial ESAT-6 antigen) and negative controls. The top 4 candidates out of the 34 proteins displayed good accuracy ranging from 50% to 80% compared with the commercial ESAT-6 antigen. Subsequent epitope examination confirmed that a pool of peptides, including a 25aa peptide from Rv1198, demonstrated significant tuberculosis-specific cellular interferon-γ production. Overall, the current study draws significant attention to novel M. tuberculosis antigens, many of which have not been previously reported. This discovery provides a large amount of useful information for the diagnosis of tuberculosis and the development of vaccines to provide protection against tuberculosis. Molecular & Cellular Proteomics 13: 10.1074/mcp.M113.032623, 897–906, 2014.

Despite great efforts to improve its diagnosis, prevention, and treatment, tuberculosis (TB) remains a serious burden on global health, especially in developing countries (1). Diagnostic inaccuracy and delays, vaccine failure, and drug resistance are the main contributors to the current TB epidemic (2). The World Health Organization (WHO) reports that only 60% of the estimated total TB cases have been detected; nearly half of active TB cases remain undetectable, and the pathogen continues to be transmitted (3). A critical step in the control of TB is the early and sensitive diagnosis of infection and disease; however, antigen biomarkers in Mycobacterium tuberculosis that are able to induce antigen-specific immune responses in diverse patients are not yet comprehensively understood. Scientists have been working to identify novel antigens in order to develop new, efficacious, and long-lasting vaccines; to improve the accuracy and specificity of diagnostic tools; and to fill gaps in knowledge regarding TB (4–7). It is well known that cell-mediated immunity plays a central role in controlling the proliferation of bacteria and that IFN-γ is the dominant cytokine that indicates infection. T-cell-based IFN-γ release assays were developed several decades ago to assess IFN-γ production after in vitro stimulation with M. tuberculosis antigens and are among the methods used to identify

1 The abbreviations used are: TB, tuberculosis; WHO, World Health Organization; ORF, open reading frame; ELISPOT, enzyme-linked immunospot; ESAT-6, early secretory antigenic target-6; IFN-γ, interferon γ; PBMC, peripheral blood mononuclear cell; SFC, spot-forming cell; HRCT, High Resolution Computed Tomography; PPD, purified protein derivation of tuberculin; LTBI, latent tuberculosis infection.
M. tuberculosis infection, although their reliability is still controversial (8). Among the over 4,000 predicted open reading frames (ORFs) in the M. tuberculosis genome, only three M. tuberculosis antigens, ESAT-6, CFP-10, and Ag85, have been commonly studied and are used in practice. Furthermore, the diagnostic methods based on ESAT-6 and CFP-10 are still in dispute and have at least two disadvantages: (i) neither is significantly superior to tuberculin skin tests or able to differentiate latent M. tuberculosis infection from active TB (9), and (ii) both miss diagnoses, as only 60% to 80% of active pulmonary TB is diagnosed as positive by the present established methods (10–13). Thus, the discovery of novel M. tuberculosis antigens is vital in order for antigenic epitopes to be identified that have better accuracy and specificity for the diagnosis of active TB. In addition to cell-mediated immune response, emerging evidence indicates that the B-cell-mediated humoral immune response plays a critical role in defending against M. tuberculosis (14, 15). Although the current commercial serodiagnostic kits are used in developing countries, antibody-based diagnosis is controversial, and current test performance is poor. WHO recommends against using these inaccurate TB serological tests because most lack sensitivity and specificity (16, 17). But the identification of new serum biomarkers is still highly recommended by WHO, and the study of novel M. tuberculosis serum biomarkers is still important. Moreover, the demand for serodiagnostic methods for TB diagnosis is still huge in developing and undeveloped countries because they are cost-effective and easy to use. Thus, it is worthwhile to examine the effectiveness of providing more antigen choices and a better panel, rather than using a single antigen, when diagnosing TB using these methods.

High-throughput multi-omics technologies have made it feasible to do genome-wide studies of certain pathogens. Though this necessitates translating a large amount data from biological studies into a form that can be used clinically, it is necessary to meet urgent clinical requirements. A few proteome-scale studies have been performed, and several antigenic proteins of M. tuberculosis have been identified using serum antibody-based screening. However, certain limitations still exist. Inconsistent results have been obtained from different groups using various methods, and systemic, functional antigen screening, based on the cellular immune response, is missing because of the experimental complexity and difficulty of recruiting enough patients to obtain fresh blood specimens that contain viable white blood cells.

