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T Cell Expression Cloning of a *Mycobacterium tuberculosis* Gene Encoding a Protective Antigen Associated with the Early Control of Infection[1]

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Infection of C57BL/6 mice with *Mycobacterium tuberculosis* results in the development of a progressive disease during the first 2 wk after challenge. Thereafter, the disease is controlled by the emergence of protective T cells. We have used this infection model in conjunction with direct T cell expression cloning to identify Ags involved with the early control of the disease. A protective *M. tuberculosis*-specific CD4 T cell line derived from mice at 3 wk postchallenge was used to directly screen an *M. tuberculosis* genomic expression library. This screen resulted in the identification of a genomic clone comprising two putative adjacent genes with predicted open reading frames of 10 and 41 kDa, MTB10 and MTB41, respectively (the products of Rv0916c and Rv0915c, respectively, in the TubercuList H37Rv database). MTB10 and MTB41 belong to the PE and PPE family of proteins recently identified to comprise 10% of the *M. tuberculosis* genome. Evaluation of the recombinant proteins revealed that MTB41, but not MTB10, is the Ag recognized by the cell line and by *M. tuberculosis*-sensitized human PBMC. Moreover, C57BL/6 mice immunized with MTB41 DNA developed both CD4- (predominantly Th1) and CD8-specific T cell responses to rMTB41 protein. More importantly, immunization of C57BL/6 mice with MTB41 DNA induced protection against infection with *M. tuberculosis* comparable to that induced by bacillus Calmette-Guérin. Thus, the use of a proven protective T cell line in conjunction with the T cell expression cloning approach resulted in the identification of a candidate Ag for a subunit vaccine against tuberculosis. *The Journal of Immunology,* 2000, 165: 7140–7149.

Approximately two billion of the world's population are infected with *Mycobacterium tuberculosis*, the causative agent of tuberculosis, and an estimated three million deaths due to this disease occur annually (1, 2). This, compounded with the emergence of drug-resistant strains and the HIV epidemic in both developing and industrialized countries, underscores the need for an effective vaccine against this disease (3–5). Although combination chemotherapy is effective in the treatment of tuberculosis, the treatment is arduous and requires stringent compliance to avoid the development of multidrug-resistant strains of *M. tuberculosis*. The attenuated strain of *Mycobacterium bovis*, bacillus Calmette-Guérin (BCG)[3] is currently the only available vaccine against tuberculosis (6, 7). However, the efficacy of BCG in the control of tuberculosis has shown considerable variation in different clinical trials and geographically distinct populations (6, 8). Moreover, BCG can cause disseminated disease in immunocompromised individuals (9, 10). Thus, current efforts are directed toward the development of a safer and more effective vaccine against tuberculosis and these include BCG overexpressing *M. tuberculosis* Ags, attenuated *M. tuberculosis*, and recombinant Ag-based subunit vaccines.

M. tuberculosis is an intracellular pathogen, and, as such, cell-mediated immunity plays a key role in the control of the bacterial replication and the subsequent protection against tuberculosis. In animal studies, acquired resistance against tuberculosis is mediated by sensitized T lymphocytes, and, in particular, IFN-γ secreting $CD4^+$ and $CD8^+$ lymphocytes are critical in mediating protection against tuberculosis in the murine model of this disease (11–16). The central role of IFN-γ in the control of tuberculosis has been further demonstrated by the high susceptibility to mycobacterial infections in mice with a disrupted IFN-γ gene and in humans with a mutated IFN-γ receptor (9, 10, 17, 18). Thus, the identification of mycobacterial Ags that preferentially activate T cells to proliferate and secrete IFN-γ is critical in the development of subunit vaccines against tuberculosis.

Much effort has been spent on the identification of candidate Ags from culture filtrate proteins (CF) derived from in vitro-cultivated *M. tuberculosis*. This has been an attractive source of Ags, primarily because CF has been shown to induce protection when used as vaccines in animal models of tuberculosis (19–23). In addition, the ability of CF to stimulate the proliferation and cytokine production from T cells of infected mice, guinea pigs, and PBMC from purified protein derivative (PPD)-positive human donors (24–26) have led to the conclusion that CF is an important source of candidate Ags for a subunit vaccine against tuberculosis.

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[3] Abbreviations used in this paper: BCG, bacillus Calmette-Guérin; CF, *M. tuberculosis* culture filtrate Ags; PPD, purified protein derivitized of tuberculin; ORF, open reading frame; IPTG, isopropyl β-D-thiogalactoside.

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Other approaches have also been used to successfully identify candidate T cell Ags. These include the biochemical fractionation of complex mixtures of mycobacterial Ags and serological expression cloning of *M. tuberculosis* expression libraries (26–35). More recently, Alderson et al. (36) reported on the development of a rapid and sensitive T cell expression cloning methodology to directly identify T cell Ags using human $CD4^+$ T cell lines generated from healthy PPD-positive donors.

In this report, we describe an alternative strategy to use the direct T cell expression cloning approach to selectively clone gene/Ags associated with the early control of *M. tuberculosis* infection in the mouse model. A protective $CD4^+$ T cell line, generated from spleen cells of C57BL/6 mice, harvested at a time point coinciding with the early control of the infection, was used to screen a genomic *M. tuberculosis* library. This led to the identification of several polypeptides, one of which has been extensively studied and is reported here. This Ag, MTB41, strongly stimulates human PBMC from healthy PPD-positive donors to proliferate and to produce IFN-γ and induces in the murine model of tuberculosis protection comparable to that obtained with BCG.

Materials and Methods

Animals

C57BL/6 mice were obtained from Charles River Laboratories (Wilmington, MA). The mice were maintained under pathogen-free conditions and used at 8–12 wk of age.

Bacteria and mice infections and immunizations

Virulent *M. tuberculosis* H37Rv strain (American Type Culture Collection, Manassas, VA; ATCC 35718) suspended in PBS Tween 80 (0.05%) were pushed through a 26-gauge needle six times and delivered either i.v. at 2×10^5 CFU/mouse, or using the aerosol route at ~200 CFU/mouse. After infection, organ (spleen and lung) homogenates in PBS Tween 80 (0.05%) were prepared and plated at 5- or 10-fold serial dilution on Middlebrook 7H11 Bacto Agar (Becton Dickinson Microbiology Systems, Cockeysville, MD). CFU were enumerated 3 wk later. Mice were immunized intramuscularly, three times, 1 mo apart, with 100 μg of plasmid DNA containing the gene of interest or with DNA alone ("empty vector"). Positive control mice were immunized with BCG (5×10^4 CFU) in the base of the tail (once), and negative control animals were injected with saline. Thirty days after the last immunization with DNA (3 mo after BCG), the mice were challenged with *M. tuberculosis* H37Rv.

M. tuberculosis *CF*

CF was prepared from 2-wk-old cultures of *M. tuberculosis* H37Rv strain grown in defined medium as described (26). CF was centrifuged at $2000 \times g$ for 20 min, and the supernatant was sterilized by passing through a 0.2-μm filter. The filtrate was concentrated with an Amicon 3 Centriprep concentrator (Beverly, MA) to 1/100 of the original volume, and the protein content was determined with a bicinchoninic acid protein assay (Pierce, Rockford, IL). Secreted proteins from *M. tuberculosis* Erdman and H37Rv strains were also provided by Dr. John Belisle (Colorado State University, Fort Collins, CO) produced through National Institute of Allergy and Infectious Diseases/National Institutes of Health "Tuberculosis Research Materials" Contract N01-AI-25147.

