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

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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

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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 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'-CCTCTAGAAATGGCCCAT ACGAGAACCTGA-3' and reverse primer 5'-CAGGCTCTAGACATCGGCA CCTGGCTAGC-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.dcrt.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 10⁶ 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 [RL^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% NaN₃ 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 Cytofix/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^dD^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 (Gilbertville, 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.dcert.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-

A

B

C

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.

TABLE 1. Candidate T-cell epitopes on the p21 peptide of the MPT51 molecule

Peptide	Amino acid sequence ^a	Estimated scores for restriction molecules ^b		
		K ^d	D ^d	L ^d
p21	FLAGGPHAVYLLDAFNAGPD			
p22-32	LAGGPHAVYLL			
p25-32	<u>G</u> PHAVYLL	57.6	—	—
p23-32	AGGPHAVYLL	<u>11</u>	—	—
p22-31	LAGGPHAVYL	40, <u>11</u>	—	—
p23-31	AGGPHAVYL	57, <u>16</u>	20	<u>15</u>
p24-32	GGPHAVYLL	48, <u>12</u>	400	4.5, <u>12</u>
p29-37	<u>VYLLDAFNA</u>	120	—	—
p25-35	GPHAVYLLDAF			
p27-35	<u>H</u> AVYLLDAF	—	—	10
p25-34	<u>G</u> PHAVYLLDA	—	—	30

^a Boldface type indicates peptide sequences that were synthesized and used for experiments. Underlining indicates anchor residues.
^b Scores (binding affinity) were estimated by BIMAS or SYFPEITHI (underlined) epitope prediction (algorithm). —, no binding score.

haps because p22-32 and p25-35 share 8 of 11 aa. Thus, we prepared two typical nine-mer candidate peptides, p23-31 and p24-32, which had higher theoretical binding stability (Table 1). As shown in Fig. 3B, p24-32, but not p23-31, could induce vigorous intracellular IFN- γ synthesis in the immune CD8⁺ T cells, indicating that the p24-32 nine-mer peptide is a bona fide CD8⁺ T-cell epitope on the MPT51 molecule for BALB/c mice. By using intracellular IFN- γ staining we detected the p24-32-specific CD8⁺ T cells in the immune spleen cells (Fig. 3C).

Identification of an MHC class Ia restriction molecule for p24-32. Since p24-32 was found to be a CD8⁺ T-cell epitope for BALB/c mice, we tried to determine which MHC class Ia molecule binds and presents the peptide to CD8⁺ T cells. First, the binding and stabilization of BALB/c MHC class Ia molecules (K^d, D^d, and L^d) by the peptide were investigated by using the TAP2-deficient RMA-S-K^d, RMA-S-D^d, and RMA-S-L^d cell lines. Remarkably, the p24-32 peptide appeared to up-regulate and stabilize D^d molecules on RMA-S-D^d cells, and the other molecules were not stabilized by this peptide (Fig. 4A). As a control, we utilized the p25-35 peptide, which contained two L^d-binding motifs. As expected, this peptide stabilized only L^d molecules on RMA-S-L^d cells (data not shown). Listeriolysin O 91-99 (LLO91-99) is a well-known CTL epitope which binds to K^d molecules. The LLO91-99 peptide stabilized K^d molecules but not D^d or L^d molecules on RMA-S transformants (data not shown).

To gain insight into the functional activity of the p24-32-D^d complex on RMA-S-D^d cells, we examined T-cell responses to the peptide-MHC complex. As shown in Fig. 4B, the peptide-pulsed RMA-S-D^d cells, but not RMA-S-K^d and RMA-S-L^d cells, were capable of stimulating MPT51-immunized splenocytes to secrete IFN- γ . We also examined whether the peptide-MHC complex was recognized by CTL. As shown in Fig. 4C, in vitro-stimulated splenic T cells from BALB/c mice immunized with MPT51 DNA vaccine appeared to lyse the peptide-pulsed RMA-S-D^d and P815 target cells substantially. However, neither peptide-pulsed RMA-S-K^d cells nor peptide-pulsed RMA-S-L^d cells were lysed by the same effector cells. Collec-