In the current study, we screened and identified M. tuberculosis antigens that were specific to serological and cellular responses in one study of active pulmonary TB patients. After genome-based high-throughput cloning and expression, 1,250 purified candidate proteins were obtained for the identification of M. tuberculosis T and B cell antigens. The serological IgG level of each protein was determined by means of semi-quantitative Western blot. In addition, antigen-specific IFN-γ secretion was evaluated in 1,250 proteins using a cell-based ELISPOT assay. To our knowledge, this is the first time that cellular antigenic response has been examined on a large scale in TB patients, and the results provide an overview of a broad range of novel antigen biomarkers that could be beneficial for improving TB diagnosis and vaccine development.

EXPERIMENTAL PROCEDURES

Bacterial Strains and Plasmids—E. coli DH5α cells were used for plasmid construction. E. coli DH5α cells carrying entry plasmids derived from pDONR221 (Invitrogen) were cultured with 50 μg/ml kanamycin. E. coli BL21 (DE3) (Novagen, Darmstadt, Germany) and BL21-CodonPlus® (DE3)-RP cells (Stratagene, Santa Clara, CA) carrying pDEST17-derived (Invitrogen) plasmids for protein expression were cultured with 50 μg/ml ampicillin and 50 μg/ml ampicillin plus 34 μg/ml chloramphenicol, respectively.

DNA Cloning—All coding sequences for H37Rv were obtained from NCBI (NC_000962.3) (18). Gateway Recombination Cloning Technology (19) (Invitrogen) was used for site-specific cloning following the manufacturer’s instructions. Briefly, the target genes (between 150 bp and 2,800 bp in length) were amplified with attB PCR primers, including an attB site plus a generic-specific sequence of 15 to 20 bp. The PCR products were cloned into the entry vector (pDONR221) and transferred into the expression vector (pDEST17) using Gateway® BP and LR Clonase, respectively. Experiments were done in 96-well plates following the manufacturer’s instructions.

Protein Expression and Purification—Two bacterial strains of BL21 (DE3) and BL21-CodonPlus® (DE3) RP, under two inducible expression temperatures (37 °C and 28 °C), were used for expression of all target ORF proteins. The bacterial strains were cultured and induced overnight in 96-deep-well plates with 1 mm isopropl β-D-1-thiogalactopyranoside at 37 °C or 28 °C. Extraction of target proteins was performed using BugBuster Protein Expression Reagent (Novagen, San Diego, CA) according to the instruction manual. Protein purification was performed in 96-well plates from the His MultiTrap FF purification kit (GE Healthcare, Uppsala, Sweden) following the manufacturer’s instructions. Proteins for second-round evaluation experiments were purified on a large scale using His tag gravity flow columns (GE Healthcare). Purified proteins were examined via SDS-PAGE and quantified using the Thermo Scientific Pierce BCA Protein Assay Kit. The purified proteins were over 90% pure as determined by Quality One software and were used at a concentration of 50 to 500 μg/ml for examination of function.

Patient Characterization—Patients and healthy controls were recruited from Shenzhen Third People’s Hospital and the Beijing Tuberculosis & Thoracic Tumor Research Institute in China. A total of 370 patients with pulmonary TB (before treatment) were diagnosed and classified according to the 1990 edition of Diagnostic Standards and Classification of Tuberculosis (38). Diagnosis also included clinical signs and symptoms and radiographic findings (chest x-ray and/or HRCT) (20). All recruited individuals were also examined for antigen-specific IFN-γ secretion using a well-established ELISPOT kit (21). Patients determined to be positive based on bacteriological examination were recruited for a second or third round of experiments to identify antigenicity. Patients with extrapulmonary TB (n = 5) including lumbar spine, cervical vertebrae, and pelvic TB were recruited for some experiments. Hospitalized patients (n = 21) who were clinically diagnosed as not having TB (no TB history, no contact with TB patients, and no mycobacterial infection as determined by IFN-γ ELISPOT assay) served as disease controls. Healthy individuals (n = 5) who had no TB history, no contact with TB patients, and no clinical symptoms of TB and who tested negative for TB in both a PPD test and IFN-γ ELISPOT assay were also recruited. Individuals with HIV, hepatitis infections, or autoimmune disorders were excluded. Clinical
samples, including serum and peripheral blood mononuclear cells (PBMCs), were collected from subjects and used for serological and cellular function examinations, respectively. Patient details are listed in Table I (humoral immune antigen discovery) and Table III (cellular immune antigen discovery). This study was approved by and performed under the guidelines of the Ethical Committee of the Shenzhen-Hong Kong Institute of Infectious Disease, Shenzhen Third People’s Hospital. Written informed consent was directly obtained from each participant.

