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Identification of Human T Cell Antigens for the Development of Vaccines against Mycobacterium tuberculosis

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Development of a subunit vaccine for Mycobacterium tuberculosis (Mtb) depends on the identification of Ags that induce appropriate T cell responses. Using bioinformatics, we selected a panel of 94 Mtb genes based on criteria that included growth in macrophages, up- or down-regulation under hypoxic conditions, secretion, membrane association, or because they were members of the PE/PPE or EsX families. Recombinant proteins encoded by these genes were evaluated for IFN-γ recall responses using PBMCs from healthy subjects previously exposed to Mtb. From this screen, dominant human T cell Ags were identified and 49 of these proteins, formulated in CpG, were evaluated as vaccine candidates in a mouse model of tuberculosis. Eighteen of the individual Ags conferred partial protection against challenge with virulent Mtb. A combination of three of these Ags further increased protection against Mtb to levels comparable to those achieved with bacillus Calmette-Guérin vaccination. Vaccine candidates that led to reduction in lung bacterial burden following challenge-induced pluripotent CD4 and CD8 T cells, including Th1 cell responses characterized by elevated levels of Ag-specific IgG2c, IFN-γ, and TNF. Priority vaccine Ags elicited pluripotent CD4 and CD8 T responses in purified protein derivative-positive donor PBMCs. This study identified numerous novel human T cell Ags suitable to be included in subunit vaccines against tuberculosis. The Journal of Immunology, 2008, 181: 7948–7957.

Tuberculosis (TB) is a chronic infectious disease caused by Mycobacterium tuberculosis (Mtb) and is one of the leading causes of mortality due to infectious disease worldwide. Nearly one-third of the world’s population is infected with Mtb, with ~25 million people actively infected and 8.8 million new cases arising each year. Upon infection with Mtb, active disease develops in ~10% of subjects within 1–2 years of the initial exposure. The remainder of those infected with Mtb enter a state of latent infection, which can reactivate at a later stage, particularly in the elderly or in immunocompromised individuals.

The high mortality associated with Mtb infection occurs despite the widespread use of a live, attenuated TB vaccine Mycobacterium bovis, bacillus Calmette-Guérin (BCG). BCG appears to be effective at preventing disease in newborns and toddlers, but not pulmonary tuberculosis in adults. The variable efficacy afforded by BCG vaccination and the absence of a TB vaccine protective in adults have been the primary rationale for our approach to identify immunodominant Mtb Ags that could be used in a subunit vaccine to boost immune responses leading to improved protection. There is a new urgency for a TB vaccine as the World Health Organization recently reported alarming rates of “multidrug resistant” and “extensively drug-resistant” TB, mostly because of improper observance of a lengthy and costly drug regimen treatment.

Protective immunity to TB is conferred by Th1 CD4 and effector CD8 T cells, and it is recognized that an effective TB vaccine requires the generation of a T cell-mediated immune response. Thus far, many of the dominant Mtb T cell Ags have been associated with proteins expressed by Mtb growing in macrophages, up- or down-regulated under hypoxic conditions, required for reactivation, membrane associated, secreted, or represented by virulence factors such as PE/PPE or EsX. Several promising subunit vaccine candidates have been developed using Ags among these protein classes (reviewed in Refs. 9, 10).

Choosing the right adjuvant or delivery platform is critical to the success of a subunit vaccine. In this study, we used the oligodeoxynucleotide CpG, a TLR9 ligand, as an adjuvant based on its reported ability to potentiate Th1 CD4 and CD8 T cell responses (reviewed in Ref. 11).

Our approach to vaccine Ag discovery has been to identify relevant human T cell Ags that elicit dominant Th1 responses and to evaluate these Ags in appropriate adjuvant formulations in animal models. Taking advantage of the published genome sequence of Mtb (12), we used bioinformatic selection criteria to identify genes of interest, including protein secretion, induction during growth in macrophages, up- or down-regulation in response to low oxygen or carbon source, and/or selection of members in the EsX or PE/PPE families. Recombinant proteins were made for 94 Mtb genes and evaluated with human PBMCs from healthy purified protein derivative (PPD) and PPD+ individuals for IFN-γ responses. A subset of 49 Ags adjuvanted with CpG oligonucleotides was tested...
prophylactically in mice using the Mtb aerosol challenge model. Ab and cytokine responses to the vaccine Ags were characterized.

Materials and Methods

Cloning and purification of target Ags

DNAs encoding selected Mtb genes were PCR amplified from HRe37 genomic DNA using PF DNA polymerase (Invitrogen). PCR primers were designed to incorporate specific restriction enzyme sites 5′ and 3′ of the gene of interest for directional cloning into the expression vector pET28a (Novagen). Purified PCR products were digested with restriction enzymes, ligated into pET28a, and transformed into XL10G cells (Stratagene). Recombinant pET28a plasmid DNA was recovered from individual colonies and sequenced to confirm the correctly cloned coding sequence. The recombinant clones contained an N-terminal six-histidine tag followed by a thrombin cleavage site and the Mtb gene of interest.

