Expression Cloning of an Immunodominant Family of *Mycobacterium tuberculosis* Antigens Using Human CD4+ T Cells

By Mark R. Alderson,* Teresa Bement,* Craig H. Day,‡ Liqing Zhu,* David Molesh,‡ Yasir A.W. Skeiky,‡ Rhea Coler,§ David M. Lewinsohn,§‖ Steven G. Reed,‡§ and Davin C. Dillon‡

*From the *Department of Immunology and the ‡Department of Antigen Discovery, Corixa Corporation, Seattle, Washington 98104; the §Infectious Disease Research Institute, Seattle, Washington 98104; and the ‖Division of Pulmonary and Critical Care Medicine, University of Washington, Seattle, Washington 98104*

Abstract

Development of a subunit vaccine for *Mycobacterium tuberculosis* (Mtb) is likely to be dependent on the identification of T cell antigens that induce strong proliferation and interferon γ production from healthy purified protein derivative (PPD)+ donors. We have developed a sensitive and rapid technique for screening an Mtb genomic library expressed in *Escherichia coli* using Mtb-specific CD4+ T cells. Using this technique, we identified a family of highly related Mtb antigens. The gene of one family member encodes a 9.9-kD antigen, termed Mtb9.9A. Recombinant Mtb9.9A protein, expressed and purified from *E. coli*, elicited strong T cell proliferation and IFN-γ production by peripheral blood mononuclear cells from PPD+ but not PPD− individuals. Southern blot analysis and examination of the Mtb genome sequence revealed a family of highly related genes. A T cell line from a PPD+ donor that failed to react with recombinant Mtb9.9A recognized one of the other family members, Mtb9.9C. Synthetic peptides were used to map the T cell epitope recognized by this line, and revealed a single amino acid substitution in this region when compared with Mtb9.9A. The direct identification of antigens using T cells from immune donors will undoubtedly be critical for the development of vaccines to several intracellular pathogens.

Key words: *Mycobacterium tuberculosis* • intracellular pathogens • antigen presentation • interferon γ • expression cloning

Introduction

Tuberculosis (TB)[1] is the leading cause of mortality among the infectious diseases afflicting humans, with an estimated three million deaths annually (1). This alarming statistic has been compounded in recent years by the emergence of drug-resistant strains of TB and coinfections with HIV. The attenuated strain of *Mycobacterium bovis*, bacillus Calmette-Guérin (BCG), is currently the only vaccine for TB approved for use in humans. Despite its widespread use, BCG has variable efficacy in human populations (2) and can cause disseminated disease in immunocompromised individuals. Thus, the development of an improved TB vaccine is a high priority from both efficacy and safety viewpoints. One such approach to achieve this goal is the identification of immunodominant Mtb antigens that could be incorporated into a subunit vaccine.

The causative agent of TB, *Mycobacterium tuberculosis* (Mtb), is an intracellular pathogen, and as a result the cellular arm of the immune response predominantly mediates protective immunity. In particular, CD4+ T cells have been demonstrated to be critical for containment of Mtb infection in both mice and humans. In addition, the cytokine IFN-γ appears to be critical in the development of immunity to TB. Both mice with a disrupted IFN-γ gene and humans with a mutated IFN-γ receptor are highly susceptible to mycobacterial infections (3–6). Thus, in the development of subunit vaccines to Mtb, it is important to identify immunodominant CD4+ T cell antigens that are capable

Address correspondence to Mark R. Alderson, Department of Immunology, Corixa Corporation, 1124 Columbia St., Suite 200, Seattle, WA 98104. Phone: 206-754-5744; Fax: 206-754-5715; E-mail: alderson@corixa.com

[1]*Abbreviations used in this paper:* ATCC, American Type Culture Collection; BCG, bacillus Calmette-Guérin; CFP, culture filtrate protein; DC, dendritic cell; Mtb, *Mycobacterium tuberculosis*; PBS-T, PBS/0.1% Tween 20; PPD, purified protein derivative; SI, stimulation index; TB, tuberculosis.

of inducing strong IFN-γ responses. A logical source of T cells to identify such antigens is healthy PPD^{+} (non–BCG-vaccinated) donors who presumably have contained their infection because of protective CD4^{+} T cell responses.

It is difficult at this time to assess the number and identity of the immunodominant Mtb antigens that are recognized by T cells from TB-infected individuals. This is because Mtb comprises thousands of proteins, and most Mtb antigens characterized previously were identified using serological reagents or by biochemical purification (7–9). However, antigens that induce strong antibody responses are not necessarily the most potent T cell antigens. Moreover, most of the CD4^{+} T cell clones derived from PPD^{+} donors do not react with previously identified Mtb antigens, such as the antigen 85 family or heat shock protein 65 (10). Various biochemical purification techniques have been developed to identify antigens directly using T cells, including T cell blotting (11). Although these approaches have met with some success with the identification of antigens such as 6-kD early secretory antigenic target (ESAT-6 [12]) and Mtb8.4 (13), it is likely that there are T cell antigens not easily identified by this methodology because of low expression in Mtb preparations, making them difficult to purify to homogeneity.

To overcome the potential difficulties in identifying T cell antigens by their purification, as described above, we have developed a rapid, simple, and sensitive technique for the identification of antigens that have been cloned from Mtb into an *Escherichia coli* expression library using T cells from healthy PPD[+] donors. These donors have been infected with Mtb and were able to control the infection, and are therefore a good source of T cells that are presumably reactive with protective antigens. In this study, we have used T cells from one such donor to isolate a family of genes from Mtb, and have subsequently demonstrated that one member of this family is recognized by T cells from the majority of healthy PPD[+] individuals. As such, these antigens may be important for the development of a subunit vaccine for Mtb.

Materials and Methods

Bacterial Strains. Mtb strains H37Rv and Erdman were gifts from the Seattle Veterans Administration Hospital. Mtb "C" strain was a gift from Dr. Lee Riley (University of California at Berkeley, Berkeley, CA); *Mycobacterium bovis* BCG and *Mycobacterium leprae* were obtained from Genesis Corporation, and the following mycobacterial strains were obtained from American Type Culture Collection (ATCC): *M. vaccae* (ATCC 15483), *M. avium* (ATCC 35718), *M. chelonae* (ATCC 14472), *M. fortuitum* (ATCC 6841), *M. gordonae* (ATCC 14470), *M. scrofulaceum* (ATCC 19981), and *M. smegmatis* (ATCC 19420).