*Generation of anti-*M. tuberculosis *murine cell line*

For the generation of murine anti-*M. tuberculosis*-specific T cell line, standard procedures were followed. Spleen cells, obtained from C57BL/6 mice 3–4 wk after i.v. infection with *M. tuberculosis*, were stimulated in vitro with CF of *M. tuberculosis* H37Rv for 4–5 days and then with recombinant human IL-2 (2 ng/ml) for ~7 days. Cells were then restimulated with irradiated syngeneic APC (adherent spleen cells) plus Ag. This cycle of stimulation was repeated an additional two to three times. The cells were then rested in the absence of Ag stimulation and in the presence of IL-7 (10 ng/ml). Cells were subsequently tested for Ag specificity by proliferation/IFN-γ/IL-4 production assays. Surface markers were analyzed by FACScan for expression of CD4 or CD8 surface Ags using specific FITC-labeled mAbs (PharMingen, San Diego, CA) or isotype-matched monoclonal mouse control Ig. For adoptive transfer of immunity studies, C57BL/6 mice were inoculated i.v. with $\sim 10^7$ cells and subsequently (24 h later) challenged i.v. with 2×10^5 CFU of *M. tuberculosis* H37Rv. CFU were enumerated in the mice lungs and spleens 3 wk after the challenge.

Construction of the plasmid expression library

Genomic DNA from *M. tuberculosis* Erdman strain was randomly sheared to an average size of 2 kb, blunt ended with Klenow polymerase, and followed by the addition of *Eco*RI adaptors. The insert was subsequently ligated into the λScreen phage vector predigested with *Eco*RI (Novagen, Madison, WI) and packaged in vitro using the PhageMaker extract (Novagen). The phage library (Erd λScreen) was amplified, and a portion converted into a plasmid expression library. The *M. tuberculosis* Erd λScreen phage library was converted into a plasmid library (pScreen) by autosubcloning using the *E. coli* host strain BM25.8 as suggested by the manufacturer (Novagen). Plasmid DNA was purified from BM25.8 cultures containing the pScreen recombinants and used to transform competent cells of the expressing host strain BL21(DE3)pLysS. Transformed cells were aliquoted into 96-well microtiter plates with each well containing a pool size of ~50–60 recombinant colonies. Replica plates of the 96-well plasmid library format were induced with isopropyl β-D-thiogalactoside (IPTG) to allow recombinant protein expression. Following induction, the plates were centrifuged to pellet the *Escherichia coli*, and the bacterial pellet was suspended in 200 μl of PBS

Screening of M. tuberculosis *expression library with specific T cell line*

This strategy was recently described for rapid cloning of *M. tuberculosis* genes using human $CD4^+$ T cells (36), and a diagram of it is outlined in Fig. 1. Its general principle is based on the direct recognition by the T cells of Ags presented by APCs that have internalized a library of *E. coli*-containing expressed recombinant Ags. The *M. tuberculosis* library was initially divided in pools containing ~50–60 transformants/well distributed in 96-well microtiter plates and stored in a replica plate manner. Adherent spleen cells (APCs) were fed with the *E. coli* pools and incubated for processing for 2 h. After washing, the APCs were exposed to a specific T cell line in the presence of gentamicin (50 μg/ml) to inhibit the bacterial growth. T cell recognition of pools containing *M. tuberculosis* Ags was then detected by proliferation assay ($[^3H]$thymidine incorporation). Wells that scored positive were then broken down using the same protocol until a single clone was detected. The gene was then sequenced, subcloned, expressed, and the recombinant protein evaluated.

High level expression and affinity purification of recombinant mycobacteria Ags

Oligonucleotide PCR primers were designed to amplify the full-length sequences of MTB10 and MTB41 from genomic DNA of the virulent Erdman strain. MTB10 was amplified using the oligonucleotide primers 5′ (5′-CAA TTA CAT ATG **CAT CAC CAT CAC CAT CAC** *ATG TCT TTT GTG ACC ATC CAG*-3′) and 3′ (5′-CAT GGA ATT CTT AAC CGG TCG CGA CCA CAT T-3′) The full-length coding portion of MTB41 was PCR amplified using the following primer pairs: 5′ (5′-CAA TTA CAT ATG **CAT CAC CAT CAC CAT CAC** *ATG GAT TTC GGG CTT TTA CCT*) and 3′ (5′-CAT GGA TAT CGT TGC CGG ACT TTT ACC GCG G-3′). The 5′ oligonucleotides contain an *Nde*I restriction site preceding the ATG initiation codons (underlined) followed by nucleotide sequences encoding six histidines (bold) and sequences derived from the gene (italic). The 3′ oligonucleotides contain protein coding sequences followed by stop codons (underlined) and *Eco*RI or *Eco*RV restriction sites. The resultant PCR products were digested with *Nde*I and *Eco*RI or *Nde*I and *Eco*RV (MTB10 and MTB41, respectively) and subcloned into the pET17b vector similarly digested with *Nde*I and *Eco*RI or *Nde*I and *Eco*RV for directional cloning. Ligation products were initially transformed into *E. coli* XL1-Blue competent cells (Stratagene, La Jolla, CA) and were subsequently transformed into *E. coli* BL-21 (pLysE) host cells (Novagen) for expression.

The recombinant proteins were expressed in *E. coli* with six histidine residues at the amino-terminal portion using the pET plasmid vector (pET-17b) and a T7 RNA polymerase expression system (Novagen). *E. coli* strain BL21 (DE3) pLysE (Novagen) was used for high level expression. Recombinant (His-Tag) Ags were purified from the insoluble inclusion body of 500 ml of IPTG-induced batch cultures by affinity chromatography using the one step QIAexpress Ni-NTA Agarose matrix (Qiagen, Chatsworth, CA) in the presence of 8 M urea as previously described (37). The yield of recombinant protein varied from 25–50 mg per liter of induced bacterial culture. Purity of the recombinant proteins were assessed by SDS-PAGE, followed by Coomassie blue staining, and N-terminal sequencing using Edman chemistry with a Procise 494 protein sequencer (Perkin-Elmer/Applied Biosystems, Foster City, CA). The recombinant proteins