Recombinant plasmids were transformed into the Escherichia coli BL21 derivative Rosetta2(DE3)pLySs (Novagen). Recombinant strains were cultured overnight at 37°C in 2× yeast tryptone containing appropriate antibiotics, diluted 1/25 into fresh culture medium, grown to mid-log phase (OD at 600 nm of 0.5–0.7), and induced by the addition of 1 mM isopropyl β-D-thiogalactoside. Cultures were grown for an additional 3–4 h, cells were harvested by centrifugation, and bacterial pellets were stored at −20°C. Bacterial pellets were thawed and disrupted by sonication in 20 mM Tris (pH 8.0), 150 mM NaCl, 1 mM PMSF, followed by centrifugation to fractionate the soluble and insoluble material. Recombinant His-tagged protein products were isolated under native (soluble recombinant proteins) or denaturing (8 M urea) conditions using Ni-nitrilotriacetic acid metal ion affinity chromatography according to the manufacturer’s instructions (Qia-gen). Protein fractions were eluted with an increasing imidazole gradient and analyzed by SDS-PAGE. Affinity-purified protein fractions were combined and dialyzed against 20 mM Tris (pH 8.0), concentrated using Amicon Ultra 10-kDa molecular mass cutoff centrifugal filters (Millipore), and quantified using a bicinchoninic acid protein assay (Pierce). LPS contamination was evaluated by the Limulus amoebocyte lysate assay (Cambrex). All the recombinant proteins used in this study showed residual endotoxin levels <100 EU/mg of protein.

Human PBMC assay

PBMCs were purified from heparinized blood obtained from 7 PPD− and 18 PPD+ healthy subjects. Informed consent was obtained from all the subjects and the study was approved by Western Institutional Review Board (Seattle, WA). PBMCs were plated in triplicate at 2–2.5 × 10^5 cells/well and cultured with medium, 10 µg/ml Con A, 10 µg/ml PPD, or 0 µg/ml of each recombinant protein for 72 h. Supernatants were harvested by centrifuging the plates with deionized water, and spots were counted on a automated ELISPOT reader (CTL series 3A analyzer, Cellular Technology) and analyzed with ImmunoSpot (CTL Analyzers).

Flow cytometry

Splenocytes from immunized animals or PBMCs from healthy PPD+ subjects were plated at 1–2 × 10^6 cells/well in 96-well V-bottom plates and stimulated with anti-CD28/CD49d (1 µg/ml each, ebioscience) and recombinant proteins (20 µg/ml) for 6–12 h at 37°C in the presence of GolgiStop (BD Biosciences). The cells were then fixed for 10 min with Cytotix/Cytoperm (BD Biosciences), washed in 0.1% PBS-BSA, incubated with FITC- or PE-labeled anti-IgG1, or APC-labeled anti-IgG2c (eBioscience) for 15 min at 4°C, stained with fluoro-chrome-conjugated mAb anti-CD3, CD8, CD44, IFN-γ, TNF-α, IL-2, granulocyte (GrB, ebioscience) and CD4 (Caltag Laboratories) in 1× Perm/Wash buffer (BD Biosciences) for 30 min at 4°C, washed twice in Perm/Wash buffer, suspended in PBS, and analyzed on a modified three-laser LSRII flow cytometer (BD Biosciences). Viable lymphocytes were gated by forward and side scatter, and 20,000 CD3/CD8 events were acquired for each sample and analyzed with BD FACS DIVA software (version 5.0.1, BD Biosciences).

Statistical analysis

Student’s t test and standard one-way ANOVA followed by Dunnett’s multiple comparison test were used for statistical analysis; p-values of 0.05 or less were considered significant.

Results

Ag selection, expression, and purification

Mtb genes selected for testing human immune responses and as possible vaccine candidates included 1) those genes that were required for growth in macrophages as defined by Sassetti and Rubin (13) 2) those that were up- or down-regulated in response to oxygen and carbon limitation (14), 3) mycobacterial-specific genes within known immunogenic classes EsX and PE/PPE as based in TuberCulist (genolist.pasteur.fr/TuberCulList/index.html), and 4) those identified as secreted by two-dimensional PAGE and mass spectrometry analysis (www.mpib-berlin.mpg.de/2D-PAGE) and/or containing putative secretion signals. All targets were subjected to N-terminal signal sequence analysis and membrane spanning region using the SignalP (www.cbs.dtu.dk/services/SignalP) and TMpred (www.ch.embnet.org/software/TMPRED_form.html) programs. Predicted proteins were chosen containing less than three transmembrane regions and a molecular mass between 60 and 70 kDa and low (<30%) sequence homology within the human genome. DNAs encoding selected Mtb genes were PCR amplified

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from H37Rv strain genomic DNA, cloned into the expression vector PET28a, and transformed into E. coli for recombinant protein expression. His-tagged protein products were isolated, affinity-purified, identified on SDS-PAGE gel (data not shown), and tested for residual endotoxin. Mtb genes that failed in PCR, had low or no detectable expression, failed to purify to >90% homogeneity, or contained >100 EU/mg were discarded. A total of 94 recombinant proteins were used in this analysis (Table I).

