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Molecular Cloning and Immunologic Reactivity of a Novel Low Molecular Mass Antigen of *Mycobacterium tuberculosis*¹

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Polypeptide Ags present in the culture filtrate of *Mycobacterium tuberculosis* were purified and evaluated for their ability to stimulate PBMC from purified protein derivative (PPD)-positive healthy donors. One such Ag, which elicited strong proliferation and IFN-γ production, was further characterized. The N-terminal amino acid sequence of this polypeptide was determined and used to design oligonucleotides for screening a recombinant *M. tuberculosis* genomic DNA library. The gene (*Mtb 8.4*) corresponding to the identified polypeptide was cloned, sequenced, and expressed in *Escherichia coli*. The predicted m.w. of the recombinant protein without its signal peptide was 8.4 kDa. By Southern analysis, the DNA encoding this mycobacterial protein was found in the *M. tuberculosis* strains H37Rv, H37Ra, Erdman, and “C” strain, as well as in certain other mycobacterial species, including *Mycobacterium avium* and *Mycobacterium bovis* BCG (bacillus Calmette-Guérin, Pasteur). The *Mtb 8.4* gene appears to be absent from the environmental mycobacterial species examined thus far, including *Mycobacterium smegmatis*, *Mycobacterium gordonae*, *Mycobacterium chelonae*, *Mycobacterium fortuitum*, and *Mycobacterium scrofulaceum*. Recombinant *Mtb 8.4* Ag induced significant proliferation as well as production of IFN-γ, IL-10, and TNF-α, but not IL-5, from human PBMC isolated from PPD-positive healthy donors. *Mtb 8.4* did not stimulate PBMC from PPD-negative donors. Furthermore, immunogenicity studies in mice indicate that *Mtb 8.4* elicits a Th1 cytokine profile, which is considered important for protective immunity to tuberculosis. Collectively, these results demonstrate that *Mtb 8.4* is an immunodominant T cell Ag of *M. tuberculosis*. *The Journal of Immunology*, 1998, 161: 2356–2364.

*Mycobacterium tuberculosis*, the etiologic agent of tuberculosis (TB),¹ is, according to the World Health Organization, the world’s leading killer of adults. Thirty million deaths due to TB are expected in the next decade (1). Tuberculosis thus remains a major public health problem not only in developing countries, but also in industrialized countries where a resurgence has been noted, particularly in association with HIV infection (2). The majority of *M. tuberculosis* infections in humans appears to be asymptomatic, subclinical, or latent. Only 5 to 10% of infected immunocompetent individuals will develop active disease during their lifetimes, and some of these cases develop as many as 4 to 5 decades after the initial infection event. If left untreated, serious complications and death typically result.

The only currently available vaccine against TB is the live attenuated bacillus Calmette-Guérin (BCG), derived from *Mycobacterium bovis*. Based on the results of many clinical trials in developing countries, the efficacy of BCG in eliciting protective immunity has been reported to vary from 0 to 80% (3–5). In ad

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¹ Abbreviations used in this paper: TB, tuberculosis; BCG, bacillus Calmette-Guérin; PPD, purified protein derivative; CT, culture filtrate; TFA, trifluoroacetic acid; SL, stimulation index.

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healthy individuals. T cell responses against such Ags may be protective in the specific host immune response, thus providing necessary candidates for the development of an antimycobacterial subunit vaccine. This report describes the purification and biologic activity of a protein obtained from the culture filtrate of *M. tuberculosis* strain H37Rv, as well as cloning, DNA sequence, expression, and preliminary immunologic characterization of this novel immunoreactive T cell Ag of *M. tuberculosis*, Mtb 8.4.