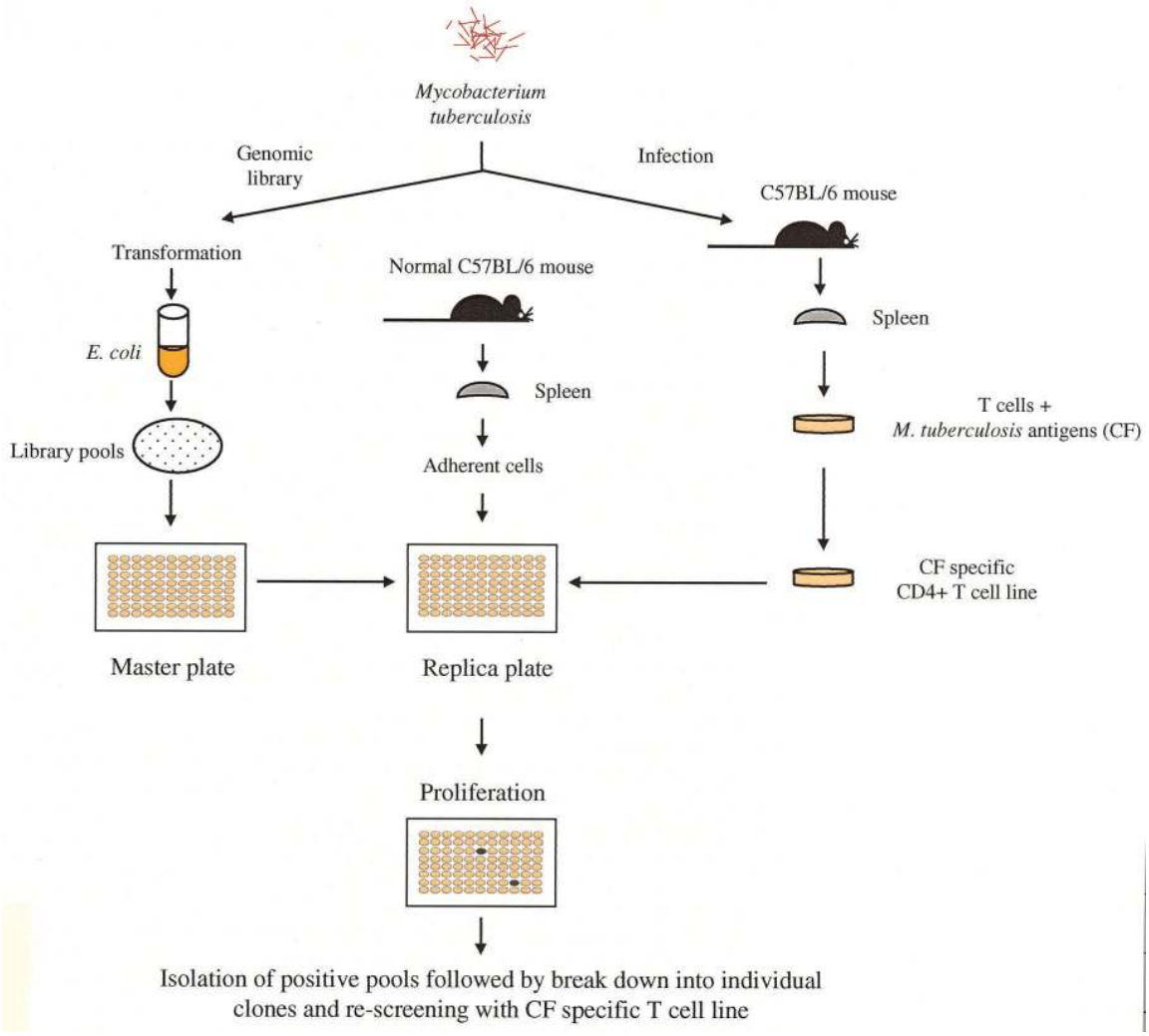


FIGURE 1. Schematic representation of the T cell expression cloning approach.

were assayed for endotoxin contamination using the *Limulus* amebocyte assay (BioWhittaker, Walkersville, MD) and were shown to contain <100 EU/mg protein.

Proliferation and cytokine assays

Spleen cells were obtained by conventional procedures, and then centrifuged over Ficoll-Hypaque to remove red cells followed by depletion of most of the phagocytic cells via passage through a Sephadex G-10 column. Mononuclear cells were cultured at 37°C + CO_2 in the presence of either medium or Ag. Proliferation was measured by [^{3}H]thymidine incorporation at day 6. For cytokine analysis, spleen cells at 10^6 cells/well (24-well tissue culture plates) were cultured in the presence of anti-IL-4 receptor mAb (Immunex, Seattle, WA), with or without Ags for 72 h. The addition of anti-IL-4 receptor mAb in these assays prevents the use of this cytokine and consequently allows its accumulation and detection in culture supernatants (38). Supernatants were harvested and analyzed for IFN-γ and IL-4 by a double sandwich ELISA using specific mAb (PharMingen). For human assays, PBMC from volunteers were isolated by gradient centrifugation and used in proliferation assays as described (26). A panel of PBMC obtained from eight healthy PPD-positive individuals (indurations of >10 mm) and six PPD-negative individuals of diverse ethnic background (African, Middle Eastern, Hispanic, Caucasian, and Asian) was used. PBMC (2×10^5 well) were incubated in 96-well round-bottom plates (Costar, Cambridge, MA) in medium only (RPMI with 10% pooled human serum and gentamicin (50 μg/ml)) or in medium containing specific Ags at the indicated concentrations. Plates were cultured for 5 days at 37°C in 5% CO_2 and were pulsed with 1 μCi of [^{3}H]thymidine (Amersham, Arlington Heights, IL) for an additional 18 h. Cells were harvested onto filter mats and counted using a Matrix 9600 direct β gas scintillation counter (Packard, Meriden, CT). The levels of supernatant IFN-γ were analyzed by sandwich ELISA, using Ab pairs and procedures available from PharMingen as described (39). For this assay, culture supernatants were obtained 72 h after the initiation of the cultures.

DNA vaccine and retroviral constructs

The full-length coding sequence of MTB41 was PCR amplified using the primer pairs: 5′-(5′-GAG AAA GCT TGC AAT C*AT G*GA *TTT CGG GCT TTT A CC*) and 3′-(5′-GAG ATC TAG A*GT TGC CGG ACT TT*T AC*C GCG G*-3′) containing sequences derived from the 5′ and 3′ coding portions, a stop codon (underlined) and 3′ untranslated sequences (italic). The 5′ primer was designed to contain a *Hin*dIII recognition site and a Kozak sequence upstream of the initiator ATG codon. The resultant PCR product was digested with *Hin*dIII/*Xba*I and subcloned into the eukaryotic expression vector pJA4304 (generous gift of James I. Mullins and Jim Arthors, University of Washington School of Medicine, Seattle, WA) similarly digested with *Hin*dIII and *Xba*I.

In addition, the MTB41 gene was also subcloned into the retroviral vector pBIB-X, a retroviral expression vector that contains a selectable marker (bsr) under translation control of an intraribosomal entry site sequence. This vector is under the control of the murine leukemia virus long terminal repeat promoter. The MTB41 encoding sequence was obtained by PCR amplification using 5′ oligonucleotides designed with the initiating methionine. The 3′ oligonucleotide included the stop codon. The PCR-amplified product was subsequently amplified with *Hin*dIII and *Not*I for directional subcloning into the pBIB-X expression vectors. The 5′ primers also included a KOZAK consensus sequence (GCCGCCACC) upstream of the initiation codon to ensure efficient translational initiation in the pBIB-X vector.

CTL assay

Target cells were EL-4 cells retrovirally transduced with the MTB41 gene essentially as described (40). Briefly, the retroviral construct was used in transfections of Phoenix-Ampho, an amphotropic retroviral packaging line. Approximately 48 h posttransfection, supernatants containing recombinant virus were harvested and used to transduce EL-4 cells. Transduction efficiency was measured by FACS using EL-4 transduced with pBIB-EGFP (enhancing green fluorescent protein) viral supernatants as a positive control. All transfectants were selected with blastocidin-S (Calbiochem, San Diego, CA) at a concentration of 10 μg/ml and cloned twice by limiting dilution. These cells were then used as target for standard ^{51}Cr release CTL assays using spleen cells isolated from mice immunized with naked MTB41 DNA as effector cells.