**Human PBMC cytokine responses to Mtb Ags**

Candidate Ags were initially prioritized based on their abilities to stimulate PBMCs from PPD+ (but not PPD−) donors to secrete IFN-γ. The donors used for the screening were tuberculosis skin test-positive, disease-free, and healthy. Ags from the RD1 region that are deleted in M. bovis BCG, such as ESAT-6 (Rv3875), were able to elicit responses from these PBMCs. Out of 94 proteins tested, 68 (72%) elicited positive IFN-γ responses by PBMCs from PPD+, but not PPD− donors (Fig. 1A). Responses to the proteins were compared with those elicited by Mtb lysate in PPD+ (2.79 ± 0.25) and PPD− (0.05 ± 0.12) and PHA (4.40 ± 0.27 and 2.59 ± 0.47, respectively). Of the PPD+ donors, 100% recognized Mtb lysate (data not shown) and a set of 28 proteins (Fig. 1B). Another set of 49 Ags was recognized by 67–90% of the subjects, and, overall, all the Ags tested were recognized by at least 44% of the PPD+ donors. Of the 28 Ags recognized by all donors, 7 were PE/PPE or EsX proteins, 6 were hypoxia-associated, 6 were identified by database searches, 5 were secreted/membrane proteins, and 4 were associated with Mtb survival in macrophages.

**Protection afforded by Mtb Ags adjuvanted with CpG**

Recombinant Mtb proteins inducing the highest levels of IFN-γ in the PBMC assays and percentage of positive responders were then tested for protective efficacy in a mouse model of TB. The number of viable bacilli (CFU) in the lungs of C57BL/6 mice immunized with Mtb proteins adjuvanted with CpG was determined 4 wk after aerosol challenge with a low dose of virulent Mtb H37Rv or Erdman strains. Twenty Mtb Ags were tested at IDRI in 7 independent experiments using H37Rv for challenge, 22 Mtb Ags were tested at Colorado State University under National Institutes of Health TB Vaccine Testing and Research Material contract in four independent experiments using the Erdman strain for challenge, and 6 Mtb Ags were tested by both laboratories. No differences in protection outcome were observed when using Mtb H37Rv or Erdman (data not shown), therefore indicating that both strains can be used interchangeably in this study. Each experiment included unvaccinated (saline) and BCG control groups, along with 8–10 groups of different Ags with CpG (seven mice per group). Mean lung log10 CFU in the various experiments ranged for the unvaccinated groups from 5.76 to 6.71 and from 4.30 to 5.82 for BCG-vaccinated mice (data not shown). Variations are often important in the aerosol model of TB, rendering direct comparison of CFU difficult due to differences in infection levels between experiments. Therefore, an accepted way of comparing the relative efficacy of a vaccine is to calculate the mean difference of CFU observed between unvaccinated and vaccinated groups.

Out of 48 proteins formulated with CpG, 30 produced no reduction in lung CFU, and 12 induced a small decrease in bacterial counts (0.1–0.3 log10). Six Ags (three secreted (Rv0577, Rv1626, and Rv2875), two PE/PPE (Rv2608 and Rv3478), and one hypoxic protein (Rv3044)) elicited >0.3 log10 reduction in lung CFU (Table II). Interestingly, the two PE/PPE proteins, Rv3478 and Rv2608, in combination with CpG resulted in the greatest decrease in viable Mtb bacilli (0.66 and 0.58 log10, respectively). Rv0577, Rv1626, Rv2875, and Rv3044 reduced lung CFU by 0.36, 0.32, 0.44, and 0.43 log10, respectively. As a comparison, three injections of Rv1886 (Ag85B, part of TB vaccines currently in clinical trials) + CpG or one injection of 104 viable BCG reduced lung CFU by 0.20 and 0.78 log10, respectively. CpG adjuvant alone did not reduce lung CFU (<0.1 log10). Reduction in CFU >0.2 log10 were reproducible and generally statistically significant (p < 0.05) by one-way ANOVA followed by Dunnett’s multiple comparison test (data not shown). Protection observed with weaker Ags was more variable from experiment to experiment and, due to the high number of animals that would have been required, experiments were not powered to detect statistical significance for CFU reduction <0.2 log10.

These results are very promising, as the levels of protection against Mtb infection achieved with two of the Ags (adjuvanted with CpG) were close to those obtained with the more complex BCG vaccine.

