

The phenotype of the TIRAP-deficient mice encouraged us to search for new TIR domain-containing adaptors, and led to the identification of a third TIR domain-containing adaptor (Yamamoto et al., 2002). Overexpression of this molecule, as well as MyD88 and TIRAP, resulted in activation of the NF- κ B reporter gene in 293 cells. Furthermore, its overexpression led to the activation of the IFN- β promoter, which was not observed when MyD88 or TIRAP was overexpressed. Therefore, this molecule was named TRIF for TIR domain-containing adaptor inducing IFN- β . The dominant negative form of TRIF inhibited TLR3-dependent activation of the IFN- β promoter. Association between TRIF and IRF-3 was also shown. These findings indicate that TRIF may act in the MyD88-independent pathway leading to the induction of IFN- β . Analysis of TRIF-deficient mice will reveal its precise role in TLR signaling in the near future.

CONCLUDING REMARKS

We are now aware that innate immunity detects microbial invasion through the recognition of specific patterns of microbial components by TLRs. However, it is still elusive how TLRs recognize them. In the signaling pathways via TLRs, MyD88 is a common adaptor leading to the induction of inflammatory cytokines. However, additional adaptors, such as TIRAP and TRIF, exist that are expected to specify the TLR signaling pathway. Elucidation of the signaling pathway that is specific to each TLR will provide us with an important clue to understanding the molecular mechanisms by which innate immunity is activated and finally lead to the development of antigen-specific adaptive immunity.

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Differential Immune Responses and Protective Efficacy Induced by Components of a Tuberculosis Polyprotein Vaccine, Mtb72F, Delivered as Naked DNA or Recombinant Protein¹

Yasir A. W. Skeiky,^{2*} Mark R. Alderson,* Pamela J. Owendale,* Jeffrey A. Guderian,* Lise Brandt,[‡] Davin C. Dillon,* Antonio Campos-Neto,^{3†} Yves Lobet,[§] Wilfried Dalemans,[§] Ian M. Orme,[‡] and Steven G. Reed^{*†}

Key Ags of *Mycobacterium tuberculosis* initially identified in the context of host responses in healthy purified protein derivative-positive donors and infected C57BL/6 mice were prioritized for the development of a subunit vaccine against tuberculosis. Our lead construct, Mtb72F, codes for a 72-kDa polyprotein genetically linked in tandem in the linear order Mtb32_C-Mtb39-Mtb32_N. Immunization of C57BL/6 mice with Mtb72F DNA resulted in the generation of IFN- γ responses directed against the first two components of the polyprotein and a strong CD8⁺ T cell response directed exclusively against Mtb32_C. In contrast, immunization of mice with Mtb72F protein formulated in the adjuvant AS02A resulted in the elicitation of a moderate IFN- γ response and a weak CD8⁺ T cell response to Mtb32_C. However, immunization with a formulation of Mtb72F protein in AS01B adjuvant generated a comprehensive and robust immune response, resulting in the elicitation of strong IFN- γ and Ab responses encompassing all three components of the polyprotein vaccine and a strong CD8⁺ response directed against the same Mtb32_C epitope identified by DNA immunization. All three forms of Mtb72F immunization resulted in the protection of C57BL/6 mice against aerosol challenge with a virulent strain of *M. tuberculosis*. Most importantly, immunization of guinea pigs with Mtb72F, delivered either as DNA or as a rAg-based vaccine, resulted in prolonged survival (>1 year) after aerosol challenge with virulent *M. tuberculosis* comparable to bacillus Calmette-Guérin immunization. Mtb72F in AS02A formulation is currently in phase I clinical trial, making it the first recombinant tuberculosis vaccine to be tested in humans. *The Journal of Immunology*, 2004, 172: 7618–7628.

Globally, approximately two billion people are infected with *Mycobacterium tuberculosis*, the causative agent of tuberculosis (TB),⁴ and an estimated three million deaths due to this disease occur annually (1, 2). Although combination chemotherapy is generally effective in the treatment of TB, the treatment is arduous and requires stringent compliance to avoid the development of multidrug resistant strains of *M. tuberculosis* (3, 4).

With respect to prophylaxis against TB, the attenuated strain of *Mycobacterium bovis*, bacillus Calmette-Guérin (BCG), is currently the only available vaccine (5, 6). However, the prophylactic use of BCG has demonstrated varying levels of efficacy in different clinical trials and geographically distinct populations (6, 7). It has been proposed that the variable efficacy in BCG vaccine trials may be due to interference caused by previous exposure to environmental mycobacteria (8, 9). A further deficiency of BCG is that it

can cause disseminated disease in immunocompromised individuals (10, 11). Thus, whereas BCG has a protective effect in children, particularly against forms such as meningeal TB, it does not consistently prevent the development of pulmonary TB in adults. Consequently, there is need for the development of more effective vaccines against TB.

M. tuberculosis is an intracellular pathogen, and, as such, cell-mediated immunity plays a key role in the control of bacterial propagation and subsequent protection against TB. In animal studies, acquired resistance against TB is mediated by sensitized T lymphocytes, in particular, IFN- γ -secreting CD4⁺ T lymphocytes are critical in mediating protection against TB in the murine model of this disease (12–16). The central role of IFN- γ in the control of TB 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 (10, 11, 17–19). In addition, MHC class I-restricted CD8⁺ T cells may be required for resistance to *M. tuberculosis* as an alternative source of IFN- γ . Recent studies have suggested that whereas CD8⁺ T cells do not appear to be critical during acute TB, they may play an important role in preventing the reactivation of latent TB infection (10, 11, 17–20).

The identification of mycobacterial Ags that preferentially activate T cells to proliferate and secrete IFN- γ by both CD4 and CD8 T cells, is critical to the development of subunit vaccines against TB. We have identified several *M. tuberculosis* Ags in the context of controlled infection in humans and C57BL/6 mice characterized by their ability to elicit T cell and Ab responses (21–28). Of these, two proteins, Mtb32 (generated as overlapping amino and carboxyl fragments) and Mtb39 (Rv0125 and Rv1196, respectively) (23,

*Corixa Corp. and [†]Infectious Disease Research Institute, Seattle, WA 98104; [‡]Colorado State University, Fort Collins, CO 80523; and [§]GlaxoSmithKline Biologicals, Rixensart, Belgium

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² Address correspondence and reprint requests to Dr. Yasir A. W. Skeiky at the current address: Aeras Global TB Vaccine Foundation, 7500 Old Georgetown Road, Suite 800, Bethesda, MD 20814. E-mail address: yskeiky@aeras.org

³ Current address: The Forsyth Institute, Boston, MA 02115.

⁴ Abbreviations used in this paper: TB, tuberculosis; BCG, bacillus Calmette-Guérin; 3D-MPL, 3-deacylated monophosphoryl lipid A; IPTG, isopropyl- β -D-thiogalactoside; ORF, open reading frame; PPD, purified protein derivative.

26), were developed further, and their open reading frames (ORFs) were expressed as a single recombinant polyprotein with a predicted size of 72 kDa (Mtb72F). We report in this study that immunization of C57BL/6 mice with Mtb72F DNA or recombinant protein formulated in two different adjuvant systems, AS01B and AS02A, resulted in the elicitation of differential immune responses (both qualitative and quantitative) to the components of Mtb72F, with the AS01B formulation eliciting the broadest range of immune response. Despite these immunogenicity differences, all three forms of immunization protected mice against TB infection. Most significantly, immunization of guinea pigs with Mtb72F, delivered either as DNA or as a rAg-based vaccine, prolonged the survival of the animals after aerosol challenge with virulent *M. tuberculosis*.

Materials and Methods

Generation of a tandemly linked ORF encoding Mtb72F

Mtb72F was generated by the sequential linkage in tandem of the ORFs of the ~14-kDa C-terminal fragment of mtb32 (26) (residues 192–323; 132 aa) to the full-length ORF of mtb39 (23), followed at the C terminus with the ~20-kDa N-terminal portion (residues 1–195) of mtb32. These two genes correspond to the ORFs Rv0125 and Rv1196, respectively, as defined in the TubercuList H37Rv database (<http://genolist.pasteur.fr/TubercuList/>). This was accomplished using sequence-specific oligonucleotides containing unique restriction sites (*EcoRI* and *EcoRV*) and devoid of the authentic stop codons at the C-terminal ends (in the case of Mtb32-C and Mtb-39) by PCR using genomic DNA from the *M. tuberculosis* strain H37Rv as template. The details of the process were as follows.

Generation of Mtb32c construct devoid of a stop codon

The 5' and 3' oligonucleotides to the C-terminal portion of mtb32 (mtb32c) were designed as follows: 5' (5'-CAA TTA CAT ATG CAT CAC CAT CAC CAT CAC ACG GCC GCG TCC GAT AAC TTC-3') and 3' (5'-CTA ATC GAA TCC GGC GCG GGG TCC CTC GGC CAA-3'). The 5' oligonucleotide contains an *NdeI* restriction site (underlined) preceding an ATG initiation codon, followed by nucleotide sequences encoding six histidine residues (italics) and sequences derived from the first seven amino acid residues of Mtb32c (bold). The 3' oligonucleotide contains an *EcoRI* restriction site (underlined), followed immediately by sequences comprising the last seven amino acid residues (bold) and devoid of the termination codon. These oligos were used to amplify Mtb32c, the carboxyl 396-nt portion (aa residues 192–323; a 14-kDa 132 aa residues) of Mtb32 and the resulting PCR-amplified product digested with *NdeI* and *EcoRI*, followed by subcloning into the pET117b expression vector similarly digested with *NdeI* and *EcoRI*. Ligated products were then transformed into *Escherichia coli*, and transformants with the correct insert were identified by restriction digest and verified by DNA sequencing. The mtb32c-pET plasmid was subsequently linearized by digestion with *EcoRI* and *EcoRV*. The latter cuts within the polylinker sequence of the pET vector that is located downstream of the *EcoRI* site.

PCR amplification of the full-length coding sequences of Mtb39 and subcloning into the Mtb32c-pET plasmid

The 5' and 3' oligos of Mtb39 contain the following sequences: 5' (5'-CTA ATC GAA TTC ATG GTG GAT TTC GGG GCG TTA-3') and 3' (5'-CTA ATC GAT ATC GCC GGC TGC CGG AGA ATG CGG-3'). The 5' oligonucleotide contains an *EcoRI* restriction site (underlined) preceding the first seven amino acid residues of Mtb39 (bold). The 3' oligonucleotide contains an *EcoRV* restriction site (underlined), followed immediately by sequences comprising the last seven amino acid residues (bold) and devoid of the termination codon. These were used to amplify the full-length coding sequence of Mtb39 (1173 bp; a 391-aa stretch with a predicted size of ~39 kDa), and the resulting PCR-amplified product was digested with *EcoRI* and *EcoRV*, followed by subcloning in-frame with the predigested Mtb32c-pET plasmid. The ligated products were then transformed into *E. coli*, and transformants with the correct insert were identified by restriction digest and verified by DNA sequencing. For expression of the recombinant Mtb32c-Mtb39, the pET plasmid construct was transformed into the bacterial host (BL-21; pLysE), and expression of the protein resulted in a single recombinant.

Cloning of the N-terminal 195-aa sequence of mtb32 into the mtb32c-mtb39 pET construct

The 5' and 3' oligonucleotides of the N-terminal fragment of Mtb32 were designed as follows: 5' (5'-CTA ATC GAT ATC GCC CCG CCG GCC TTG TCG CAG GAC-3') and 3' (5'-CTA ATC GAT ATC CTA GGA CGC GGC CGT GTT CAT AC-3'). Both sets of oligonucleotides contain an *EcoRV* restriction site (underlined) preceding the first eight amino acid residues of Mtb32 (bold) and immediately following the sequences upstream of the stop codon (italics). The 3' oligonucleotide also includes sequences comprising the last 20 nt (bold) of Mtb32n. They were designed to amplify the N-terminal 585-bp (195-aa residue) portion of Mtb32. The resulting PCR-amplified product was digested with *EcoRV*, followed by subcloning into the Mtb32c-Mtb39 fusion pET plasmid (similarly digested with *EcoRV*). Ligated products were then transformed into *E. coli*, and transformants with the correct insert and orientation were identified by restriction digest and verified by DNA sequencing. The final construct, a 72-kDa polyprotein (Mtb72F), comprises a single ORF organized in the linear order, Mtb32_C-Mtb39-Mtb32_N.

