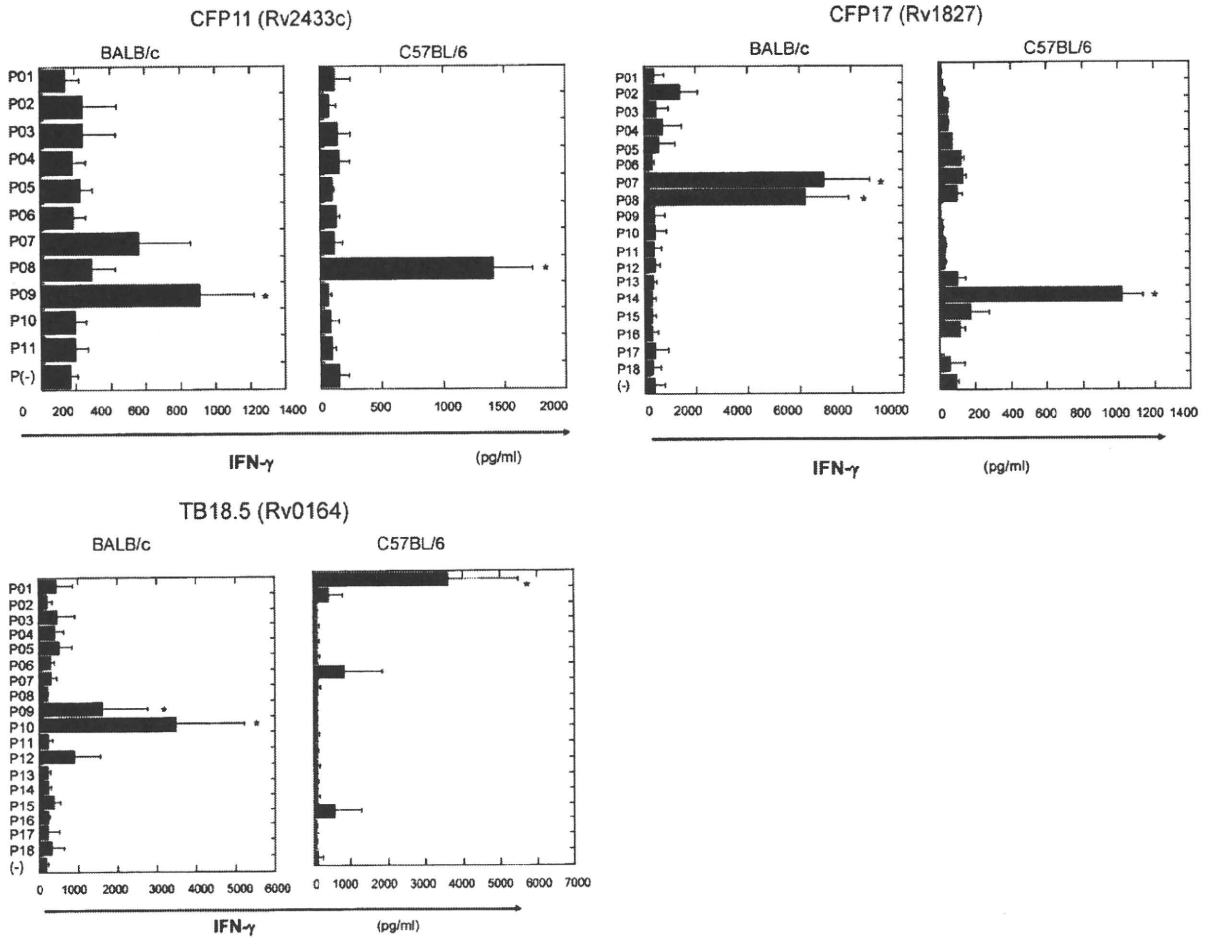

Fig. 2. IFN-γ production by splenocytes stimulated with overlapping peptides of CFP11, CFP17, and TB18.5 proteins. Inbred mice (BALB/c and C57BL/6) were immunized with plasmid DNA encoding CFP11, CFP17, or TB18.5 proteins using gene gun four times at 1-week intervals. The splenocytes were stimulated with overlapping peptides (7.5 μM) 2 weeks after the last immunization. IFN-γ amounts in the supernatants were analyzed by ELISA 72 h later. The means and SD from three mice are shown. Asterisks indicate statistical significance compared with the value without peptides ($P \leq 0.0001$).

2 μg of plasmid DNA and the injection was performed with a single shot of 0.5 mg gold cartridge (device helium discharge pressure: 400 lb/in.^{2}). Mice were injected with plasmid DNA four times at 1-week intervals.

2.4. Peptides

Peptides spanning the entire CFP11 (Rv2433c; 96 aa), CFP17 (Rv1827; 162 aa), and TB18.5 (Rv0164; 161 aa) aa sequences of *M. tuberculosis* were synthesized as approximately 20-mer peptides overlapping by 10 residues (Fig. 1) by Bio Synthesis (Lewisville, TX, USA). Short peptides used for minimal T-cell epitope determination were synthesized by Hayashi kasei (Osaka, Japan). All peptides were dissolved in phosphate-buffered saline (PBS) at a concentration of 1 mM and stored at −80 °C until use.

2.5. Prediction of T-cell epitopes by MHC binding peptide prediction algorithms

For the prediction of potential murine T-cell epitopes that could bind to MHC class I molecules, the following MHC binding peptide prediction algorithms were used through their web sites. These are the National Institutes of Health BioInformatics and Molecular Analysis Section (BIMAS) [31] (http://bimas.dcrt.nig.gov/cgi-bin/molbio/ken_parker_comboform), the SYFPEITHI program [32] (http://www.syfpeithi.de/), and the RANKPEP program [33] (http://bio.dfci.harvard.edu/Tools/rankpep.html).

2.6. Preparation of splenocyte culture supernatants

Two weeks after the last immunization, spleen cells were aseptically harvested from DNA-immune mice. A single cell suspension was prepared and red blood cells were lysed with ACK lysis solution (0.15 M NH_4Cl, 1 mM $KHCO_3$, and 0.1 mM EDTA, pH 7.2). Cells were cultured in RPMI 1640 medium supplemented with 10% heat-inactivated fetal calf serum (RPMI/10FCS) in 96-well plates at 1×10^6 cells (for BALB/c mice) or 2×10^6 cells (for C57BL/6 mice) per well in the presence or absence of 7.5 μM of each peptide at 37 °C in a 5% CO_2 atmosphere. Supernatants were harvested 72 h later and stored at −20 °C until they were assayed. The levels of IFN-γ in the culture supernatants were determined by a mouse-specific sandwich enzyme-linked immunosorbent assay (ELISA), as shown in our previous work [30].