**Immunogenicity of vaccine candidate Ags**

Experiments were conducted to investigate the T and B cell responses induced against the vaccine candidates. C57BL/6 mice

<table>
<thead>
<tr>
<th>Secreted/Membrane</th>
<th>PE/PPE</th>
<th>EsX</th>
<th>Database</th>
<th>Growth in Macrophages</th>
<th>Hypoxia</th>
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<tr>
<td>Rv0054 Rv1860</td>
<td>Rv0915</td>
<td>Rv0287</td>
<td>Rv0952</td>
<td>Rv0523c</td>
<td>Rv0363c</td>
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<td>Rv0068 Rv1886c</td>
<td>Rv1196</td>
<td>Rv1793</td>
<td>Rv1009</td>
<td>Rv0655</td>
<td>Rv0570</td>
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<td>Rv1789</td>
<td>Rv3020</td>
<td>Rv1174</td>
<td>Rv0716</td>
<td>Rv1240</td>
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<td>Rv1818c</td>
<td>Rv3619</td>
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<td>Rv2608</td>
<td>Rv3620x</td>
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<td>Rv1288</td>
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<td>Rv2032</td>
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<td>Rv1589</td>
<td>Rv2558</td>
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<tr>
<td>Rv0455c Rv2873</td>
<td>Rv2389c</td>
<td>Rv1590</td>
<td>Rv2623</td>
<td></td>
<td></td>
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<td>Rv0496 Rv2875</td>
<td>Rv2450</td>
<td>Rv1908</td>
<td>Rv2624c</td>
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<td>Rv2554c</td>
<td>Rv2626c</td>
<td></td>
<td></td>
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<tr>
<td>Rv0733 Rv3246</td>
<td>Rv3204</td>
<td>Rv3587</td>
<td>Rv2801c</td>
<td></td>
<td></td>
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<tr>
<td>Rv0831c Rv3310</td>
<td>Rv3211</td>
<td>Rv3611</td>
<td>Rv2866</td>
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</tr>
<tr>
<td>Rv0909 Rv3628</td>
<td>Rv3407</td>
<td>Rv3614</td>
<td>Rv3044</td>
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</tr>
<tr>
<td>Rv0934 Rv3804c</td>
<td>Rv3876</td>
<td>Rv3129c</td>
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<tr>
<td>Rv1411 Rv3841</td>
<td>Rv3133</td>
<td></td>
<td></td>
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<tr>
<td>Rv1511 Rv3881</td>
<td>Rv3810</td>
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were immunized three times 3 wk apart with Ag and CpG. Ag-
specific Ab titers and cytokine responses were determined 1 wk
and 3 wk, respectively, after the last boost.

At the Ab level, higher IgG2c responses were measured in re-
sponse to 17 Ags, 18 Ags showed mixed IgG2c/IgG1 Abs, and 1
Ag induced higher IgG1 (Table III). Rv0577, Rv1886, Rv2608,
Rv2875, Rv3044, Rv3478, and Rv3619 induced log_{10} IgG2c Ab
titers ranging from 4.2 to 5.9 and log_{10} IgG1 titers ranging from
3.8 to 5.7 (Fig. 2A), while no IgG2c or IgG1 to Rv1626 could be
detected (data not shown). The Ag-specific IgG2c responses were

**Table II. Vaccine-induced protection against Mtb**

<table>
<thead>
<tr>
<th>CFU Reduction (log_{10}) ± SEM</th>
<th>&lt;0.1</th>
<th>0.1–0.3</th>
<th>&gt;0.3</th>
</tr>
</thead>
<tbody>
<tr>
<td>Rv0164b</td>
<td>Rv2450</td>
<td>Rv0496</td>
<td>0.11 ± 0.08</td>
</tr>
<tr>
<td>Rv0410</td>
<td>Rv2623</td>
<td>Rv0733</td>
<td>0.23 ± 0.10</td>
</tr>
<tr>
<td>Rv0455</td>
<td>Rv2626</td>
<td>Rv0831</td>
<td>0.13 ± 0.06</td>
</tr>
<tr>
<td>Rv0655</td>
<td>Rv2801</td>
<td>Rv1411</td>
<td>0.11 ± 0.11</td>
</tr>
<tr>
<td>Rv0952</td>
<td>Rv2866</td>
<td>Rv1569</td>
<td>0.12 ± 0.05</td>
</tr>
<tr>
<td>Rv1211</td>
<td>Rv2945</td>
<td>Rv1789</td>
<td>0.15 ± 0.16</td>
</tr>
<tr>
<td>Rv1270</td>
<td>Rv3029</td>
<td>Rv1813</td>
<td>0.14 ± 0.14</td>
</tr>
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<td>Rv1410</td>
<td>Rv3133</td>
<td>Rv1860</td>
<td>0.19 ± 0.07</td>
</tr>
<tr>
<td>Rv1590</td>
<td>Rv3204</td>
<td>Rv1886</td>
<td>0.20 ± 0.04</td>
</tr>
<tr>
<td>Rv1738</td>
<td>Rv3407</td>
<td>Rv2220</td>
<td>0.25 ± 0.11</td>
</tr>
<tr>
<td>Rv1818</td>
<td>Rv3541</td>
<td>Rv3020</td>
<td>0.17 ± 0.07</td>
</tr>
<tr>
<td>Rv1884</td>
<td>Rv3620</td>
<td>Rv3619</td>
<td>0.24 ± 0.05</td>
</tr>
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<td>Rv1926</td>
<td>Rv3628</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Rv1984</td>
<td>Rv3810</td>
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<td></td>
</tr>
<tr>
<td>Rv2032</td>
<td>CpG</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Rv2389</td>
<td></td>
<td></td>
<td>(-0.09 ± 0.05)</td>
</tr>
</tbody>
</table>

* Rv were immunized s.c. three times, 3 wk apart, with 8 μg Mtb Ags (Rv no.) + 25 μg CpG, or intradermally once with 5 × 10^4 BCG.