Generation of Mtb-specific T Cell Lines from PPD[+] Donors. PBMCs were obtained from the apheresis product of healthy PPD[+] donors by density centrifugation over Ficoll. HLA typing was performed at the Puget Sound Blood Center (Seattle, WA). Donor 160 is a health care worker who became PPD skin test positive after exposure to a patient with TB and is HLA-DR13, 15 and HLA-DQ1, 7. Other donors used in this study were HLA typed as follows. Donor 7: HLA-DR13, 15, HLA-DQ1; donor 103: HLA-DR4, 15, HLA-DQ1, 3; donor 184: HLA-DR4, 7, HLA-DQ2, 4; and donor 201: HLA-DR3, HLA-DQ2. Dendritic cells (DCs) were generated by culture of autologous adherent PBMCs with GM-CSF and IL-4 for 7 d as described (14). DCs were infected with Mtb by overnight culture at a multiplicity of infection of 10 as described (15). Mtb-infected DCs were cultured at 10^4 cells per well in 96-well round-bottomed plates with varying numbers of monocyte-depleted PBMCs as responder cells (10^2–10^4). Wells that showed obvious growth of T cells were then expanded with CD3 antibody and tested for reactivity with culture filtrate proteins (CFPs) from Mtb (provided by Dr. John Belisle, Colorado State University, Fort Collins, CO; produced through National Institutes of Health, National Institute of Allergy and Infectious Diseases Tuberculosis Research Materials contract N01 AI-25147), using autologous PBMCs as APCs. The T cell lines used in this study (DC-4 and DC-6) were from wells plated at 10^3 lymphocytes per well, and at this seeding density were predicted to be oligoclonal.

Construction and Screening of an Mtb Expression Library. Mtb Erdman genomic DNA was isolated and sheared by sonication to a size range of 1–4 kb. H37Rv genomic DNA was partially digested with Sau3A and was size fractionated. Libraries were constructed in Lambda ZapII (Stratagene) as described (13). Both phage libraries were excised according to the manufacturer's protocol, using ~10^6 recombinant phages. Transfected bacteria were plated to give ~50–80 transfectants per plate, and colonies were pooled to establish glycerol stocks. The glycerol stocks were used to establish overnight cultures (2XYT/100 μg/ml ampicillin) that were split 1:5 or 1:10 the next morning. Plates were grown for an additional 1 h and then induced by the addition of isopropyl-β-d-thio-galactopyranoside (IPTG). After induction for 3–4 h, plates were centrifuged and the supernatant was discarded. Bacterial pellets were resuspended in 200 μl antibiotic-free RPMI/10% FCS, and 10 μl was added in duplicate to wells containing DCs, also plated in antibiotic-free medium. The plates were cultured for 90 min at 37°C and washed to remove excess bacteria. The medium was replaced with antibiotic-containing complete medium (RPMI/10% pooled human serum/50 μg/ml gentamicin), and 10^4 T cells were added. The plates were cultured for an additional 3 d, after which 50 μl was removed for assessment of IFN-γ by ELISA, and the plates were pulsed with tritiated thymidine (1 μCi per well). After culture for an additional 18 h, cells were harvested and tritium uptake was determined using a gas scintillation counter. IFN-γ levels in culture supernatants were determined by ELISA as described (15).

Expression of rMtb9.9A. Clone TB46R10C10, isolated from pool 46, was used as a template to PCR amplify the region encoding *mtb9.9A*. The 5′ primer was designed to include an NdeI site, which also contained the starting ATG, followed by residues encoding an NH$_2$-terminal six-histidine tag. The 3′ primer included a HindIII site following the residues encoding the termination codon. Amplified product was digested with NdeI and HindIII and ligated into pET17b. This clone was referred to as pETMtb9.9A, and the recombinant protein encoded as rMtb9.9A.

Expression and purification of rMtb9.9A was performed essentially as described for other Mtb proteins (16). In brief, induced *E. coli* BL-21 (pLysE) pellets were lysed, and the recombinant proteins were recovered in the inclusion bodies. Purification was via affinity chromatography on an Ni^{2+} nitrilotriacetic acid (NTA)-agarose column. Purity of the recombinant protein was assessed by SDS-PAGE, followed by Coomassie blue staining and then NH$_2$-terminal sequencing using traditional Erdman chemistry with a Procise® 494 protein sequencer (Perkin Elmer/Applied Biosystems). Endotoxin was determined to be <100 EU/mg of protein by the *Limulus* amebocyte lysate (LAL) assay (BioWhittaker).

Engineering, expression, and purification of rMtb 85B were performed as described (16).

All DNA manipulations of the various clones were confirmed by DNA sequencing to eliminate the possibility of the introduction of mutations by restriction, ligation, and PCR.

Mtb9.9 Peptide Synthesis. Peptides derived from the predicted amino acid sequences from the *mtb9.9* gene family were synthesized on a Rainin/PTI Symphony peptide synthesizer using 9-fluorenyl-methoxycarbonyl batch chemistry with 2-(1H-benzotriazole-1-yl)-1,1,3,3-tetramethyluronium hexafluorophosphate activation. Peptides were analyzed by reverse phase HPLC using a Vydac C18 column. Peptide molecular masses were verified using a matrix-assisted laser desorption ionization time-of-flight (MALDI-TOF) mass spectrometer.

Molecular Analysis of Mtb Clones. DNA was prepared according to the manufacturers' protocols (Qiagen and Promega). DNA sequencing was performed using an Applied Biosystems Automated Sequencer (model 373). DNA sequences and deduced amino acid sequences were used in database searches (GenBank nonredundant DNA and protein databases). The Mtb H37Rv nucleotide sequences of *mtb9.9a*, *mtb9.9c*, *mtb9.9d*, and *mtb9.9e* are available from GenBank under accession nos. CAA17714, CAB07821, CAB06842, and CAB06161, respectively. The Mtb Erdman nucleotide sequence of *mtb9.9b* is available from Gen-Bank under accession no. AF226277.

Genomic DNA from mycobacterial strains was digested with PstI, separated by agarose gel electrophoresis, and blotted on Ny-tran® (Schleicher & Schuell). The *mtb9.9a* gene was labeled with [^{32}P]dCTP by random oligonucleotide primers (Boehringer Mannheim) and used as a probe. Hybridization was performed at 65°C in 0.2 M NaH$_2$PO$_4$, 3.6 M NaCl, 0.2 M EDTA overnight and washed to a stringency of 0.075 M NaCl, 0.0075 M C$_6$H$_5$Na$_3$O$_7$, pH 7.0, 0.5% SDS at the temperature of hybridization.

Immunoblot Analysis. Antisera to rMtb9.9A and r85B were raised using adult New Zealand white rabbits (R & R Rabbitry) by an initial subcutaneous immunization of 200 μg of recombinant antigen in 1 ml IFA and 100 μg muramyl dipeptide (Calbiochem), followed by two successive subcutaneous immunizations of 100 μg antigen in 1 ml IFA at 3-wk intervals. A final intravenous boost of 100 μg antigen was delivered after another 4 wk, and serum was collected 2 wk later.

Mtb H37Rv lysate, CFPs, and purified rMtb9.9A were subjected to SDS-PAGE and transferred to nitrocellulose. Filters were blocked with PBS containing 5% nonfat dried milk at 4°C overnight, washed three times in PBS/0.1% Tween 20 (PBS-T), and incubated for 1 h in rabbit sera (1:250 in PBS-T) on a rocker at room temperature. Filters were washed three times with PBS-T, and bound antibody was detected with 10^5 cpm/ml ^{125}I-labeled protein A, followed by autoradiography.