2.7. Detection of IFNγ-producing cells by enzyme-linked immunospot (ELISPOT) assay

Single cell suspensions were tested for Ag-induced IFN-γ secretion using a standard cytokine BD ELISPOT system (BD Biosciences, San Jose, CA, USA). Briefly, cells were stimulated in RPMI/10FCS

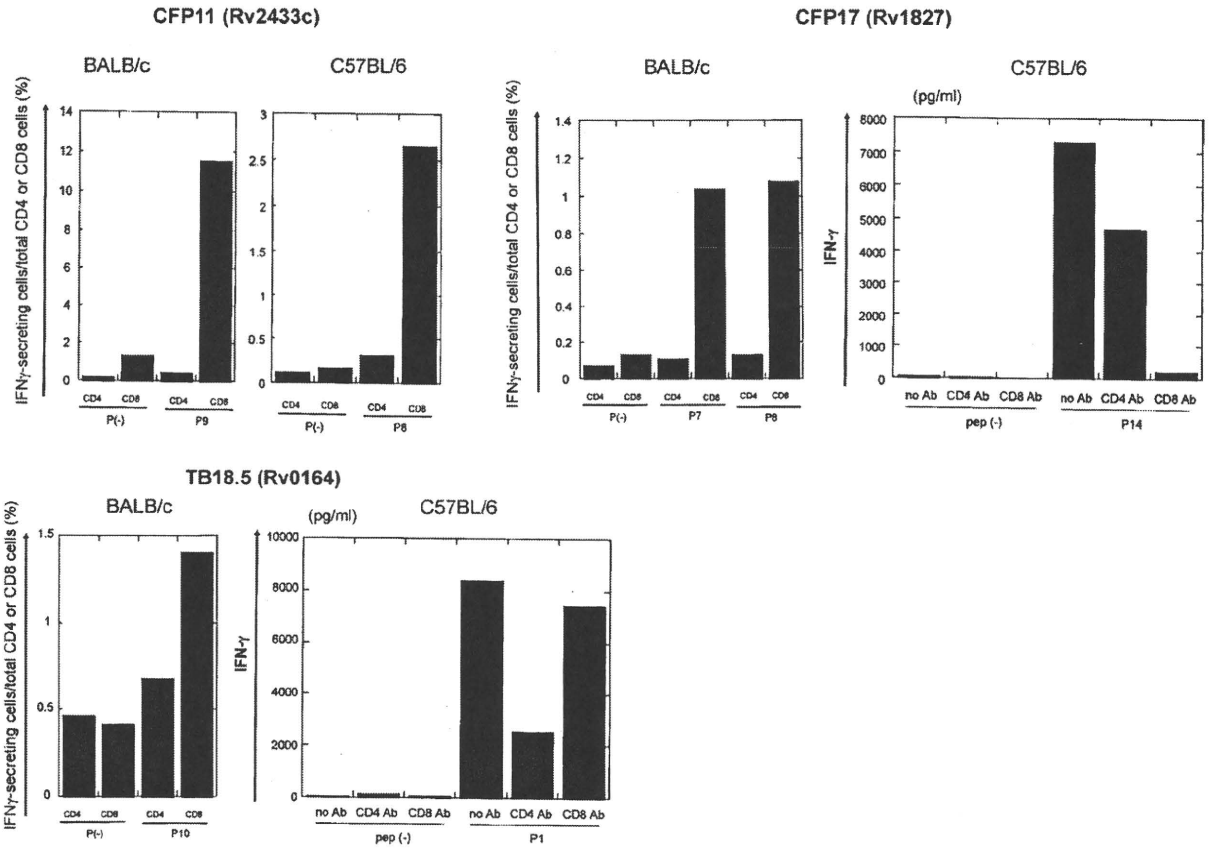

Fig. 3. Analysis of T-cell subsets responsive to CFP11, CFP17, and TB18.5 peptides. Mice were immunized with CFP11, CFP17, or TB18.5 DNA on the same schedule as in Fig. 2. Immune splenocytes were subjected to intracellular IFN-γ staining and cell-surface CD4/CD8 staining with respective mAbs. In the case of CFP17 and TB18.5 in C57BL/6, immune splenocytes were treated with magnetic beads specific for CD4 or CD8. Cells of the negative fraction were stimulated with respective peptides (7.5 μM). IFN-γ amounts in the supernatants were analyzed by ELISA 72 h later. The representative data from two to six mice are shown.

medium with 7.5 μM of each peptide in nitrocellulose-backed 96-well plates (MultiScreen 96-well plates; Millipore, Billerica, MA, USA) coated with 2.5 μg ml^{-1} anti-murine IFN-γ monoclonal antibody (mAb) (R4-6A2; BD Biosciences). The plates were incubated at 37 °C in a 5% CO_2 humidified incubator for 18–24 h. IFN-γ was detected by incubation with 0.75 μM of biotin-labeled anti-murine IFN-γ mAb XMG1.2 (BD Biosciences) for 2 h at room temperature followed by binding to horseradish peroxidase-conjugated streptavidin (eBioscience, San Diego, CA, USA). BD AEC (3-amino-9-ethyl-carbazole) substrate reagent (BD Biosciences) was used to detect bound horseradish peroxidase-conjugated streptavidin. Spots developed on the nitrocellulose filters were enumerated manually under a dissecting microscope.

2.8. Intracellular IFN-γ staining

The intracellular IFN-γ staining procedure was described in our previous work [30]. In brief, immune splenocytes were treated with ACK lysis solution to remove red blood cells, washed twice with RPMI 1640 medium and resuspended in RPMI/10FCS at a concentration of 10^7 cells ml^{-1}. The cells (200 μl) were incubated for 2 h at 37 °C in the presence or absence of 7.5 μM peptide. Golgistop solution (BD Biosciences) was added, further, the cells were incubated for an additional 4 h and then washed twice with FACS buffer (PBS supplemented with 1% FCS), stained with fluorescein isothiocyanate-conjugated anti-CD8 and PerCP-Cy5.5-conjugated anti-CD4 mAbs (BD Biosciences) on ice for 30 min and washed twice. Subsequently, intracellular IFN-γ staining was performed with phycoerythrin-conjugated anti-IFN-γ mAb (clone XMG1.2, BD Biosciences) using a Cytofix/Cytoperm kit (BD Biosciences) according to the manufacturer's instructions. These were analyzed with an EPICS digital flow cytometer (EPICS XL: Beckman Coulter, Miami, FL, USA).

2.9. Depletion of the CD4+ or CD8+ T-cell subset

CD4+ or CD8+ T-cell subsets of peptide-reactive T cells were examined by depletion of CD4+ or CD8+ T cells, respectively, using a BD IMag system (BD Biosciences). Briefly, immune splenocytes were resuspended and centrifuged at 1200 rpm with 1× BD IMag buffer. The supernatants were then carefully aspirated and 50 μl of anti-mouse CD4 particles-DM or anti-mouse CD8a particles-DM (BD Biosciences) was added for every 10^7 cells and the mixture was placed at 12 °C for 30 min. The labeled cells were adjusted to 5×10^7 cells ml^{-1} with 1× BD IMag buffer and immediately placed on BD IMagnet and incubated at room temperature for 8 min. The supernatants were carefully aspirated and confirmed to contain the fraction with either CD4+ or CD8+ T cells depleted by flow cytometry using fluorescent anti-CD4 or CD8 mAbs (data not shown).

2.10. MHC stabilization assay

MHC stabilization assay was originally described by Ljunggren and Kärre [34]. Wild-type RMA-S cells [35], or transfected RMAS-K^d, RMAS-D^d, or RMAS-L^d cells [30], were cultured in RPMI/10FCS at 26 °C overnight and then incubated for 1 h in the presence or

Table 1
T-cell epitope candidates in CFP11, CFP17, and TB18.5.