**Materials and Methods**

**Mycobacterial strains**

*M. tuberculosis* strains H37Rv and Erdman were gifts from Dr. Sean Skerritt, Seattle Veterans Affairs Hospital, Seattle, WA; “C” strain was a gift from Dr. Lee Riley, University of California, Berkeley CA; *M. bovis* BCG and *Mycobacterium leprae* (Pasteur) were obtained from Genesis, Auckland, New Zealand. Other species of mycobacteria were obtained from the American Type Culture Collection (ATCC, Manassas, VA): *M. tuberculosis* H37Ra (ATCC 25177), *Mycobacterium vaccae* (ATCC 15483), *Mycobacterium avium* avium (ATCC 35718), *Mycobacterium chelonae* (ATCC 14472), *Mycobacterium fortuitum* (ATCC 6841), *Mycobacterium gordonae* (ATCC 14470), *Mycobacterium scrofulaceum* (ATCC 19981), and *Mycobacterium smegmatis* (ATCC 19420).

**Purification of secreted polypeptides**

Culture supernatants were prepared from 2- to 3 wk log phase cultures of *M. tuberculosis* H37Rv grown in a synthetic medium (1.5% glucose, 1% basal medium Eagle’s amino acid solution (Life Technologies, Grand Island, NY), 1% MEM nonessential amino acid solution (Life Technologies), 1% basal medium Eagle’s vitamin solution (Life Technologies), 0.05g/L ferric ammonium citrate, 4g/L K,HPO₄, 2g/L citric acid, 1.2g/L MgCl₂, 0.6g/L K,SO₄, 2g/L NH₄Cl, 0.72g/L NaOH) at 37°C. Culture supernatant was harvested and filtered through a 0.22-μm cellulose acetate membrane (Corning Glassworks, Corning, NY). The filtrate was concentrated 50× with an Amicon Centriprep-3 concentrator (Beverly, MA) and submitted for quantitation of bacterial endotoxin using a *Limulus* amebocyte lysate assay (LAL; BioWhittaker, Walkersville, MD). The protein concentration of the culture filtrate (CF) was determined using a commercial bicinchoninic acid assay (BCA; Pierce, Rockford, IL).

Fractionation of the CF was performed using a microbore Vydac C18 column (Vydac, Hesperia, CA) and a standard linear gradient of 0.5%/min. increase from 20 to 50% buffer B (80% acetonitrile/0.05% trifluoroacetic acid (TFA)). Aliquots of different fractions were submitted for immunologic assay and for N-terminal protein sequencing.

**Amino-terminal sequencing**

Amino acid identifications were performed on polypeptide(s) in positive fractions. Individual fractions were dried onto Biobrene-treated glass fiber filters (Perkin-Elmer/Applied Biosystems, Foster City, CA). The filters were washed twice at room temperature for 20 min in 6M urea solution (ATCC 14470), Mycobacterium vaccae (ATCC 15483), Mycobacterium avium avium (ATCC 35718), Mycobacterium chelonae (ATCC 14472), Mycobacterium fortuitum (ATCC 6841), Mycobacterium gordonae (ATCC 14470), Mycobacterium scrofulaceum (ATCC 19981), and Mycobacterium smegmatis (ATCC 19420).

**Screening of bacteriophage libraries**

Degenerate oligonucleotides were synthesized (Life Technologies) based on the deduced N-terminal amino acid sequence and used as probes to screen the M. tuberculosis Erdman library. Two micrograms of purified oligonucleotides designed to make a PCR product that would begin 3′ to the possible secretory sequence and would continue to the stop codon. The 5′ primer included sequence coding for six histidine residues for ease of purification with Ni-NTA resin (Qiagen), as well as an NdeI restriction enzyme site for subcloning. The 3′ primer sequence (Mtb 8.4 5′ His) was 5′-CAATTACATATGCATCACCATCACCATCACGATCCCGTGGACGCG-3′.

**Production of rabbit polyclonal serum against the rMtb 8.4 Ag**

The coding portion of Mtb 8.4 was PCR amplified using two oligonucleotides designed to make a PCR product that would begin 3′ to the possible secretory sequence and would continue to the stop codon. The 5′ primer included sequence coding for six histidine residues for ease of purification with Ni-NTA resin (Qiagen), as well as an NdeI restriction enzyme site for subcloning. The 3′ primer sequence (Mtb 8.4 3′ End) was 5′-CAAGAATCTTACTTATGTTGTCGAGGA-3′.