FIG. 4. p24-32 peptide binds to H2-D^d and can be recognized by immune T cells in the context of H2-D^d. (A) MHC class I stabilization assay indicated that the p24-32 peptide binds to H2-D^d. The p24-32 peptide was able to stabilize expression of D^d molecules. The MFI ratio was calculated as described in Materials and Methods. (B) p24-32 peptide-pulsed RMA-S-D^d cells, but not RMA-S-K^d and RMA-S-L^d cells, were able to stimulate immune spleen cells to produce IFN- γ . peptide(-), medium alone. (C) p24-32 peptide-pulsed RMA-S-D^d and P815 cells were lysed by spleen cells from MPT51 DNA-vaccinated BALB/c mice. Immune spleen cells (effectors) were incubated with target cells by using the effector/target cell ratios (E/T ratio) indicated on the x axis.

FIG. 5. p171 and p191 are CD4⁺ Th1 epitopes in C57BL/6 mice. (A) Depletion of CD4⁺ T cells with MAb plus complement, but not CD8⁺ T cells, eliminated IFN-γ production in response to the p171 and p191 peptides. (B) Three-color flow cytometric analyses for staining of intracellular IFN-γ and T-cell subsets indicated that CD4⁺ T cells, but not CD8⁺ T cells, synthesized intracellular IFN-γ in response to the p171 and p191 peptides. peptide(-), medium alone.

tively, these data clearly indicate that the p24-32 peptide is an H2-D^d-restricted CD8⁺ T-cell epitope.

Dominant and subdominant Th1 epitopes on MPT51 in C57BL/6 mice. As shown in Fig. 2B, p171 (aa 171 to 190) and p191 (aa 191 to 210) seem to contain T-cell epitopes for C57BL/6 (H-2^b) mice. To determine the T-cell subset responsible for IFN-γ production, T-cell subset depletion and flow cytometry analyses were performed. As shown in Fig. 5A, depletion of CD4⁺ T cells with an MAb and complement almost completely eliminated IFN-γ production in response to both p171 and p191. However, CD8⁺ T-cell depletion had no effect on IFN-γ production, suggesting that both p171 and p191 contain Th1 epitopes. This was confirmed by three-color flow cytometric analysis, which demonstrated that only CD4⁺ T cells were capable of synthesizing intracellular IFN-γ in response to not only p171 but also p191 (Fig. 5B). Since CD4⁺ T-cell epitopes (MHC class II ligands) consist of 12 to 25 aa (35) and their lengths are more heterogeneous than the lengths of CD8⁺ T-cell epitopes (18), the minimal epitopes were not determined.

FIG. 6. p171 and p191 are dominant and subdominant Th1 epitopes in C57BL/6 mice. The immune spleen cells were stimulated with serially twofold diluted p171 and p191 peptides, and the IFN-γ concentrations in the supernatants were determined.

We observed that p171 always induced greater IFN-γ production than p191 induced (Fig. 2 and 5). In attempt to confirm this, the immune splenocytes were stimulated with serially dilutions of both peptides, and the IFN-γ concentrations were measured. As shown in Fig. 6, clearly different levels of IFN-γ production were observed after stimulation with the p171 and p191 peptides at peptide concentrations ranging from 1.53×10^{-9} to 3.81×10^{-10} M. Therefore, we concluded that p171 and p191 are dominant and subdominant Th1 epitopes, respectively.

DISCUSSION

From the data described above, we were able to draw the following conclusions about T-cell epitopes on the mature MPT51 molecule: (i) the p24-32 peptide is an H2-D^d-restricted CD8⁺ T-cell epitope; and (ii) p171-190 and p191-210 contain dominant and subdominant H2-A^b-restricted Th epitopes, respectively.

A greater understanding of the nature of protective immunity to TB would facilitate the development of a future vaccine. The cellular arm of the immune response mediated by Th1 cells has been determined to be a pivotal component of the protective immunity against TB (22). However, there is now mounting evidence from murine models and human studies that CD8⁺ T cells also play a pivotal role in the protection (21, 41). The precise mechanism of MHC class Ia-restricted CD8⁺ T-cell-mediated protection is not known. Mice deficient in perforin, granzyme, or Fas show no more susceptibility to *M. tuberculosis* infection than wild-type mice show (6). IFN-γ secreted by CD8⁺ T cells is probably involved in the protection (43).