Results

Generation and specificity of a protective CD4$^+$ T cell line from splenocytes of M. tuberculosis*-infected C57BL/6 mice*

An anti-CF CD4$^+$ T cell line was generated from spleen cells of C57BL/6 mice infected for ~3 wk with *M. tuberculosis*. CF was chosen because this material contains protective Ags (19–23). The time point after the infection was deliberately selected because, in the murine model of tuberculosis, there is the initial burst of the *M. tuberculosis* in the spleen, lungs, and liver during the first 2 wk after the infection followed by a decline in the number of the microorganisms during the subsequent 2 wk. The reduction of the bacterial burden coincides with the emergence of specific and protective CD4$^+$ T cells (12, 41). Therefore, an anti-CF T cell line generated from splenocytes obtained from C57BL/6 mice infected for 3–4 wk with *M. tuberculosis* should contain protective T cell clones. Indeed, the resulting cell line was strongly reactive with CF, comprised of exclusively CD4$^+$ T cells, and, more importantly, it contained protective T cells as indicated by adoptive transfer experiments (Fig. 2). This cell line was then used for the T cell expression cloning experiments.

CD4$^+$ T cell expression cloning and molecular characterization of the Ags MTB10 and MTB41

The *M. tuberculosis* library was initially divided in pools containing ~60 recombinant colonies per well in a 96-well microtiter plate format. Adherent spleen cells from naive C57BL/6 mice were

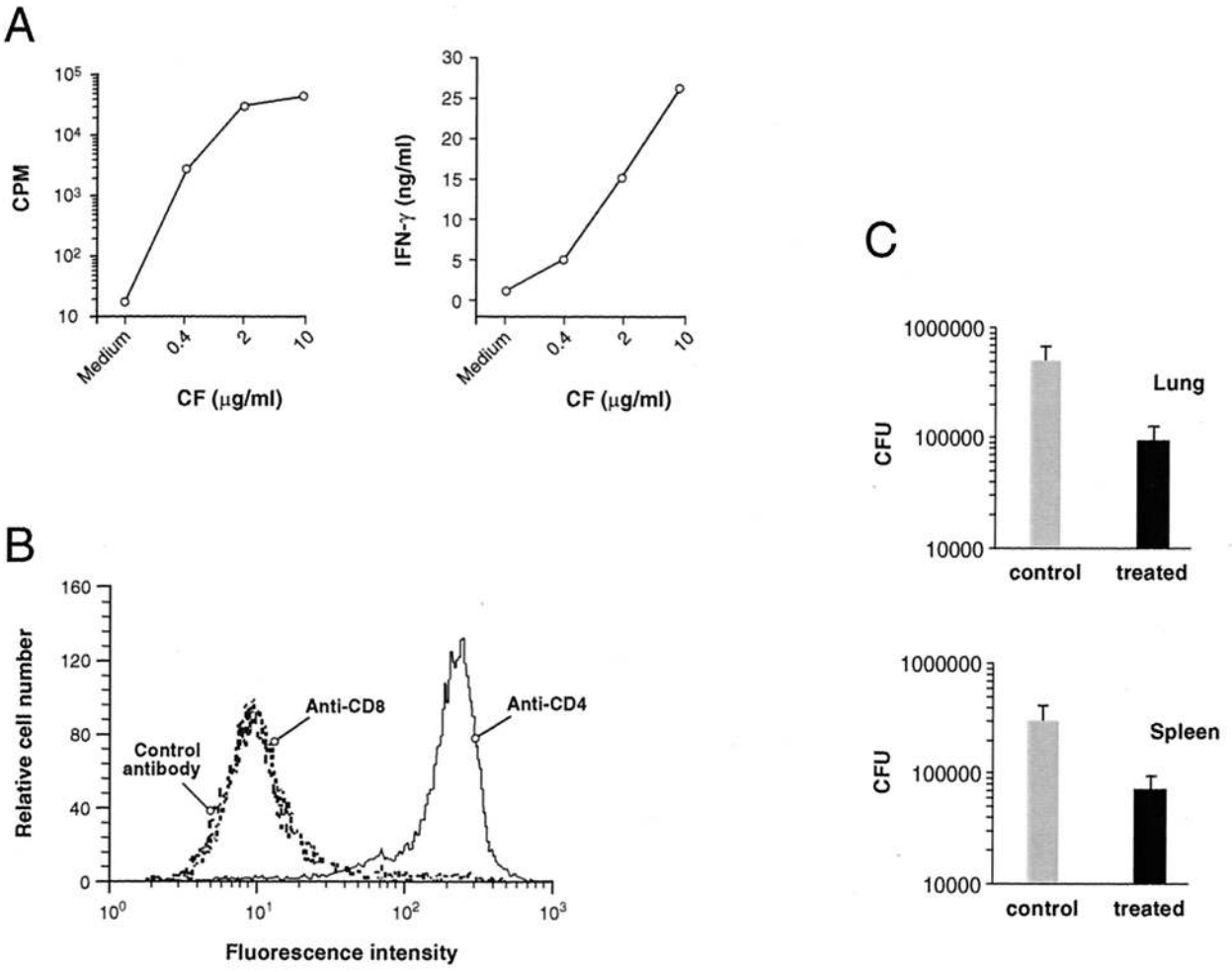
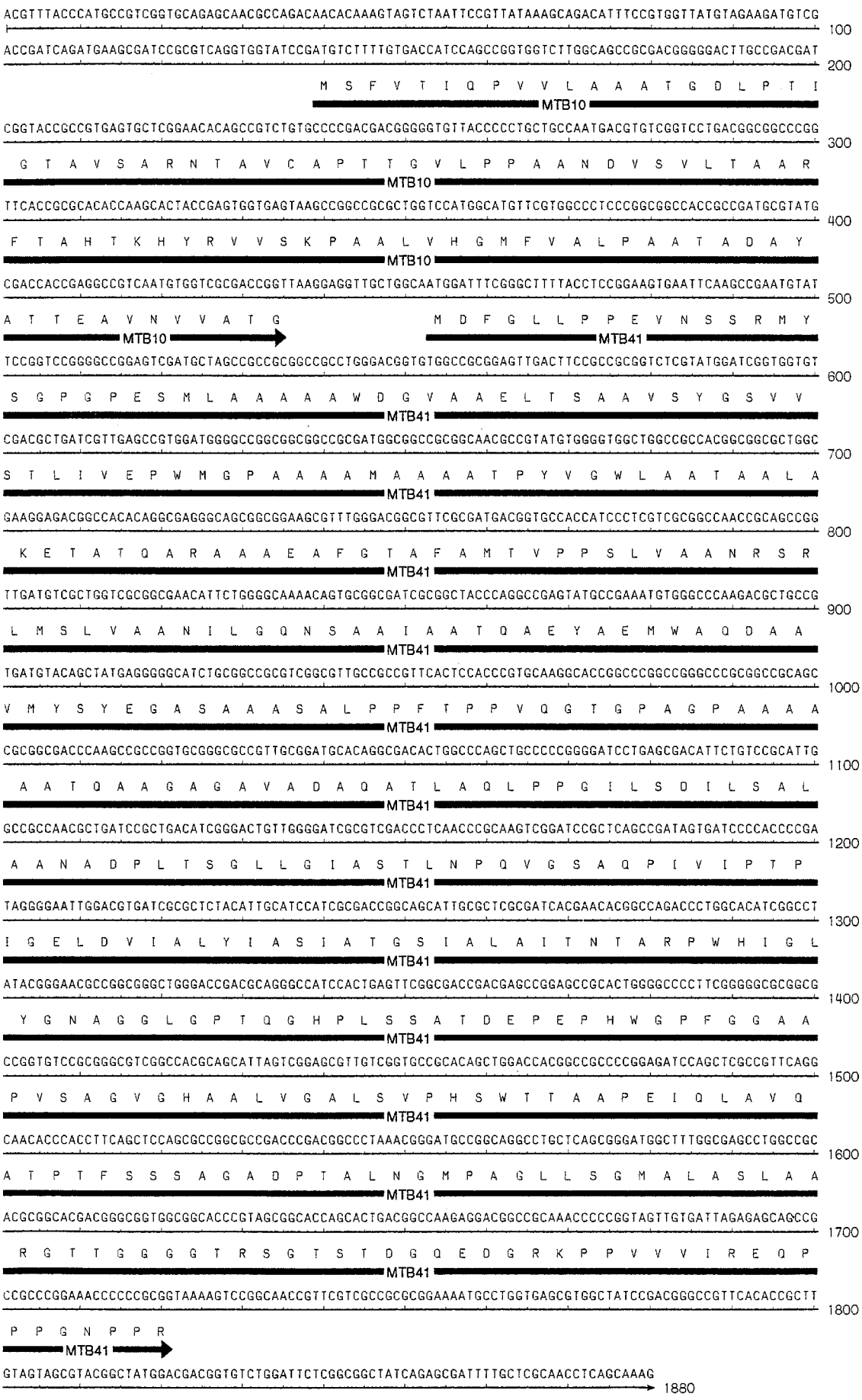