Recombinant protein expression and purification of Mtb72F

The recombinant (His-Tag) Ag was purified from the insoluble inclusion body of 500 ml of isopropyl- β -D-thiogalactoside (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. Briefly, 20 ml of an overnight-saturated culture of BL21 containing the pET construct was added to 500 ml of 2 \times YT medium containing 50 μ g/ml ampicillin and 34 μ g/ml chloramphenicol and grown at 37°C with shaking. The bacterial cultures were induced with 2 mM IPTG at an OD 560 of 0.3 and grown for an additional 3 h (OD, 1.3–1.9). Cells were harvested from 500-ml batch cultures by centrifugation and resuspended in 20 ml of binding buffer (0.1 M sodium phosphate (pH 8.0) and 10 mM Tris-HCl (pH 8.0)) containing 2 mM PMSF and 20 μ g/ml leupeptin. *E. coli* was lysed by adding 15 mg of lysozyme and rocking for 30 min at 4°C after sonication (four times, 30 s each time), then spun at 12,000 rpm for 30 min to pellet the inclusion bodies.

The inclusion bodies were washed three times in 1% 3-[(3-cholamidopropyl)dimethylammonio]-1-propanesulfonate in 10 mM Tris-HCl (pH 8.0). This step greatly reduced the level of contaminating LPS. The inclusion body was finally solubilized in 20 ml of binding buffer containing 8 M urea, or 8 M urea was added directly to the soluble supernatant. Recombinant Ags with His-Tag residues were batch-bound to Ni-NTA-agarose resin (5 ml of resin/500 ml of inductions) by rocking at room temperature for 1 h, and the complex was passed over a column. The flow through was passed twice over the same column and the column washed three times with 30 ml each of wash buffer (0.1 M sodium phosphate and 10 mM Tris-HCl (pH 6.3)) also containing 8 M urea. Bound protein was eluted with 30 ml of 100 mM imidazole in wash buffer, and 5-ml fractions were collected. Fractions containing the rAg were pooled, dialyzed against 10 mM Tris-HCl (pH 8.0), bound one more time to the Ni-NTA matrix, eluted, and dialyzed in 10 mM Tris-HCl (pH 7.8). The yield of purified recombinant protein varied from 50–75 mg/l induced bacterial culture with >98% purity. Endotoxin levels were typically <10 endotoxin U/mg protein (i.e., <1 ng of LPS/mg).

Mice, immunizations, and cytokine assays

Female C57/BL6 mice were obtained from Charles River and age-matched (4–6 wk) within each experiment. Mice were immunized three times (3 wk apart) with 8 μ g of rMtb72F formulated with the adjuvants AS02A or AS01B or with a 100- μ g dose of Mtb72F-DNA. For protein formulations, the required immunization dose of rMtb72F (typically 8 μ g) was brought up to either 50 or 43 μ l with 1 \times PBS (pH 6.8) and mixed with 50 μ l of AS01B or 57 μ l of AS02A, respectively. Mice were injected with a total volume of 100 μ l/mouse via the i.m. (tibialis) route with 50 μ l/leg. Three weeks after the last boost, animals designated for immunogenicity studies were killed, and spleen cells were obtained by conventional procedures. Mononuclear cells were cultured at 37°C in 5% CO₂ in the presence of either medium (containing 10% FBS, 50 μ M 2 β -ME, and 50 μ g/ml gentamicin), or medium plus rAg. For cytokine analysis, spleen cells were plated in duplicate 96-well tissue culture plates at 2.5 \times 10⁵ cells/well and cultured with or without Ags for 72 h. Supernatants were harvested and analyzed for IFN- γ by a double-sandwich ELISA using specific mAb (BD PharMingen, San Diego, CA) as previously described (22, 27).

ELISPOT assay

An ELISPOT assay was used to determine the relative number of IFN- γ -expressing cells in the single-cell spleen suspensions. A MultiScreen 96-well filtration plate (Millipore, Bedford, MA) was coated with 10 $\mu\text{g/ml}$ rat anti-mouse IFN- γ capture Ab (BD PharMingen) and incubated overnight at 4°C. Plates were washed with PBS, blocked with RPMI 1640 and 10% FBS for at least 1 h at room temperature, and washed again. Spleen cells were plated in duplicate at 1×10^5 cells/well in 100 μl and stimulated with the specific rAg at a 10 $\mu\text{g/ml}$ dose mixed with 0.2 ng/ml IL-2 for 48 h at 37°C. The plates were subsequently washed with PBS and 0.1% Tween and incubated overnight at 4°C with a biotin-conjugated, rat anti-mouse IFN- γ secondary Ab (BD PharMingen) at 5 $\mu\text{g/ml}$ in PBS, 0.5% BSA, and 0.1% Tween. The filters were developed using the Vectastain ABC avidin peroxidase conjugate and Vectastain AEC substrate kits (Vector Laboratories, Burlingame, CA) according to the manufacturer's protocol. The reaction was stopped by washing the plates with deionized water, plates were dried in the dark, and spots were counted.

IgG isotype ELISA

Mice were bled 3 wk after the last immunization, and sera were stored at -20°C until use. The specific serum IgG isotype Ab response was measured by conventional ELISA. Recombinant Ags were coated onto 96-well Immulon-4 ELISA plates at a concentration of 100 ng/well and incubated overnight at 4°C. The plates were blocked with 200 $\mu\text{l/well}$ PBS containing 0.05% Tween 20 and 1% BSA for 2 h at room temperature and washed with PBS-0.05% Tween, followed by the addition of 50 $\mu\text{l/well}$ of diluent (PBS-0.05% Tween and 0.1% BSA). Sera were added at serial 2-fold dilutions (beginning at a 1/200 dilution) incubated in a humidified box on a rocking platform for 2 h at room temperature and washed (as described above), followed by the addition of 100 $\mu\text{l/well}$ of biotinylated isotype-specific secondary Abs (rat anti-mouse IgG1 or IgG2a; Southern Biotechnology Associates, Birmingham, CA) diluted at 1/4000 in PBST-0.1% BSA. Plates were incubated for 1 h at room temperature, washed, and developed with for ~5 min with 100 $\mu\text{l/well}$ of tetramethylbenzidine peroxidase substrate (Kirkegaard & Perry Laboratories, Keene, NH) mixed 1/1 with peroxidase solution B. Reactions were stopped by the addition of 50 $\mu\text{l/well}$ of 1 N H₂SO₄ and were read on an ELISA plate reader (Dynatech Laboratories, Chantilly, VA) at 450 nm with 570 nm as the reference wavelength.

DNA vaccine and retroviral constructs

The full-length coding sequence of Mtb72F was PCR amplified from the protein expression vector (pET-72F) using primer-specific pairs. Except for the N-terminal six histidine residues, the sequence of the entire ORFs of Mtb72F was identical in the protein and DNA constructs. The 5' primer was designed to contain a *Hind*III recognition site and a Kozak sequence upstream of the initiator ATG codon. The resultant PCR product was digested subcloned into the eukaryotic expression vector pJA4304 (gift from J. I. Mullins and J. Arthurs, University of Washington School of Medicine, Seattle, WA).

In addition, the three subcomponents of Mtb72F (mtb32-C, Mtb39, and Mtb32-N) were 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 sequences of all three genes were obtained by PCR amplification using 5' oligonucleotides designed with the initiating methionine. The 3' oligonucleotide included the stop codon. 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 mtb32-C, mtb39, and Mtb32-N, essentially as previously described (27). Briefly, the retroviral constructs (described above) were used in transfections of Phoenix-Ampho, an amphotropic retroviral packaging line. Approximately 48 h post-transfection, supernatants containing recombinant virus were harvested and used to transduce EL-4 cells. Transduction efficiency was measured by flow cytometry using EL-4 transduced with pBIB-enhanced green fluorescent protein viral supernatants as a positive control. All transductants were selected with blastocidin-S (Calbiochem, San Diego, CA) at a concentration of 10 $\mu\text{g/ml}$. These cells were then used as targets in standard ⁵¹Cr release CTL assays. For analysis of effector CTL, spleen cells from immunized mice were cultured in 24-well Costar (Coming Glass, Corning, NY) tissue culture plates at a density of 5×10^6 /well and stimulated with 2.5×10^5 /well of EL-4 cells expressing Mtb-specific Ags in complete me-

dium for 6 days, harvested, and tested in a standard 5-h ⁵¹Cr release assay as previously described (27).

The Mtb32c-specific CTL line was generated from spleen cells from mice that were immunized with Mtb72F DNA and restimulated in vitro with EL4 cells transduced with mtb32c. The CTL line was maintained by weekly stimulation of 4×10^5 CTL with 2×10^5 irradiated EL4 transductants and 2.5×10^6 irradiated syngeneic spleen cells in 2 ml of medium containing 1 ng/ml IL-2 in 24-well plates.

CTL epitope mapping and MHC restriction analysis

Peptides used in this study were synthesized using a Symphony system (Rainin Instrument, Woburn, MA) according to the guidelines of the manufacturer. P815 (H-2^d) cells and H-2D^b and H-2K^b transfectants of P815 were gifts from M. Bevan (University of Washington, Seattle, WA). Either EL4 (H-2^b) or P815 (parental or H-2D^b or H-2K^b transfectant) stimulator cells were pulsed with peptides at various concentrations for 1.5 h at 37°C and then incubated with Mtb32C-specific CTL. After 48 h, culture supernatants were assayed for IFN- γ by ELISA.

Protection experiment

Mice were immunized i.m., three times, 3 wk apart, with 8.0 μg of the rAg formulated in the indicated adjuvant or 100 μg of plasmid DNA containing the gene of interest. 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, adjuvant alone, or DNA vector. Thirty days after the last immunization, mice were challenged by low dose aerosol exposure with *M. tuberculosis* H37Rv strain (ATCC 35718; American Type Culture Collection, Manassas, VA) using a Glas-Col (Terre Haute, IN) aerosol generator calibrated to deliver 50–100 bacteria into the lungs. Four weeks later, mice were euthanized, and lung and spleen homogenates were prepared in PBS/Tween 80 (0.05%). Bacterial counts were determined by plating serial dilutions of individual whole organs on nutrient Middlebrook 7H11 Bacto Agar (BD Biosciences, Cockeysville, MD) and counting bacterial colony formation after 21-day incubation at 37°C in humidified air.

Guinea pigs were similarly immunized via the i.m. route, three times, 3 wk apart, with either a 200- μg dose of Mtb72F-DNA (brought up to 250 μl with $1 \times$ PBS, pH 7.0) or a 20- μg immunization dose of rMtb72F (or a mixture of the three components on a molar basis) formulated in AS02A in a final volume of 250 μl . The protocol for rMtb72F formulation in AS02A (Ag:adjuvant) was identical with those described for the mouse studies, but was adjusted for a 20- μg immunization dose in a final volume of 250 μl . Animals were immunized with 125 μl of the final formulation per leg. BCG (a single dose of 10^3 CFU) was used as the positive control and administered via the intradermal route. Negative control groups include adjuvant and saline alone groups. Thirteen weeks after the third immunization, the animals were challenged with the virulent H37Rv strain via the aerosol route by calibrating the nebulizer compartment of the Middlebrook airborne-infection apparatus (using the same Glas-Col aerosol generator used in the mouse experiments) to deliver ~20–50 bacteria into the lungs. All challenge studies reported in this paper were performed in the same laboratory. Animals were killed at the indicated time. At necropsy, all lung lobes were removed from the thorax individually to enable separate manipulations with each lobe. The number of viable bacteria in the lungs was determined by plating serial 10-fold dilutions of right cranial lung lobe homogenates onto nutrient Middlebrook 7H11 agar, and bacterial colony formations were counted after 21 days of incubation at 37°C under 5% CO₂. Data are expressed as log₁₀ of the mean number of bacteria recovered.

Results

Generation, expression, and purification of recombinant Mtb72F

Two Mtb Ags, Mtb32 and Mtb39 (Rv0125 and Rv1196, respectively), previously identified by serological and T cell expression cloning and demonstrated to stimulate CD4 and CD8 responses in PBMC of healthy, purified protein derivative-positive (PPD⁺) donors and in infected or immunized mice (23, 27), were selected for the development of a TB vaccine. An initial, straightforward approach was to link in tandem the ORFs of both genes such that the final construct would result in the generation of a single rAg comprising both Ags. However, attempts to express and purify Mtb32 in *E. coli* (either as the full-length or mature form) or as a fusion with Mtb39 (at either the N or C end) were unsuccessful. This was

attributed to the inherent serine protease activity of Mtb32 probably leading to toxicity of the expressing host cell (26). In contrast, it was relatively easy to express and purify high levels of two separate fragments of Mtb32 (N- and C-terminal portions). Therefore, we reasoned that it should be feasible to generate a chimeric construct by fusing at either end of Mtb39, the N- and C-terminal portions of Mtb32 (designated Mtb32_N and Mtb32_C). Our initial experience with the expression level and purification profile of the C-terminal portion of Mtb32 (Mtb32_C) revealed that when expressed in *E. coli*, the recombinant protein partitions into the soluble fraction. In addition, Mtb32_C has been demonstrated to drive the high level expression of heterologous proteins in *E. coli* when fused to their N-terminal end (29). We therefore engineered for expression in *E. coli* a polyprotein construct organized in the linear order Mtb32_C-Mtb39-Mtb32_N coding for an ORF with a predicted molecular mass of 72 kDa (Mtb72F).