**Serum Sample Preparation**—Serum samples were pretreated with *E. coli* cell lysate (mixed using equal parts *E. coli* BL21 and BL21-CodonPlus) to remove nonspecific IgG antibodies as previously described (22). Briefly, the heat-denatured *E. coli* cell lysate was separated using SDS-PAGE and transferred onto nitrocellulose membranes. Serum samples were diluted 500 times and incubated with the membranes overnight at 4 °C, with gentle shaking. 20 ml serum samples were incubated with pretreated serum was diluted 1:500, and secondary goat antibody in 20 μl. E. coli culture lysate was optimized to absorb nonspecific *E. coli* antibody in 20 μl of serum.

**Serological Detection**—A semi-quantitative Western blot assay (23) was used to examine the *M. tuberculosis*-specific serum antibody response. During the first round of experiments, equal volumes of sera from 200 patients were pooled and pretreated, as described above, to screen 1,250 proteins. Serum samples from another 10 patients (5 pulmonary TB and 5 extrapulmonary TB) were prepared separately for evaluation of the 12 selected protein candidates. In the Western blot assay, each reaction was performed with 100 ng of purified protein, which was then transferred onto nitrocellulose membranes. Pretreated serum was diluted 1:500, and secondary goat anti-human IgG antibody, conjugated with IRDye680 Fluorescence (GENE, Hong Kong, China), was diluted 1:10,000 as the final concentration. The fluorescence signal was analyzed using the LICOR Odyssey Infrared imager and Odyssey software 3.0.x. Experiments were run in parallel using a commercial 38-kDa protein (IMMUNO, Woburn, MA) as the positive control and bovine serum albumin as the negative control. The ratio between the intensities of the reaction band of the tested protein and of the 38-kDa protein was calculated and used to evaluate the humoral response of tested proteins.

**Cellular Antigen-specific IFN-γ ELISPOT Assay**—An IFN-γ ELISPOT assay was performed using a human IFN-γ cytokine pre-coated ELISPOT kit (Dakewe Biotech Company, Shenzhen, China) as previously described (21). Briefly, the plates were seeded with 2.5 × 10^5 freshly purified PBMCs per well. Each individual sample was tested in parallel with a positive control (phytohemagglutinin), a negative control (blank and buffer for protein purification), the ESAT-6 antigen from the *M. tuberculosis* infection detection ELISPOT kit mentioned above, and purified ESAT-6 protein from our system. The final concentration of proteins was 10 μg/ml (21). After overnight incubation at 37 °C with 5% CO₂, the plates were developed following the manufacturer’s instructions. The spot-forming cells (SFCs) were counted with the BIOREADER® 4000 PRO-X (Biosys, Karben, Germany). The SFC number in the buffer control was subtracted from the number of all test wells. Assays were deemed positive using the criteria for *M. tuberculosis* infection as the established kit. For epitope confirmation, 25-mer peptides with 8-mer overlap were designed and synthesized (Table V) that covered the Rv2016 and Rv1198 sequences, respectively (ChinaPeptides Shanghai, Co., Ltd., Shanghai, China). Polypeptides were dissolved in dimethyl sulfoxide to make a 2 mg/ml stock solution. Pool 1 and pool 2 were composed of five equally mixed polypeptides, respectively. The polypeptides or the pool of polypeptides was used for ELISPOT assay at a concentration of 10 μg/ml.

**Protein Sequence Confirmation**—Protein candidate sequences were confirmed by mass-spectrum analysis. Briefly, protein bands were excised from the SDS-PAGE gel, washed, and digested at 37 °C with sequencing-grade trypsin (1:50 w/w, Promega, Madison, WI) overnight. The digestion mixtures were desalted using the ZipTipC18 (Millipore, Billerica, MA) according to the manufacturer’s instructions. MALDI-TOF-MS/MS and nano-LC-MS/MS analyses were performed as previously described (24, 25). Raw data were searched against the H37Rv genome (3,988 entries) combined with 247 known contaminant protein sequences using MASCOT v2.2.