FIGURE 2. Generation of a protective anti-*M. tuberculosis* CF-specific CD4$^+$ T cell line. Spleen cells were obtained from C57BL/6 mice 3 wk after i.v. infection with 2×10^5 CFU of *M. tuberculosis*. Cells were stimulated in vitro with crude culture filtrate (CF) of *M. tuberculosis* H37Rv for 4–5 days and then with 2 ng/ml recombinant human IL-2 for ~7 days. Cells were then restimulated with irradiated syngeneic APC (adherent spleen cells) plus Ag. This cycle of stimulation was repeated an additional two to three times. The cells were then rested in the absence of Ag stimulation and in the presence of IL-7 (10 ng/ml). Cells were subsequently tested for Ag specificity by proliferation/IFN-γ/IL-4 production assays after stimulation with various concentrations of CF (*A*). No IL-4 was detected in the culture supernatants (data not shown). Cells were analyzed by FACScan for expression of CD4 or CD8 surface Ags (*B*) using specific mAbs or isotype-matched monoclonal mouse control Ig. For protection (*C*), C57BL/6 mice were inoculated i.v. with ~10^7 of CF-specific CD4-positive T cells (treated) and subsequently (24 h later) challenged i.v. with 2×10^5 CFU of *M. tuberculosis* H37Rv. CFU were enumerated in the mice spleen and lungs 3 wk after the challenge. Bars represent SEM of the results given by five mice per group.



fed with the library containing pools of *E. coli* expressing *M. tuberculosis* Ags followed by incubation with the anti-$CD4^+$ T cell line. Recognition of specific *M. tuberculosis* Ags were detected by proliferation of the T cells ($[^3H]$thymidine incorporation). The screening of two 96-well microtiter plates resulted in the identification of five positive pools of which two were pursued further because they elicited strong proliferative responses. These pools were broken down until single positive recombinant colonies (Y1–44-A11 and Y2-88-C10) were identified. Sequencing of both plasmid clones revealed that they were identical and code for two adjacent, nonoverlapping open reading frames (ORFs). Their predicted full-length ORFs code for proteins with molecular mass of 10 and 41 kDa, respectively, and are thus referred to as MTB10 and MTB41. These two Ags correspond exactly to the gene products of Rv0916c and Rv0915c on the TubercuList H37Rv database (http://genolist.pasteur.fr./TubercuList/). Fig. 3 shows the nucleotide sequence and predicted ORFs of the locus comprising the full-length sequences of MTB10 and MTB41. Both genes contain an ATG initiation codon preceded by the ribosome binding sequences (Shine-Dalgarno sequence, SD) AGGTGG and AG GAGG at 7 and 9 bp upstream of the initiator methionine residue of MTB10 and MTB41, respectively. The intergenic region between the stop codon of MTB10 and start codon of MTB41 is only 15 bp. The ORF of MTB10 codes for a 10-kDa (99 amino acids) hydrophobic protein with a predicted isoelectric point of 8.23 and a net charge of 1.38 at pH 7.0. About 50% of this polypeptide comprises hydrophobic residues (Ala (22 residues), Ile (2 residues), Leu (2 residues), Phe (3 residues), and Val (15 residues)). In addition, MTB10 contains five prolines (which are known to generate kinks within protein structures and alter their mobilities on denaturing gels) and a single cystein that could generate protein-protein interactions via disulfide linkage. The ORF of MTB41 codes for 423 amino acids with a predicted molecular mass of 41.4 kDa, an isoelectric point of 4.45 and a net charge of −13.22 at pH 7.0

Protein expression of rMTB10 and rMTB41

The ORFs of the full-length genes of MTB10 and MTB41 were amplified by PCR with 5′- and 3′-specific oligonucleotides and cloned into the pET17b expression vector. Both constructs were designed to contain six N-terminal histidine residues for ease of purification by affinity chromatography over Ni-NTA matrix. The recombinant proteins were purified from inclusion bodies with yields ranging from 20 to 45 mg of purified protein per liter of induced culture. Fig. 4, *A* and *B*, shows Coomassie blue-stained SDS-PAGE gels of the *E. coli* cultures before and after induction and the respective purified recombinant Ags. MTB10 migrated as multimers even though it contains a single cysteine residue, whereas MTB41 migrated with its predicted size of 41 kDa.

Recognition of MTB10 and MTB41 by human T cells

To evaluate the potential use of the Ags MTB10 and MTB41 as possible vaccine candidates for humans, these Ags were tested using a panel of PBMC from healthy PPD-positive and -negative donors of diverse ethnic backgrounds. Using a stimulation index cutoff >5 as a positive response, seven of eight PPD-positive donors responded to MTB41 (Fig. 5) and none of them responded to MTB10 (data not shown). In addition, none of the PPD-negative donors (six) responded to either Ag (data not shown). In parallel, evaluation of the IFN-γ production by these PBMC revealed a direct correlation between the proliferative responses with the secretion of this cytokine. Thus, IFN-γ was detected in the culture supernatants of the PBMC stimulated with MTB41 but not with MTB10. It is interesting to note that the same pattern of reactivity was observed when the murine anti-CF cell line was used to validate these two Ags, i.e., this cell line readily recognized the Ag MTB41 but did not do so for Ag MTB10 (data not shown). In view of these results, the experiments aiming to investigate protection were conducted only with the Ag MTB41.