Fig. 1A is a diagrammatic representation of Mtb72F showing the physical organization and the restriction enzyme sites used to link the three subunits. The length of the predicted ORF of Mtb72F is 2187 nt, coding for a 729-aa polypeptide with a predicted molecular mass of ~72 kDa, an isoelectric point of 4.98, and a net charge of -15.88 at pH 7.0. Because of the way the construct was generated, the addition of the hinge sequences (*EcoRI* and *EcoRV*) resulted in the introduction of six nucleotides (two amino acid

residues) at each of the junction sites. In addition, the construct was designed such that the N terminus contained six histidine residues for ease of purification by affinity chromatography over Ni-NTA matrix. After expression in *E. coli*, the recombinant protein was purified from inclusion bodies with yields ranging from 50–75 mg of purified protein/l induced culture. Fig. 1B shows a Coomassie Blue-stained SDS-PAGE gel of the *E. coli* culture before and after induction along with the final purified recombinant Mtb72F (migrating at its predicted size of ~72 kDa).

Immune responses in mice immunized with Mtb72F DNA

To investigate the specificity and breadth of the immune response to Mtb72F in C57BL/6 mice, we initially used the naked DNA approach because this form of immunization is known to stimulate both CD4⁺ and CD8⁺ T cell responses. The Mtb72F ORF was subcloned into the eukaryotic expression vector pJA4304, which is under control of the CMV promoter. Plasmid DNA was prepared with >95% migrating as a supercoiled form, as visualized by ethidium bromide staining (not shown). Mice (typically eight to 10/group) were immunized with three doses of 100 μg of Mtb72F-DNA via the i.m. route at 3-wk intervals. Three weeks after the third immunization, three mice from each group were killed for evaluation of anti-Mtb72F Ab and T cell responses (CD4⁺ and CD8⁺). IgG1 and IgG2a Ab responses were evaluated by ELISA

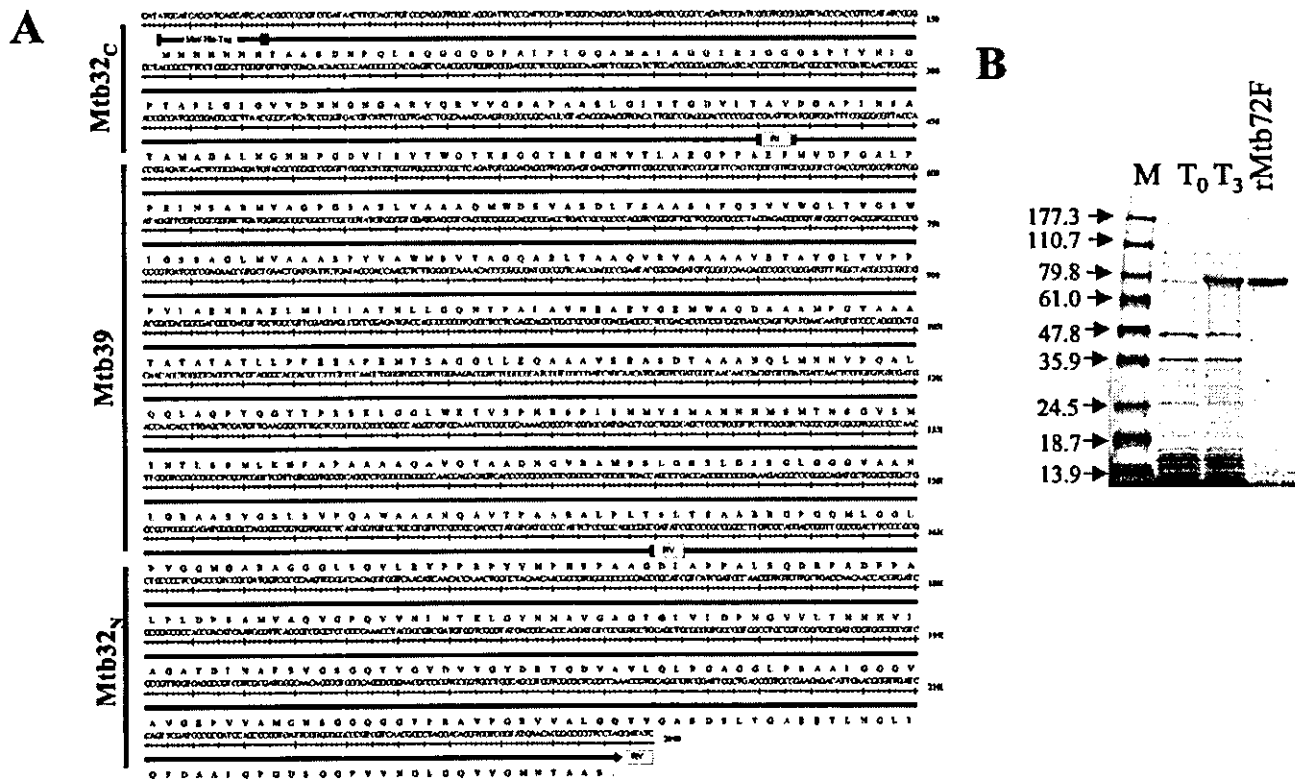


FIGURE 1. A, Generation of recombinant Mtb72F. Mtb72F was generated by the sequential linkage in tandem of the ORFs beginning at the amino end with Mtb32c linked to the full-length ORF of Mtb39 and terminating with sequences comprising Mtb32_N. The final construct comprises a single polyprotein in the linear order Mtb32_C-Mtb39-Mtb32_N. The size of the predicted ORF of Mtb72F is 2187 nt and codes for a 729-aa polypeptide with a predicted molecular mass of ~72 kDa. The figure depicts a diagrammatic representation of Mtb72F, showing the physical organization of the three components along with the hinge sequences (*EcoRI* and *EcoRV*), resulting in the generation of two amino acid residues each at the joining junction. B, Expression and purification of recombinant Mtb72F. *E. coli* (BL-21/pLysE) transformed with the expression vector harboring Mtb72F was grown and induced with IPTG. The figure is a Coomassie Blue-stained 12% SDS-PAGE of the *E. coli* lysates before (lane T₀) and 3 h after (lane T₃) induction with IPTG. The resulting protein, Mtb72F (rAg), was purified from the inclusion fraction by affinity chromatography over Ni-NTA agarose matrix (lane 3). The faint and lower bands were verified by both N-terminal sequencing and immunoblot analysis to represent proteolytic breakdown products of the full-length Mtb72F. Molecular mass markers (lane M; in kilodaltons) are indicated on the left.

using specific mouse IgG isotype antibodies. The results revealed that mice immunized with DNA developed an Mtb72F Ab response of the IgG2a, but not the IgG1, subclass, and the response was exclusively directed against the N-terminal (Mtb32_C) portion of the molecule (Fig. 2A). No Mtb72F-specific Ab was detected in sera of mice immunized with the empty vector or injected with saline (data not shown).

CD4⁺ T cell responses were evaluated by stimulating spleen cells in vitro with either the full-length rMtb72F protein or with each of the three separate protein components, rMtb32_C, rMtb39, and rMtb32_N, at 10, 2, and 0.4 μg/ml Ag. Supernatants were harvested and assayed for IFN-γ at 72 h poststimulation. The results revealed that immunization of C57BL/6 mice with Mtb72F DNA (but not the vector or saline control groups) induced the production of high amounts of IFN-γ (>20 ng/ml) after in vitro stimulation with rMtb72F (Fig. 2B). Stimulation of the same splenocyte culture with each of the three components of Mtb72F revealed that the IFN-γ response is elicited predominantly by sequences comprising the first two components (Mtb32_C and Mtb39) of the polyprotein construct, with little or no response directed against the C-terminal portion (Mtb32_N) of the molecule. Finally, PPD (at a 10 μg/ml dose) also stimulated the splenocyte cultures from Mtb72F DNA-immunized mice to produce IFN-γ.

Because DNA immunization has been shown to be an effective method for the induction of a CD8⁺ T cell response, we evaluated

whether immunization with Mtb72F DNA could result in the generation of CTL responses. Splenocytes from immunized mice were stimulated for 6 days with EL4 cells that had been transduced with Mtb32_C, Mtb32_N, or Mtb39. The cells were subsequently washed and evaluated for cytotoxicity against specific targets or EL-4 cells transduced with enhanced green fluorescent protein as a negative control. Fig. 2C shows that immunization of mice with Mtb72F-DNA induced the generation of Mtb72F-specific CTL, and the CTL response was directed exclusively against the N-terminal portion (Mtb32_C) of the polyprotein. The lysis of Mtb32_C targets was specific to immunized mice, because no lysis was observed in control groups immunized with either saline or the empty vector (data not shown).

Mapping of the CD8 T cell epitope of Mtb32C

We next analyzed the CTL response to Mtb32_C in more detail. First, we generated an Mtb32_C-specific CTL line from spleen cells of mice immunized with Mtb32_C DNA and stimulated in vitro with EL-4 cells retrovirally transduced with Mtb32_C. Epitope-mapping studies were performed by stimulation of the Mtb32_C-specific CTL with 23 peptides (15-mer overlapping by 10) spanning the entire length of Mtb32_C (Fig. 3A). This analysis revealed that the Mtb32_C-specific CTL made a strong IFN-γ response to peptide 18 (residues 92-DGAPINSATAMADAL-106) even at the low stimulation dose of 1 ng/ml (Fig. 3A). The adjacent sequence (peptide