Proliferation and IFN-γ Assays. PBMCs from PPD$^+$ and PPD$^-$ donors were cultured in complete medium in 96-well round-bottomed plates (Corning Costar) at 2 × 10^5 cells per well in a volume of 200 μl. After 5 d of culture at 37°C in 5% CO$_2$, 50 μl of culture supernatant was removed for determination of IFN-γ levels, and the plates were pulsed with tritiated thymidine as described above. Data are presented as the mean of triplicate cultures.

Results

Recognition of E. coli–expressed Antigen by T Cells. To establish the feasibility of detecting Mtb antigens expressed in *E. coli*, we used a T cell clone of known specificity for an Mtb antigen, Mtb11, identified previously in our laboratory (Dillon, D.C., M.R. Alderson, C.H. Day, T. Bement, A. Campos-Neto, Y.A.W. Skeiky, T. Vedvick, R. Badaro, S.G. Reed, and R. Houghton, manuscript in preparation). This T cell clone, 4E4, proliferated and produced IFN-γ in response to rMtb11 and CFPs derived from Mtb. 4E4 T cells were assessed for their ability to recognize whole *E. coli* expressing the Mtb11 antigen. *E. coli* expressing either Mtb11 or vector alone were incubated with either autologous DCs or autologous macrophages for 90 min under antibiotic-free conditions. Nonphagocytosed and nonbound *E. coli* were then washed away and replaced with medium containing gentamicin to kill remaining free bacteria. 4E4 T cells were then added and cultured for an additional 72 h before assay for IFN-γ production. As shown in Fig. 1, 4E4 cells produced IFN-γ in response to *E. coli* expressing Mtb11 but not *E. coli* expressing vector alone. In addition, the responses using DCs as APCs were far stronger than with macrophages. The data also demonstrate that the *E. coli* expressing Mtb11 could be
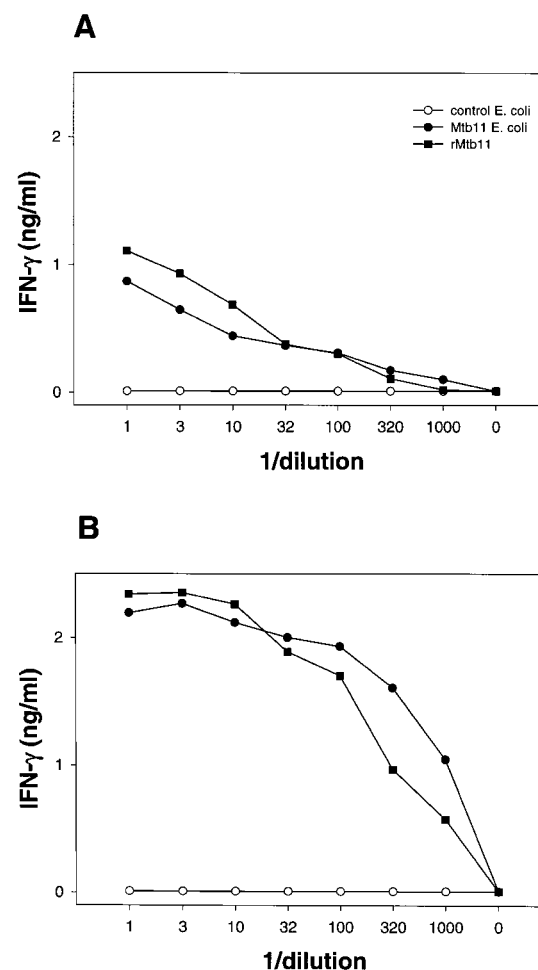

Figure 1. Responses of Mtb11-specific T cell clone (4E4) to either purified rMtb11 or varying numbers of *E. coli* expressing either vector alone or rMtb11. 10^4 4E4 cells were cultured for 3 d with either (A) 10^4 autologous macrophages or (B) 10^4 autologous monocyte-derived DCs that had been incubated with either *E. coli* or purified rMtb11. Supernatants were assessed for IFN-γ levels by ELISA. The highest concentrations of *E. coli* and rMtb11 were ~10^6 bacteria per well and 10 μg/ml, respectively.

diluted out as far as 1:1,000 (~10^3 bacteria per well) and still induce strong antigen-specific IFN-γ production.

Generation of T Cell Lines to Mtb Antigens. Having established that *E. coli*–expressed antigens could be presented to T cells efficiently by monocyte-derived DCs, we attempted next to determine whether this technique could be used to identify novel Mtb antigens. We have established a panel of PBMCs from healthy PPD+ individuals with no history of TB as a source of Mtb-reactive T cells. Because these individuals were most likely infected with Mtb, but did not develop TB, they have presumably acquired a protective immune response, and as such their T cells are predicted to recognize protective antigens. Furthermore, to bias the T cells towards antigens expressed by Mtb-infected cells rather than antigens that may be overexpressed in broth cultures of Mtb, we generated T cell lines to autologous blood-derived DCs that had been infected with Mtb. We and others have shown previously that these DCs are amenable to infection with Mtb and are extremely potent APCs (15, 17). Limiting dilution techniques were used to establish Mtb-reactive T cell lines that were subsequently screened for reactivity with CFPs and a panel of rMtb antigens. The reactivity of two such T cell lines, DC-4 and DC-6, which were established from a recent PPD converter (donor 160), is shown in Fig. 2. The DC-4 cell line showed strong reactivity with CFPs and rMtb39A (16) but not with any of the other recombinant antigens, whereas DC-6 reacted strongly with CFPs and Mtb11 and weakly with
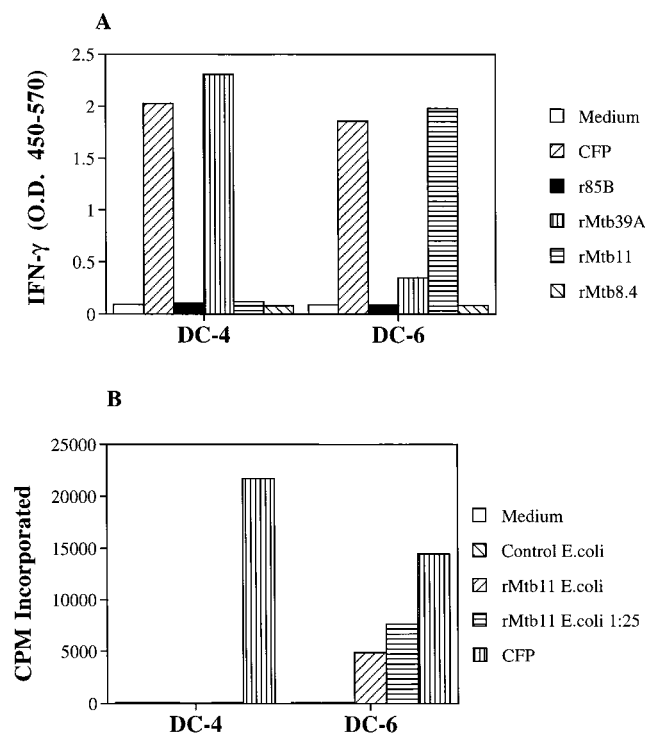
Mtb39A. In addition, DC-6 but not DC-4 reacted strongly with whole *E. coli* expressing Mtb11 (Fig. 2 B). We chose to use the DC-6 line to screen an expression library to try and identify the Mtb11 and Mtb39A genes as a test of the system (proof of principal), as well as to identify potentially novel Mtb genes due to the established oligoclonal nature of the T cell line.