T-cell epitope candidates in CFP11, CFP17, and TB18.5				
Peptide	Amino acid sequence	Estimated scores for restriction molecules[a]		
CFP11 (BALB/c)		K^d	D^d	L^d
p69-86 (P9)	PFLVASVGYLGARRGVRR			
p76-84 (9mer)	GYLGARRGV	**1000** 24 17.9	—[b]	**2.0**
p70-78 (9mer)	FLVASVGYL	**115.2** 16	**1.2**	**5.0** 13
CFP11 (C57BL/6)		K^b	D^b	
p60-78 (P8)	VRMVINYLVPFLVASVGY			
p64-71 (8mer)	INYLVPFL	**6.6** 18 10.7	—	—
p61-69 (9mer)	RMVINYLVP	**0.2**	**3.9** 13	—
CFP17 (BALB/c)		K^d	D^d	L^d
p52-70 (P7)	PPGSALLVVKRGPNAGSRF			
p61-79 (P8)	KRGPNAGSRFLLDQAITSA			
p62-70 (9mer)	RGPNAGSRF	5	**120** 20.8	**15** 12
p62-69 (8mer)	RGPNAGSR	—	**4**	—
p63-71 (9mer)	GPNAGSRFL	**57.6** 9	**1**	**195** 23
CFP17 (C57BL/6)		K^b	D^b	
p112-130 (P14)	DVGSLNGTYVNREPVDSAV			
p113-121 (9mer)	VGSLNGTYV	—	**14.3** 23	
p113-122 (10mer)	VGSLNGTYVN	—	13	
p116-123 (8mer)	LNGTYVNR	**0.38** 13	13	
p117-126 (10mer)	NGTYVNREPV	—	**14.3**	
p118-126 (9mer)	GTYVNREPV	—	**12** 12.9	
TB18.5 (BALB/c)		K^d	D^d	L^d
p82-100 (P10)	AVYYPGENQIQTVMQQGEL			
p83-91 (9mer)	VYYPGENQI	**3456** 23 19.5	—	—
p84-91 (8mer)	YYPGENQI	**2000** 14.1	—	—

Epitopes predicted by computer algorithms are shown.
[a]Estimated scores are derived from BIMAS (bold), SYFPEITHI (underlined), or RANKPEP (plain).
[b]—; score not shown in the program.

absence of 50 μM of the respective peptides at 26 °C. The cells were then transferred to 37 °C for 2 h, washed with FACS buffer, and cell-surface expression of H2-K^b, H2-D^b, H2-K^d, H2-D^d, or H2-L^d molecules was detected by flow cytometry using FITC-conjugated mouse mAbs specific for H2-D^bL^d (28-14-8; eBioscience), H2-K^dD^d (34-1-2S; eBioscience), or H2-K^b (9013F; Cedarlane, Hornby, Ontario, Canada). To allow comparison between multiple experiments and to reduce interexperimental variations, the mean fluorescence intensity (MFI) values, which are direct measures of peptide binding, were converted to percent maximal stabilization values. The values were calculated using the following formula: (experimental MFI − control MFI)/(maximal MFI − control MFI) × 100. Control MFI was obtained from cells incubated without peptide at 37 °C while MFI of cells at 26 °C was taken as the maximal MFI.

2.11. Statistics

Statistical analyses were performed using the StatView-J 5.0 program (Abacus Concepts, Berkeley, CA, USA). Data from multiple experiments were expressed as mean and SD. Data were analyzed by analysis of variance (ANOVA) followed by a post-hoc Fisher protected least significant difference (PLSD) test.

3. Results

3.1. IFN-γ production by splenocytes of immune mice in response to overlapping synthetic peptides from CFP11 (Rv2433c), CFP17 (Rv1827), and TB18.5 (Rv0164)

Splenocytes of mice immunized with plasmid DNAs encoding CFP11, CFP17, or TB18.5 (pCI-CFP11, pCI-CFP17, or pCI-TB18.5, respectively) were stimulated with the overlapping peptides of the corresponding protein for 72 h and the IFN-γ concentration of the culture supernatants was measured by ELISA. As shown in Fig. 2, robust IFN-γ production was observed in splenocytes from the immune BALB/c mice ($H2^d$ haplotype) in response to peptides P9 (aa 69–86), P7 (aa 52–70)/P8 (aa 61–79), or P9 (aa 72–89)/P10 (aa 82–100) for CFP11, CFP17, or TB18.5, respectively. Similarly, significantly high IFN-γ production was observed in splenocytes of the immune C57BL/6 mice ($H2^b$ haplotype) in the presence of peptides

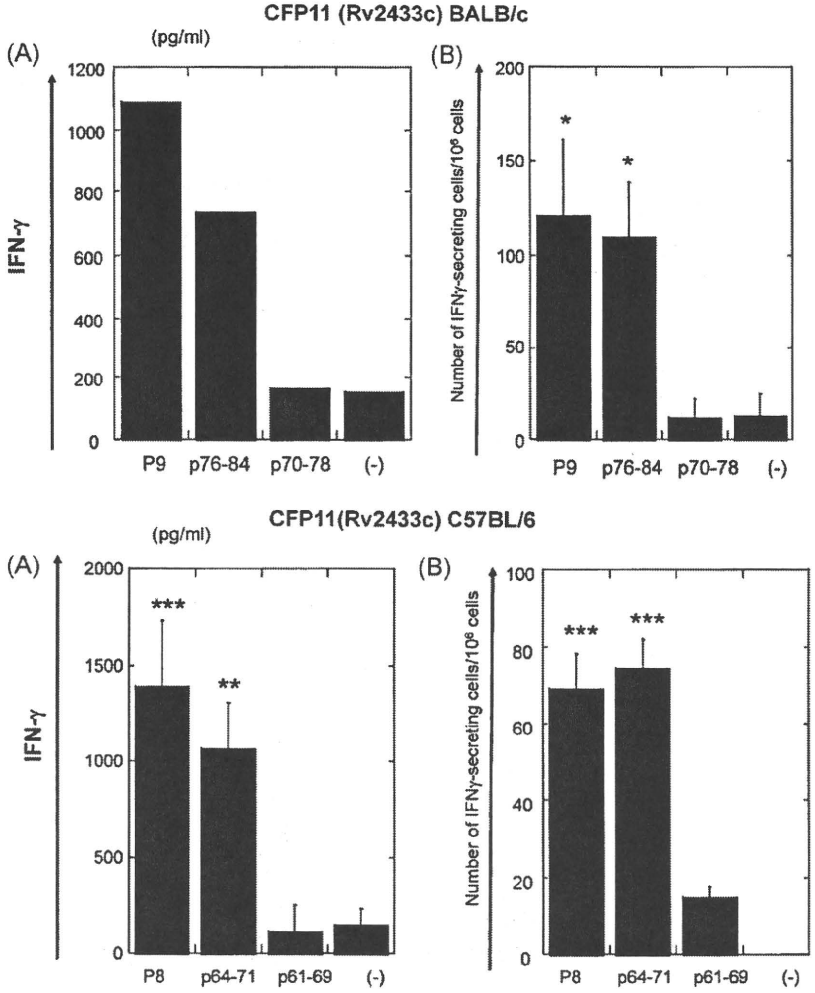

Fig. 4. Determination of minimal T-cell epitopes in CFP11 (Rv2433c) with ELISA and ELISPOT analyses. Inbred mice (BALB/c and C57BL/6) were immunized with CFP11 DNA on the same schedule as in Fig. 2. Immune splenocytes were stimulated with respective peptides (7.5 μM). IFN-γ amounts in the supernatants were analyzed by ELISA 72 h later, whereas the number of IFN-γ-producing cells was examined using ELISPOT 18-24 h later. The results of ELISA (A) and ELISPOT (B) are shown. The means ± SD from three mice are shown except for BALB/c ELISA, for which, the mean of two mice are shown. Asterisks indicate statistical significance compared with the value without peptides (***, $P \leq 0.0001$; **, $P \leq 0.001$; *, $P \leq 0.005$).