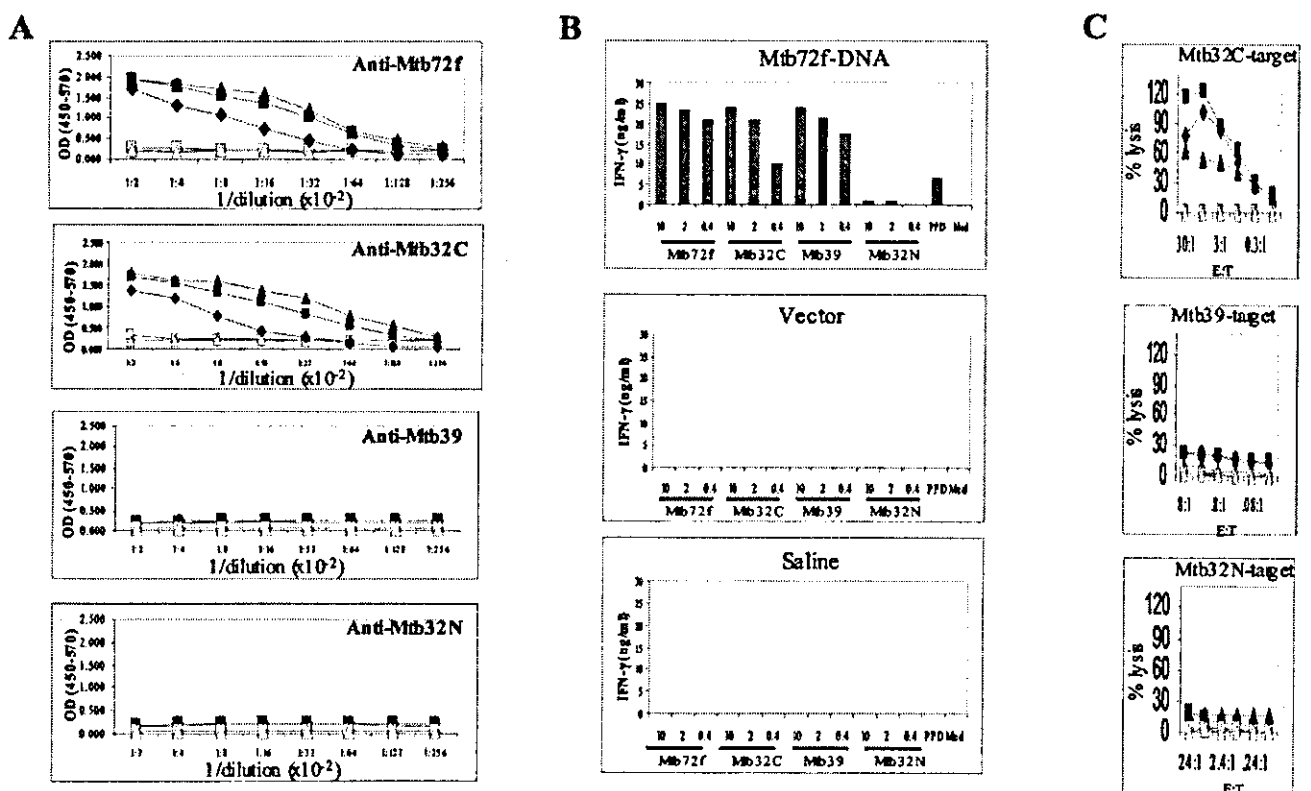


FIGURE 2. Immune responses in C57BL/6 mice immunized with Mtb72F-DNA. *A*, IgG1 and IgG2a Ab responses. Mice were immunized three times i.m. with 100 μg/dose of either Mtb72F-DNA or the empty vector or saline control (not shown). Three weeks after the third immunization, the animals were bled, and sera were tested for anti-Mtb72F and for each of the three components for Ab responses of both IgG1 (□ and △) and IgG2a (■, ▲, and ◆) isotypes by ELISA. *B* and *C*, For IFN-γ responses (*B*), spleen cells were obtained from the same mice and stimulated in vitro for 3 days with a 5-fold serial dilution (10, 2, and 0.4 μg) of either rMtb72F or each of the three components (Mtb32_C, Mtb39, and Mtb32_N) as indicated. As controls, splenocyte cultures were also stimulated with either PPD (10 μg/ml) or medium alone. IFN-γ production was subsequently measured by ELISA. For evaluation of CTL activity (*C*), the mononuclear spleen cells were stimulated for 6 days with Mtb32_C-, Mtb39-, or Mtb32_N-transfected EL-4 cell targets. Effector cells were washed and tested for cytotoxicity in a 4-h ⁵¹Cr release assay against both control transduced EL-4 cells (EGFP; □ and △), and Mtb-transduced EL-4 cells (■, ▲, and ◆). Results are expressed as the percent specific cytotoxicity against the respective transduced targets. The curves express the results obtained for each individual mouse. These data are representative of five experiments.

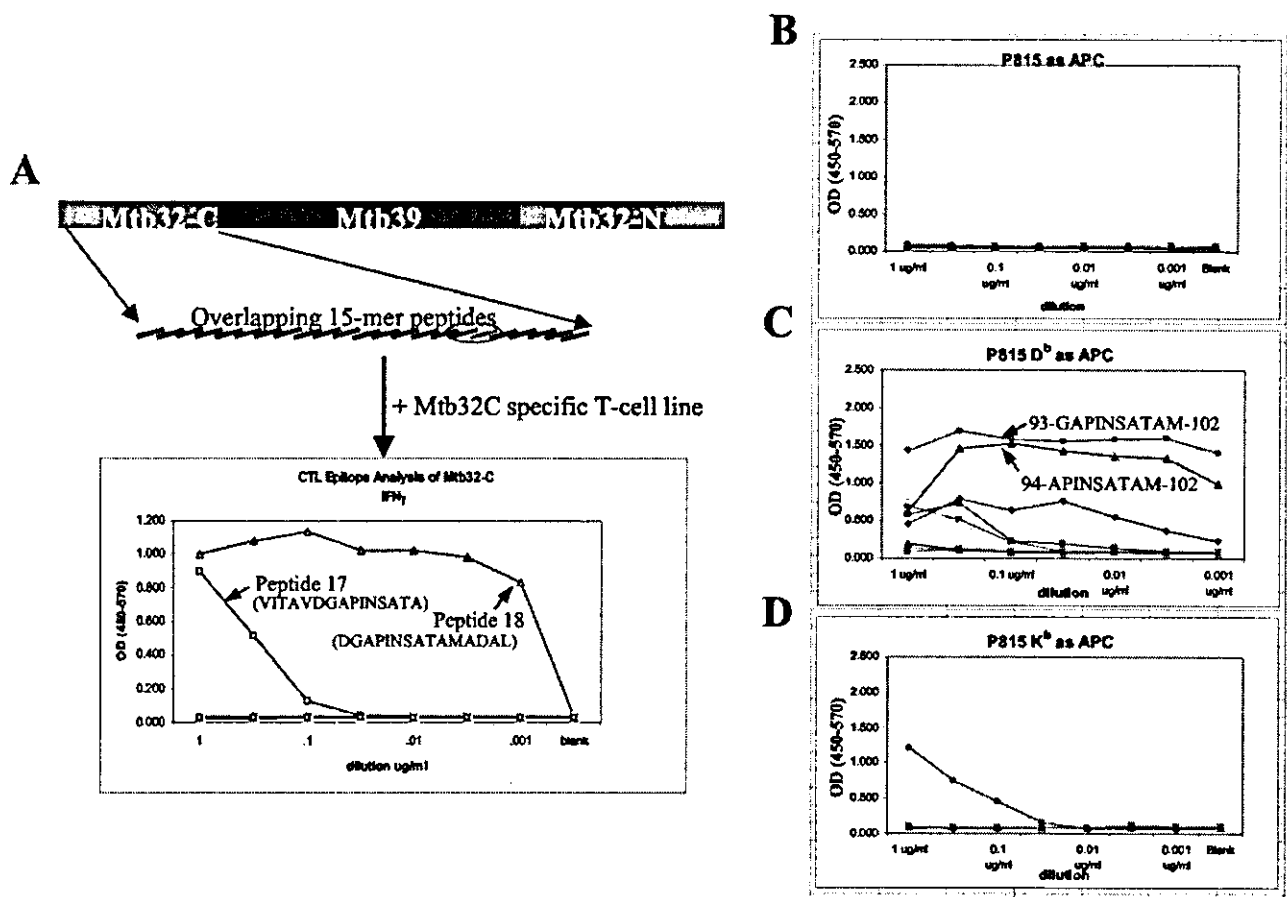


FIGURE 3. Mapping of the CTL epitope of Mtb32C and MHC restriction analysis. *A*, Stimulation of Mtb32_C-specific CTL with overlapping 15-mer peptides corresponding to the sequence of Mtb32. EL4 cells were pulsed with the peptides at the indicated concentrations for 1.5 h at 37°C and then incubated with Mtb32C-specific CTL. After 48 h, culture supernatants were assayed for IFN- γ by ELISA. *B*, Stimulation of Mtb32C-specific CTL with peptides derived from the peptide sequence DGAPINSATAM derived in *A*. P815 cells or P815 cells transfected with either D^b or K^b gene K41 cells were pulsed with the indicated peptides at the indicated concentrations and then incubated with Mtb32C-specific CTL. After 48-h culture, supernatants were assayed for IFN- γ by ELISA.

17, residues 87-VITAVDVGAPINSATA-101) read out at the high dose, but rapidly titrated out at 0.1 μ g/ml. Thus, the sequence of overlap between the two positive peptides was identified as DGAPINSATAM. Given these results, the deduced sequence of the CD8 T cell epitope was further dissected into overlapping 9- and 10-mer peptides. In addition, we wanted to determine which MHC allele was responsible for presentation of the peptide to the Mtb32_C-specific CD8 T cells. Therefore, we used P815 (H-2d-restricted) cells that had been transfected with either H-2Kb or H-2Db as APCs (Fig. 3, *B–D*). The data showed that only the peptide-pulsed H-2Db, not H-2Kb-transduced, P815 cells stimulated strong responses by the Mtb32_C-specific CTL line. The two peptides that stimulated the strongest IFN- γ responses were the 10-mer (93-GAPINSATAM-102) and 9-mer (94-APINSATAM-102) sequences (Fig. 3C). In agreement with these data, both peptides are predicted to bind with high affinity to Db, as determined by class I MHC prediction algorithms.

Immune responses to Mtb72F protein formulated in two adjuvant systems, AS02A and AS01B

The immune response to Mtb72F protein were subsequently evaluated in two adjuvants, AS01B and AS02A (GlaxoSmithKline Biologicals, Rixensart, Belgium). AS01B contains monophosphoryl lipid A (MPL) and QS-21 in a liposomal formulation, whereas

AS02A has the same components formulated in an oil-in-water emulsion. Mice were immunized three times, 3 wk apart, with 8 μ g of rMtb72F formulated in AS01B or AS02A via the i.m. route. Three weeks after the third immunization, three animals from each group were killed, and anti-Mtb72F Ab and T cell responses were evaluated. With regard to Ab responses, mice immunized with rMtb72F formulated in either AS01B or AS02A mounted strong and comparable IgG1 and IgG2a responses against Mtb72F (Fig. 4A) and to each of the three components comprising the construct (data not shown). No Mtb72F-specific responses were detected in the adjuvant-alone control groups.

CD4⁺ T cell responses were evaluated by IFN- γ ELISA of the supernatant culture or by the more sensitive IFN- γ ELISPOT assay. Immunization of mice with Mtb72F formulated in AS02A resulted in the production of a strong IFN- γ response after in vitro stimulation of splenocyte cultures with rMtb72F or rMtb39, a relatively weaker response to Mtb32_C, and a low to undetectable response to Mtb32_N (Fig. 4B). Immunization of mice with rMtb72F formulated in AS01B stimulated a robust production of IFN- γ (5- to 10-fold higher) against all three components of the polyprotein. Of particular interest, the adjuvant AS01B induced a strong IFN- γ response specific for the Mtb32_N portion of the molecule, a response not readily observed after immunization with

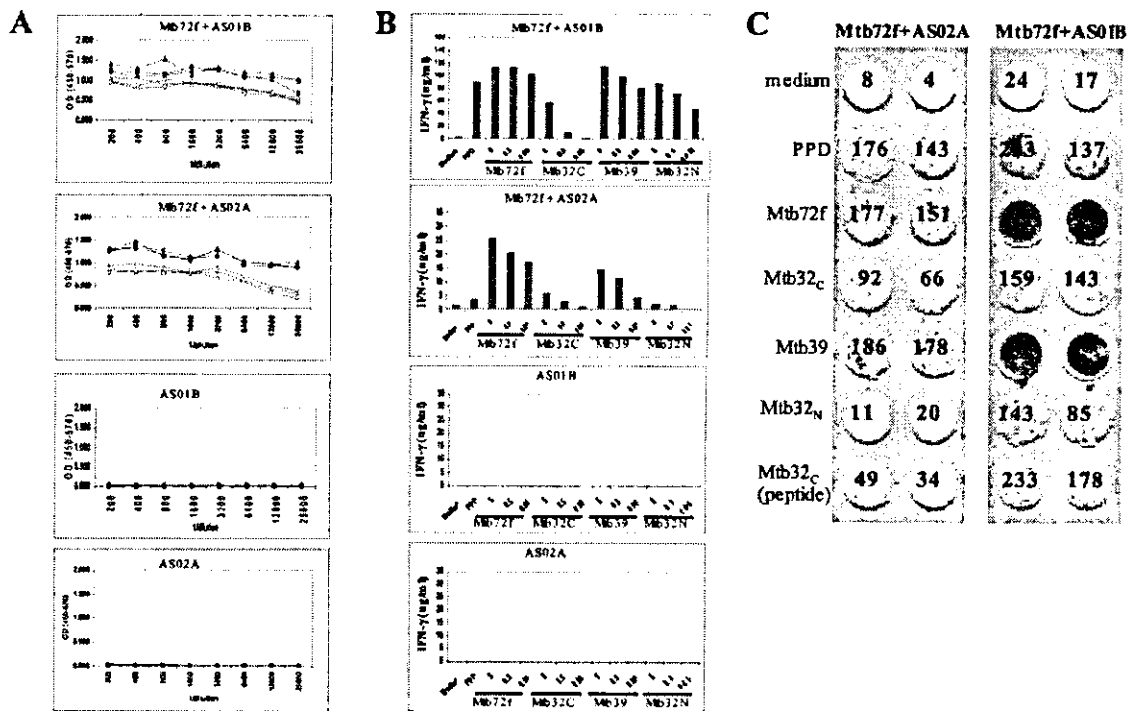


FIGURE 4. Immune responses in C57BL/6 mice immunized with rMtb72F formulated in either AS02A or AS01B adjuvants. C57BL/6 mice were injected three times (at 3-wk intervals) i.m. with 8 μ g of rMtb72F formulated in either AS02A or AS01B. Control groups include animals immunized with adjuvant alone or saline. Three weeks after the third immunization the animals were bled and killed. Sera were obtained and tested for specific anti-Mtb72F (and all three components; not shown) Ab response of both IgG1 (■ and ▲) and IgG2a (□ and Δ) isotypes by ELISA (A). For cytokine production (B), mononuclear spleen cells were obtained from mice immunized with rMtb72F formulated in AS01B and AS02A or with the adjuvants alone (control groups) as indicated and stimulated for 3 days with the indicated dose of rMtb72F protein or each of the three components at 5, 0.5, or 0.05 μ g/ml. Control groups were stimulated with either medium alone or PPD (10 μ g/ml). Supernatants were harvested and assayed for the presence of IFN- γ by ELISA. C, ELISPOT assay. An ELISPOT assay was used to determine the relative numbers of IFN- γ -expressing cells in single-cell spleen suspensions of mice immunized with rMtb72F formulated in AS01B or AS02A and stimulated with the indicated rAg or the CD8⁺ 10-mer peptide from Mtb32_C.

rMtb72F formulated in AS02A or with Mtb72F-DNA. Qualitatively, the corresponding IFN- γ ELISPOT is in agreement with the ELISA data, in that AS01B stood out as inducing the broadest and most robust T cell response to all three components of Mtb72F (Fig. 4C).