The 1.4-kb DNA fragment encoding the Mtb 8.4 gene was purified from an agarose gel after EcoRI digestion and used as template for subcloning. Standard PCR reactions were conducted in a Peltier thermal cycler (DNA Engine PTC-200; MJ Research, Watertown, MA). The reaction amplifications were performed for 30 cycles at 94°C for 1 min, 60°C for 30 s, and 72°C for 1.5 min. The resulting PCR fragment was ligated with T4 ligase in NdeI/EcoRI-digested pET 17b plasmid vector (Novagen, Madison, WI) and was transformed into *Escherichia coli* XL-1 Blue and BL-21 (DE3) ploye (Novagen) by the standard procedures for DNA manipulation and protein expression, respectively (27).

To obtain *E. coli* BL21 lysates, single colonies were inoculated into 2× yeast tryptone (YT) broth and grown to an OD of 0.5 at 560 nm. isopropyl-β-D-thiogalactopyranoside was then added, and growth was continued for an additional 3 h. The bacteria were harvested by centrifugation and lysed in the presence of protease inhibitors and lysozyme, using a sonicator. The induced target protein was identified by SDS-PAGE in the insoluble inclusion body. The purification of rMtb 8.4 was performed by metal chelate column chromatography using Ni-NTA resin according to the manufacturer’s recommendations (Qiagen).

**Southern analysis**

Genomic DNA from several mycobacterial species was prepared using the detergent cetyltrimethylammonium bromide (CTAB, Sigma, St. Louis, MO). DNA was extracted using phenol:chloroform and precipitated. One microgram of DNA was then digested using *PstI* or *SfiI*, electrophoresed in a 1.5% agarose gel, and transferred to a nitrocellulose membrane (Schleicher and Schuell). The Mtb 8.4 insert DNA was 32P radiolabeled by the random hexamer priming method (28) and used to probe the mycobacterial DNAs. After hybridization for 18 h at 65°C, the blots were washed at 60°C, twice for 15 min with 2× SSC, once for 30 min with 2× SSC/0.2% SDS, and once for 10 min with 0.2× SSC. After drying at room temperature, blots were mounted for autoradiography at −80°C for 24 h.

**Production of rabbit polyclonal serum against the rMtb 8.4 Ag**

The rMtb 8.4 Ag (150 μg) was emulsified in a mixture of 100 μg of muramyl dipeptide and 1 ml of IFA (Life Technologies) as adjuvants and injected intramuscularly at multiple sites into a New Zealand rabbit (R&R Rabbity, Stanwood, WA). A s.c. booster injection of 100 μg of rAg with 1 ml of IFA was given 6 wk later, and 25 μg in PBS was given 3 wk later. The rabbit was sacrificed 1 wk following the last boost, and serum was collected and stored at −20°C.

**PAGE and immunoblotting**

Samples of crude *E. coli* protein extracts were separated by SDS-15% PAGE before being stained with Coomassie brilliant blue or transferred onto nitrocellulose sheets. The samples blotted on nitrocellulose were probed with polyclonal rabbit serum against the purified rMtb 8.4 protein or against *M. tuberculosis* H37Rv CF proteins. The detecting agent was protein A-conjugated 125I.
Cytokine assays