Isolation of a Novel Family of T Cell–stimulating Mtb Antigens by Expression Cloning. To screen for novel Mtb T cell antigens, we constructed a genomic Mtb library in the pBSK expression vector (see Materials and Methods). Based on the data shown in Figs. 1 and 2, we chose to screen pools of 50–80 recombinants using the DC-6 T cell line and autologous DCs as APCs. The data obtained from the screening of 48 pools of recombinants in duplicate are shown in Fig. 3, A and B. Six pools (6, 8, 29, 32, 34, and 46) elicited strong proliferation and IFN-γ responses, and two pools (37 and 47) gave weak but positive responses. Similar data were obtained when these pools were screened with the DC-4 T cell line (data not shown). Individual colonies were generated from the positive pools, and the assay
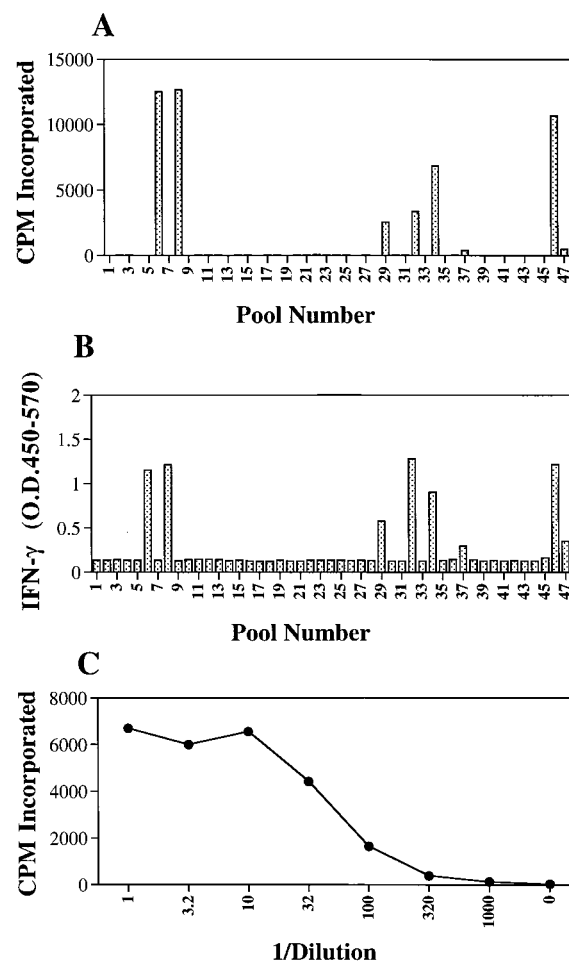

Figure 2. Reactivities of donor 160 DC-4 and DC-6 T cell lines generated to autologous DCs infected with Mtb to (A) purified rMtb antigens (10 μg/ml) and (B) *E. coli* expressing rMtb11 either neat or diluted 1:25 in control *E. coli*.

Figure 3. Screen of 48 pools of ~50–80 recombinants per pool from Mtb Erdman library with donor 160 DC-6 T cell line. Data are the mean of duplicate wells. (A) Proliferation. (B) IFN-γ. (C) Confirmation of single positive clone derived from pool 31.

Table I. *Identity of Isolated Recombinants from Positive Pools Detected by T Cell Line Donor 160 DC-6*

Pool no.	Identity
6	Novel gene, termed *mtb9.9b*
8	Novel gene, termed *mtb9.9c*
29	Unable to identify reactive gene
32	*mtb39* NH$_2$ terminus
34	Novel gene, termed *mtb9.9a*
37	Unable to identify reactive gene
46	Novel gene, termed *mtb9.9a* (overlaps with clone in pool 34)
47	*mtb39* COOH terminus

was repeated with less complex pools until single recombinant positive clones were identified. Single recombinants were assayed an additional time to confirm that they were indeed positive (Fig. 3 C), and the DNA inserts were sequenced. Single recombinant clones were successfully identified in six of the eight positive pools. Two of the single recombinants contained fragments of the previously identified Mtb39A antigen (Table I). The other four clones contained
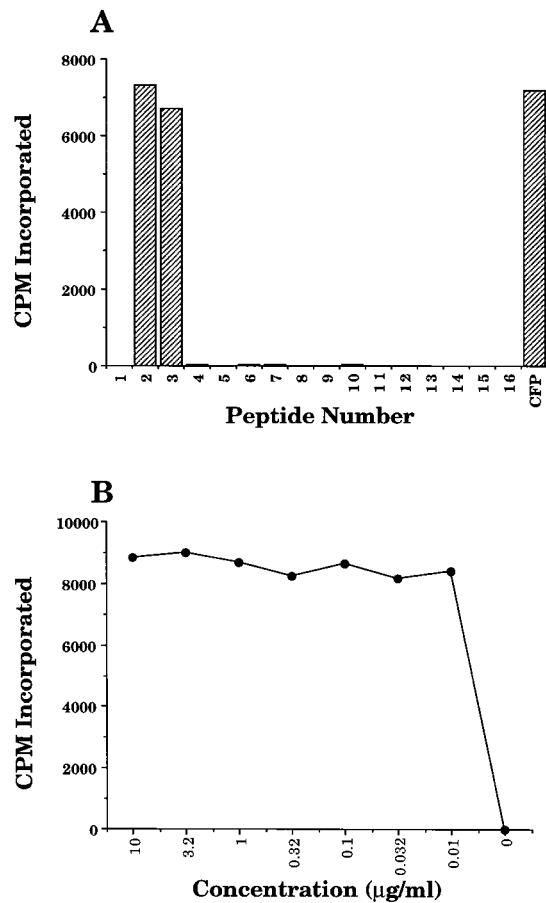
three distinct open reading frames that were highly homologous with one another and were predicted to encode 94 amino acid proteins of molecular mass of 9.9 kD, and were therefore designated Mtb9.9A, Mtb9.9B, and Mtb9.9C. Subsequent screening of additional pools also identified a fourth family member, designated Mtb9.9D, and the Mtb11 gene (data not shown).

Synthetic Peptides Generated Using the mtb9.9a Gene Sequence and Purified rMtb9.9A Stimulate DC-6 Cells. To establish conclusively that the *mtb9.9* genes were responsible for stimulation of the DC-6 T cell line, we synthesized peptides (15 amino acids that overlapped by 10 amino acids) corresponding to the protein sequence predicted to be encoded by the *mtb9.9a* gene. As shown in Fig. 4 A, peptides 2 and 3 from Mtb9.9A stimulated a strong response in the DC-6 T cell line. The overlapping sequence between these two peptides contained the amino acid sequence DAH-GAMIRAQ. In addition, the *mtb9.9a* gene was subcloned into the pET17b expression plasmid (Novagen), and the recombinant protein was expressed and purified (data not shown). Purified rMtb9.9A also stimulated a strong response in the DC-6 line (Fig. 4 B).