**Data Analyses**—Figures were made using GraphPad Prism version 5.00 and Excel 2007. The accuracy was defined as the percentage of the number of clinically diagnosed TB patients that reacted positively to the testing antigen out of the number of clinically diagnosed TB patients that reacted positively to antigen ESAT-6.

**RESULTS**

**Cloning and Expression of *M. tuberculosis* ORF Proteins—**Gateway® Technology was used to obtain *M. tuberculosis* ORF proteins because it is a rapid and highly efficient way to move a target DNA sequences to a multivector system. In the current study, 3,536 moderately sized (150 to 2,800 bp) ORFs were designed from the 3,988 ORFs in the H37Rv strain. After PCR, 3,206 genes were successfully obtained and inserted into pDONR221 as entry clones and 2,532 ORFs were then successfully transferred into pDEST17 as expression clones, covering 63.5% of the *M. tuberculosis* ORFs. Reasons for missing certain genes are as follows: (1) some genes lacked the appropriate sequence for the design of qualified primers using the high-throughput PCR method; (2) it was difficult to successfully obtain positive PCR products using one condition; (3) three colonies were picked from the entry clones and three colonies from expressing clones for sequencing confirmation; and (4) the cloning experiment was performed only once because of a large workload. Four expression conditions were used to achieve high expression efficacy, including two *E. coli* strains (BL21 and BL21-CodonPlus®) under expressing temperatures of 37 °C and 28 °C, respectively. Finally, 1,895 proteins were expressed in different quantities. After purification, 1,250 proteins were determined to be at a concentration sufficient and reasonable (50 to 500 μg/ml) for functional examination (supplemental Table S1). Another 645 proteins were purified, but quantities were rather low (less than 50 μg/ml) and they were not included in this study. The distribution of the 1,250 purified proteins was almost identical to the whole genome ORF protein as predicted by PSORT (Fig. 1A) and covered 25.0%, 20.0%, 18.8%, 38.3%, and 29.9% of the secreted proteins, cytoplasmic membrane proteins, cell wall proteins, cytoplasmic proteins, and proteins with an unknown localization, respectively. The data guaranteed that the functional analysis was representative of each classification of H37Rv. The humoral and cellular immune reactivity of the purified proteins were screened and identified separately, as shown in Fig. 1B.

**Identification of *M. tuberculosis* Serological Antigens via Semi-quantitative Western Blot—**Although previous publications have reported proteome-scale screening and identification of *M. tuberculosis* antigens that react serologically in TB patients, results are still controversial because of the incon-
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Fig. 1. Overview of *M. tuberculosis* H37Rv antigenic protein screening. A, comparison of the protein compositions of the H37Rv genome and ORFeome. B, *M. tuberculosis* H37Rv proteins were cloned, expressed, and purified. The antigenic proteins were screened by two means: semi-quantitative Western blot was used to screen the antigenic proteins thought to be involved in humoral reactions, and ELISPOT was used to screen the antigenic proteins that might induce the specific cellular secretion of IFN-γ. The sequences of protein candidates were further confirmed by mass spectrometry.
Fig. 2. Serum IgG levels of 1,250 purified proteins in pulmonary TB patients. Each column represents one H37Rv protein. The height and color of the columns indicate the reactivity of each protein, evaluated based on the ratio of the reactivity between the tested protein and the commercial 38-kDa protein. The red column represents the commercial 38-kDa protein, which has a ratio set at 1. The dark blue columns represent 29 proteins that had a higher response than the 38-kDa protein. The yellow columns represent proteins that had a reactivity ratio between 40% and 100%. The light gray columns represent proteins that had a response to sera from active TB patients but a reactivity ratio less than 40%.