P8 (aa 61–77), P14 (aa 112–130), or P1 (aa 1–19) for CFP11, CFP17, or TB18.5, respectively.

3.2. Determination of T-cell subsets responsive to peptides of CFP11, CFP17, and TB18.5

Next, we examined the T-cell subsets that reacted to the peptides of CFP11, CFP17, and TB18.5. Intracellular IFN-γ staining analyses revealed that CD8+ T cells produced high amounts of IFN-γ in response to CFP11 P9 in BALB/c mice and P8 in C57BL/6 mice (Fig. 3). Similarly, CD8+ T cells produced high amounts of IFN-γ in response to CFP17 P7 and P8 peptides in BALB/c mice (Fig. 3). In TB18.5 DNA-immune BALB/c mice, CD8+ T cells produced high amounts of IFN-γ in response to TB18.5 P10 peptide (Fig. 3). In CFP17 and TB18.5 DNA-immune C57BL/6 mice, results of intracellular IFN-γ staining assays were not clear. Therefore, a CD4/CD8 depletion assay was performed. In the assay, CD4+ or CD8+ T cells were removed with magnetic beads and residual cells were stimulated with the relevant peptide. In the case of CFP17 DNA-immune C57BL/6 mice, treatment with CD8 mAb significantly reduced IFN-γ amounts in the culture supernatants whereas treatment of CD4 mAb did not affect the IFN-γ amounts, indicating that CD8+ T cells are the main producer of IFN-γ in the presence of CFP17 P14 peptide in C57BL/6 mice. In the case of TB18.5 DNA-immune C57BL/6 mice, treatment with CD4 mAb significantly reduced IFN-γ amounts in the culture supernatants whereas treatment of CD8 mAb did not affect the IFN-γ amounts, indicating that CD4+ T cells are the main producers of IFN-γ in the presence of TB18.5 P1 peptide in C57BL/6 mice (Fig. 3).

3.3. Identification of minimal T-cell epitopes in the responsive peptide regions of CFP11, CFP17, and TB18.5

Generally, CD8+ T cells recognize peptides of 8–11 aa residues on MHC class I molecules and CD4+ T cells recognize peptides of 12–18 aa residues on MHC class II molecules [36]. Several MHC binding peptide prediction algorithms are available on the Internet. We employed the BIMAS, SYFPEITHI, and RANKPEP programs for predicting CD8+ T-cell epitopes. Results of the analyses using these algorithms are summarized in Table 1.

In the CFP11 antigen, the p76-84 9-mer peptide (GYLGARRGV) in the P9 region showed the highest score (1000) for H2-K^d binding in the BIMAS program. Therefore, we examined the p76-84 peptide for induction of IFN-γ production using ELISA (Fig. 4). The

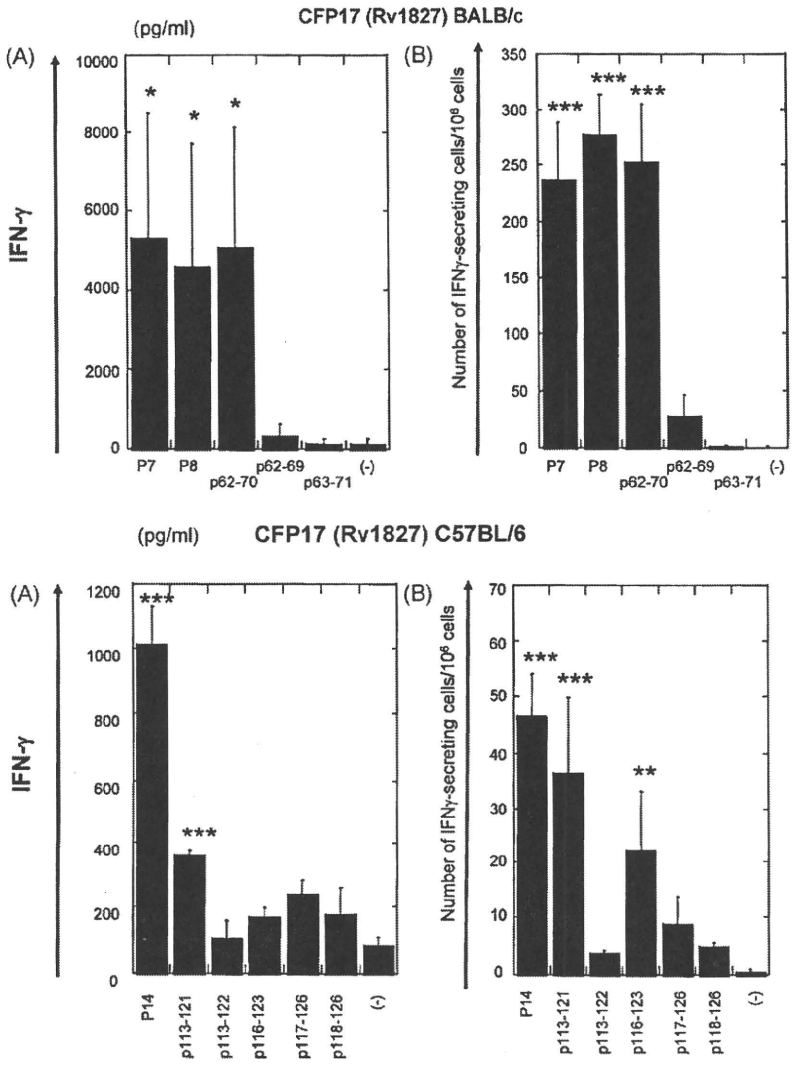

Fig. 5. Determination of minimal T-cell epitopes in CFP17 (Rv1827) with ELISA and ELISPOT analyses. Inbred mice (BALB/c and C57BL/6) were immunized with CFP17 DNA on the same schedule as in Fig. 2. ELISA and ELISPOT were performed as in Fig. 4 and the results of ELISA (A) and ELISPOT (B) are shown. The means and SD from three mice are shown. Asterisks indicate statistical significance compared with the value without peptides (***, $P \leq 0.0001$; **, $P \leq 0.005$; *, $P \leq 0.05$).

peptide induced robust IFN-γ production compared with the p70-78 peptide, which indicates that the p76-84 peptide is a minimal T-cell epitope. We detected a significantly high number of IFN-γ-secreting cells in immune splenocytes in the presence of P9 or the p76-84 peptide in ELISPOT assay (Fig. 4). In C57BL/6 mice, the algorithms gave the highest score for binding to H2-K^b to the p64-71 peptide (INYLVPFL; Table 1). The p64-71 peptide stimulation showed significantly high IFN-γ levels on ELISA and significantly high IFN-γ-producing cell numbers on ELISPOT assay (Fig. 4).