Characterization of the Mtb9.9 Genes and Proteins. To further characterize the *mtb9.9* gene family, Southern blot analysis was performed on a variety of mycobacterial strains using the *mtb9.9a* gene as a probe (Fig. 5). The data demonstrated the presence of a highly related gene family containing between three and five members within different isolates of Mtb, including two from clinical sources. The
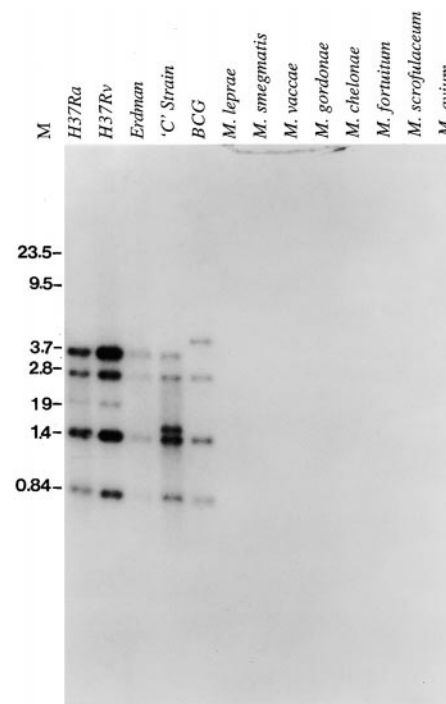

Figure 4. Responses of donor 160 DC-6 T cell line to either (A) synthetic peptides generated from Mtb9.9A (10 μg/ml) or (B) rMtb9.9A.

Figure 5. Southern blot analysis of Mtb9.9 genes. Genomic DNA from various mycobacterial strains was digested with PstI, separated by agarose gel electrophoresis, and blotted on Nytran®. The *mtb9.9a* gene was labeled with ^{32}P and used as a probe. Size markers (M) are in kb.

data also indicate that these genes are well conserved in *M. bovis* BCG, but significantly less conserved or absent in all other mycobacterial species tested.

Examination of the Mtb H37Rv genome sequence (18) revealed four *mtb9.9* gene family members, including three that we had isolated from the Erdman strain. The H37Rv genome did not contain the *mtb9.9b* form that we isolated from the Erdman library, but did contain an additional form that was designated *mtb9.9e*. A comparison of the predicted amino acid sequences encoded by the five *mtb9.9* genes is shown in Fig. 6. Interestingly, it was observed that *mtb9.9c* is located near *mtb39a* (16), a member of a gene family encoding a potent T cell antigen also recognized by DC-6 cells (Table I).

Purified rMtb9.9A was used to generate a high titer rabbit antiserum that was used for Western blot analysis of the native Mtb9.9 protein. As demonstrated in Fig. 7, Mtb9.9 protein is predominantly located in the cell fraction of Mtb, with low but detectable quantities in the culture filtrate of Mtb. As a control, a rabbit antiserum to antigen 85B showed abundant expression of this protein in both Mtb lysate and CFPs (Fig. 7 C).

Response of PBMCs from PPD⁺ and PPD⁻ Donors to rMtb9.9A. rMtb9.9A was next assessed for its ability to stimulate T cell proliferation and IFN-γ production from a panel of PBMCs derived from normal PPD⁻ and healthy PPD⁺ donors, including those of donor 160, from which the Mtb9.9-reactive T cell line (DC-6) was generated. Using a stimulation index (SI) of 5 as a measure of a positive response, 10/12 (83%) of the PPD⁺ donors responded to rMtb9.9A compared with 8/12 (67%) that responded to recombinant antigen 85B (Fig. 8). In addition, the magnitude

A

```
Mtb9.9A  MTINYQFGDVDAHGAMIRAQAASLEAEHQAIVRDVLAAGDFWGGAGSVACQEFITQLGRN  60
Mtb9.9B  ...................L.GL.........IS....T.S.........A...G........  60
Mtb9.9C  ...................Q.GL.........IR...T.S.........A...G........  60
Mtb9.9D  ...................Q.GS.........IS....T.S.........A...G........  60
Mtb9.9E  ...................Q.GL.........VR....A.G.........V...E........  60

Mtb9.9A  FQVIYEQANAHGQKVQAAGNNMAQTDSAVGSSWA   94
Mtb9.9B  ..................................   94
Mtb9.9C  ..................................   94
Mtb9.9D  ..................................   94
Mtb9.9E  ..................................   94
```

B

```
PEP1    MTINYQFGDVDAHGA        PEP8    LAAGDFWGGAGSVACQ
PEP2    QFGDVDAHGAMIRAQ        PEP8B   LTASDFWGGAGSAACQ
PEP3    DAHGAMIRAQAASLE        PEP9    FWGGAGSVACQEFIT
PEP3B   DAHGAMIRALAGLLE        PEP9B   FWGGAGSAACQGFIT
PEP3C   DAHGAMIRAQAGLLE        PEP10   GSVACQEFITQLGRN
PEP4    MIRAQAASLEAEHQA        PEP10B  GSAACQGFITQLGRN
PEP4B   MIRALAGLLEAEHQA        PEP11   QEFITQLGRNFQVIYEQA
PEP4C   MIRAQAGLLEAEHQA        PEP11B  QGFITQLGRNFQVIY
PEP5    AASLEAEHQAIVRDV        PEP12   RNFQVIYEQANAHGQ
PEP5B   AGLLEAEHQAIISDV        PEP13   IYEQANAHGQKVQAA
PEP5C   AGLLEAEHQAIIRDV        PEP14   NAHGQKVQAAGNNMA
PEP6    AEHQAIVRDVLAAGD        PEP15   KVQAAGNNMAQTDSA
PEP6B   AEHQAIISDVLTASD        PEP16   GNNMAQTDSAVGSSWA
PEP6C   AEHQAIIRDVLTASD
PEP7    IVRDVLAAGDFWGGA
PEP7B   IISDVLTASDFWGGA
PEP7C   IIRDVLTASDFWGGA
```

Figure 6. Predicted amino acid sequence of Mtb9.9 proteins. (A) Comparison of the predicted amino acid sequences of the five Mtb9.9 family members, and (B) overlapping synthetic peptides generated comprising portions of these sequences (peptides derived from Mtb9.9A denoted by number only). The Mtb H37Rv nucleotide sequences of *mtb9.9a, mtb9.9c, mtb9.9d,* and *mtb9.9e* are available from GenBank under accession nos. CAA17714, CAB07821, CAB06842, and CAB06161, respectively. The Mtb Erdman nucleotide sequence of *mtb9.9b* is available from GenBank under accession no. AF226277.
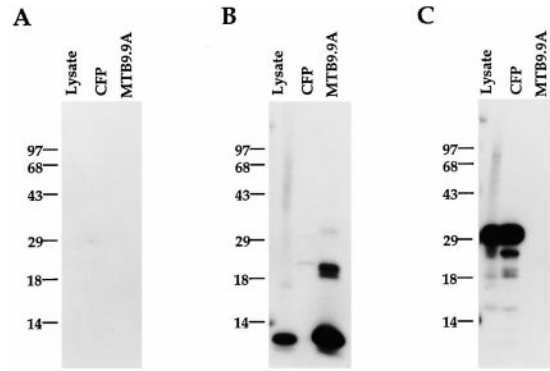
Figure 7. Western blot analysis of Mtb9.9 protein. The blots were probed with (A) preimmune rabbit serum, (B) antiserum to rMtb9.9A, and (C) antiserum to antigen r85B. Mtb lysate and CFPs were run at 5 μg/lane and rMtb9.9A at 50 ng/lane.