**FIGURE 1.** Levels of IFN-γ released by Ag-stimulated human PBMCs were measured in vitro. PPD⁺ and PPD⁻ PBMCs were incubated for 72 h in medium,
PHA (10 μg/ml), Mtb lysate (10 μg/ml), or Mtb recombinant proteins (50 μg/ml). Ags are grouped according to the selection criteria: m, membrane-associated;
S, secreted proteins; P, PE/PPE; E, EsX; M, macrophage growth required; H, hypoxic response; D, database searches. A, IFN-γ in supernatants was measured by
sandwich ELISA. Mean (mean_{Ag}–mean_{medium}) ± SEM are shown for PPD⁺ (n = 18) and PPD⁻ (n = 7) PBMCs. B, Percentage of PPD⁺ positive responders.

Antigens inducing <0.1 log reduction in lung CFU in the first screening were considered not protective and were not repeated for ethical concerns to
reduce the number of animals used in these studies.

Ag category: S, secreted; P, PE/PPE; E, EsX; M, growth in macrophages; H, regulated under hypoxic growth.

a Reduction of viable bacteria (CFU) in the lungs of immunized animals compared to saline controls 4 wk after a low-dose aerosol challenge with *M.
tuberculosis* H37Rv and/or Erdman strains. Data represent the mean of two to three independent experiments ± SEM for Ags inducing >0.1 log reduction.
determined by ELISPOT 3 wk after the last injection. 

Table III. Immunogenicity of Mtb Ags

<table>
<thead>
<tr>
<th>Ag</th>
<th>IFN-γ</th>
<th>TNF</th>
<th>IgG</th>
</tr>
</thead>
<tbody>
<tr>
<td>Rv0577</td>
<td>523 ± 8</td>
<td>388 ± 297</td>
<td>0.98</td>
</tr>
<tr>
<td>Rv1626</td>
<td>20 ± 21</td>
<td>268 ± 117</td>
<td>1.19*</td>
</tr>
<tr>
<td>Rv2608</td>
<td>798 ± 11</td>
<td>175 ± 105</td>
<td>1.09</td>
</tr>
<tr>
<td>Rv2875</td>
<td>428 ± 172</td>
<td>137 ± 60</td>
<td>1.05*</td>
</tr>
<tr>
<td>Rv3044</td>
<td>331 ± 161</td>
<td>57 ± 1</td>
<td>1.05*</td>
</tr>
<tr>
<td>Rv3478</td>
<td>453 ± 4</td>
<td>149 ± 73</td>
<td>1.03</td>
</tr>
</tbody>
</table>

Reduction in CFU of >0.3 Log10

<table>
<thead>
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<th>Ag</th>
<th>IFN-γ</th>
<th>TNF</th>
<th>IgG</th>
</tr>
</thead>
<tbody>
<tr>
<td>Rv0496</td>
<td>68 ± 52</td>
<td>24 ± 5</td>
<td>1.21*</td>
</tr>
<tr>
<td>Rv0733</td>
<td>ND</td>
<td>ND</td>
<td>ND</td>
</tr>
<tr>
<td>Rv0831</td>
<td>24 ± 12</td>
<td>24 ± 8</td>
<td>1.19*</td>
</tr>
<tr>
<td>Rv1411</td>
<td>187 ± 36</td>
<td>ND</td>
<td>1.00</td>
</tr>
<tr>
<td>Rv1569</td>
<td>ND</td>
<td>ND</td>
<td>ND</td>
</tr>
<tr>
<td>Rv1789</td>
<td>ND</td>
<td>ND</td>
<td>ND</td>
</tr>
<tr>
<td>Rv1813</td>
<td>388 ± 103</td>
<td>32 ± 13</td>
<td>1.18*</td>
</tr>
<tr>
<td>Rv1860</td>
<td>ND</td>
<td>ND</td>
<td>ND</td>
</tr>
<tr>
<td>Rv1886</td>
<td>590 ± 106</td>
<td>102 ± 37</td>
<td>1.00</td>
</tr>
<tr>
<td>Rv2220</td>
<td>ND</td>
<td>ND</td>
<td>ND</td>
</tr>
<tr>
<td>Rv3020</td>
<td>48 ± 27</td>
<td>20 ± 16</td>
<td>1.18*</td>
</tr>
<tr>
<td>Rv3619</td>
<td>604 ± 184</td>
<td>1261 ± 319</td>
<td>1.13*</td>
</tr>
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</table>