In the CFP17 antigen, the p62-70 9-mer peptide (RGPNAGSRF) in the P7/P8 region showed high scores (120 in BIMAS; 20.8 in RANKPEP) for H2-D^d binding (Table 1). Therefore, we examined the p62-70 peptide for induction of IFN-γ production using ELISA and ELISPOT assays. The p62-70 peptide stimulation showed significantly high IFN-γ amounts on ELISA and significantly high numbers of IFN-γ-producing cells on ELISPOT assay (Fig. 5), indicating that the p62-70 peptide is a minimal T-cell epitope. In C57BL/6 mice, SYFPEITHI algorithm gave a high score for binding to H2-D^b to the p113-121 peptide (VGSLNGTYV; Table 1), and the peptide showed significantly high levels of IFN-γ production and significantly high numbers of IFN-γ-producing cells with ELISA and ELISPOT assays, respectively (Fig. 5).

In the TB18.5 antigen, the p83-91 9-mer peptide (VYYPGENQI) in the P10 region has a high score (3456) for H2-K^d binding in the BIMAS program (Table 1). Therefore, we examined the p83-91 peptide for induction of IFN-γ production using ELISA and determined the number of IFN-γ-producing cells via ELISPOT assay. The p83-91 peptide induced robust IFN-γ production compared with p84-91, which indicates that the p83-91 peptide is a minimal T-cell epitope (Fig. 6). In C57BL/6 mice, no peptides were predicted to bind to K^b or D^b with high scores, although P1 of TB18.5 elicited robust IFN-γ production (Fig. 2) and CD4+ T cells were the main IFN-γ producer (Fig. 3). Since C57BL/6 mice have a deletion of the H2-Eα gene, therefore, they do not express H2-E molecules on the cell surface [37], the identified CD4+ T-cell epitopes in C57BL/6 mice are considered to be exclusively presented on H2-A^b.

3.4. Identification of MHC class I restriction molecules for CFP11, CFP17, and TB18.5 minimal epitopes

Finally, we investigated the binding and stabilization of MHC class I restriction molecules by each identified minimal epitope of CFP11, CFP17, or TB18.5. As shown in Fig. 7, the p76-84 peptide of CFP11 and p83-91 of TB18.5 were strongly bound to the H2-K^d

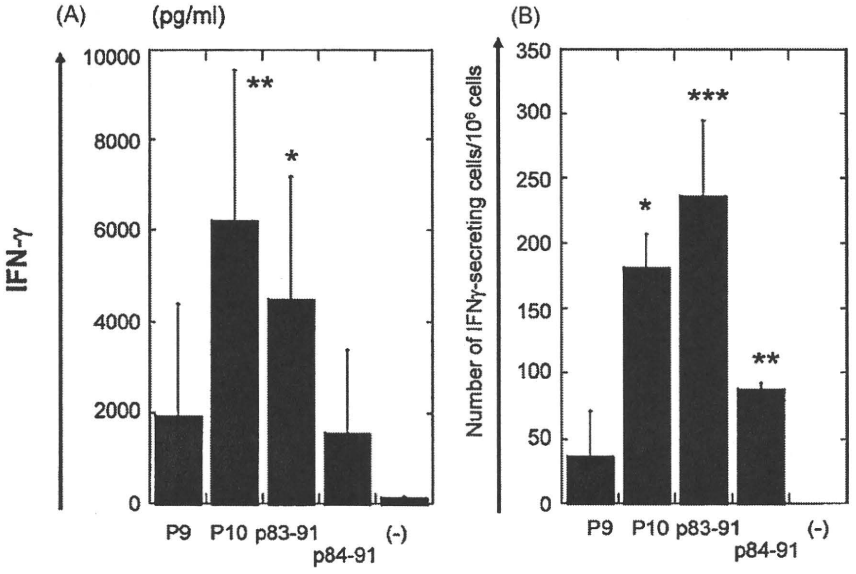

Fig. 6. Determination of minimal T-cell epitopes in TB18.5 (Rv0164) with ELISA and ELISPOT analyses. BALB/c mice were immunized with TB18.5 DNA on the same schedule as in Fig. 2. ELISA and ELISPOT were performed as in Fig. 4 and the results of ELISA (A) and ELISPOT (B) are shown. The means and SD from three mice are shown. Asterisks indicate statistical significance compared with the value without peptides (***, $P \leq 0.0001$; **, $P \leq 0.01$; *, $P \leq 0.05$).

molecule, whereas p62-70 of CFP17 was strongly bound to the H2-D^d molecule. In addition, p64-71 of CFP11 was bound to H2-K^b and p113-121 of CFP17 was bound to the H2-D^b molecule.

4. Discussion

T cells have several features that are important for protective immunity against *M. tuberculosis*. These include production of proinflammatory cytokines such as IFN-γ and tumor necrosis factor-α. IFN-γ has been regarded as an important correlate of protective immune responses [38]. Activation of macrophages harboring *M. tuberculosis* by IFN-γ has been considered to be crucial for eradication of the intracellular bacterium. In addition, the majority of IFN-γ-producing CD8+ T cells have cytotoxic activity for cells harboring the bacterium, which also contribute to protective ability. Analyses of the protective ability of the CD8+ T-cell epitope peptides identified in this study is required to clarify their physiological functions.

We have performed ELISA and ELISPOT assays for the identification of minimal epitopes in this study. Basically, both ELISA and ELISPOT assays showed similar results. However, we noticed that in some cases these results differed. For example, P7 of CFP17 in BALB/c mice (Fig. 5) induced higher IFN-γ production than P8 on ELISA, but P8 induced a higher number of IFN-γ-producing cells than P7 on ELISPOT assay. Although the differences were not very high, similar results were obtained each time we performed the experiments. The difference in the additional flanking aa residues next to the common minimal epitope aa sequence in the peptides may affect the stability of peptides and the kinetics of IFN-γ production during the different incubation times of ELISA (72 h) and ELISPOT (18–24 h). This may account for the differences observed between the ELISA and ELISPOT results.

The T-cell epitopes identified in this study were all CD8+ T-cell epitopes except for one CD4+ T-cell epitope in the TB18.5 protein in C57BL/6 mice. This may be caused in part by the fact that DNA immunization is an efficient method for producing cytoplasmic antigens that are consequently detected by CD8+ T cells. In addition, low-molecular-mass proteins may be easily degraded to smaller peptides that tend to be subject to the antigen presentation pathway through MHC class Ia molecules. Of note, among mouse MHC binding motifs, the H2-K^d binding motifs have been shown to be quite similar to those of HLA-A24 [39], which is the most popular subtype of HLA-A (approximately 60%) of the Japanese population [40]. Therefore, the H2-K^d-restricted T-cell epitopes identified in this study may also function as HLA-A24-restricted epitopes although further investigation is needed to confirm this.