Immunogenicity studies with the Ag MTB41

Because immunity to tuberculosis is apparently dependent on both $CD4^+$ and $CD8^+$ T cell responses, experiments were designed to investigate the protection potential of MTB41 delivered in naked DNA format, a type of immunization known to stimulate these two arms of the immune system. The MTB41 gene was initially subcloned into the eukaryotic expression vector pJA4304, which is under the control of a CMV promoter. Mice were immunized i.m. with 100 μg of MTB41-DNA three times, 1 mo apart. Anti-MTB41 Ab responses and T cell responses ($CD4^+$ and $CD8^+$) were evaluated 3 wk after the last immunization. Both IgG1 and IgG2a Ab responses were evaluated by ELISA using specific anti-mouse isotype Abs. The results indicated that the mice immunized with MTB41-DNA developed higher titers of IgG2a than IgG1 anti-MTB41 responses (Fig. 6*A*). No anti-MTB41 Ab was detected in the sera of mice immunized with the vector control (data not shown).

To measure the $CD4^+$ T cell response, mononuclear spleen cells were obtained 3 wk after the last DNA immunization and stimulated in vitro with the recombinant MTB41 protein for 3 days. To increase the sensitivity of the assay, a monoclonal anti-IL-4 receptor Ab was added to the cultures (38). Supernatants were harvested and assayed for both IFN-γ and IL-4. The results are depicted in Fig. 6*B* and indicate that MTB41 stimulates high quantities of IFN-γ. In contrast, no IL-4 could be detected in these supernatants (data not shown). These results are in synchrony with the preferential IgG2a Ab response, and suggest that the $CD4^+$ T cell response induced by MTB41-DNA immunization is preferentially of the Th1 phenotype.

To measure the $CD8^+$ T cell response in the MTB41-DNA-immunized mice, their mononuclear spleen cells were stimulated for 5 days in vitro with irradiated EL-4 cells transduced with MTB41 gene. Stimulated cells were washed and tested for cytotoxicity (^{51}Cr release assay) against EL-4/EGPF- and EL-4/MTB41-transfected targets. Fig. 6*C* clearly shows that immunization of mice with MTB41-DNA induces the generation of MTB41-specific CTL.

Induction of protection against challenge with M. tuberculosis *by MTB41 DNA immunization*

In view of the fact that the immunogenicity experiments revealed that MTB41-DNA immunization resulted in the induction of strong Ag-specific CD4 and CD8 responses, we next assessed whether this form of Ag delivery could provide protection against

FIGURE 3. Nucleotide and deduced amino acid sequences of the predicted ORF of MTB10 and MTB41. The figure shows a 1.88-kb locus containing the full-length ORFs of the linked PE-PPE gene pair (Rv0916c-Rv0915c), MTB10 and MTB41. Both genes contain an ATG initiation codon preceded by the ribosome binding sequences (Shine-Dalgarno sequence, SD) AGGTGG and AGGAGG at 7 and 9 bp upstream of the initiator methionine residue of MTB10 and MTB41, respectively. The intergenic region between the stop codon of MTB10 and start codon of MTB41 is only 15 bp. The ORF of MTB10 codes for a 10-kDa (99 amino acids) hydrophobic protein and MTB41 codes for 423 amino acids with a predicted molecular mass of 41.4 kDa.

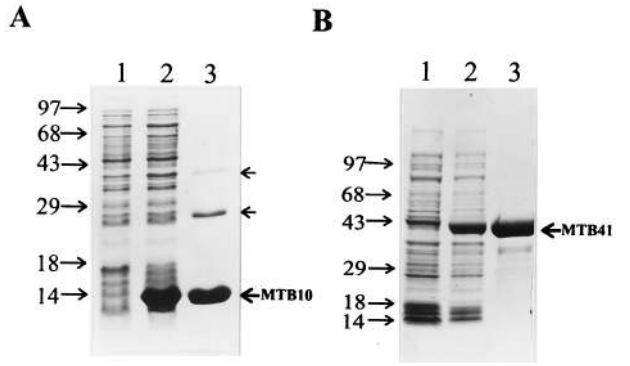


FIGURE 4. Overexpression and purification of recombinant MTB10 and MTB41 Ags. Recombinant MTB10 and MTB41 (*A* and *B*, respectively) were expressed in *E. coli* with six His-tag amino-terminal residues and the proteins purified by affinity chromatography using Ni-NTA agarose matrix. Coomassie blue-stained SDS/15% polyacrylamide gel of 10 μl each of *E. coli* lysates from noninduced (*lane 1*) and IPTG induced (*lane 2*) cultures and 5 μg each of the purified recombinant proteins (*lane 3*).

aerosol challenge with *M. tuberculosis*. For this purpose, C57BL/6 mice were immunized as above i.m., three times, 1 mo apart, with 100 μg of MTB41-DNA. As negative controls, groups of mice were also immunized with DNA alone (empty vector), or injected with saline only. As positive controls, mice were immunized once with BCG. Thirty days after the last DNA immunization, the mice were challenged with 200 CFU of *M. tuberculosis* H37Rv using the aerosol route. Bacteriological burden (CFU) was measured in the mice lungs 3 wk after the challenge. Fig. 7 illustrates that the level of protection induced by this regime of immunization was similar to the protection induced by BCG. Immunization of mice with control DNA resulted in no reduction in the *M. tuberculosis* CFU in the lungs of these animals as compared with the CFU given by the mice injected with saline only.

Discussion

Acquired resistance to tuberculosis has long been shown to be strongly dependent on the emergence of *M. tuberculosis*-specific $CD4^+$ T cells (12–16). This concept is supported by solid evidence obtained from observations from both the human disease and from experiments conducted in the murine model of tuberculosis. In humans, particularly in AIDS patients, reactivation of tuberculosis is commonly associated with low levels of $CD4^+$ T cells. In the murine model, resistance to tuberculosis can be achieved by adoptive immunity with $CD4^+$ T cells obtained from mice infected with sublethal doses of *M. tuberculosis* (12, 41). In addition, mice deficient in either MHC class II molecules or $CD4^+$ T cells are highly susceptible to infection with *M. tuberculosis* (42, 43). Among the $CD4^+$ T cells, both activated and memory $CD4^+$ T cells can transfer immunity to tuberculosis in this model (12, 41). The emergence of protective activated cells occurs early on after the infection (~1 wk), peaks around 3 wk, and declines thereafter. The protective memory cells emerge around 2 wk after infection and can be detected both functionally and morphologically for several months (12). Therefore, a cell line generated from spleens harvested at 3 wk postinfection should contain protective T cell clones of both phenotypes. In addition, several lines of evidence suggest that the *M. tuberculosis* Ags that induce protection, in both mouse and guinea pig models, are proteins found in the mycobacteria CF (19–23).