The production of cytokines was quantified by sandwich ELISA. Briefly, ELISA plates (Corning) were coated for 4 h at room temperature with 50 μg/well cytokine capture mAb (1 μg/ml for IFN-γ, IL-5, and IL-10; 2 μg/ml for TNF-α; PharMingen, San Diego, CA) in 0.1 M NaHCO3/Na2CO3 buffer (pH 9.6). After blocking overnight at 4°C (5% [W/V] non-fat dried milk for IFN-γ and 1% BSA for IL-5, IL-10, and TNF-α, samples or standards were added for 2 h at room temperature. Plates were washed with PBS, 0.05% Tween (PBS-Tween) and then incubated for 2 h at room temperature with 100 μl/well of second Ab (rabbit anti-human polyclonal Ab; ImmuneX, Seattle, WA) diluted 1:3000 in PBS-10% normal goat serum for IFN-γ, biotinylated detection Ab in PBS-Tween, 0.1% BSA at 0.5 μg/ml for IL-5 and IL-10; and at 1 μg/ml for TNF-α (PharMingen). After washing, plates were incubated with goat anti-rabbit horseradish peroxidase in PBS-Tween, 5% nonfat dried milk (IFN-γ) or with streptavidin-peroxidase in PBS-Tween, 0.1% BSA (IL-5, IL-10, and TNF-α). Plates were developed using TMB substrate (3,3′-5,5′-tetramethylbenzidine, Kirkegaard and Perry, Gaithersburg, MD). OD was determined at 450 nm using 570 nm as a reference wavelength. Cytokine concentration was evaluated using the respective standard curves.

Immunogenicity studies

BALB/c and C57BL/6 mice were immunized in the footpads with 15 to 30 μg of the rMtb 8.4 Ag formulated in IFA as adjuvant. Mice were also immunized with saline or IFA alone as control. Draining lymph nodes were removed 9 days later, and cells were plated at 2.5 × 10^5/well for proliferation assays and at 2.5 × 10^4/well for cytokine assays. The lymph node cells were cultured in the presence of anti-IL-4R (Immunex) at 3 μg/ml. The anti-IL-4R Ab was added to the cultures because it presumably blocks the uptake of secreted IL-4 by activated T cells, thereby increasing the accuracy of quantitating IL-4 without affecting proliferation or IFN-γ production. The lymph node cells were restimulated in vitro with rMtb 8.4 at 1, 5, and 25 μg/ml. Plates were cultured for 3 days at 37°C in 5% CO2. Supernatants were then taken for cytokine ELISA, or plates were pulsed.
with 1 μCi of [3H]thymidine for 18 h, harvested, and [3H] thymidine incorporation was counted in a gas scintillation counter.

Results
Identification of T cell Ags in M. tuberculosis culture filtrate
The CF of M. tuberculosis has previously been reported to contain Ags that elicit specific immune responses or protection in animals infected with M. tuberculosis (12–17). To study the PBMC responses to such proteins, CF from M. tuberculosis that tested negative for endotoxin by Limulus amebocyte lysate assay was separated into 16 fractions on a microbore column using a slow gradient (Fig. 1) and evaluated immunologically using PBMC from PPD+ individuals (Fig. 2). As shown, fractions 8 through 16 at a 1:40 dilution were able to stimulate proliferation of PBMC from both of the PPD+ individuals to a strong degree (Fig. 2).

Direct determination of N-terminal amino acid sequences on individual protein fractions was performed. Several fractions revealed amino acid sequences of proteins that have already been described, such as the Ag 85 complex (F8) (29, 30), MPT64 (F16) (31–33), and the 45/47-kDa Ag complex (F14) (9, 34, 35). The amino-terminal analysis of native protein(s) from one such fraction of the M. tuberculosis H37Rv culture filtrate (F 13) revealed the novel sequence Asp-Pro-Val-Asp-Ala-Val-Ile-Asn-Thr-Thr-Cys-Asn-Tyr-Gly-Gln-Val-Val-Ala-Ala-Leu-Asn. The protein was named Mtb 8.4, based on the molecular mass in its mature form.