Formulation of Mtb72F in AS01B elicits a robust CD8⁺ (CTL and IFN- γ) T cell response in C57BL/6 immunized mice

CD8⁺ T cell responses are typically associated with DNA or viral delivery approaches. In contrast, rAg based formulations do not readily induce such responses. Given that the quality and strength of the immune response to rMtb72F were remarkably affected by the adjuvant system (AS01B or AS02A) used in the formulation, we extended our study to include CD8⁺ T cell responses to rMtb72F in immunized C57BL/6 mice. Fig. 5A shows that immunization of C57BL/6 mice with rMtb72F formulated in AS01B resulted in a strong CTL response directed exclusively against Mtb32_C. As with Mtb72F-DNA immunization, this CTL response was directed against the same CD8 10-mer peptide (residues 92-GAPINSATAM-102; data not shown). In contrast, mice immunized with a formulation of Mtb72F in AS02A mounted a relatively weaker CTL response (Fig. 5B). Spleen cells from rMtb72F-immunized mice were also stimulated with the CTL peptide derived above and assayed for IFN- γ by ELISPOT. In agreement with the cytotoxicity data, strong IFN- γ responses to peptide GAPINSATAM were observed in mice immunized with rMtb72F formulated in AS01B (Fig. 4C). Responses in mice immunized with rMtb72F in AS02A were weaker, but stronger than those in the nonstimulated cultures.

Mtb72F protects C57BL/6 mice against M. tuberculosis infection

Given the results of the above immunogenicity experiments, we sought to determine whether the way in which Mtb72F is delivered (naked DNA or recombinant protein formulated in AS01B or AS02A) impacts the outcome of protection against infection of C57BL/6 mice with *M. tuberculosis*. Briefly, C57BL/6 mice were immunized three times i.m. (at 3-wk intervals) with Mtb72F-DNA (100 μ g/dose) or with three doses (8 μ g/dose) of recombinant protein formulated in AS01B or AS02A. As controls, groups of mice were immunized with saline, adjuvant, or BCG. Four weeks after the last immunization, mice were challenged via the aerosol route with ~100 CFU of the virulent *M. tuberculosis* strain H37Rv. Bacteriological burden (CFU) was measured in the lungs of mice at 4 wk postchallenge. The results from three independent challenge experiments revealed that immunization of C57BL/6 mice with Mtb72F-DNA consistently led to a 0.7- to 1.0-log reduction in bacterial burden approaching the protective efficacy observed with BCG (Fig. 6A). We also compared the protective efficacy of rMtb72F formulated in AS02A with the DNA delivery approach in the same experiment (Fig. 6A). Mtb72F in AS02A has been evaluated exhaustively, and in six independent experiments we found that this immunization with this formulation resulted in a 0.4- to 0.6-log reduction of bacterial burden in the lung. This level of protection was comparable to that seen after immunization with a formulation comprising a mixture of the three components of Mtb72F (data not shown). Interestingly, despite the qualitative and quantitative differences observed in the immune responses to

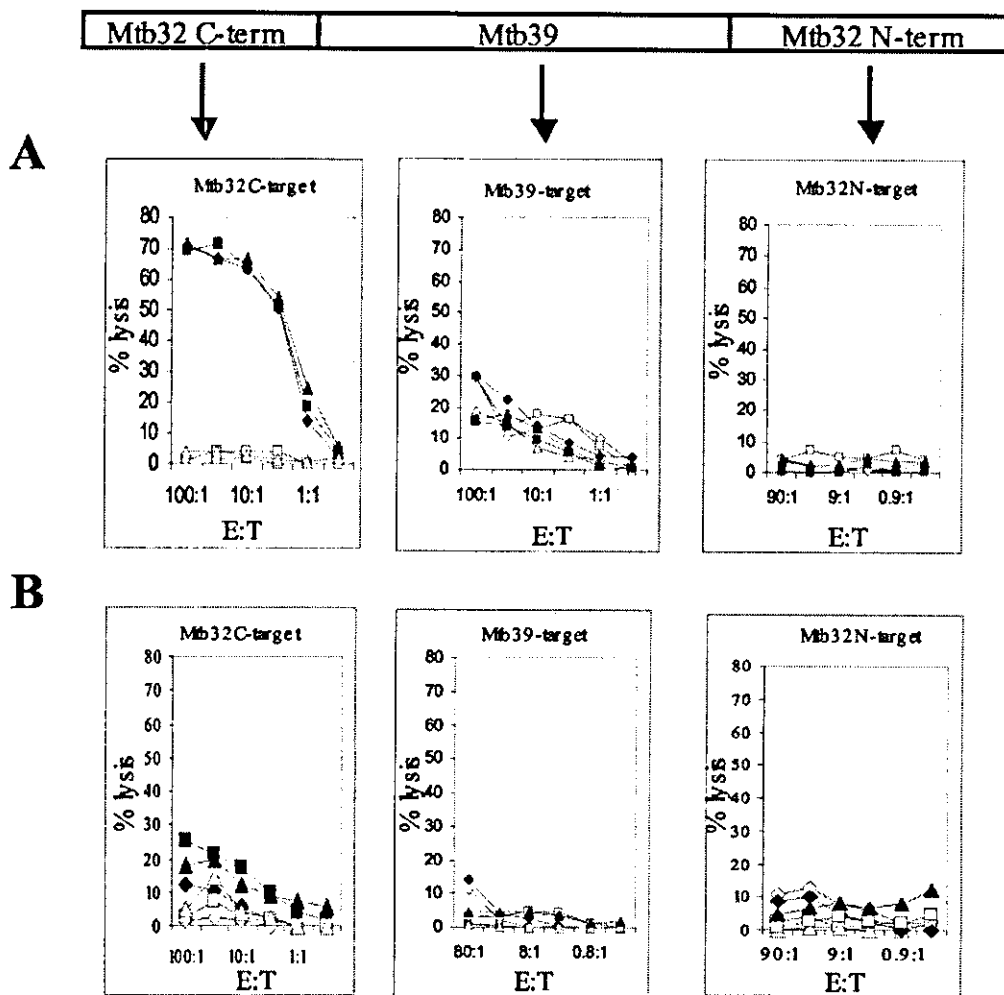


FIGURE 5. CD8⁺ T cell responses to Mtb72F components after immunization of mice with rMtb72F formulated in either AS01B or AS02A. For evaluation of CTL activity, mononuclear spleen cells of mice immunized with rMtb72F formulated in AS01B (*A*) or AS02A (*B*) were stimulated for 6 days with EL4 cells transduced with Mtb32_C, Mtb39, or Mtb32_N. Effector cells were washed and tested for cytotoxicity in a 4-h ⁵¹Cr release assay against both control transduced EL-4 cells (enhanced green fluorescent protein; □, △, and ◇), or against specific target cells (■, ▲, and ◆). Results are expressed as the percent specific cytotoxicity against Mtb32C-transfected cells for each of the three animals per group as indicated.

rMtb72F formulated in AS01B Vs AS02A, immunization of mice with three doses of 8 μg of rMtb72F in either of the two adjuvant systems resulted in a comparable reduction in bacterial burden in the lung (~0.6 log; Fig. 6*B*).

Mtb72F protects guinea pig against aerosol challenge with virulent M. tuberculosis

Having demonstrated that in the mouse model immunization with Mtb72F-DNA or rMtb72F formulated in AS02A protected against TB, we next evaluated whether these vaccination approaches would also protect against TB infection in the guinea pig model. Groups of five guinea pigs were immunized with three doses each of 200 μg of Mtb72F-DNA or 20 μg of rMtb72F formulated in AS02A. Control groups were immunized with AS02A adjuvant alone or saline via the i.m. route or with a standard dose of 10³ CFU BCG administered via the i.d. route. Thirteen weeks after the third immunization, the animals were aerosol-challenged with 20–50 CFU of the virulent Mtb strain H37Rv. Protection was monitored by outward signs of infection (difficulty in breathing and weight loss), with survival as an end point. One of the five animals in the BCG control group died at ~7 wk postchallenge, but of causes not related to TB infection. Therefore, in the case of

the BCG control group, the percent survival was based on *n* = 4. The results from this study revealed that at 30 wk postchallenge (Fig. 7) although all animals in the saline and adjuvant control groups were moribund and had to be euthanized, three of four (75%) of the guinea pigs immunized with BCG, four of five (80%) of those immunized with rMtb72F, and three of five (60%) of the animals immunized with Mtb72F-DNA were still alive. At 40 wk (~3 mo after all animals in the control groups succumbed), three of five (60%) of the animals in each of the Mtb72F-vaccinated groups were still alive. By 70 wk (~15 mo) postchallenge, two of five (40%) animals in the DNA-immunized groups and one of four (25%) animals in the BCG group were still alive. Taken together, the results revealed that in the guinea pig model, Mtb72F, delivered either as naked DNA or recombinant protein formulated in AS02A, protected guinea pigs against virulent TB challenge to an extent comparable to that seen with BCG and for periods lasting >1 year.

Discussion

The development of an efficacious subunit-based recombinant vaccine against TB would require a multivalent mixture of Ags for a broad coverage of a heterogeneous MHC population. Despite the fact that a single Ag could by itself induce protection in inbred

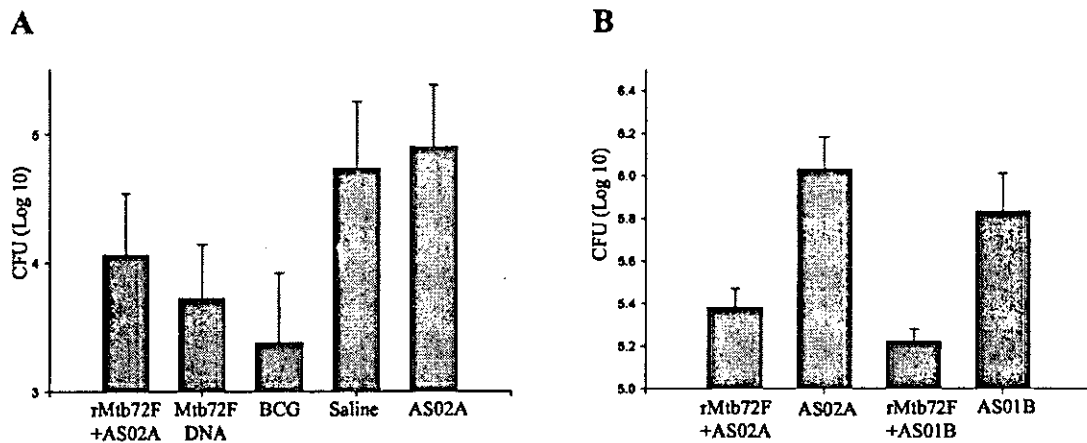


FIGURE 6. Mtb72F protects C57BL/6 mice against aerosol challenge with Mtb. C57BL/6 mice (five animals per group) were injected three times i.m. with 100 μ g of Mtb72F-DNA or with 8 μ g of rMtb72F formulated in AS02A (A) or AS02A vs AS01B (B). Control groups were injected once (s.c.) with 5×10^4 CFU of BCG (Aventis Pasteur, Lyon, France) or with saline or adjuvant alone. One month after the last immunization, the animals were challenged with 100 viable aerosolized *M. tuberculosis*, and CFU in the lungs were enumerated 3 wk later.

strains of mice, a mixture comprising several Ags is conceivably a better vaccine for applications in humans because it is less likely to suffer from MHC-related unresponsiveness in a heterogeneous population. In fact, using *in vitro* responses (proliferative and IFN- γ production) of PBMC from healthy PPD⁺ donors as a measure of the extent of coverage of infected responders to a panel of defined TB Ags, a broad-range coverage could only be accomplished with Ag combinations. Therefore, although the murine model is an important first step to determine the nature of the immune responses as well as the protective capacity of Ags, this model does not necessarily predict the outcome in the context of human MHC restriction.