The Ag85 complex is known to comprise immunodominant antigens in CD4⁺ and CD8⁺ T-cell responses to *M. tuberculosis* infections in mice and humans. Vaccination with plasmid DNA encoding Ag85A or Ag85B reveals Th1 CD4⁺ T cells and CD8⁺ T cells in mice (25), and these cells confer protection against *M. tuberculosis* challenge (3, 17). Evidence that the Ag85 complex has T-cell epitopes has accumulated. Ag85A

was found to contain several CD4⁺ T-cell epitopes and at least one CD8⁺ T-cell epitope in BALB/c mice (10). In C57BL/6 mice, a cross-reactive T-cell response against two peptides spanning aa 241 to 260 and aa 261 to 280 of Ag85A and Ag85B has been detected (12). Furthermore, aa 240 to 254 of Ag85B has been reported to be an H2-A^b-restricted CD4⁺ T-cell epitope which is recognized by V β 11⁺ T cells (47). T-cell epitope mapping of Ag85A was also performed in humans (24). For example, HLA-A*0201-restricted CD8⁺ T-cell epitopes were identified in Ag85B (14). Another major secreted protein, MPT51, has been reported. The MPT51 molecule exhibits 37 to 43% homology to the mycolyl transferase component of the Ag85 complex (29) and is secreted as much as Ag85A (30). However, it seems unlikely that the MPT51 molecule possesses mycolyl transferase activity since two of three amino acid residues essential for enzymatic mycolyl transferase function (37) were replaced (S126A and H262N). Very recently, we observed that vaccination with plasmid DNA encoding MPT51 is capable of inducing specific protective cellular immunity against TB (27). This observation prompted us to identify T-cell epitopes on the MPT51 molecule.

In BALB/c mice, we identified an H2-D^d-restricted CD8⁺ T-cell epitope, p24-32 (GGPHAVYLL), by using gene gun DNA vaccination and an overlapping peptide library with biometric analysis. DNA vaccination is a powerful tool for identifying T-cell epitopes, as previously reported (10). IFN- γ responses were clearly seen in the spleen cells from mice immunized with plasmid DNA compared to the spleen cells from mice immunized with BCG. In addition, a computer-assisted algorithm is useful for identifying minimal epitopes after epitope mapping. In fact, p24-32, which we identified as a CD8⁺ T-cell epitope, had a high binding score (half-time dissociation score), 400, for D^d in BIMAS. However, various factors other than MHC-binding affinity are used to determine T-cell epitopes; these factors include (i) antigen processing (cleavage preference of the proteasome), (ii) TAP-dependent peptide transport, and (iii) the response to the T-cell repertoire. For example, p29-37 in p21-40, which was thought to contain a T-cell epitope(s), exhibits a high binding score for K^d, 120, in BIMAS. However, the p29-37 peptide failed to induce IFN- γ synthesis in spleen cells from immunized BALB/c mice. In addition, p25-35 contains two L^d-binding peptide motifs and can bind to L^d molecules on RMA-S-L^d cells (data not shown). However, this peptide could not elicit significant intracellular IFN- γ synthesis in immune spleen cells. Three-color flow cytometric analysis demonstrated that the p24-32 peptide can induce intracellular IFN- γ synthesis in immune CD8⁺ T cells, indicating that the p24-32 peptide is a CD8⁺ T-cell epitope in BALB/c mice. We further examined the restriction molecule of the epitope by using TAP2-deficient RMA-S cells transfected with the K^d, D^d, or L^d gene. We observed that p24-32 stabilized only H2-D^d molecules on RMA-S-D^d cells, indicating that the peptide binds to H2-D^d molecules. One could envision that only RMA-S-D^d cells can express D^d molecules on the cell surface and that neither RMA-S-K^d nor RMA-S-L^d cells can express K^d or L^d molecules. The uncertainty can be eliminated by our observations that LLO91-99 stabilized the expression of K^d molecules on RMA-S-K^d cells and that the p25-35 peptide stabilized L^d expression on RMA-S-L^d cells. We also demonstrated that the