sistent data from different groups using various methods (26, 27). In the current study, a semi-quantitative Western blot assay was used to detect M. tuberculosis antigen-specific serum IgG antibody for the following reasons: (1) all positive reactions could be judged by reading visible bands, which guaranteed an accurate and specific reaction; (2) it is easy to calculate the reaction degree relative to the well-studied and commercialized M. tuberculosis 38-kDa protein, which is the most used M. tuberculosis protein for serum antibody detection at present (28, 29); and (3) the results are not influenced by denatured proteins. Before screening, nonspecific E. coli–IgG antibody depletion was performed in all serum samples. For screening the 1,250 proteins, equal volumes of serum from 200 patients were pooled before nonspecific E. coli–IgG depletion. The intensities of all visible reaction bands were calculated based on comparison to that of the 38-kDa protein. As shown in Fig. 2, 514 out of the 1,250 proteins had positive reactions with clearly visible bands in the corresponding places (colored bars; original Western blot results of the 514 proteins from the screening experiment of 1,250 proteins are presented individually in supplemental Fig. S1). The ratio of the serological IgG reactivity of tested proteins to that of the 38-kDa protein ranged from 0.2% to 181.4%. Among the tested proteins, 119 had greater reactivity than the 38-kDa protein (above 40%; light blue and dark blue bars), including 29 proteins that had stronger antigen-specific serological reactions than the 38-kDa protein (above 100%; dark blue bars). Equal volumes of serum from 10 other pulmonary TB patients were pooled to further confirm the reaction. The 29 protein candidates were checked again, and 12 protein candidates were selected for further evaluation because these proteins had the highest reaction levels relative to the 38-kDa protein (ranging from 155.6% to 181.4%) and none of the 12 proteins had been previously reported. Serum samples were prepared from another 10 patients (5 pulmonary TB and 5 extrapulmonary TB; Table I) for case-by-case detection. The results showed that the top 12 protein candidates displayed strong antibody reactions in patients with a maximum test to 38-kDa protein ratio of 175% (Table II). Moreover, the 12 proteins presented similar serum antibody responses in extrapulmonary TB patients and in pulmonary TB patients (Table II, supplemental Fig. S2). Original data of the 1,250 proteins, including serological reaction ratios and cellular IFN-γ secretion levels, are summarized in supplemental Table S1. Mass spectrometry analysis was used to test whether the 119 proteins that showed higher reactivity (above a ratio of 40%) relative to the 38-kDa protein expressed the correct amino acid sequences (supplemental Table S2 and supplemental File S1).

Identification of M. tuberculosis Antigenic Proteins by Examining Cellular IFN-γ Secretion—T-cell-mediated immunity plays a central role in protecting the host and controlling bacteria proliferation. Presently, antigens of Rv3875 (ESAT-6) and Rv3874 (CFP-10) are commercially used in M. tuberculosis–specific ELISPOT assays to diagnose TB infection. However, not all clinically diagnosed TB patients test positive, and meta-analysis has shown a maximum 60% to 80% positive test rate in TB patients (10–13). Only 2 of the 4,000 M. tuberculosis ORF proteins have been studied well enough to meet clinical needs. Cellular antigen-stimulated IFN-γ secretion was examined using the ELISPOT assay to screen the purified 1,250 ORF proteins in order to identify novel antigens. IFN-γ is the only immunological marker presently used to assist in the diagnosis of M. tuberculosis infection, and ESAT-6 is a good stimulator of IFN-γ secretion (13). In the current study, commercial ESAT-6 and purified ESAT-6 protein from the Gateway system, in addition to positive, blank, and negative controls, were used in parallel with test antigens (supplemental Fig. S3). To ensure a unified standard, the ratio of SFCs of examined proteins to SFCs of the commercial ESAT-6 protein was calculated and analyzed. An assay was deemed positive only when the number of SFCs of the parallel ESAT-6 protein from our expression system was

| Characteristic                  | Pulmonary TB | Extrapulmonary TB |
|--------------------------------|--------------|-------------------|
| Number of patients             | 5            | 5                 |
| Age, median (range) (yr)       | 33.0 (17–57) | 31.0 (25–36)      |
| Male/female                    | 2/3          | 2/3               |
| Bacteriological test positive   | 5            | ND                |
| ELISPOT, a median (range)      | 306.0 (217–377) | 85.2 (28–176)    |

a Note: The ELISPOT was judged by the SFCs for the antigen ESAT-6.
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TABLE II