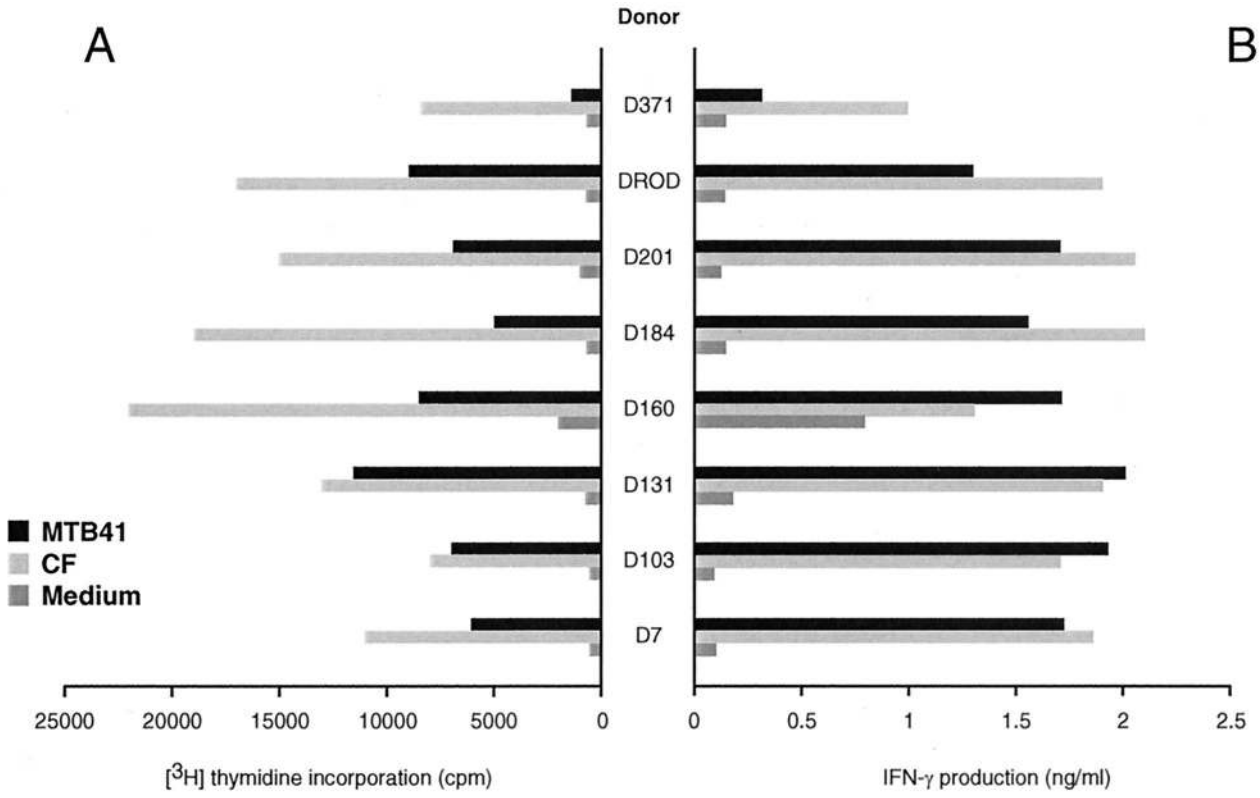


FIGURE 5. Recognition of MTB10 and MTB41 by human PBMC. Proliferative responses (*A*) and IFN-γ production (*B*) of PBMC from PPD-positive healthy donors following stimulation with 10 μg/ml CF or 5 μg/ml rMTB41. Proliferation was measured by [^{3}H]TdR incorporation. IFN-γ was measured by sandwich ELISA in the culture supernatants.

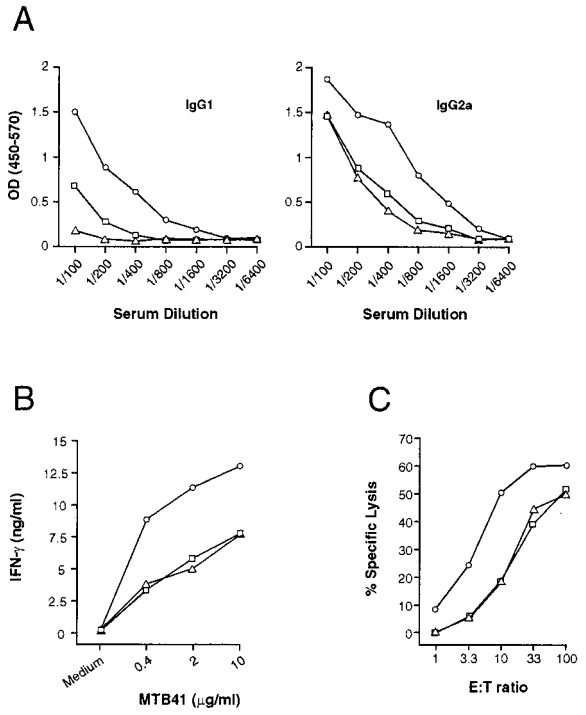


FIGURE 6. Induction of specific $CD4^+$ T cell (Th1) and CTL responses in mice immunized with MTB41-DNA. Three C57BL/6 mice were injected three times i.m. with 100 μg of MTB41-pJA4304 DNA (1-mo interval). One month after the last immunization, the animals were bled and sacrificed. Sera were obtained and tested for specific anti-MTB41 Ab response of both IgG1 and IgG2a isotypes by ELISA (*A*); for cytokine production (*B*), mononuclear spleen cells were obtained and stimulated for 3 days with 10 μg/ml of recombinant MTB41 protein. In addition, cells were cultured in the presence of anti IL-4 receptor mAb (1 μg/ml). Supernatants were harvested and assayed for the presence of IFN-γ and IL-4 by ELISA. No IL-4 could be detected (data not shown); for evaluation of CTL activity (*C*), the mononuclear spleen cells were stimulated for 6 days with MTB41-transfected EL-4 cell targets. Effector cells were washed and tested for cytotoxicity in a 4-h ^{51}Cr release assay against both control transduced EL-4 cells (EGPF), and against MTB41 transduced EL-4 cells. Results are expressed as % specific cytotoxicity against MTB41 transfected cells (cytotoxicity against EL-4 control targets has been subtracted). The curves express the results obtained for each individual mouse. This is one representative experiment of three separate experiments with virtually the same results.

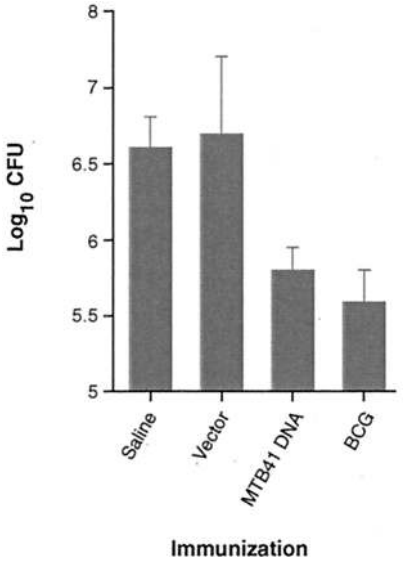


FIGURE 7. Vaccination of mice against tuberculosis with MTB41-DNA. C57BL/6 mice (five animals per group) were injected three times i.m. with 100 μg of either MTB41-pJA4304 DNA or control vector (1 mo interval), or once (s.c.) with 5×10^4 CFU of BCG (Aventis Pasteur, Swiftwater, PA), or with saline. One month after the last immunization, the animals were challenged with 200 viable aerosolized *M. tuberculosis*, and CFU in the lungs were enumerated 3 wk later. This is one representative experiment of three separate experiments with virtually the same results, i.e., in two other experiments, MTB41-DNA conferred 0.8 and 1 log protection, respectively. Bars represent SEM of the results given by five mice per group.