Cloning of the Mtb 8.4 gene
Several overlapping degenerate oligonucleotides were designed based on the amino-terminal sequence data of the native Mtb 8.4 polypeptide. One of the oligonucleotides, TGYAAYTAYGGTGRCARGT (where Y = C + T; I = deoxynosine; R = A + G; S = C + G), representing the amino acids Cys-Asn-Tyr-Gly-Gln-Val-Val-Ala-Ala-Leu, was used to clone a recombinant gene with an insert size of \( \frac{1}{2} \) kb. The nucleotide sequence of Mtb 8.4 and the predicted amino acid sequence coding for the Mtb 8.4 protein are shown in Figure 3. An open reading frame of 330 nucleotides coded for a protein of 110 amino acids with a consensus signal peptide of 28 amino acids (residues 969–1052) and a predicted molecular mass of 10.8 kDa. The DNA sequence contained an open reading frame starting with an ATG codon at nucleotide number 969. The mature amino terminus is indicated at position 1053. The potential ribosome-binding site at position 953 is denoted by SD for Shine-Dalgarno. The putative Mtb 8.4 Pribnow boxes (–35 and –10 sequences) are marked at positions 919 and 945, respectively. The stop codon is at position 1299.

FIGURE 3. Nucleotide sequence and the deduced amino acid sequence of the translated product of the Mtb 8.4 gene. The 110-residue amino acid sequence is shown below the corresponding open reading frame beginning at nucleotide number 969. The molecular mass of the predicted protein is 10.8 kDa. The translation initiation codon ATG is indicated at position 969, and the mature amino terminus is indicated at position 1053. The potential ribosome-binding site at position 953 is denoted by SD for Shine-Dalgarno. The putative Mtb 8.4 Pribnow boxes (–35 and –10 sequences) are marked at positions 919 and 945, respectively. The stop codon is at position 1299.

FIGURE 5. Expression and purification of E. coli expressed rMtb 8.4 Ag. Crude 15% SDS-PAGE separated proteins of noninduced, induced, and purified Mtb 8.4 (1 μg/track) were stained with Coomassie brilliant blue. Molecular weight markers in kDa, lane M; recombinant E. coli lysates before (lane 1) and after (lane 2) induction with isopropyl-β-D-galactopyranoside to express rMtb 8.4 containing an amino-terminal six-histidine affinity tag and the purified r8.4 Ag (lane 3).

purified culture filtrate polypeptide and the deduced amino acid sequence of the cloned DNA corresponding to Mtb 8.4 without its hydrophobic secretory region were identical. Given the known amino terminal sequence of the purified culture filtrate Ag and the characteristics of the signal peptide sequence, it is feasible that the signal peptidease recognition sequence (Ala-X-Ala) (31) is located in front of the N-terminal region of the mature form of the protein at nucleotide 1052. The structural gene encoding the mature Mtb 8.4 protein, Mtb 8.4, derived from M. tuberculosis Erdman is thus found at nucleotide residues 1053–1298. A potential ribosome-binding site (GGAGGG) is located at nucleotide residue 953–958. Putative Pribnow boxes (~35 and ~10 sequences), similar to the E. coli promoter-like consensus sequences, are located at nucleotide residues 919 and 945, respectively.

Presence of Mtb 8.4 in various mycobacterial species
To determine the distribution of Mtb 8.4 within species belonging to the M. tuberculosis complex, M. avium and M. bovis BCG, as well as in “environmental” mycobacterial species, the 279-bp NdeI/EcoRI Mtb 8.4 fragment from pET/Mtb 8.4 was used as a probe in a Southern hybridization analysis of digested total genomic DNA from various mycobacterial strains (Fig. 4). This experiment indicated that the Mtb 8.4 gene is present as a single copy in the mycobacterial genome. The probe hybridized to SauI fragments of approximately 4.4 kb in the M. tuberculosis sub-strains H37Rv, H37Ra, Erdman, and the “C” strain, as well as in M. bovis BCG (Pasteur). Hybridization to a fragment of ~8 kb was observed in M. avium avium, but the probe did not hybridize to any SauI fragments from M. leprae, M. smegmatis, M. vaccae, M. gordonaie, M. cheloneae, M. fortuitum, or M. scrofulaceum.