| Antigen     | Pulmonary TB | Extrapulmonary TB | Annotation                  |
|-------------|--------------|-------------------|-----------------------------|
| Rv0388c     | 0.993 ± 0.443| 0.983 ± 0.220     | PPE family protein          |
| Rv0415      | 0.855 ± 0.164| 0.985 ± 0.214     | Thiamine biosynthesis oxidoreductase Thio |
| Rv0582      | 0.923 ± 0.162| 0.990 ± 0.246     | Hypothetical protein Rv0582 |
| Rv0813c     | 1.263 ± 0.252| 1.272 ± 0.346     | Hypothetical protein Rv0813c|
| Rv0819      | 1.163 ± 0.376| 1.246 ± 0.309     | Hypothetical protein Rv0819 |
| Rv1455      | 1.101 ± 0.237| 1.180 ± 0.277     | Hypothetical protein Rv1455 |
| Rv1773c     | 0.831 ± 0.223| 0.830 ± 0.276     | Transcriptional regulatory protein |
| Rv1853      | 1.366 ± 0.170| 1.348 ± 0.325     | Urease accessory protein ureD|
| Rv1864c     | 0.940 ± 0.283| 0.920 ± 0.202     | Hypothetical protein Rv1864c|
| Rv2234      | 1.133 ± 0.308| 1.106 ± 0.253     | Phosphatase protein phosphatase PTPA |
| Rv3102c     | 0.908 ± 0.232| 0.956 ± 0.217     | Putative cell division ATP-binding protein |
| Rv3242c     | 0.992 ± 0.207| 0.918 ± 0.245     | Hypothetical protein Rv3242c|

Note: The values are presented as the mean ± S.D. The antigenic proteins are listed in order of the gene locus.

TABLE III

| Characteristic | E⁺ pulmonary TB patients | Non-tuberculosis patients |
|----------------|--------------------------|---------------------------|
| Number         | 31                       | 21                        |
| Age, median (range) (yr) | 36.0 (24–76)           | 45.8 (21–90)            |
| Male/female    | 17/14                    | 14/7                      |
| Bacteriological test positive | 31                      | 0                         |
| ELISPOT, median (range) | 137.0 (50–338)       | 7.1 (0–21)               |

a “E⁺” denotes active TB patients with a positive response to ESAT-6 antigen in ELISPOT.

b ELISPOT was judged by the SFCs of the antigen ESAT-6.

TABLE IV

| Antigen | Accuracy relative to commercial kit (%) | SFC ratio to ESAT-6 kit, average (range) (%) | SFC ratio to ESAT-6, average (range) (%) |
|---------|----------------------------------------|---------------------------------------------|----------------------------------------|
| Rv0232  | 50                                     | 69.4 (35.1–160.4)                           | 58.9 (34.2–89.4)                       |
| Rv1031  | 50                                     | 70.9 (30.6–134.2)                           | 38.5 (29.0–47.6)                       |
| Rv1198  | 80                                     | 55.9 (16.7–137.8)                           | 64.7 (19.1–113.8)                      |
| Rv2016  | 70                                     | 72.6 (20.5–131.5)                           | 63.8 (35.3–92.3)                       |

Note: Only the four proteins with the best accuracy relative to the clinically used ESAT-6 protein are listed.

greater than the cutoff recommended in the manual. Our data showed that the ESAT-6 protein expressed by our system displayed 100% accuracy relative to the commercial ESAT-6 antigen, indicating that the protein obtained from the Gateway expression system had good reactivity.

Because freshly separated PBMC cells are required for detecting cellular IFN-γ secretion, 120 pulmonary TB patients were recruited so we could examine the 1,250 proteins individually. On average, each protein was tested in at least two pulmonary TB patients during the first round of screening. Results are shown in Fig. 3, with each column representing the SFC ratio of an individual protein. Among the 1,250 proteins, 1,216 proteins (presented as gray columns) had an SFC ratio of less than 30%, including 1,195 proteins of which the reaction to PBMCs from TB patients was not detectable and 21 proteins that had a very weak reaction (supplemental Table S1). Thirty-four proteins had positive results in pulmonary TB patients with SFC ratios ranging from 30% to 93%. Among these, 11 proteins, besides ESAT-6, had an SFC ratio greater than 50% relative to the commercial ESAT-6, indicating potential antigenicity. Mass spectrometry analysis was performed to verify that the 11 proteins had correct amino acid sequences (supplemental Table S2 and supplemental File S1). Detailed information on the standardized SFC ratios of the 1,250 proteins is summarized in supplemental Table S1.