Although the nature of an effective immune response to TB is incompletely understood, particularly in humans, the most effective vaccination strategies in animal models are those that stimulate T cell responses, both CD4⁺ and CD8⁺, to produce Th1-associated cytokines. Therefore, formulations that induce the production of enduring Th1 responses are desirable and probably are an essential element of a successful vaccine. Of about a dozen T cell Ags initially identified in the context of host response to infection in infected donors and C57BL/6 mice (21–28), we prioritized Mtb32 (Rv0125) and Mtb39 (Rv1196) as our lead candidates for the development of a TB vaccine. Their potential was further corroborated in animal protection studies suggesting that the combination of Ags was more effective at protecting mice and guinea pigs than the individual subunits (not shown). However, from a practical standpoint for the development of a TB vaccine for developing countries, a vaccine consisting of multiple recombinant proteins would be too expensive to manufacture and formulate. For this reason, together with the difficulties encountered in the expression of a stable form of the full-length secreted version of Mtb32, we engineered for expression in *E. coli* a genetic fusion construct encoding a 72-kDa polyprotein in a contiguous ORF organized in the linear order Mtb32_C-Mtb39-Mtb32_N (Mtb72F). In addition to simplifying the manufacturing process, we reasoned that immunization with a single construct may ensure equivalent uptake of the components by APCs and, in turn, generate an immune response that is broadly specific.

Because genetic delivery approaches of vaccine candidates are efficient methods for the elicitation of both CD4⁺ and CD8⁺ T cell responses and, as well, an effective vaccination protocol for conferring protection against several infectious disease targets, we

used the naked DNA approach to initially determine the nature of the immune responses and the protective efficacy of Mtb72F in C57BL/6 mice. Indeed, we found that immunization of C57BL/6 mice with Mtb72F-DNA elicited both CD4⁺ and CD8⁺ IFN- γ responses. The CD4⁺ T cell response was predominantly directed against Mtb39, whereas the CD8⁺ T cell response (as assessed by both IFN- γ and CTL) was directed against a single epitope within Mtb32_C. We next evaluated the immune responses and protective efficacy of rMtb72F formulated in two novel adjuvant systems, AS01B and AS02A (30–34).

The active ingredients of both adjuvants are 3-deacylated MPL (3D-MPL) (35–38), a nontoxic derivative of LPS and QS21 (a triterpene glycoside purified from the bark of *Quillaja saponaria*, (39–41), and both components have a good clinical safety record (31, 32, 42, 43). The biological properties of MPL are attributed to its immunostimulatory effects on the innate immune system (via activation of the Toll-like receptor 4) and the direct activation of

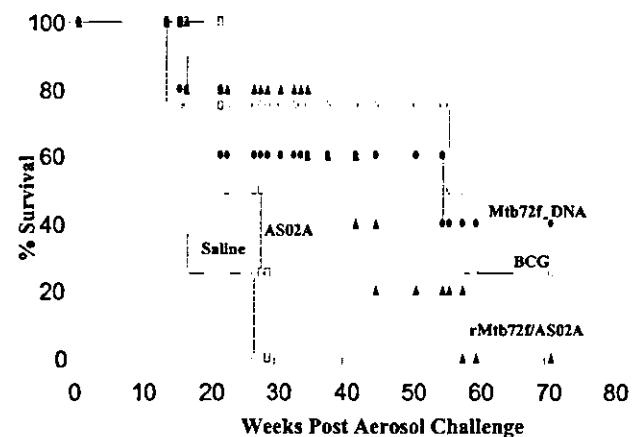


FIGURE 7. Mtb72F protects in the guinea pig model of TB. Guinea pigs were immunized three times i.m. with 20 μ g of rMtb72F formulated in AS02A or 200 μ g of Mtb72F-DNA. Control groups include BCG, saline, and AS02A. Approximately 13 wk after the last immunization, animals were challenged with a virulent strain of Mtb via the aerosol route. Protection was assessed by weight loss and survival.

APCs resulting in enhanced phagocytosis and microbicidal activities as a consequence of the production of IL-12, TNF- α , GM-CSF, and IFN- γ (36, 38, 44, 45). The immunostimulant activity of QS21 extract resides in the saponic fraction and acylation appears to be critical for adjuvant activity (46). QS21 promotes both humoral and cell-mediated immunity when added to parenteral or mucosal vaccine formulations (46). Analysis of cytokine secretion by Ag-specific T cells demonstrated that QS21 augmented Th1 and Th2 responses, whereas addition of 3D-MPL resulted in preferential induction of type 1 T cells (46–49). The distinction between AS01B and AS02A formulations resides in their liposome or oil-in-water emulsion properties, respectively. Both adjuvants as well as their components are currently under clinical evaluation for various vaccines and has been tested in thousands of patients in several clinical trials, including infectious disease vaccines such as malaria (31, 33), hepatitis B (50, 51), and allergy desensitization (52–54). Furthermore, the use of MPL-stable emulsion as an alternate adjuvant to IL-12, known for its Th1-inducing properties, in conjunction with a polyprotein Ag was recently demonstrated as a safe and effective vaccine against *Leishmania* infection (55).

The immunogenicity studies revealed that immunization of C57BL/6 mice with rMtb72F formulated in AS01B elicited an immune response profile that is stronger and broader than those observed with AS02A formulation or with naked DNA delivery. AS01B stood out at eliciting a robust and comprehensive (CD4⁺, CD8⁺ T cells and Ab) immune response profile encompassing all three components of the vaccine. Therefore, with an appropriate adjuvant formulation, it is, in fact, possible to generate a CD8⁺ T cell response (typically associated with DNA or recombinant viral delivery systems) using a recombinant-based vaccine. In addition, with AS01B, a relatively robust CD4⁺ T cell IFN- γ response was generated against the N-terminal portion of Mtb72F (Mtb32_C). This was not detected after immunization with rMtb72F formulated in AS02A or with naked DNA or a recombinant Mtb72F adenovirus. Given this unique aspect of AS01B, experiments are currently underway to determine the strength of AS01B by varying the Ag dose and coupling the immunological readout with protection against TB challenge.

There is a general consensus that the guinea pig is currently the best animal model of human TB, and for this reason, it has been widely used as a model to evaluate new vaccines and vaccine delivery approaches. The central reason for this assumption is the fact that guinea pigs develop granulomas similar to those seen in humans with active TB. Guinea pigs can be used for both short term protection studies (CFU) or as longer term disease models (56–59). As lung tissue necrosis progresses, guinea pigs, like humans, begin to undergo weight loss and eventually die from TB. Successful immunization leads to reduced necrosis, with small lesions characterized by infiltrating lymphocytes, decreased weight loss, and prolonged survival (56, 60). In this model, BCG protects guinea pigs from disease and death for periods of >1 year compared with nonvaccinated animals (56, 58, 60, 61). Thus, more recent and rigorous vaccine evaluation in guinea pigs has focused on using disease and survival as end points instead of only short term bacterial counts, which are not always predictive of long term disease outcome. Of importance to the development of Mtb72F as a vaccine against TB, we report that in this model the delivery of Mtb72F as recombinant protein or DNA protected guinea pigs from death against infection with TB. The extent of protection with Mtb72F represents the longest documented survival end point reported to date for any defined subunit vaccine. In addition, given that the animals in this study were aerosol-challenged with TB at ~13 wk after the third immunization (instead of the standard 4–6 wk after the third immunization), the protective outcome confirms

that the immune responses elicited by Mtb72F, delivered as either rAg formulated in AS02A or naked DNA, are long lasting. The protection with Mtb72F DNA immunization was comparable to that with BCG, whereas the efficacy of rMtb72F formulated in AS02A approached that of BCG. At the time these experiments were initiated, the adjuvant AS01B was not available for evaluation. Based upon more recent data obtained with AS01B, experiments are currently underway in the guinea pig model aimed at comparing the protective efficacy of rMtb72F formulated in either AS01B or AS02A.

Even though DNA and viral delivery approaches are good and effective first methods for the screening of vaccine candidates, particularly in small animal models, the apparent ineffectiveness of most DNA vaccines in humans and nonhuman primates and, from a regulatory standpoint, the lack of a sufficient safety record on DNA vaccines make the recombinant subunit-plus-adjuvant approach more desirable. Further, there is sufficient proof of concept for the latter approach given that it has already been demonstrated to be safe and efficacious in several human clinical trials. Therefore, the use of AS01B and AS02A in conjunction with the feasibility of manufacturing Mtb72F polyprotein under GMP conditions represent major developments toward the realization of an affordable and safe vaccine against TB. The rMtb72F formulated in AS02A adjuvant is currently in phase I clinical trial in the U.S., making it the first recombinant TB protein vaccine ever to be tested in humans.

Acknowledgments

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Identification of Murine H2-D^d- and H2-A^b-Restricted T-Cell Epitopes on a Novel Protective Antigen, MPT51, of *Mycobacterium tuberculosis*

Mina Suzuki, Taiki Aoshi, Toshi Nagata, and Yukio Koide*

Department of Microbiology and Immunology, Hamamatsu University School of Medicine, Hamamatsu 431-3192, Japan

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Both CD4⁺ type 1 helper T (Th1) cells and CD8⁺ cytotoxic T lymphocytes (CTL) play pivotal roles in protection against *Mycobacterium tuberculosis* infection. Here, we identified Th1 and CTL epitopes on a novel protective antigen, MPT51, in BALB/c and C57BL/6 mice. Mice were immunized with plasmid DNA encoding MPT51 by using a gene gun, and gamma interferon (IFN- γ) production from the immune spleen cells was analyzed in response to a synthetic overlapping peptide library covering the mature MPT51 sequence. In BALB/c mice, only one peptide, p21-40, appeared to stimulate the immune splenocytes to produce IFN- γ . Flow cytometric analysis with intracellular IFN- γ and the T-cell phenotype revealed that the p21-40 peptide contains an immunodominant CD8⁺ T-cell epitope. Further analysis with a computer-assisted algorithm permitted identification of a T-cell epitope, p24-32. In addition, a major histocompatibility complex class I stabilization assay with TAP2-deficient RMA-S cells transfected with K^d, D^d, or L^d indicated that the epitope is presented by D^d. Finally, we proved that the p24-32/D^d complex is recognized by IFN- γ -producing CTL. In C57BL/6 mice, we observed H2-A^b-restricted dominant and subdominant Th1 epitopes by using T-cell subset depletion analysis and three-color flow cytometry. The data obtained are useful for analyzing the role of MPT51-specific T cells in protective immunity and for designing a vaccine against *M. tuberculosis* infection.

The world-wide problem of tuberculosis (TB) is increasing due to several factors, including multi-drug-resistant strains and coinfection with human immunodeficiency virus (32). An attenuated strain of *Mycobacterium bovis* BCG is the only currently available anti-TB vaccine, yet its efficacy, particularly its efficacy against pulmonary TB in adults, is controversial (42). It is evident that there is an urgent need for a new and more reliable anti-TB vaccine (23).

Although the mechanisms of protection against TB have not been completely determined, cell-mediated immunity plays a pivotal role in the control of *Mycobacterium tuberculosis* infection. There is mounting evidence that CD4⁺ type 1 helper T (Th1) cells are involved in the development of resistance to the disease, primarily through the production of macrophage-activating cytokines, such as gamma interferon (IFN- γ) (22). In addition, CD8⁺ cytotoxic T lymphocytes (CTL) contribute to disease resistance since susceptibility to *M. tuberculosis* is greater in mice deficient in CD8⁺ T cells (21, 41). In fact, mice with a β 2-microglobulin deficiency are susceptible to *M. tuberculosis* infection (13, 36, 40). However, these mice are more susceptible than mice deficient in major histocompatibility complex (MHC) class Ia molecules, perhaps due to derangement of β 2-microglobulin-dependent systems other than the MHC class Ia system, such as the iron uptake system of the host macrophages that depends on β 2-microglobulin (38). Human CD8⁺ CTL recognize mycobacterial cell wall-derived lipid and glycolipid antigens in the context of group 1 CD1 molecules (CD1a, -b, and -c) and β 2-microglobulin-dependent MHC class Ib molecules expressed almost exclusively on den-

dritic cells (34), but the in vivo function of these cells remains unknown.