The T-cell epitope repertoire determined by DNA immunization method, using plasmids encoding mycobacterial antigens has been reported to be broader than that stimulated by live mycobacterial infection. A variety of peptides induced IFN-γ and/or IL-2 production in mice immunized with plasmid DNA encoding Ag85A, Ag85B, or Ag85C [27,28]. However, in our study, only one peptide or two overlapping peptides of CFP11, CFP17, or TB18.5 in BALB/c or C57BL/6 mouse strains induced IFN-γ production from immune splenocytes. This may be due to the small size of the proteins examined here compared with the Ag85 family proteins. Previously, we observed that only a restricted peptide region (one or two 20-mer peptides) induced IFN-γ production in mice immunized with DNA encoding MPT51 that has a molecular mass similar to that of the Ag85 family proteins [29,30]. In addition, our previous work [41] showed that only one immunodominant epitope of Ag85A was detected in the dendritic cell immunization system of BALB/c mice, although at least three epitope peptides have been reported [27]. The mechanism of optimum epitope selection from several predicted peptides that fit well with the MHC binding motifs remains to be clarified.

Concerning the importance of T-cell epitope mapping in mice, immunodominant antigens in mice are also generally immunodominant in humans. However, precise, specific T-cell analyses are possible only in mice. Therefore, T-cell epitope mapping in mice and analyses using the identified epitope peptides will provide important information that can be used for better understanding of T cells specific for antigens of *M. tuberculosis*, although T-cell epitope peptides themselves are, in principle, different between mice and humans. T-cell epitope mapping is critical for the accurate analysis of T cells specific for antigens and for the design of epitope-based vaccines [42,43].

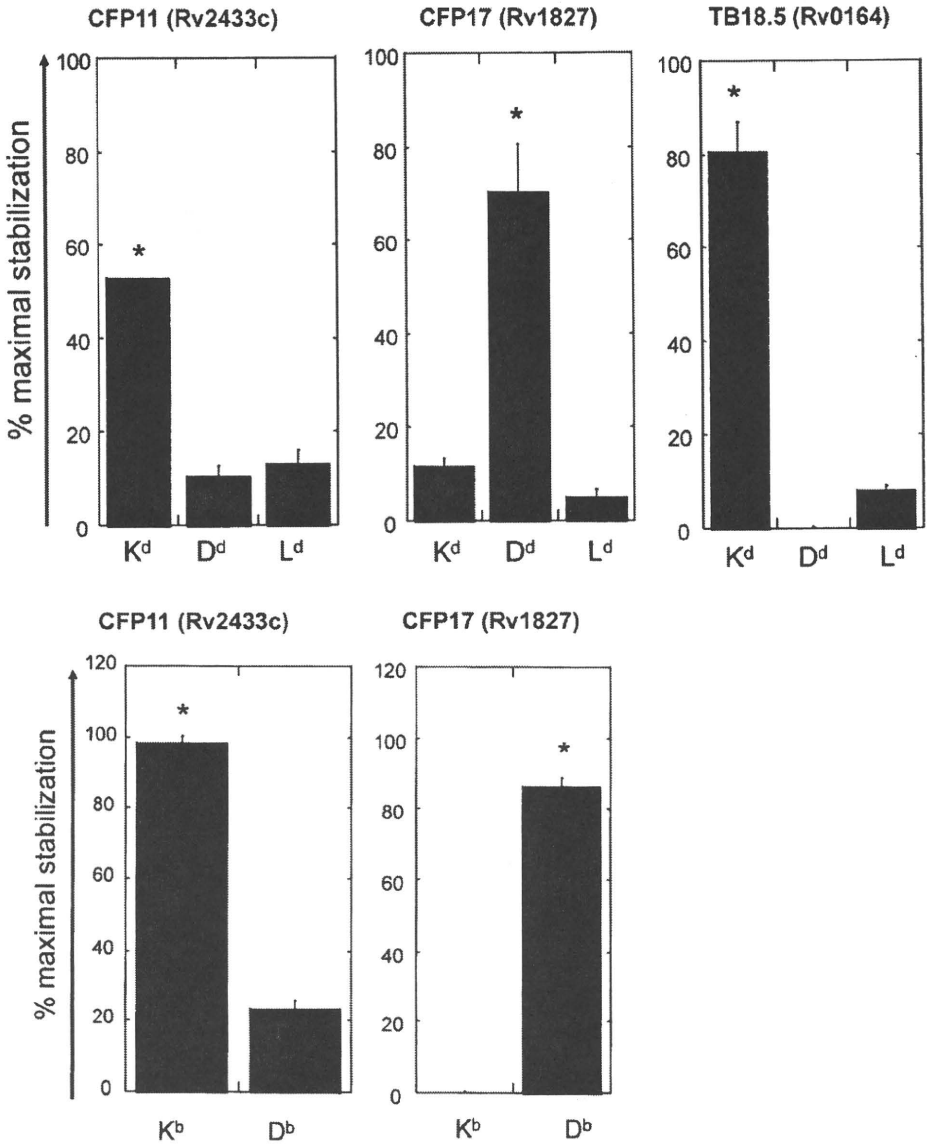

Fig. 7. MHC binding assay of peptides from CFP11, CFP17, and TB18.5. The ability of CD8+ T-cell epitope candidate peptides to bind to MHC class I molecules was measured by MHC stabilization assay using RMA-S cells (for H2-K^b and D^b molecules) or the transfectants, RMAS-K^d, RMAS-D^d, or RMAS-L^d cells (for H2-K^d, D^d, and L^d molecules, respectively). The results are expressed as percent maximal stabilization values and SD. Asterisks indicate statistical significance compared with the value without peptides ($P \leq 0.0001$).

In conclusion, we identified murine T-cell epitopes of CFP11, CFP17, and TB18.5, which are immunoreactive low-molecular-mass antigens of *M. tuberculosis*. We identified two CD8+ T-cell epitopes on CFP11 and CFP17, one in BALB/c mice and the other in C57BL/6 mice, respectively. On TB18.5, we identified a CD8+ T-cell epitope in BALB/c mice and a CD4+ T-cell epitope in C57BL/6 mice. With the aid of computer algorithms, we could identify the minimal CD8+ T-cell epitopes. T-cell epitopes of CFP11, CFP17, and TB18.5, as well as MPT51, would be feasible for analysis of T-cell responses to *M. tuberculosis* infection.

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Identification of HLA-DR4-restricted T-cell epitope on MPT51 protein, a major secreted protein derived from *Mycobacterium tuberculosis* using MPT51 overlapping peptides screening and DNA vaccination

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ABSTRACT

We identified a novel HLA-DR4-restricted CD4+ T-cell epitope on a secreted antigen of *Mycobacterium tuberculosis*, MPT51, in 004149-MM HLA-DR4-transgenic mice which express HLA-DRB1*0401, but not murine MHC class II molecules. The mice were immunized with plasmid DNA encoding MPT51 using gene gun and interferon (IFN)-γ production from the immune splenocytes was analyzed. In response to overlapping synthetic peptides covering the mature MPT51 sequence, only one peptide, p191–210, stimulated the splenocytes to produce IFN-γ. Further analysis using flow cytometry and computer-assisted algorithm, ProPred, narrowed down the region of CD4+ T-cell epitope to p191–202. The CD4+ T-cell epitope would be feasible for vaccine design against tuberculosis as well as for analysis of MPT51-specific T-cells in *M. tuberculosis* infection.