p24-32 peptide-H2-D^d complex can be recognized by CD8⁺ T cells producing IFN- γ and exhibiting CTL activity. Corr et al. (8) demonstrated that the dominant peptide binding motif of H2-D^d consists of glycine at position 2 (P2), proline at P3, a positively charged residue (lysine or arginine) at P5, and a hydrophobic C-terminal residue (leucine, isoleucine, or phenylalanine) at P9 or P10. p24-32 (GGPHAVYLL) is consistent with this motif except at P5. However, P5 contributes ancillary support for high-affinity binding, so that the positively charged residue seems to be not always necessary. Ag85A and Ag85B sequences corresponding to MPT51 p24-32 do not possess proline at P3 but possess glycine at P2 and leucine at P9, implying that substitution of P3 residues may make the homologous region of Ag85A and Ag85B unable to bind D^d molecules. It is especially noteworthy that a hydrophobic C-terminal residue is critical for the CD8⁺ T-cell epitope since the immune CD8⁺ T cells were able to recognize p22-32 and p24-32 but not p23-31. The importance of the C-terminal residue in the CD8⁺ T-cell epitope was also observed in an HLA-A*0201-restricted MPT51 epitope (unpublished data), in H2-K^b-restricted Moloney murine leukemia virus (4), and in HLA-A*0201-restricted MAGE-A epitopes (15).

In BALB/c mice, the level of epitope-specific IFN- γ production by CD8⁺ T cells was below the detection level in BCG-vaccinated mice. This is consistent with the observation that in BALB/c mice, the level of Ag85-specific IFN- γ -producing T cells, which can be easily elicited by DNA vaccines, is below the detection level in BCG-vaccinated or TB-infected mice (10). Since a DNA vaccine encoding MPT51 is capable of inducing protective immunity against infection with *M. tuberculosis* in BALB/c mice (27) and the DNA vaccine induces CD8⁺ T cells recognizing the p24-32 in the context of H2-D^d, it is possible that low-level expression of the CD8⁺ T-cell epitope on *M. tuberculosis*-infected cells might be recognized by the DNA vaccine-induced CD8⁺ T cells in BALB/c mice.

In C57BL/6 mice immunized with a DNA vaccine encoding MPT51, we found one dominant Th1 epitope and one subdominant Th1 epitope, which are located in p171-190 and p191-210, respectively. Since C57BL/6 mice have a deletion in the E α gene and do not express H2-E molecules on the cell surface (26), these epitopes are considered to be exclusively presented in the context of H2-A^b. Spleen cells from BCG-vaccinated C57BL/6 mice produced a significant level of IFN- γ only in response to p171-190. DNA vaccination is suitable for mapping of T-cell epitopes since this type of vaccination is more potent than vaccination with BCG or infection with *M. tuberculosis*, as previously reported (10, 11). p171-190 shows no sequence homology to corresponding sequences of Ag85A and Ag85B except for the last 2 aa. However, 5 aa at the N terminus of p191-210 in MPT51 are identical to the corresponding residues in Ag85A and Ag85B, indicating that the 5 aa of MPT51 are not required for H2-A^b binding and do not play a role in the binding to H2-A^b molecules in the context of the rest of the sequence. In contrast to the prediction for MHC class I ligand motifs, the highly degenerate anchor positions in most class II motifs make it rather difficult to predict MHC ligands (35). Again, it is noteworthy that binding to MHC molecules is a necessary but not sufficient element for a T-cell epitope. We demonstrated that the MPT51 molecule does not possess MHC class Ia-restricted epitopes in C57BL/6 mice like an

Ag85A molecule (11). It has been demonstrated that vaccination with plasmid DNA encoding Ag85A does not protect H2^b CD4 knockout mice but significantly decreases bacterial replication in the lung and prolongs survival of H2^b β 2-microglobulin knockout mice (11). MPT51 may also induce CD4⁺ T-cell-mediated protective immunity in C57BL/6 mice. Thus, identification of T-cell epitopes may contribute to elucidation of the role of the molecules in the protective immunity induced in different strains of mice.

In conclusion, we identified one H2-D^d-restricted CD8⁺ CTL epitope in BALB/c mice and two H2-A^b-restricted Th1 epitopes in C57BL/6 mice, which are thought to play pivotal roles in protection against *M. tuberculosis* infection. Identification of these T-cell epitopes will be very useful for further elucidation of the role of MPT51-specific T cells in the protective immunity by tetramer staining or ICS.

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