of the responses to rMtb9.9A was very strong (mean SI 39.0) compared with antigen r85B (mean SI 15.3). All 12 of the PPD⁺ donors made a significant response to CFPs. Of the PPD⁻ donors, 0/12 responded to rMtb9.9A compared with 2/12 that responded to antigen r85B, suggesting that rMtb9.9A has greater specificity for Mtb infection than r85B. rMtb9.9A also induced strong IFN-γ responses in the majority of the PPD⁺ donors (Fig. 8).

Response of PBMCs from PPD⁺ Donors to Overlapping Synthetic Peptides Derived from the Mtb9.9A, Mtb9.9B, and Mtb9.9C Sequences. Four donors that made strong responses to rMtb9.9A were selected for epitope mapping studies of their T cell responses to peptides derived from the Mtb9.9 gene family sequences. Donor 160 PBMCs responded predominantly to the number 2 and 3 peptides, in agreement with the reactivity of the DC-6 T cell line generated from this donor. Donor 7 PBMCs responded weakly to peptides 2 and 3, and made a strong response to peptide 6 from Mtb9.9A and Mtb9.9C, but not from Mtb9.9B. Donor 103 PBMCs responded primarily to peptides 12 and 13, whereas PBMCs from donor 184 responded to peptides 14 and 15. Collectively, these data suggest that there is a minimum of four T cell epitopes contained within the Mtb9.9 sequences.

Identification of an Mtb9.9C-specific T Cell Line. We next generated a T cell line from donor 201, whose PBMCs failed to react with rMtb9.9A. This T cell line was used to screen the same pools as were screened with the DC-6 line. Interestingly, the only positive pool detected using the donor 201 T cell line with autologous DCs as APCs was number 8, which was known to contain the *mtb9.9c* gene. Despite showing strong reactivity with pool 8, this T cell line failed to react with other pools known to contain *mtb9.9a* and *mtb9.9b* genes (pools 6, 34, and 46). This suggested that either this T cell line reacted with a novel clone contained within pool 8, or it reacted with Mtb9.9C but not Mtb9.9A or Mtb9.9B. To test the latter hypothesis, we assessed the donor 201 line for reactivity with the synthetic peptides derived from the Mtb9.9 sequences. This T cell line reacted only with peptides 3 and 4 from Mtb9.9C, and not with either rMtb9.9A or peptides derived from Mtb9.9A
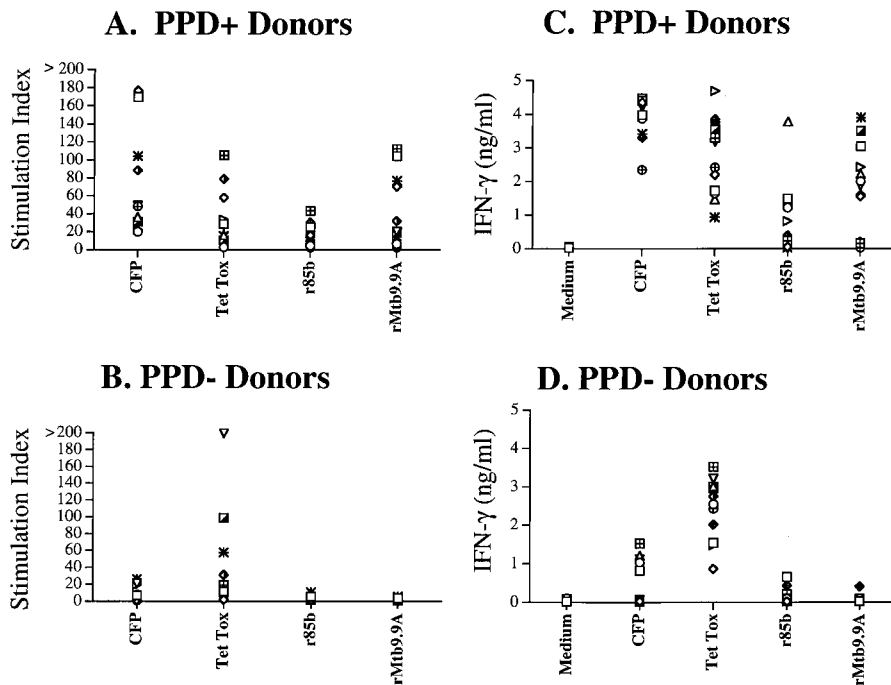
A. PPD+ Donors

C. PPD+ Donors

B. PPD- Donors

D. PPD- Donors

Figure 8. Response of PBMCs from (A and C) 12 healthy PPD$^+$ and (B and D) 12 normal PPD$^-$ donors to rMtb9.9A. A and B show proliferation data; C and D show IFN-γ data. CFPs, r85B, and rMtb9.9A were used at 10 μg/ml, and Tetanus Toxoid (Tet Tox) was used at 0.1 LP/ml.

or Mtb9.9B (see Fig. 10). Interestingly, the peptide sequence for this region (the overlap between peptides 3 and 4) only differs by two amino acids between Mtb9.9C and Mtb9.9A, and one amino acid between Mtb9.9C and Mtb9.9B (Fig. 6).

Discussion

The identification of antigens from Mtb that stimulate CD4$^+$ T cells has been hampered in the past by the use of techniques that relied on non–T cell components of the immune response for their identification. For example, the majority of Mtb antigens characterized thus far were originally identified based on their reactivity with antibodies, or as a consequence of their abundance and subsequent ease for purification (7–9). The potential problem with these approaches is that antigens that stimulate potent T cell responses are not necessarily the same as those that stimulate strong antibody responses, nor are they necessarily the most abundant antigens. Indeed, of five Mtb antigens that we recently isolated using human serum from patients with TB, only one induced strong T cell responses in PPD$^+$ donors (16). The technique we describe in this paper minimizes this problem because it uses T cells to directly identify antigens that are expressed as recombinant antigens in *E. coli.*

Previous studies have demonstrated that antigenic proteins expressed by recombinant *E. coli* can be presented by class II MHC molecules to antigen-specific T cells (19). Indeed, Shastri and colleagues (20, 21) demonstrated that this property of *E. coli*–expressed antigens could be applied to identify two previously unknown antigens from *Listeria monocytogenes* using murine CD4$^+$ T cells. This technique relied on the fusion of the antigen-specific T cells with a hybridoma partner containing a reporter gene (lacZ), and on screening for T cell activation at the single cell level.