Here, we used these two concepts, i.e., protective spleen cells and protective Ags (CF), to generate a protective T cell line that could be used as the readout of the T cell expression cloning approach. The obtained cell line was phenotypically characterized as $CD4^+$ T cells and strongly reacted with CF with both proliferation and production of high levels of IFN-γ and nondetectable IL-4. Moreover, adoptive immunity experiments clearly demonstrated that this cell line contained protective T cell clones. For these experiments, in contrast to former publications (41, 44), normal uncompromised mice were used. Even under these unfavorable conditions, the cell line conferred protection to the recipients as indicated by ~0.3 log protection in the spleen and 0.6 log protection in the lungs. These results confirmed the initial proposal, and the protective anti-CF CD4-positive T cell line was used to successfully clone *M. tuberculosis* genes encoding proteins that might be associated with protection. The use of a proven protective T cell line as the readout of the T cell expression cloning approach is an attractive alternative. This approach, initially developed for the identification of a leishmanial and a listerial gene encoding T cell Ags (45, 46), was recently described as a powerful strategy for the direct screening and cloning of genes from a *M. tuberculosis* genomic expression library (36) using human *M. tuberculosis* reactive T cell lines as readouts. Here, the use of this technology, employing a proven protective murine $CD4^+$ T cell line, resulted in the cloning of several potentially protective genes. We further pursued the two strongest reactive *E. coli* clones and identified that they contain identical adjacent ORFs (MTB10 and MTB41) belonging to the PE and PPE family of proteins. The PE and PPE family of proteins represent two large unrelated families comprising about 10% of the coding capacity of the MtbH37Rv genome (47). There are about 100 members of the PE protein family characterized by the presence of a highly conserved N-terminal hydrophobic globular domain (95–100 amino acid residues). Most of the members have C-terminal extensions ranging in size from 100 to 1400 residues. MTB10 is an example of a PE protein that contains only the N-terminal hydrophobic domain and lacks a C-terminal extension. The sequenced H37Rv genome also revealed the presence of 66 members of the PPE protein family characterized by a conserved ~180 residue N-terminal domain and C-terminal extensions ranging in size from 200 to >3500 amino acid residues (47). The adjacent organization of MTB10 and MTB41 (Rv0916c-Rv0915c) represent an example of a PE-PPE gene pair in which a short PE (MTB10 has only the N-terminal hydrophobic domain) is linked to a PPE ORF (MTB41) by a short intergenic segment.

The fact that only MTB41 was identified as the dominant Ag in the murine protection model argues that the cloning of this gene resulted from the recognition of MTB41 sequence-specific epitopes. Subsequent analysis demonstrated that MTB41 is also a potent T cell Ag recognized by PBMC from PPD-positive but not PPD-negative donors. Thus, members of the PPE family constitute potent immunogens comprising of specific as well as shared antigenic epitope(s). In this regard, another PPE protein, MTB39A,

with little sequence homology to MTB41, was also recognized by T cells from healthy PPD-positive donors (33).

The evaluation of MTB41 as a potential vaccine candidate was accomplished by experiments conducted in mice and by studies done with human PBMC. The mouse model was used to investigate the immunogenicity and ability of MTB41 to induce protection. The human cells were used to evaluate the ability of T cells from healthy PPD-positive individuals to recognize MTB41, to support the use of this Ag in humans. These results indicated that MTB41 is recognized by a high percentage of the healthy PPD-positive individuals (presumably resistant) and suggests that this recognition is mediated preferentially by Th1 cells because in all cases, the proliferative response was invariably accompanied by the production of high levels of IFN-γ. Because IFN-γ production is essential for resistance to tuberculosis in both mice and humans (9, 10, 17, 18), these findings lend support to the possibility that MTB41 might be associated with protection against this disease in humans as well.

The use of a murine cell line in the T cell expression cloning approach as an alternative to human cell lines is justified simply based of the fact that in the murine system one can have the assurance of initiating the cloning strategy with a protective cell line. However, this important premise for the cloning of genes encoding protective Ags, cannot be easily achieved with human cells. In other words, the screening of a *M. tuberculosis* library with a protective murine cell line may be more successful for the identification of protective Ags than with the use of a cell line that is unknown to contain protective T cell clones. In the well-defined murine system, as stated above, the disease progression is timely contained by the emergence of protective T cells. This fact per se is an important asset for the successful generation of a cell line containing several protective T cell clones. Perhaps more important, is the fact that the premise of a protective cell line can be easily achieved and proven in the murine system using adoptive immunity experiments. In contrast, a cell line generated from healthy PPD-positive individuals is only presumably protective. Nonetheless, one cannot exclude that human cells lines may contain protective T cell clones and therefore are also suitable for T cell expression cloning of protective Ags.

The protection experiments in the mouse model were conducted primarily using DNA vaccination because protection against tuberculosis in these animals has been generally achieved with this form of immunization rather than with the conventional immunization with proteins (48–51). The reason for this disparity is not clear. However, it is generally accepted that the low efficiency of protein immunization is related to the lack of an appropriate adjuvant to help the stimulation of the ideal balance of responses by the various subsets of T cells engaged in protection against tuberculosis. Apparently, DNA immunization, in general, induces both CD4 responses predominantly of the Th1 phenotype and CD8-mediated responses. Therefore, this mode of Ag delivery seems to work better in vaccination protocols against tuberculosis because resistance to this disease is apparently mediated by both CD4 and CD8 T cell responses. Indeed, when C57BL/6 mice were immunized with MTB41 DNA, they developed high titers of IgG2a anti-MTB41 Abs and their spleen cells produced high levels of IFN-γ and no IL-4 after in vitro stimulation with the recombinant protein. Thus, a typical CD4 Th1 response. Moreover, immunization of these mice with MTB41 DNA induced the generation of anti-MTB41 specific CD8 CTL response as assayed by the ability of these cells to specifically lyse EL-4 targets that had been transduced with MTB41. More importantly, this regime of immunization induced protection comparable to that induced by BCG.

Despite the fact that several Ags from *M. tuberculosis* have been isolated, cloned, and shown to be protective, our results clearly demonstrate that a protein encoded by a gene of the PE-PPE family induces protection in the murine model of tuberculosis. As stated earlier, these genes comprise ~10% of the *M. tuberculosis* genome. Therefore, this work points to this family of proteins as important targets for a systematic evaluation of vaccine candidates. Corroborating with this possibility is the recent observation (52) that indicates that members of the PE-PPE family are virulence factors for *M. marinum*, therefore supporting the idea that vaccine candidates may be present among these proteins in *M. tuberculosis* as well.

In conclusion, these results support the premise of the approach used in these studies, i.e., the use of a proven protective T cell line to directly screen a pathogen expression library to clone genes encoding protective Ags.

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