Reduction in CFU of <0.1 Log10

<table>
<thead>
<tr>
<th>Ag</th>
<th>IFN-γ</th>
<th>TNF</th>
<th>IgG</th>
</tr>
</thead>
<tbody>
<tr>
<td>Rv2389</td>
<td>39 ± 49</td>
<td>92 ± 31</td>
<td>1.02</td>
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<tr>
<td>Rv2450</td>
<td>148 ± 104</td>
<td>4 ± 4</td>
<td>0.97</td>
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<tr>
<td>Rv2623</td>
<td>21 ± 12</td>
<td>2 ± 1</td>
<td>1.14*</td>
</tr>
<tr>
<td>Rv2626</td>
<td>95 ± 30</td>
<td>7 ± 4</td>
<td>0.95</td>
</tr>
<tr>
<td>Rv2801</td>
<td>ND</td>
<td>ND</td>
<td>ND</td>
</tr>
<tr>
<td>Rv2866</td>
<td>104 ± 56</td>
<td>32 ± 12</td>
<td>1.31*</td>
</tr>
<tr>
<td>Rv2945</td>
<td>ND</td>
<td>ND</td>
<td>ND</td>
</tr>
<tr>
<td>Rv3029</td>
<td>116 ± 29</td>
<td>6 ± 2</td>
<td>1.25*</td>
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<tr>
<td>Rv3133</td>
<td>24 ± 8</td>
<td>12 ± 8</td>
<td>1.95*</td>
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<tr>
<td>Rv3204</td>
<td>8 ± 13</td>
<td>5 ± 7</td>
<td>2.10*</td>
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<tr>
<td>Rv3407</td>
<td>ND</td>
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<td>Rv3541</td>
<td>ND</td>
<td>ND</td>
<td>ND</td>
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<tr>
<td>Rv3620</td>
<td>184 ± 44</td>
<td>72 ± 33</td>
<td>1.13*</td>
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<tr>
<td>Rv3628</td>
<td>16 ± 8</td>
<td>ND</td>
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<td>Rv3810</td>
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<tr>
<td>Rv3810</td>
<td>ND</td>
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Note: * = Student’s t test.

Reduction in CFU by ELISPOT 3 wk after the last injection per million cells ± SD. Mice were immunized subcutaneously three times, 3 wk apart, with Mtb Ags (Rv no.) + CpG. Cytokine responses to the Ags were determined by ELISPOT 3 wk after the last injection. 

* Ags are displayed based on efficacy at reducing lung bacterial burden.

** Spot-forming units (SFU) per million cells ± SD. Mice were immunized s.c. three times, 3 wk apart, with Mtb Ags (Rv no.) + CpG. Cytokine responses to the Ags were determined by ELISPOT 3 wk after the last injection.

† IgG2c/IgG1 ratio, * = p < 0.05, Student’s t test.

‡ ND, not done.

Predominant for all the proteins but two (Rv0577, Rv1886), as indicated by an IgG2c/IgG1 ratio >1 (Fig. 2B). Differences in Ag-specific IgG2c vs IgG1 Ab titers were found statistically significant (p < 0.05) for Rv2875, Rv3044, and Rv3619 using Student’s t test.

FIGURE 2. Ab responses to Mtb recombinant Ags were measured in sera isolated from mice vaccinated with Rv0577, Rv2875, Rv1886, Rv2608, Rv3478, Rv3619, Rv3620, Rv1813, and Rv3044 + CpG. Sera were tested for Ag-specific IgG1 and IgG2c Ab. A, Mean of reciprocal titers ± SEM. B, Endpoint titers and IgG2c/IgG1 ratios are shown. * p < 0.05, Student’s t test. Data shown are representative of two independent experiments.

FIGURE 3. IFN-γ responses to Mtb recombinant Ags were determined in vaccinated mice. Splenocytes were prepared from animals immunized with Rv0577, Rv1626, Rv1813, Rv1886 (85B), Rv2875, Rv2608, Rv3619, Rv3620, Rv3044, and Rv3478 + CpG or saline, 3 wk after the last injection, and tested for IFN-γ in vitro recall responses. Cells were plated in duplicate at 2 × 10^5 cells/well and cultured with medium, Con A (3 μg/ml), PPD (10 μg/ml), or each recombinant Ag (10 μg/ml) for 48 h. Frequencies of IFN-γ-secreting cells (spot-forming units, SFU) were determined by ELISPOT. Means ± SD (n = 5 mice) shown are representative of two independent experiments.
Combination of multiple Ags increased protection against challenge with Mtb