Expression and immunoblotting analysis of rMtb 8.4
Recombinant Mtb 8.4 lacking its hydrophobic, putative signal peptide sequence was expressed in E. coli with six consecutive His residues at the amino-terminal portion immediately following the initiator Met residue (N-terminal HIS-Tag) of the pET plasmid vector (pET-17b) and a T7 RNA polymerase expression system (Novagen). Crude protein extracts of Mtb 8.4 and purified rMtb 8.4 were subjected to SDS-PAGE and stained with Coomassie brilliant blue (Fig. 5). To further characterize native and recombinant Mtb 8.4, a Mtb 8.4 antiserum was raised in a rabbit and used as a probe in an immunoblot assay. Figure 6A shows that although the rabbit anti-Mtb 8.4 serum reacted with a single band in the CF, it did not detect the Mtb 8.4 molecule in the M. tuberculosis lysate H37Rv. This finding suggests that the Mtb 8.4 protein may be rapidly processed and exported from the bacilli after synthesis. The absence of general protease degradation of the lysate during preparation was substantiated by probing another blot with a rabbit polyclonal antiserum raised against the M. tuberculosis protein Ag 85B. This antiserum recognized recombinant 85B, native 85B in CF, and a single band in the M. tuberculosis H37Rv lysate (data not shown). A rabbit antiserum raised against M. tuberculosis H37Rv CF protein was also used in Western blot analysis. This antiserum reacted with numerous proteins in the M. tuberculosis H37Rv lysate and the CF, as well as with rMtb 8.4, thus confirming the presence of the native Mtb 8.4 in the culture supernatant during growth (Fig. 6B). Preimmune rabbit serum had no reactivity with rMtb 8.4 (data not shown).

PBMC reactivity to rMtb 8.4
To determine the immunologic properties of rMtb 8.4, PBMC isolated from PPD + healthy individuals were stimulated with a range of concentrations of purified rMtb 8.4 (0.0002–20 μg/ml). Individual donors were found to elicit different responses, but a 10 μg/ml concentration was found to be optimum for a number of donors (data not shown). The data in Figure 7A demonstrate that PBMC from 7 of the 10 donors with prior exposure to M. tuberculosis proliferated in response to rMtb 8.4, with stimulation indices (SI) > 5. The mean proliferative response of the PPD + healthy PBMC to rMtb 8.4 was SI = 25.6. The differences between the mean responses of PPD + healthy donors to the medium control and to the rMtb 8.4 Ag were statistically significant (p < 0.05). All of the PPD + healthy donors responded to CF with an SI of >5, and 90% of these donors stimulated the PBMC with an SI of >10. None of the 10 healthy PPD − donors responded to rMtb

FIGURE 6. Immunoblot analysis of M. tuberculosis H37Rv lysate, CF, and rMtb 8.4. Mycobacterial Ags were separated by 15% SDS-PAGE and transferred to nitrocellulose. These proteins were probed with polyclonal rabbit serum produced against rMtb 8.4 (A); M. tuberculosis H37Rv-secreted protein sera (B). Lanes: 1, M. tuberculosis H37Rv lysate (10 μg); 2, CF (2 μg); 3, rMtb 8.4 (50 ng). The results from one of three comparable experiments are shown.
controls. In vitro proliferation was measured by \[^{3}\text{H}]\text{TdR}\) incorporation and PPD ELISA from 120 h cultures of donor PBMC (\(B_6\) (2798, CF (10 \(\mu\)g/ml) and PPD 8.4 (10 \(\mu\)g/ml) with an SI of >5 (Fig. 7A). The mean levels of proliferative responses to rMtb 8.4 were significantly different in the PPD\(^-\) and PPD\(^+\) healthy donors \((p < 0.05)\).