1. Introduction

Tuberculosis (TB) has been a major cause of illness and death worldwide. There were estimated 9.2 million new causes and 1.7 million deaths from TB in 2006 [1]. Although WHO's "The Stop TB Strategy" approach seems to work, especially in the African region, multi-drug resistant strains and co-infection with human immunodeficiency virus (HIV) remains difficult issues in TB prevention (0.7 million cases and 0.2 million deaths from TB occurred in HIV-positive people in 2006 [1]). The only TB vaccine currently available is the attenuated *Mycobacterium bovis* strain Bacillus Calmette-Guérin (BCG), the effect of which has been questioned for preventing pulmonary TB in adults [2] and also reported to wane with time since vaccination [3]. Therefore, the improved vaccine is an urgent need against TB [2,4].

It has been widely accepted that cell-mediated immunity plays a pivotal role in the control of *Mycobacterium tuberculosis* infection [5,6]. 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 interferon (IFN)-γ and tumor necrosis factor-α. In addition, CD8+ cytotoxic T-lymphocytes (CTL) have also been reported to contribute to disease resistance [5,6].

Secreted and surface-exposed cell wall proteins, virulence factors such as PE/PPE and EsX have been reported to be immunodominant antigens, which are able to induce protective cellular immunity against TB [7–9]. The mouse model of TB infection revealed that memory cells from immune mice produced substantial amounts of IFN-γ in response to two fractions of culture filtrate of *M. tuberculosis* represented by 6–10 kDa proteins and antigen 85 (Ag85) complex, a 30–32 kDa protein family [7,8,10,11].

Ag85 complex (Ag85A, Ag85B, and Ag85C), which possesses mycolyltransferase activity in cell wall synthesis and in the biogenesis of cord factor [12] and has been shown to be a major fraction of the secreted proteins of *M. tuberculosis* [13]. Another major secreted protein, termed MPT51, has primary structure similarity (38–43%) with those components and the antibody (Ab) against MPT51 was demonstrated to cross-react with the three components of Ag85 complex [13,14]. Using DNA vaccine encoding MPT51, we found that MPT51 can induce specific cellular immune responses and the protective immunity against challenge with *M. tuberculosis* in murine infection model [15]. We identified murine T-cell epitopes using C57BL/6 and BALB/c mouse strains [16,17] and an HLA-A*0201-restricted CD8+ T-cell epitope in MPT51 molecule [18].

Here, we identified an HLA-DRB1*0401-restricted CD4+ T-cell epitope on MPT51 with a strategy using HLA-DRB1*0401-transgenic (Tg) mice, gene gun immunization with expression plasmid DNA encoding MPT51, overlapping synthetic peptides spanning the entire mature MPT51 amino acid (aa) sequence, and the computer-assisted major histocompatibility complex (MHC) binding peptide prediction algorithms.

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2. Materials and methods

2.1. Animals

HLA-DRB1*0401-Tg mice (004149-MM) [19] were purchased from Taconic Farms, Inc. (Hudson, NY, USA). The 004149-MM mice do not express H2 class II molecules, but express HLA-DRB1*0401 composed of *HLA-DRA-IEα* and *HLA-DRB1*0401-IEβ* chimeric genes [19]. In 004149-MM mice, the HLA-DRB1*0401 is the only species of MHC class II molecule expressed. The mice 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 to three-month-old female mice were used in all experiments. Animal experiments were performed according to the Guidelines for Animal Experimentation, Hamamatsu University School of Medicine.

2.2. Peptides

Peptides spanning the entire mature MPT5l aa sequence of *M. tuberculosis* (266 aa residues) were synthesized as 20-mer peptides overlapping by 10 residues, with the exception of the carboxy-terminal 12-mer from aa 255 to 266, were described in our previous work [16]. Briefly, lyophilized peptides were purchased from Invitrogen Corporation (Carlsbad, CA, USA), purity of which was confirmed by mass spectrometry. All peptides were dissolved in distilled water to a concentration of 1 mM and stored at −80 °C until use.

2.3. Immunization of mice

For DNA immunization with Helios gene gun system (Bio-Rad Laboratories, Hercules, CA, USA), preparation of DNA-coated gold particle cartridge was followed to the manufacturer's instruction manual. Finally, 0.5 mg of gold particles was coated with 2 μg of plasmid DNA and the injection was carried out with 0.5 mg gold per shot twice. Mice were injected with 4 μg of plasmid DNA four times at 1-week intervals. Mice were also immunized subcutaneously with 1×10^6 CFU of *M. bovis* BCG (Tokyo strain; Japan BCG Inc., Tokyo, Japan) twice with a 2-week interval.

2.4. Preparation of splenocyte culture supernatants and measurement of IFN-γ amounts

Spleen cells were harvested from MPT51 DNA-immune mice. Recovered cells were plated in 96-well plates at 1×10^6 cells per well in the presence or absence of 5 μM of each MPT51 peptide at 37 °C with 5% CO_2 atmosphere. Purified protein derivative (PPD; Japan BCG Inc.) (1 μg ml^{-1}) was used as a positive control. Supernatants were harvested 72 h later and stored at −20 °C until they were assayed. Concentration of IFN-γ in the culture supernatants was determined by a sandwich enzyme-linked immunosorbent assay (ELISA). The ELISA was carried out as described in our previous work [16] with some modifications. The method is as follows. The 96-well ELISA plates (EIA/RIA Plate A/2; Costar, Cambridge, MA, USA) were coated with 2 μg ml^{-1} of capture antibody (Ab) (anti-murine IFN-γ monoclonal Ab [mAb] R4-6A2; BD Biosciences, San Jose, CA, USA) at 4 °C overnight, washed with PBS supplemented with 0.05% Tween 20 (PBS-Tween), and blocked with Block One Blocking solution (Nacalai Tesque, Kyoto, Japan) at room temperature for 45 min. After washed with PBS-Tween, the culture supernatants were added to the plates and the plates were incubated at 4 °C overnight. After washed with PBS-Tween, 0.5 μg ml^{-1} of biotin-labeled anti-murine IFN-γ mAb XMG1.2 (BD Biosciences) was added to the plates, and the plates were incubated for 2 h at room temperature. After washed with PBS-Tween, horseradish peroxidase (HRP)-conjugated avidin (Bio-Rad Laboratories) was added and incubated for 30 min at room temperature. After washed, the plates were added with TMB one component HRP microwell substrate (BioFX Laboratories, Owings Mills, MD, USA). After 10 min, the enzyme reaction was stopped by adding 2 M H_2SO_4, followed by measuring the absorbance at 450 nm using an EZS-ABS Microplate Reader (Asahi Techno Glass Tokyo, Japan).

2.5. Computer-assisted algorithms for prediction of Th epitope

Th epitope prediction of MPT51 was performed using ProPred HLA-DR binding peptide prediction algorithm ([20], http://www.imtech.res.in/raghava/propred/) at the default setting (threshold value of 3.0). This server has been used for searching the promiscuous binding regions which bind to a total of 50 alleles of HLA-DR molecules. The annotated 50 alleles are composed of 9 serologically defined HLA-DR molecules, namely, HLA-DR1 (2 alleles), DR3 (7 alleles), DR4 (8 alleles), DR7 (2 alleles), DR8 (6 alleles), DR11 (9 alleles), DR13 (11 alleles), DR15 (3 alleles) and DR51 (2 alleles). In addition, RANKPEP MHC binding peptide prediction algorithm ([21], http://immunax.dfci.harvard.edu/Tools/rankpep.html) was also used in this study.