We hypothesized that combining multiple Ags would lead to increased reduction in bacterial burden following Mtb infection. We used Rv2608, Rv1813, and Rv3620, which were the first Ags we identified as inducing partial protection against Mtb. Mice were injected three times 3 wk apart with single Ags + CpG, a mixture of the three Ags + CpG, CpG alone, or BCG. The number of viable bacilli (CFU) in the lung of mice from the different immunized groups was compared (Fig. 6A). In this experiment, Rv1813 and Rv2608 adjuvanted with CpG reduced the number of lung bacteria by 0.30 log_{10} (p < 0.01) and 0.25 log_{10} (p < 0.05), respectively, while immunization with Rv3620 + CpG was ineffective (0.05 log_{10}, p > 0.05). The weak protection we observed with Rv3620 in our initial experiment that led to its inclusion in the combination was not confirmed in this or subsequent experiments. Nevertheless, with 0.67 log_{10} reduction (p < 0.01), the effect of the three Ags combined was greater than that seen with any single Ag and comparable to vaccination with BCG (0.71 log_{10}, p < 0.01).

FIGURE 5. Ag-specific CD4 and CD8 T cell cytokine responses were analyzed in vaccinated mice. Splenocytes, isolated from animals immunized with Rv1813, Rv3620, Rv2608, Rv3619, Rv3478, or Rv1886 + CpG or saline, were plated at 2 x 10^{6} cells/well and cultured with medium or each recombinant Ag (20 μg/ml) for 12 h in the presence of GolgiStop. A, CD4 T cells were identified by ICS based on CD3 and CD4 expression and further gated as CD4^{high} T cells (Ag experienced) or CD4^{low} (naive). The percentages of cells expressing two cytokines or more of IFN-γ, TNF, and/or IL-2 were determined. Means (n = 3) are shown. B, CD8 Ag-specific T cells were identified by ICS as CD3/CD4/CD44^{high}/CD8 cells also positive for IFN-γ and/or TNF, and further analyzed for their content in GrB. Numbers of cytokine+ (IFN/TNF) and cytokine+ GrB+ per million splenocytes were determined. Means ± SD (n = 3 mice) shown are representative of two independent experiments.
Human T cell responses to vaccine candidate Ags

Human PBMCs from six healthy PPD\(^+\) subjects with documented prior exposure to \(Mtb\) were HLA typed (Pudget Sound Blood Center, Seattle, WA): D160 (HLA-A2/3, B14/44, DR4/13, DQ1/7), D204 (HLA-A3/68, B27/35, DR7/15, DR51/53, DQ6/9), D336 (HLA-A2/3, B7/44, DR4/13, DQ1/7), D365 (HLA-A1/2, B7/8, DR2/3, DQ1/2), D366 (HLA-A2/24, B60, DR4, DQ3), and D368 (HLA-A2/29, B44, DR15/4, DQ0602/0301). CD4 and CD8 T cell cytokine responses to PPD and the nine most protective Ags were analyzed by ICS at the single cell level. We also looked at responses to Rv1813 and Rv3620 because of their possible additive effect when used in combination with Rv2608. Memory T cells were gated on expression of CD3/CD4/CD45RO or CD3/CD8/CD45RO molecules, and the data reflect the percentage of cells staining positive for IFN-\( \gamma \), TNF, or the two cytokines after background (media control) removal. PPD and PMA+Ionomycin stimulation induced CD4 T cells staining positive for IFN-\( \gamma \), TNF, and/or the two cytokines in all subjects (Fig. 7). CD4 T cell responses to the different \(Mtb\) Ags were more variable among donors, with mixed profiles consisting most of the time of cells positive for a single cytokine (IFN-\( \gamma \) or TNF), as well as cells expressing both cytokines. For CD8 T cells, PMA+Ionomycin stimulation receiving the three Ags compared with animals injected with Rv3620 only. Additional T-dependent help provided by the other two Ags might explain this difference, as Rv3620 is a small (10 kDa) protein. Ag-specific IgG1 was also detected (data not shown). IgG2c/IgG1 ratios were \(>1\) for all groups showing Ag-specific Abs, consistent with a predominant Th1 response. Mice immunized with CpG alone did not show IgG2c- or IgG1-specific responses to either Ag. In contrast, IFN-\( \gamma \) and TNF responses were essentially restricted to Rv2608 and diminished in the group receiving the Ag combination compared with the single Ag (Fig. 6, C and D). The reduced cytokine responses in the group receiving the three Ags + CpG can at least partially be attributed to the fact that these animals were immunized with less protein (6 vs 8 \( \mu \)g for the single Ag groups) to use a dose of \(<20 \mu \)g Ag total per injection. We observed in other infection models that immunizing mice with \(>20 \mu \)g of Ag was detrimental (R. Coler, unpublished observations).
induced positive responses for IFN-γ, TNF, and the two cytokines in all subjects, while PPD induced IFN-γ and IFN-γ + TNF, but not TNF alone (Fig. 8). Most of the CD8 T cell responses to Mtb Ags were IFN-γ or IFN-γ + TNF. Overall, each Ag was recognized by subjects with a diversity of HLA by either CD4 or/and CD8 T cells.