To assay cytokine production elicited by rMtb 8.4, supernatants were removed from 24-h or 5-day cultures of donor PBMC (PPD\(^-\) and PPD\(^+\) healthy individuals) stimulated with rMtb 8.4 to assay for IFN-\(\gamma\), as well as for TNF-\(\alpha\), IL-5, and IL-10 production. IFN-\(\gamma\) production by PBMC from PPD\(^+\) healthy individuals in response to rMtb 8.4 varied between individuals \((<5 – 870 \text{ pg/ml})\), with 8 of the 10 PPD\(^+\) donor PBMC eliciting IFN-\(\gamma\) levels > 10 \text{ pg/ml}. The difference in mean levels of IFN-\(\gamma\) production in the reactivity of PPD\(^+\) healthy donors to the medium control and to the rMtb 8.4 was statistically significant by the paired Student’s \(t\) test \((p < 0.05)\). In contrast, this cytokine was undetectable in the culture supernatants of cells from PPD\(^-\) individuals. A large variation in IFN-\(\gamma\) production \((11 – 1028 \text{ pg/ml})\) was also observed in the PPD\(^+\) PBMC stimulated with CF (Fig. 7B).

Concentrations of IL-5 were undetectable above background in the culture supernatants of rMtb 8.4-stimulated PBMCs from PPD\(^+\) healthy donors (Fig. 8C). Concentrations of IL-10 in supernatants of 10 \(\mu\)g/ml rMtb 8.4-stimulated PPD\(^+\) PBMCs were high (mean, 2135 pg/ml \(\pm\) 80) compared with the levels of <1000 pg/ml reported in the literature for \(M.\) \(tuberculosis\)-stimulated PBMC from healthy tuberculin reactors (36, 37). The elicitation of IFN-\(\gamma\), TNF-\(\alpha\), and IL-10 was shown to be dependent on the concentration of rMtb 8.4 used to stimulate the PPD\(^+\) healthy PBMC (Fig. 8, A–C). Interestingly, the overall profile of cytokine production (IFN-\(\gamma\), TNF-\(\alpha\), and IL-10) elicited by rMtb 8.4 was very similar to that observed for CF (data not shown). Mtb 8.4-specific TNF-\(\alpha\), IL-5, and IL-10 cytokine production was undetectable in PBMC from PPD\(^-\) donors (data not shown).

**Immunogenicity of rMtb 8**

Immunization of C57BL/6 mice with rMtb 8.4 formulated in IFA demonstrated that this \(M.\) \(tuberculosis\) protein is a potent immunogen. SI of 8.9 to 45 were measured in popliteal lymph node cells (Fig. 9A). Additionally, IFN-\(\gamma\) elicitation was substantial and varied between 3 and 68 \(\text{ng/ml}\) according to the concentration of rMtb 8.4 used for in vitro restimulation (Fig. 9B). The Th2 cytokine, IL-4, could not be detected in any of the supernatants taken from lymph node cells cultured with Mtb 8.4.

**Discussion**

Our understanding of the immunologic basis for protective immunity to TB and the experimental observation that immunization of mice with live but not heat-killed \(M.\) \(tuberculosis\) can induce protection (9) has provided us with a valuable approach to screening for potential protective Ags that would comprise a subunit vaccine against \(M.\) \(tuberculosis\). We have applied an Ag discovery approach involving identification of native Ags released by live mycobacteria using PBMC from healthy PPD\(^+\) individuals, followed by a similar evaluation of purified recombinant proteins. We thus report herein the identification of native Ags released by live mycobacteria using purified recombinant proteins. We have applied an Ag discovery approach involving identification of native Ags released by live mycobacteria using PBMC from healthy PPD\(^+\) individuals, followed by a similar evaluation of purified recombinant proteins. We thus report herein the identification of native Ags released by live mycobacteria using PBMC from healthy PPD\(^+\) individuals, followed by a similar evaluation of purified recombinant proteins.
the Th1 phenotype that are considered to play an important role in the response against virulent *M. tuberculosis*.

Our preliminary screening has identified Mtb 8.4 as an Ag that is recognized with T cell proliferation and IFN-γ production by 70% of the donors tested. Given that IFN-γ can be produced by both T cells and NK cells, it was interesting to observe correlation between proliferation and IFN-γ levels, suggesting that the IFN-γ recorded was T cell derived. Equally important was the finding that PPD+ healthy donors who elicited proliferation SI < 10 to rMtb 8.4 also correlated with relatively low levels (<100 pg/ml) of IFN-γ production, indicating that the former test was representative of the active T cell repertoire. The ability of rMtb 8.4 to elicit cytokine production from PPD+ donors even at extremely low Ag concentrations is impressive and suggests that at low expression levels some Ags are able to trigger vigorous immune responses.