The antigenicity of the top 11 proteins was further examined case by case in samples from 10 pulmonary TB patients that...
showed positive reactions to ESAT-6 in ELISPOT (defined as E/H11001 active TB patients; Table III). The results showed that four of the proteins—Rv0232, Rv1031, Rv2016, and Rv1198—had the best accuracy (varying from 50% to 80%) and the average of their SFC ratios ranged from 55.9% to 72.6% (Table IV). Strikingly, the ESAT-6-like protein Rv1198 (EsxL) and hypothetical protein Rv2016 showed SFC ratios of 55.9% and 47.4% relative to ESAT-6, respectively.

Although the individual proteins identified were not markedly superior to the existing, well-characterized ESAT-6 antigen in stimulating M. tuberculosis–specific T cells, results of this study are still informative as far as the discovery of new biomarkers. Epitopes of the two best proteins of Rv2016 and Rv2198 were then investigated. We designed 25-mer peptides with 8-mer overlap covering the sequences of Rv2016 (11 peptides) and Rv1198 (5 peptides) (Table V and Fig. 4A). The 11 peptides of Rv2016 all showed negative on the ELISPOT assay of PBMCs from 15 pulmonary TB patients (data not shown). Interestingly, peptide P2–3 (VLTASDFWGAGSAACQGFITQLGR) from Rv1198 showed a significantly greater SFC count than the other four peptides. Furthermore, the pools of peptides (Pool 1 contained P2–2, P2–3, and P2–4, and Pool 2 contained all five peptides derived from Rv1198; Fig. 4A) displayed a marked increase in IFN-γ SFCs (Fig. 4B), indicating that peptide 3 may be a useful epitope but multi-epitopes could improve antigenicity and reactivity. To investigate whether the stimulation was M. tuberculosis antigen specific, 21 patients with respiratory syndrome but not TB infection were recruited as disease controls (Table III). Importantly, the pool of antigens including peptide 3 from Rv1198 stimulated significant IFN-γ production in pulmonary TB patients but not in the disease controls, indicating M. tuberculosis antigen-specific stimulation (Fig. 4C).

**DISCUSSION**

The current study aimed to closely examine omics-based M. tuberculosis biology and identify novel M. tuberculosis antigens. A total of 1,250 purified M. tuberculosis ORF pro-
teins were obtained, and their serological and antigen specific cellular responses were studied. Using a semi-quantitative Western blot assay, we found that 29 of the 1,250 proteins exhibited higher levels of antibodies than the commercialized 38-kDa protein and 12 proteins reacted to patient serum at ratios ranging from 155.6% to 181.4% relative to the 38-kDa protein. Antigen-specific cellular IFN-γ secretion was also evaluated using a cell-based ELISPOT assay. The top four candidates from the original 1,250 proteins displayed good accuracy relative to commercial ESAT-6. The following epitope examination confirmed that a pool of peptides, including a 25-mer peptide from Rv1198, had significant cellular IFN-γ production. Interestingly, a combination of pooled peptides, including the above peptide, had even more reactivity. Overall, our results present a panel of new antigen biomarkers for *M. tuberculosis*. These biomarkers have the potential to improve TB diagnosis and/or may be used in the development of a more effective vaccine.

Accurate and quick diagnosis of TB is key for the prevention and control of this disease. Over recent decades, many *M. tuberculosis* biomarkers have been studied, and substantial progress has been made in some areas to advance our knowledge of the infection, disease development, and prognosis (30–34). With the increased understanding of *M. tuberculosis* infection, people now realize that a single antigen or sole biomarker may not provide sufficient sensitivity and specificity for the diagnosis of such a complicated disease. Increasingly, scientists have recognized that a set of biomarkers should be used in combination to obtain the desired sensitivity and specificity for diagnosis. Advances in molecular techniques and genome-scale translational and proteomic research have accelerated our understanding of immunity against *M. tuberculosis* and the identification of novel antigens.