**Discussion**

With the goal of identifying Ags for use in a human TB vaccine, we performed the most comprehensive analysis to date of human T cell Ags of Mtb. From selected genome mining, we chose ~100 potential candidates to be expressed as recombinant proteins and evaluated for IFN-γ recall responses using PBMCs from healthy PPD+ subjects. Based on PBMC results, a subset of 48 Ags was selected to be administered to mice in a CpG formulation, with 18 of them conferring partial protection against challenge with virulent Mtb H37Rv and/or Erdman strains. A combination of three of these Ags further increased protection against TB to levels comparable to those achieved with BCG vaccination. Induction of predominant Th1 CD4 responses by vaccine Ags were observed in mice with reduced lung bacterial burden.

Recall secretion of IFN-γ by pathogen-exposed cells is a relevant marker for cellular immune responses in Mtb infections, and it has been used as a rationale for Ag selection for potential vaccines (reviewed in Refs. 8, 15). With 94 Mtb proteins evaluated for antigenicity on PPD+ donor PBMCs, our study considerably expands the number of Mtb proteins tested so far. Most of the proteins (79) were widely recognized as indicated by positive IFN-γ production by PBMCs from >70% of the subjects. Of the 79 proteins, 28 were recognized by all PPD+ donors tested. HLA typing of a subset of donors indicated a wide heterogeneity in MHC class I and II alleles, suggesting that these Ags contain epitopes recognized by a wide range of different HLA molecules. The promiscuous recognition of Ags by individuals with a wide variety of ethnic origins is of major importance in the context of human vaccines.

We and others have demonstrated the feasibility of using protein subunit vaccines against leishmaniasis (16) and TB (17, 18) with vaccines currently in clinical trials. Unlike DNA vaccines, protein-based vaccines are immunogenic in small animal models, nonhuman primates, and humans. The addition of a CpG adjuvant further extends results obtained with CpG-adjuvanted vaccines in nonhuman primates and humans. Properly adjuvanting Mtb proteins, however, is the key to the induction of strong in vivo immune responses. In this study, we used CpG as an adjuvant based on its reported ability to potentiate Th1 CD4 and CD8 T cell responses (11, 19). To favor the selection of potent Ags, we chose a relatively low dose of CpG as an adjuvant. Under these conditions, we observed only a modest reduction in lung bacterial counts (0.20 log10) following immunization with Rv1886 (Ag85B), previously reported to have protective capacity (18), lower than some of the novel Ags reported herein.

We identified 17 previously uncharacterized Ags that were able to induce partial protection against Mtb in mice. Most of the Ags were either secreted or membrane-associated. Rv2608 and Rv3478 belong to the family of PE/PPE proteins, the members of which have been shown to induce Th1 responses (20, 21) and confer protection against Mtb (22–24). The PE/PPE proteins have also been shown to have higher levels of polymorphism among Mtb clinical isolates variants (25). However, no genetic variation was observed for Rv2608 or Rv3478 within recently published MDR and XDR genomes (www.broad.mit.edu/annotation/genome/mycobacterium_tuberculosis_spp/ToolsIndex.html). Rv3020 and Rv3619 are members of the EsX family of virulence factors, among which several potential vaccine candidates have been described (26–30). Finally, Rv1813 and Rv3044 are proteins expressed by Mtb in conditions of low oxygen and are associated with latent growth of the bacteria (14). Identifying novel vaccine Ags within families of proteins known to induce potent immune responses further validates the bioinformatic approach. In this study, adjuvanting recombinant proteins with CpG proved effective at reducing bacterial burden, further extending results obtained with CpG-adjuvanted culture filtrate proteins (31) or BCG (32). Combining multiple Mtb Ags led to increased vaccine efficacy, extending observations from us and others using combination of Ags (33) or fusion proteins like Mtb72f, Ag85B-ESAT6, and Ag85B-TB10 (17, 18, 27, 34). Fusion proteins comprised of our newly identified priority candidates are currently in development.
Strong Th1 CD4 responses and cytokines like IFN-γ and TNF are critical for protection against TB (35–39), while Ab responses are not considered to be involved in antimycobacterial adaptive immunity. In this study, we used the Ag-specific IgG2c/IgG1 ratio as an indication of a predominant Th1 (>1), mixed Th1/Th2 (=1), or Th2 (<1) response induced by vaccination. We show that vaccine-induced protection against challenge with Mtb in mice was associated with a Th1 response characterized by predominant serum IgG2c and high levels of IFN-γ and TNF. High frequencies of IFN-γ or TNF-producing cells, however, did not always correlate with lower lung bacterial counts, as exemplified by Rv1884 or Rv1984, suggesting that other factors are important to control the infection in mice, and extending observations by others (40–42).

The ability of a vaccine to induce pluriptent T cell responses has been associated with increased protection in a mouse model of Leishmania; such a mechanism has also been proposed for M. bovis BCG vaccine (43). All the vaccine candidates induced effect memory CD4 T cells expressing at least two, if not all three, array of cytokines and did not induce CD8 T cells.

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Disclosures

The authors have no financial conflicts of interest.

References