When rMtb 8.4 was titrated and used to stimulate PBMC from PPD+ donors, the production of IFN-γ, IL-10, and TNF-α, but not IL-5, were each found to be Ag dependent (Fig. 8). IL-10 is produced by both of the Th cell subpopulations in humans (38, 39). Clinical and experimental data in animals and humans suggest, likewise, that TNF-α can play both a protective and immunopathologic role in TB (40–44). Nevertheless, the overall pattern of cytokine production, indicative of a CD4+ Th1-like pattern, appears to be induced by rMtb 8.4.

Immunogenicity studies in mice have indicated that rMtb 8.4 induces a substantial amount of Ag-specific proliferation (Fig. 9A). Cytokine secretion patterns (IL-4 and IFN-γ) in mice have also indicated that a Th1 lineage of cells develops upon stimulation with rMtb 8.4, even in the presence of IFA, an adjuvant that is reported to induce both IFN-γ and IL-4 from a mixed Th cell profile with *M. tuberculosis* Ags (Fig. 9B). A central issue in the broad spectrum of T cell cytokine responses produced in response to *M. tuberculosis* and the outcome of infection is that Th1 cells are crucial for protection early in the disease process (45, 46). Later on, during the chronic phase of the disease, a mixed T cell profile is observed with concomitant production of IFN-γ, IL-4,
and IL-5 (47–49). Mechanisms by which containment of the disease occurs is incompletely understood, but IFN-γ production by Th1 CD4+ T cells is considered essential. Given that rMtb 8.4 elicits high levels of IFN-γ without any IL-4, rMtb 8.4 may be considered as a component in the development of a subunit M. tuberculosis vaccine.

The distribution of Mtb 8.4 in M. tuberculosis substrains and other mycobacterial strains was examined in this study. Southern blot experiments demonstrated the presence of Mtb 8.4 in the M. tuberculosis substrains Ra, Rv, Erdman, and “C” strain, as well as in M. avium and M. bovis BCG Pasteur. The hybridization studies showed a difference in the genomic DNA restriction enzyme pattern only within the M. avium species. The difference observed could be the result of either different localization of Mtb 8.4 on the chromosome or a chromosomal mutation of the SalI site or a product of both possibilities. Given that Southern analysis has shown that Mtb 8.4 is present in M. bovis BCG, it was important that the majority of our donors had not previously received BCG vaccinaton, to ensure that the T cell responses measured were in recognition of relevant Ags encountered by the host immune system during previous infection with M. tuberculosis.

Like Mtb 8.4, various Ags such as ESAT-6 (17, 37), the Ag 85 complex (15, 49), and MPT64 (30–32), have been previously identified in viable mycobacteria and recorded to elicit T cell responses in animal models or from healthy M. tuberculosis-infected individuals, but not TB patients (49). The question remains whether Mtb 8.4 is also recognized only by individuals with latent infection vs those with active disease. Such a finding would argue that the host immune system develops a protective response against Mtb 8.4 and would determine its utility as an agent for differentiating the two infection outcomes.

This study has revealed that Mtb 8.4 is an immunoreactive T cell Ag in individuals with latent M. tuberculosis infection. Mtb 8.4 may play an important role in determining the outcome of infection with pathogenic mycobacteria, given that it is one of the Ags encountered by the host immune system during
**M. tuberculosis** infection and given that it elicits abundant levels of the Th1 cytokine IFN-γ. As resistance to TB depends on Ag-specific T cell activation of macrophages, and as the IFN-γ pathway has been shown to be crucial in the human response to mycobacterial infection, the elicitation of high levels of IFN-γ by rMB 8.4 in *M. tuberculosis*-sensitized donors is significant.

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**References**