The technique we describe in this paper is much simpler and is amenable to high throughput screening, as it can be used with either T cell clones or lines, and does not require the generation of either hybridomas or a single cell assay. By taking advantage of the fact that blood-derived DCs are both phagocytic and are extremely potent APCs (17), we were able to use an antigen-reactive T cell line to detect a previously identified Mtb antigen (Mtb39 [16]), as well as a family of previously unknown Mtb antigens (the Mtb9.9 family). In addition, we have used T cells from a TB-immune human donor, which presumably has relevance for the development of human vaccines.

Several antigens derived from Mtb have been reported to stimulate T cell responses from Mtb-infected humans and mice. In particular, the antigen 85B has been reported to be an immunodominant T cell antigen (22). Here, we show that upon preliminary analysis, rMtb9.9A appears to be a more potent T cell antigen than antigen r85B. Of the 12 PPD$^+$ donors tested, 10 made a significant proliferative response to rMtb9.9A (83%) compared with 8 who responded to r85B (67%). In particular, the magnitude of the responses to rMtb9.9A was stronger than to r85B, such that the mean SI with rMtb9.9A from PPD$^+$ donors was 39.0 compared with 15.3 with r85B. Finally, rMtb9.9A induced strong IFN-γ responses in PPD$^+$ but not PPD$^-$ donors, further implicating Mtb9.9A as a candidate for inducing protective immune responses to Mtb.

The use of APCs infected with live Mtb to generate T cell lines ensures that the antigens that are detected are available to the immune response during infection with Mtb. Interestingly, Mtb9.9 was found to be primarily located in Mtb lysate, although low levels of protein were detected in CFPs by Western blotting. However, the quantity of Mtb9.9 in CFPs was sufficient to stimulate T
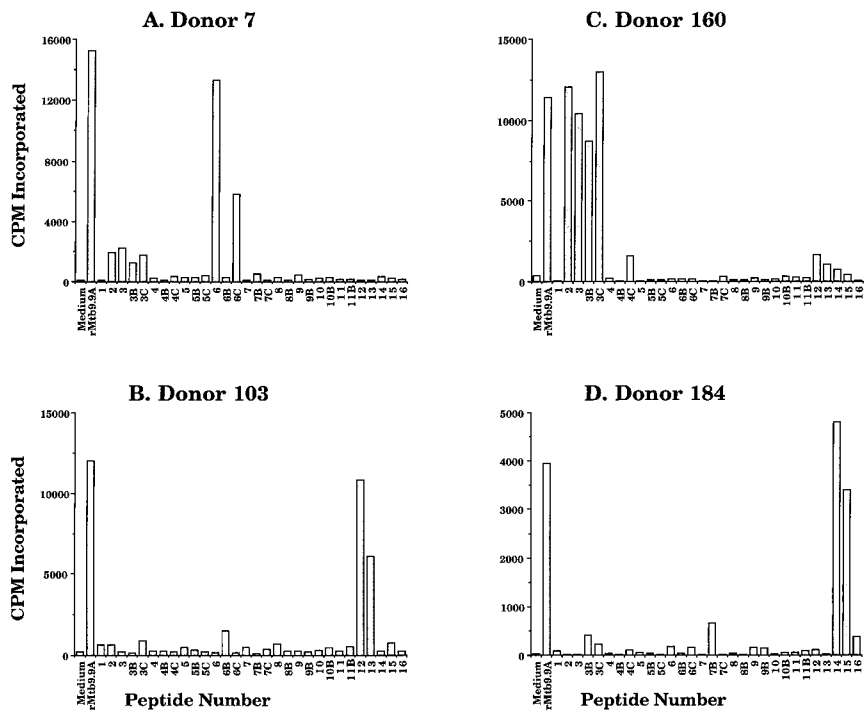
Figure 9. Response of PBMCs from four PPD[+] donors to rMtb9.9A and overlapping synthetic peptides derived from Mtb9.9A, Mtb9.9B, and Mtb9.9C sequences (peptides derived from Mtb9.9A denoted by number only). Peptides and rMtb9.9A were used at 10 μg/ml.

cell responses, as the donor 201 line that reacted with Mtb9.9C was generated using CFPs as antigen. This highlights the sensitivity of the expression cloning technique, as it was able to identify an antigen present at low levels within a complex mixture of proteins. Indeed, rMtb9.9A is able to stimulate T cell responses at extremely low protein concentrations, with as little as 10 ng/ml resulting in optimal recall responses (Fig. 4 B), and as little as 100 pg/ml able to stimulate responses above background (data not shown).

Several groups have reported a heterogeneous response to Mtb antigens among healthy PPD[+] individuals and patients with TB (10, 23). Interestingly, Boesen et al. (24) described preferential recognition of low-mass CFPs in the
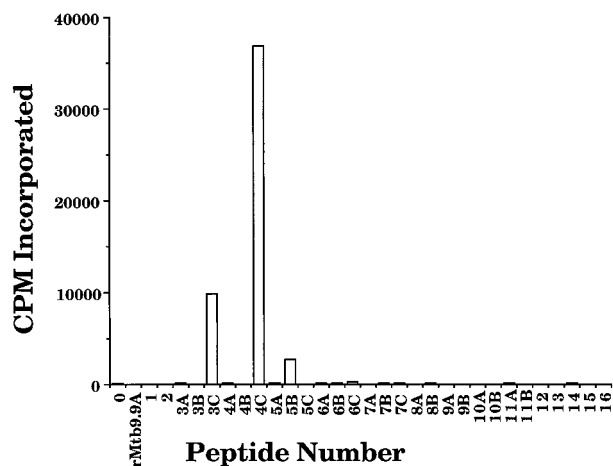

Figure 10. Response of donor 201 T cell line to rMtb9.9A and Mtb9.9A, Mtb9.9B and Mtb9.9C peptides (10 μg/ml).

majority of patients with minimal TB. This fraction contains several proteins, including 6-kD early secretory antigenic target, Mtb8.4, and Mtb9.9A. Although the Mtb9.9 family of proteins is of low mass, they were found to contain at least five distinct T cell epitopes (Figs. 9 and 10). In addition, the donors used in this study represented a variety of ethnic backgrounds and HLA types. This may be important in the design of a subunit vaccine for Mtb, where T cell responses to antigens presented by a large number of MHC alleles would be required to vaccinate the majority of humans. In addition, most PPD[+] donors (>80%) made a T cell response to rMtb9.9A, again suggesting that immunization of an outbred human population could potentially induce responses in the vast majority of individuals. Indeed, in preliminary studies, immunization of inbred mice and outbred guinea pigs with rMtb9.9A induced strong antigen-specific T cell responses (data not shown).

Despite the very high amino acid homology among the members of the Mtb9.9 family, we observed heterogeneity in the T cell response to each of the antigens. For example, T cells from donor 201 were found to react only with peptides from Mtb9.9C and not Mtb9.9A or Mtb9.9B. In addition, PBMCs from donor 7 only responded to peptide 6 from Mtb9.9A and Mtb9.9C but not from Mtb9.9B. Whether this represents a means for Mtb to evade the immune system by antigenic drift remains to be investigated.