Tuberculin skin testing has been used worldwide for more than a century to help diagnose LTBI and active tuberculosis; however, it has poor sensitivity. IFN-γ, the first identified immunological biomarker, plays a critical role in regulating cell-mediated immune responses to *M. tuberculosis* infection and has led to the development of a novel immunological assay based on *M. tuberculosis* antigens stimulating IFN-γ secretion. This assay is commonly used to detect the cellular antigenic responses for the identification of *M. tuberculosis* infection. Currently, only two antigens are used in detecting cellular antigen-specific IFN-γ secretion, ESAT-6 (Rv3875) and CFP-10 (Rv3874). The sensitivity of such tests is around 60% to 80% in patients with active TB (12) and is dependent on the immune status of the host, the complexity of the infection, the bacterial proliferation status, and the contribution of suppressive cytokines related to TB. To the best of our knowledge, this study is the first reported genome-wide investigation to screen and identify novel functional antigens that induce secretion of IFN-γ. In this study, 34 out of 1,250 proteins elicited positive IFN-γ secretion, and the top 4 proteins had 50% to 80% accuracy and specificity in pulmonary TB patients. Importantly, one epitope from Rv1198 (comprising amino acid residues 35–59) was identified that strongly stimulated IFN-γ secretion. Previously, a peptide of Rv1198 comprising amino acid residues 25–44 was identified from *Mycobacterium bovis* vaccinated cattle (35). It is possible that the different method used (ELISPOT assay in this study versus IFN-γ ELISA in Ref. 35) and the different study subjects (human patients in this study versus infected cattle in Ref. 35) might be main reasons for the difference in the epitopes identified. In addition, we demonstrated that this peptide’s reactivity and stimulation of IFN-γ secretion were markedly accelerated in pooled peptides. Therefore, using a combination of antigens might be a better way to accurately diagnose TB. Furthermore, the antigen stimulation was TB specific. These results might also lead to the discovery of useful antigens for distinguishing between latent and active TB.

Although the identification of antibodies to TB in blood samples has a limited clinical role and is still controversial, this method might be helpful in diagnosing TB, especially in developing countries (36, 37). WHO recommends against using these inaccurate TB serological tests because most lack sensitivity and specificity (16, 17), but the identification of new serum biomarkers is still highly recommended by WHO, and the study of novel *M. tuberculosis* serum biomarkers is still important. In addition, the serological detection of TB is still useful in patients who are unable to produce adequate sputum, are sputum negative, or have extrapulmonary TB (12). A commercially used recombinant 38-kDa protein has been reported to have a specificity of 94% in smear-positive TB patients, supporting the use of tests for serological reactivity (37). In previous studies, two methods, protein array and ELISA, have been used to detect antibody responses when screening for *M. tuberculosis* antigens (26, 27). In our study, 1,250 *M. tuberculosis* proteins were screened for antibody response using a semi-quantitative Western blot assay. The advantages of this method are as follows: (1) almost full-length proteins, not synthesized peptides, were used for the functional analysis; (2) this method has better specificity because the correct molecular size for the target antigens could be judged by the visible bands; and (3) the results are not influenced by denatured proteins. Detailed information regarding the 1,250 proteins examined in this study is summarized in *supplemental Table S1*. Most important, the 38-kDa protein was used to calculate the reaction intensity and to reduce errors in different experiments in our study. After two rounds of screening, the top 12 proteins displayed a higher serum IgG antibody level in pulmonary TB patients and had a nearly identical antibody level in extrapulmonary TB patients. Data from serological screening might pave the way for improvements in serodiagnostic methods and contribute to our understanding of antibody-mediated immunity against *M. tuberculosis*.

Two previous papers reported the proteome-scale identification of novel antigenic proteins in *M. tuberculosis* by means

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of various methods, but the data are still controversial (supplemental Fig. S4). According to the results of first-round screening, these two studies found that 29 out of 249 and 484 proteins were positive as determined by ELISA assay and protein array, respectively (26, 27). Other antigenic proteins were also identified in our study (31 proteins were identified in addition to those reported by Li et al. (26), and another 55 proteins in addition to those reported by Kunnath-Velayudhan et al. (27)). Among them, four proteins were presented as positive in both our results and the two reports. Unfortunately, we failed to find a protein that was reported to be positive among the top proteins in the three studies. Certainly, peripheral antibody-based screening may vary with the examination method, sample source, and unbiased judging criteria, and solid conclusions should be obtained from a larger cohort examination in the future.

In summary, a systemic ORFeome study of M. tuberculosis was done and the primary data were translated into a better understanding of the 1,250 proteins examined in the bacterium. To the best of our knowledge, this is the only study that provides data on the in vitro detection of both humoral and cellular reactivity and is the first example of cellular immune response examination performed on such a large scale. Data from this study may have an impact on TB diagnosis and vaccine development.

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