To design a new generation of vaccines, more information on the antigenic makeup of *M. tuberculosis* must be obtained in order to identify immunodominant proteins and epitopes. Secreted and surface-exposed cell wall proteins seem to play a pivotal role in the induction of protective cellular immunity against TB (31). The mouse model of TB infection has revealed that memory T cells from immune mice produce a substantial amount of IFN- γ in response to two fractions of *M. tuberculosis* culture filtrate represented by 6- to 10-kDa proteins and the antigen 85 (Ag85) complex, a family of 30- to 32-kDa proteins (2). The Ag85 complex (Ag85A, Ag85B, and Ag85C), which exhibits mycolyltransferase activity in cell wall synthesis and in the biogenesis of cord factor (5) and is able to bind fibronectin (1), is known to be a major fraction of the secreted proteins of *M. tuberculosis* (46). In fact, the Ag85 complex has been reported to induce Th1 cells and CTL in healthy individuals exposed to *M. tuberculosis* and in *M. bovis* BCG-infected mice (16, 24, 28, 39). Furthermore, vaccination of mice with plasmid DNA encoding Ag85A and Ag85B can induce strong cellular immune responses and confer protection against a challenge with *M. tuberculosis* (3, 17, 19, 25). Recently, another major secreted protein, designated MPT/MPB51 (MPT is the designation of a protein isolated from *M. tuberculosis*, and MPB indicates a protein isolated from *M. bovis* BCG), was found to cross-react with the three components of the Ag85 complex (29) and to exhibit primary structure similarity (38 to 43%) to these components (30). The homology between MPT51 and the Ag85 complex is significantly lower than the homology within Ag85 complex. The Ag85A/Ag85B/Ag85C/MPT51 ratio in *M. tuberculosis* culture filtrate is 2/3/1/2. The physiological role of MPT51 remains elusive, although the Ag85 complex has been shown to be

* Corresponding author. Mailing address: Department of Microbiology and Immunology, Hamamatsu University School of Medicine, 1-20-1 Handa-yama, Hamamatsu 431-3192, Japan. Phone: 81-53-435-2334. Fax: 81-53-435-2335. E-mail: koidclb@hama-med.ac.jp.

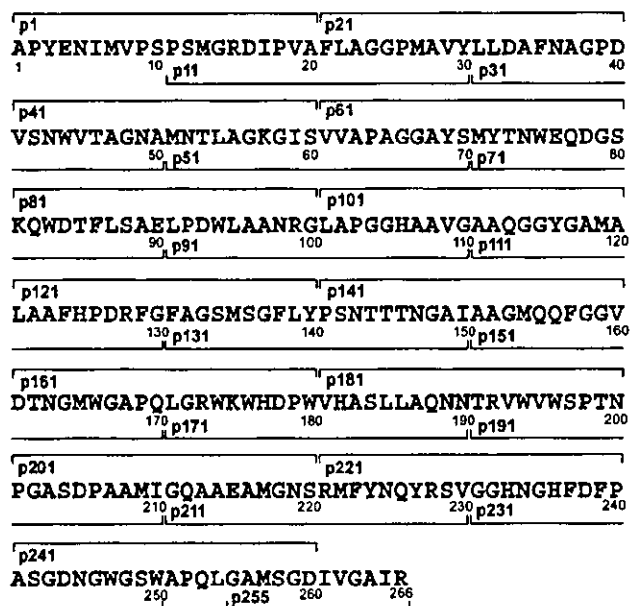


FIG. 1. Schematic representation of the 26 overlapping synthetic peptides from MPT51 of *M. tuberculosis*. All peptides were synthesized as 20-mer molecules overlapping by 10 aa with the neighboring peptides, with exception of a carboxyl-terminal 12-mer.

involved in mycolic acid metabolism, as described above. By using DNA vaccine encoding MPT51, it has been found that MPT51 can induce cellular immune responses and protective immunity upon challenge with *M. tuberculosis* (27).

Here, we identified one H2-D^d-restricted CD8⁺ T-cell epitope in BALB/c mice and two H2-A^b-restricted Th1 epitopes (one dominant epitope and one subdominant epitope) in C57BL/6 (H2^b) mice by employing gene gun immunization with plasmid DNA encoding MPT51, overlapping peptides spanning the entire mature sequence, and a computer-assisted algorithm.

MATERIALS AND METHODS

Mice. C57BL/6 and BALB/c mice (Japan SLC, Hamamatsu, Japan) were kept under specific-pathogen-free conditions and were fed autoclaved food and water ad libitum at the Institute for Experimental Animals of the Hamamatsu University School of Medicine. Two-month-old female mice were used in all experiments. All animal experiments were performed according to the Guidelines for Animal Experimentation, Hamamatsu University School of Medicine.

Construction of a plasmid DNA vaccine, pCI-MPT51. The DNA encoding the mature MPT51 molecule (Rv3803c) was amplified from a plasmid, pMB49 (29), by PCR with following primers: forward primer 5'-CCCTCTAGAAATGGCCCATACGAGAACCTGA-3' and reverse primer 5'-CAGGCTCTAGACATCGGCACTCGGCTAGC-3' (the underlined nucleotides are XbaI sites). The PCR fragment was digested with XbaI and inserted into the XbaI site located downstream of the cytomegalovirus immediate-early enhancer/promoter region of expression plasmid pCI (Promega, Madison, Wis.). The integrity of the nucleotide sequence was validated by automated DNA sequencing (ABI PRISM 310 genetic analyzer, Applied Biosystems, Foster City, Calif.) by using a dye primer cycle sequencing kit (Applied Biosystems).

Peptides. Peptides spanning the entire 266-amino-acid (aa) mature MPT51 sequence of *M. tuberculosis* were synthesized as 20-mers overlapping by 10 residues, except for the carboxyl-terminal 12-mer from aa 255 to 266 (Fig. 1). Lyophilized peptides were purchased from Invitrogen Corporation (Carlsbad, Calif.), and the purity of the peptides was confirmed by mass spectrometry. To identify the potential H2 class I-restricted CD8⁺ T-cell epitopes in the 20-mer

peptides, computer-based programs were used with access through the Bioinformatics & Molecular Analysis Section (BIMAS) HLA Peptide Binding Predictions website (http://bimas.dcrf.nih.gov/cgi-bin/molbio/ken_parker_comboform) (33) and the SYFPEITHI Epitope Prediction website (<http://www.syfpeithi.de/>) (35). All peptides were dissolved in 5% dimethyl sulfoxide in RPMI 1640 medium to a concentration of 1 mM and were stored at -80°C.

Immunization of mice. Mice were immunized with a plasmid DNA vaccine encoding the mature MPT51 molecule by using a gene gun system. For DNA immunization with the Helios gene gun system (Bio-Rad Laboratories, Hercules, Calif.), a cartridge of DNA-coated gold particles was prepared according to the manufacturer's instructions. Finally, 0.5 mg of gold particles was coated with 1 µg of plasmid DNA, and the mice were inoculated twice with 0.5 mg of gold per shot. To immunize mice, the shaved abdominal skin was wiped with 70% ethanol. The spacer of the gene gun was held directly against the abdominal skin. Then the device was discharged at a helium discharge pressure of 400 lb/in². Mice were inoculated with 2 µg of plasmid DNA four times at 1-week intervals. Mice were also vaccinated intravenously with 10⁸ CFU of *M. bovis* BCG (substrain Tokyo) twice with a 2-week interval between the vaccinations.

Cell lines. TAP2-deficient RMA-S cells are T-cell lymphomas derived from the Rauscher murine leukemia virus-induced RBL-5 cell line (20). RMA-S cell lines transfected with the H2-K^d gene (RMA-S-K^d, provided by Mike Bevan, University Washington, Seattle) (7), with the D^d gene (RMA-S-D^d, provided by David H. Raulet, University of California, Berkeley) (9), and with the L^d gene (RMA-S-L^d [R^d-E2]; provided by Keiko Uchida, Kochi Medical School, Kochi, Japan) (45) and the P815 mastocytoma cell line (H2^d) were cultured in RPMI 1640 medium supplemented with 10% heat-inactivated fetal calf serum (RPMI/10FCS) in a humidified atmosphere containing 5% CO₂ in an incubator.

Preparation of splenocyte culture supernatants for evaluation of IFN-γ production. Spleen cell suspensions (1 × 10⁶ cells/well) from mice immunized with a DNA vaccine encoding the MPT51 molecule were cultured in RPMI/10FCS in 96-well plates in the presence of 5 µM peptide at 37°C with 5% CO₂. Supernatants were harvested 24 h later and stored at -20°C until they were assayed for IFN-γ. The IFN-γ concentration was measured by a sandwich enzyme-linked immunosorbent assay (ELISA). In some experiments, RMA-S-K^d, -L^d, or -D^d cells were used as stimulator cells. RMA-S cells (4 × 10⁶ cells/ml) were kept at 26°C overnight. Then they were incubated with 5 µM peptide for 1 h at 26°C, washed three times with RPMI 1640 medium, and resuspended in RPMI/10FCS. Immune splenocytes (1 × 10⁶ cells) were stimulated with 2 × 10⁵ peptide-pulsed RMA-S cells in 200 µl of RPMI/10FCS for 24 h at 37°C, and the IFN-γ concentrations in the supernatants were determined.

Quantification of IFN-γ by a cytokine ELISA. IFN-γ production was measured by an ELISA. The 96-well ELISA plates (E.I.A./R.I.A. Plate A/2; Costar, Cambridge, Mass.) were coated with 2 µg of capture antibody (anti-murine IFN-γ monoclonal antibody [Mab] R4-6A2; BD Pharmingen, San Jose, Calif.) per ml at 4°C overnight, washed with phosphate-buffered saline containing 0.05% Tween 20, and blocked with Block Ace (Dainippon Seiyaku, Tokyo, Japan) at 37°C for 2 h. After washing, the culture supernatant to be tested and a serially diluted IFN-γ standard were added to the plates and incubated at 4°C overnight. After washing, 0.5 µg of biotin-labeled anti-murine IFN-γ Mab XMGI.2 (BD Pharmingen) per ml was added to the plates, and they were incubated at room temperature for 1 h. After the plates were washed, 0.1 µg of horseradish peroxidase-conjugated streptavidin (Vector Laboratories, Inc., Burlingame, Calif.) per ml was added. The plates were then incubated for 30 min at room temperature. After washing, bound horseradish peroxidase-conjugated streptavidin was detected by using 3,3',5,5'-tetramethylbenzene dihydrochloride (Sigma-Aldrich Japan, Tokyo, Japan). The absorbance at 450 nm was determined with an EZS-ABS microplate reader (IWAKI, Tokyo, Japan).

Depletion of CD4 or CD8 T-cell subsets. CD4 or CD8 T-cell subsets of peptide-reactive T cells were determined by depletion studies with anti-murine CD4 Mab GK1.5 or anti-murine CD8α Mab 35-17-2 (provided by Toshitada Takahashi, Aichi Cancer Center, Nagoya, Japan). The MABs were purified from supernatants of the hybridomas by ammonium sulfate precipitation coupled with a PD-10 column (Amersham Biosciences, Tokyo, Japan). The immune spleen cells (1 × 10⁷ cells/ml) were suspended in cytotoxicity buffer (RPMI 1640 medium with 25 mM HEPES buffer and 0.3% bovine serum albumin) containing the anti-CD4 or anti-CD8 MAB and incubated for 1 h at 4°C. Rabbit complement (Cedarlane, Hornby, Canada) was then added to the cell suspension, which was incubated for 1 h at 37°C. The cells were washed with RPMI 1640 medium, suspended in RPMI/10FCS, and used for experiments.

Intracellular IFN-γ staining. An antigen-specific T-cell subset was also examined by simultaneous flow cytometric assessment of the T-cell phenotype and intracellular IFN-γ synthesis. Spleen cells from the immunized mice were treated with ACK lysis buffer for 5 min at room temperature to remove red blood cells

and then washed twice with RPMI 1640 medium and resuspended in RPMI/10FCS at a concentration of 1×10^7 cells/ml. The cells (200 μ l) were incubated for 4 h at 37°C in the presence or absence of 5 μ M synthetic peptide with Golgiplug stock solution (brefeldin A solution; BD Pharmingen) diluted 1:1,000. The cells were then washed twice with fluorescence-activated cell sorting (FACS) buffer (1% fetal calf serum and 0.1% Na₂S₂O₈ in phosphate buffer solution), stained with fluorescein isothiocyanate-conjugated anti-CD8 (53-6.7; BD Pharmingen) and Cy-Chrome-conjugated anti-CD4 (RM4-5; BD Pharmingen) on ice for 30 min, and washed twice, and subsequently intracellular cytokine staining (ICS) was performed by using a Cytotfix/Cytoperm kit (BD Pharmingen) according to the manufacturer's protocol. ICS for IFN- γ was performed with phycoerythrin-conjugated anti-IFN- γ (clone XMG1.2; BD Pharmingen). Cells were washed twice and then resuspended in FACS buffer, and they were analyzed with an EPICS digital flow cytometer (EPICS XL; Beckman Coulter, Miami, Fla.).