In conclusion, we have developed a very simple and sensitive expression cloning strategy for identifying antigens that are recognized by CD4^{+} T cells. Such a strategy is likely to be applicable to the identification of CD4^{+} T cell antigens from other infectious disease microorganisms, as well as antigens from other sources.

References

1. Kochi, A. 1991. The global tuberculosis situation and the new control strategy of the World Health Organization. *Tubercle.* 72:1–6.

2. Colditz, G.A., T.F. Brewer, C.S. Berkey, M.E. Wilson, E. Burdick, H.V. Fineberg, and F. Mosteller. 1994. Efficacy of BCG vaccine in the prevention of tuberculosis. Meta-analysis of the published literature. *JAMA.* 271:698–702.

3. Flynn, J.L., J. Chan, K.J. Triebold, D.K. Dalton, T.A. Stewart, and B.R. Bloom. 1993. An essential role for interferon γ in resistance to *Mycobacterium tuberculosis* infection. *J. Exp. Med.* 178:2249–2254.

4. Cooper, A.M., D.K. Dalton, T.A. Stewart, J.P. Griffin, D.G. Russell, and I.M. Orme. 1993. Disseminated tuberculosis in interferon γ gene–disrupted mice. *J. Exp. Med.* 178:2243–2247.

5. Jouanguy, E., F. Altare, S. Lamhamedi, P. Revy, J.-F. Emile, M. Newport, M. Levin, S. Blanche, E. Seboun, A. Fischer, and J.-L. Casanova. 1996. Interferon-γ-receptor deficiency in an infant with fatal bacille Calmette-Guérin infection. *N. Engl. J. Med.* 335:1956–1961.

6. Newport, M.J., C.M. Huxley, S. Huston, C.M. Hawrylowicz, B.A. Oostra, R. Williamson, and M. Levin. 1996. A mutation in the interferon-γ-receptor gene and susceptibility to mycobacterial infection. *N. Engl. J. Med.* 335:1941–1949.

7. Lee, B.Y., S.A. Hefta, and P.J. Brennan. 1992. Characterization of the major membrane protein of virulent *Mycobacterium tuberculosis*. *Infect. Immun.* 60:2066–2074.

8. Nagai, S., H.G. Wiker, M. Harboe, and M. Kinomoto. 1991. Isolation and partial characterization of major protein antigens in the culture fluid of *Mycobacterium tuberculosis*. *Infect. Immun.* 59:372–382.

9. Kadival, G.V., S.D. Chaparas, and D. Hussong. 1987. Characterization of serologic and cell-mediated reactivity of a 38-kDa antigen isolated from *Mycobacterium tuberculosis*. *J. Immunol.* 139:2447–2451.

10. Boom, W.H., R.S. Wallis, and K.A. Chervenak. 1991. Human *Mycobacterium tuberculosis*-reactive CD4+ T-cell clones: heterogeneity in antigen recognition, cytokine production, and cytotoxicity for mononuclear phagocytes. *Infect. Immun.* 59:2737–2743.

11. Young, D.B., and J.R. Lamb. 1986. T lymphocytes respond to solid-phase antigen: a novel approach to the molecular analysis of cellular immunity. *Immunology.* 59:167–171.

12. Sorensen, A.L., S. Nagai, G. Houen, P. Andersen, and A.B. Andersen. 1995. Purification and characterization of a low-molecular-mass T-cell antigen secreted by *Mycobacterium tuberculosis*. *Infect. Immun.* 63:1710–1717.

13. Coler, R.N., Y.A. Skeiky, T. Vedvick, T. Bement, P. Ovendale, A. Campos-Neto, M.R. Alderson, and S.G. Reed. 1998. Molecular cloning and immunologic reactivity of a novel low molecular mass antigen of *Mycobacterium tuberculosis*. *J. Immunol.* 161:2356–2364.

14. Koppi, T.A., T. Tough-Bement, D.M. Lewinsohn, D.H. Lynch, and M.R. Alderson. 1997. CD40 ligand inhibits Fas/CD95-mediated apoptosis of human blood-derived dendritic cells. *Eur. J. Immunol.* 27:3161–3165.

15. Lewinsohn, D.M., M.R. Alderson, A.L. Briden, S.R. Riddell, S.G. Reed, and K.H. Grabstein. 1998. Characterization of human CD8+ T cells reactive with *Mycobacterium tuberculosis*–infected antigen-presenting cells. *J. Exp. Med.* 187:1633–1640.

16. Dillon, D.C., M.R. Alderson, C.H. Day, D.M. Lewinsohn, R. Coler, T. Bement, A. Campos-Neto, Y.A. Skeiky, I.M. Orme, A. Roberts, et al. 1999. Molecular characterization and human T-cell responses to a member of a novel *Mycobacterium tuberculosis mtb39* gene family. *Infect. Immun.* 67:2941–2950.

17. Henderson, R.A., S.C. Watkins, and J.L. Flynn. 1997. Activation of human dendritic cells following infection with *Mycobacterium tuberculosis*. *J. Immunol.* 159:635–643.

18. Cole, S.T., R. Brosch, J. Parkhill, T. Garnier, C. Churcher, D. Harris, S.V. Gordon, K. Eiglmeier, S. Gas, C.E. Barry, et al. 1998. Deciphering the biology of *Mycobacterium tuberculosis* from the complete genome sequence. *Nature.* 393:537–544.

19. Pfeifer, J.D., M.J. Wick, D.G. Russell, S.J. Normark, and C.V. Harding. 1992. Recombinant *Escherichia coli* express a defined, cytoplasmic epitope that is efficiently processed in macrophage phagolysosomes for class II MHC presentation to T lymphocytes. *J. Immunol.* 149:2576–2584.

20. Campbell, D.J., and N. Shastri. 1998. Bacterial surface proteins recognized by CD4+ T cells during murine infection with *Listeria monocytogenes*. *J. Immunol.* 161:2339–2347.

21. Sanderson, S., D.J. Campbell, and N. Shastri. 1995. Identification of a CD4+ T cell–stimulating antigen of pathogenic bacteria by expression cloning. *J. Exp. Med.* 182:1751–1757.

22. Silver, R.F., R.S. Wallis, and J.J. Ellner. 1995. Mapping of T cell epitopes of the 30-kDa alpha antigen of *Mycobacterium bovis* strain bacillus Calmette-Guérin in purified protein derivative (PPD)-positive individuals. *J. Immunol.* 154:4665–4674.

23. Munk, M.E., B. Schoel, and S.H. Kaufmann. 1988. T cell responses of normal individuals towards recombinant protein antigens of *Mycobacterium tuberculosis*. *Eur. J. Immunol.* 18:1835–1838.

24. Boesen, H., B.N. Jensen, T. Wilcke, and P. Andersen. 1995. Human T-cell responses to secreted antigen fractions of *Mycobacterium tuberculosis*. *Infect. Immun.* 63:1491–1497.