MHC stabilization assay. The abilities of peptides to bind to H2-K^d, H2-D^d, and H2-L^d were measured by determining the stabilization of class I molecules on the surfaces of RMA-S-K^d, RMA-S-D^d, and RMA-S-L^d cells, respectively. RMA-S-K^d, RMA-S-D^d, or RMA-S-L^d cells (10^6 cells/well) were cultured at 26°C overnight and then were incubated for 1 h in the presence or absence of peptide (50 or 250 μ M). The cells were then transferred to 37°C for 2 h and washed with FACS buffer, and cell surface expression of H2-K^d, H2-D^d, or H2-L^d molecules was detected by flow cytometry by using mouse MAbs specific for H2-K^d (34-1-25; Cedarlane, Honby, Ontario, Canada) or H2-D^d (34-85; BD Pharmingen), followed by fluorescein isothiocyanate-labeled anti-mouse immunoglobulin antibodies (Gilbertsville, Pa.) and a phycoerythrin-conjugated MAb specific for H2-L^d (28-14-8; eBioscience, San Diego, Calif.). The results were expressed as the mean fluorescence intensity (MFI) ratio, which was determined as follows: MFI ratio = (MFI observed in the presence of peptide at 37°C/MFI observed in the absence of peptide at 26°C - MFI observed in the absence of peptide at 37°C/MFI observed in the absence of peptide at 26°C) \times 100.

CTL assay. One week after the last immunization, immune spleen cells were cocultured in 12-well plates at a density of 2×10^7 cells/well for 5 days with 2×10^7 syngeneic splenocytes per ml; the splenocytes had been treated with 100 μ g of mitomycin C (Kyowa Hakko, Tokyo, Japan) per ml and pulsed with peptide for 2 h at 37°C. Each well also received 10 U of human recombinant interleukin-2 (Hoffmann-La Roche, Nutley, N.J.) per ml. Cell-mediated cytotoxicity was measured by using a conventional ⁵¹Cr release assay, as described previously (44). The target cells used in this study were P815 (H2^d), RMA-S-K^d, RMA-S-D^d, and RMA-S-L^d cells pulsed with peptide at a concentration of 5 μ M for 1.5 h at 37°C. Target cells at a concentration of 10^4 cells/well were incubated for 5 h in duplicate at 37°C with serial dilutions of effector cells, and the level of specific lysis of the target cells was determined by using the following equation: percentage of specific lysis = [(experimental counts per minute - spontaneous counts per minute)/(total counts per minute - spontaneous counts per minute)] \times 100.

RESULTS

IFN- γ production in response to synthetic overlapping peptides from MPT51 in BALB/c and C57BL/6 mice. Splenocytes from BALB/c mice immunized with DNA vaccine encoding mature MPT51 were stimulated with the overlapping peptides for 24 h, and the IFN- γ concentrations in the culture supernatants were determined by ELISA. As shown in Fig. 2A, substantial IFN- γ production was observed after stimulation with peptide 21 (p21) (aa 21 to 40). On the other hand, two peptides, p171 (aa 171 to 190) and p191 (aa 191 to 210), could induce significant IFN- γ production in splenocytes from the immunized C57BL/6 mice, and the former peptide always induced greater IFN- γ production than the latter peptide induced (Fig. 2B). As expected, spleen cells from both BALB/c and C57BL/6 naïve mice showed no significant IFN- γ production in response to any of the peptides in the library (data not shown). In contrast to the robust IFN- γ responses observed in mice vaccinated with DNA, spleen cells from C57BL/6 mice vaccinated with *M. bovis* BCG produced significant levels of IFN- γ only in response to p171 (Fig. 2). This is consistent with the previous observation that vaccination with DNA encoding the Ag85 complex induces a stronger and broader epitope

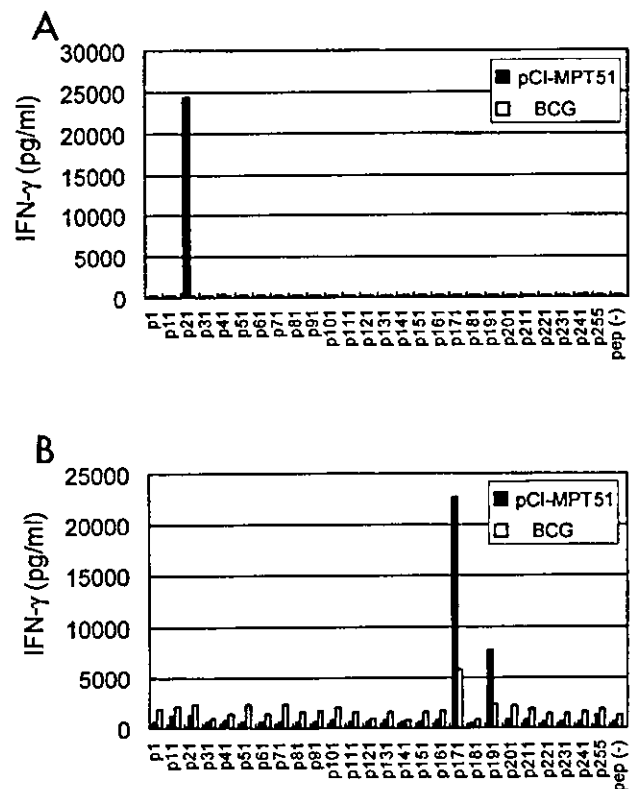


FIG. 2. IFN- γ production by spleen cells from BALB/c (A) or C57BL/6 mice (B) immunized with plasmid DNA encoding MPT51 (solid columns) or *M. bovis* BCG (open columns) in response to 1 of the 26 overlapping peptides (5 μ M) covering the MPT51 molecule or medium alone [pep(-)]. Spleen cells from naïve mice were also examined as controls. The data are representative of three independent experiments.

repertoire than vaccination with BCG or infection with *M. tuberculosis* induces (10, 12).

Identification of a nine-mer CD8⁺ T-cell epitope on p21 of MPT51. Three-color flow cytometric analysis of a T-cell subset and intracellular IFN- γ staining revealed that CD8⁺ T cells, but not CD4⁺ T cells, produced IFN- γ in response to p21 (Fig. 3A). Since the CD8⁺ T-cell epitopes presented by MHC class I molecules comprise 8 to 10 aa (generally 9 aa), we tried to identify the fine CD8⁺ T-cell epitope. Immunodominant epitopes often, but not always, display high-affinity binding for MHC molecules. Therefore, we predicted candidate peptides in the 20-mer peptides by using two computer-based programs, BIMAS HLA Peptide Binding Predictions (http://bimas.dcrf.nih.gov/cgi-bin/molbio/ken_parker_comboform) (33) and SYFPEITHI Epitope Prediction (<http://www.syfpeithi.de/>) (35). We first synthesized three peptides, p22-32, p29-37, and p25-35 (Table 1), since p22-32 and p25-35 contain five H2 class Ia-binding candidate epitopes and two L^d-binding candidate epitopes, respectively, and p29-37 exhibits a high binding score with K^d. Cytometric analysis indicated that p22-32, but not p29-37 or p25-35, could significantly stimulate the immune CD8⁺ T cells to synthesize IFN- γ (Fig. 3A). p25-35 exhibited poor IFN- γ inducer activity in the immune spleen cells, per-

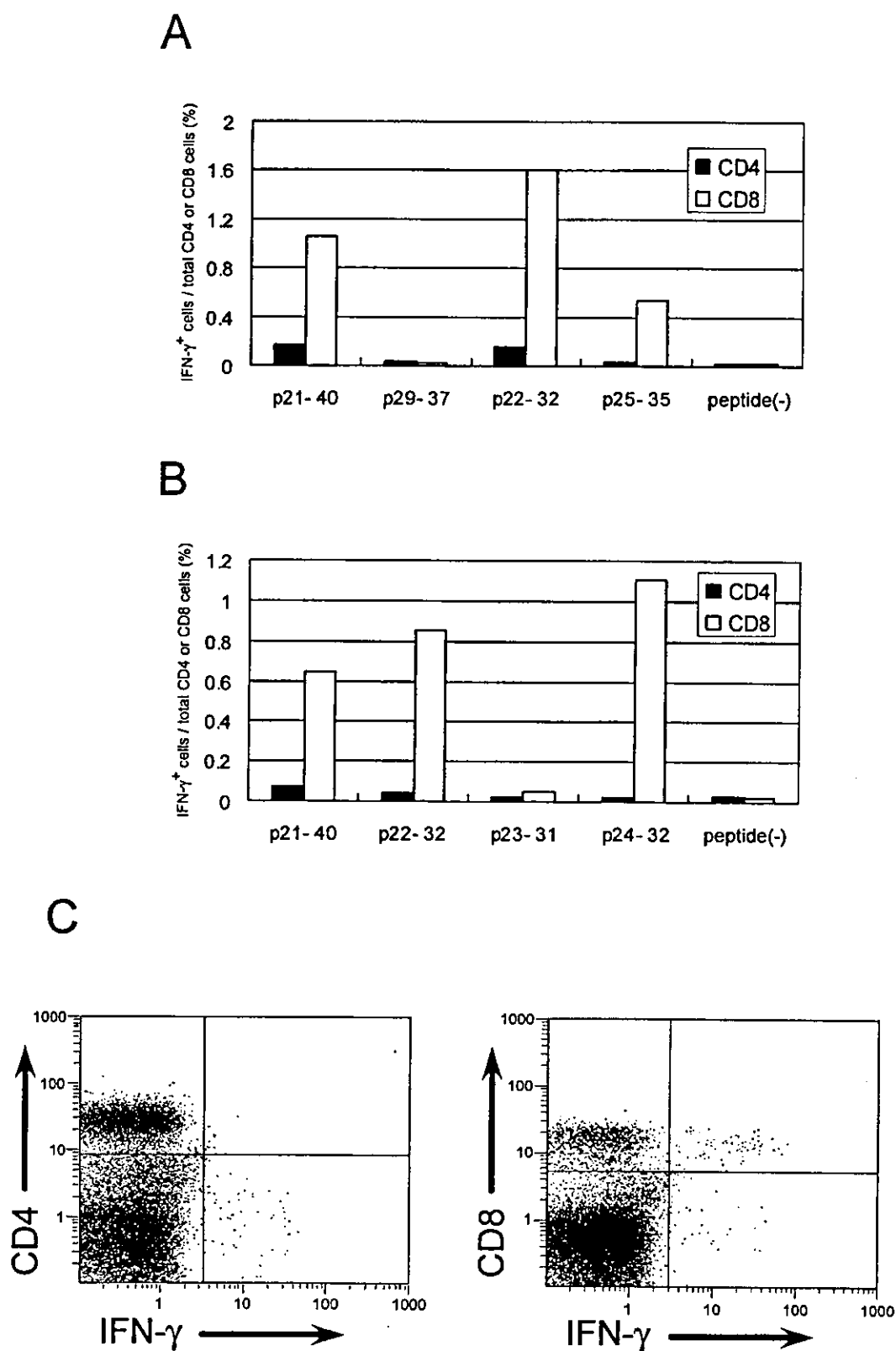


FIG. 3. Identification of a T-cell epitope on the MPT51 p21-40 peptide and a T-cell subset recognizing the epitope in BALB/c mice. Three-color flow cytometric analyses were performed to detect intracellular IFN- γ and T-cell subsets. (A) p22-32, but not p29-37 or p25-35, in the p21-40 peptide contained the CD8⁺ T-cell epitope. The graph shows the percentages of CD4⁺ or CD8⁺ cells producing IFN- γ after 4 h of stimulation with peptides. peptide(-), medium alone. (B) p24-32 nine-mer peptide was a CD8⁺ T-cell epitope on the MPT51 molecule. (C) Intracellular IFN- γ and CD8 were detected by flow cytometry in or on the immune spleen cells in response to the p24-32 peptide (right). There were, however, no intracellular IFN- γ -positive CD4⁺ T cells after *in vitro* stimulation with the p24-32 peptide (left). The results of a representative experiment are shown.