2.6. Intracellular IFN-γ staining

Antigen-specific T-cell subset was also determined with simultaneous flow cytometric assessment of T-cell phenotype and intracellular IFN-γ synthesis. Cell-surface staining of CD4 and CD8 and intracellular IFN-γ staining were described in our previous work [16,17]. Intracellular IFN-γ staining was conducted using a Cytofix/Cytoperm Plus (with GolgiStop) kit (BD Biosciences) according to the manufacturer's instruction.

2.7. Statistics

Statistical analyses were performed by using StatView-J 5.0 statistics program (Abacus Concepts, Berkeley, CA, USA). Data were analyzed with unpaired Student's *t* test.

3. Results

3.1. IFN-γ production in response to overlapping synthetic peptides from MPT51 by splenocytes of pCI-MPT51 DNA or M. bovis BCG-immune 004149-MM HLA-DRB1*0401-Tg mice

The 004149-MM mice do not express H2 class II molecules, but express HLA-DR4 transgene [19]. Expression of HLA-DR molecules on splenocytes of the mice was confirmed by flow cytometric analysis with anti-HLA DR mAb (data not shown). Splenocytes from 004149-MM HLA-DRB1*0401-Tg mice immunized with plasmid DNA encoding mature MPT5l (pCI-MPT51) were stimulated with the overlapping MPT51 peptides for 72 h and IFN-γ concentration of culture supernatants was measured by ELISA. As shown in Fig. 1, robust IFN-γ production was observed in the splenocytes only in the presence of peptide 191 (p191; aa 191–210). As expected, splenocytes from naïve 004149-MM mice showed no significant IFN-γ production in response to any MPT51 peptides. In addition, we examined the dose–response relationship by adding serial amounts of MPT51 p191 peptide to the splenocyte culture. The minimal concentration of the peptide for inducing IFN-γ production by the splenocytes was approximately 1×10^{-8} M (10 nM) (data not shown).

In order to examine whether the same peptide induce IFN-γ following natural mycobacterial infection, splenocytes from 004149-MM HLA-DRB1*0401-Tg mice immunized with *M. bovis* BCG were examined for IFN-γ production in response to MPT51

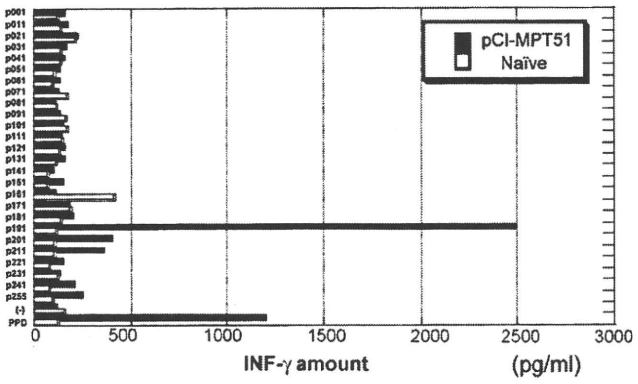

Fig. 1. IFN-γ production of spleen cells from 004149-MM HLA-DRB1*0401-Tg mice immunized with pCI-MPT51. IFN-γ production of splenocytes from the mice immunized with pCI-MPT51 plasmid in response to 1 of 26 overlapping peptides (5 μM) covering MPT51 molecule, medium alone [(−)], or PPD (1 μg ml^{-1}) was evaluated. The splenocytes from naïve mice were also examined as a control. Data are representatives of three independent experiments.

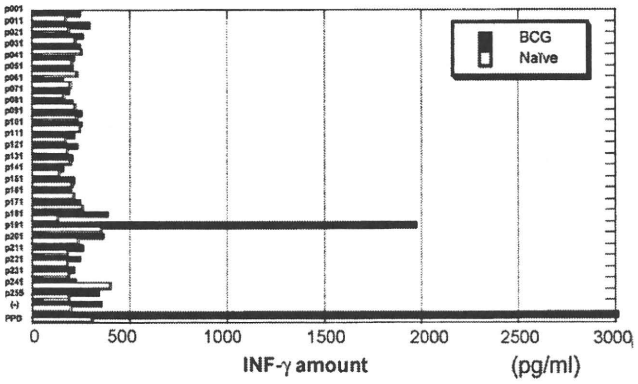

Fig. 2. IFN-γ production of spleen cells from 004149-MM HLA-DRB1*0401-Tg mice immunized with *M. bovis* BCG. IFN-γ production of splenocytes from the mice immunized with *M. bovis* BCG in response to 1 of 26 overlapping peptides (5 μM) covering MPT51 molecule, medium alone [(−)], or PPD (1 μg ml^{-1}) was evaluated. The splenocytes from naïve mice were also examined as a control. Data are representatives of three independent experiments.

peptides. Robust production of IFN-γ was observed in the presence of PPD (Fig. 2). Only MPT51 p191 among all MPT51 peptides induced production of high amounts of IFN-γ similarly to the case of MPT51 DNA-immunized mice.

3.2. Prediction of CD4+ T-cell epitope on peptide 191 of MPT51

We predicted CD4+ T-cell epitope candidate peptides in the 20-mer peptide by computer-based program, ProPred [20]. We found that a core HLA-DRB1*0401 binding motif composed of 9-mer peptide, p194–202 (WVWSPTNPG) at the default threshold setting. This amino acid region was also predicted to bind to HLA-DR4 at the highest score (17.65) in RANKPEP algorithm [21]. This core region (p194–202) was predicted to bind 20 alleles from a total of 50 alleles listed in ProPred program (Fig. 3). These HLA-DR alleles include 6 alleles in HLA-DR3 serological group, 8 alleles in HLA-DR4 group, 2 alleles in HLA-DR7 group, 2 alleles in HLA-DR11 group, and 2 alleles in HLA-DR13 group. Thus, the region (p194–202) was predicted to bind 40% HLA-DR alleles included in the ProPred program. In general, 12–20-mer antigenic peptides bind to MHC class II molecules. Therefore, we synthesized p191–202 peptide as a candidate CD4+ T-cell epitope, instead of examining jus t a core motif (p194–202) for the following experiment.

3.3. Detection of MPT51 p191–202-, or p191–210-specific CD4+ T-cells in 004149-MM HLA-DRB1*0401-Tg mice by ELISA and intracellular IFN-γ staining

We then examined splenocytes derived from MPT51 DNA-immune 004149-MM HLA-DRB1*0401-Tg mice for detection of

```
                171       181       191       201       211       220
                LGRWKWHDPWVHASLLAQNNTRVWVWSPTNPGASDPAAMIGQAAEAMGNS

DRB1*0401       LGRWKWHDPWVHASLLAQNNTRVWVWSPTNPGASDPAAMIGQAAEAMGNS
DRB1*0404       LGRWKWHDPWVHASLLAQNNTRVWVWSPTNPGASDPAAMIGQAAEAMGNS
DRB1*0405       LGRWKWHDPWVHASLLAQNNTRVWVWSPTNPGASDPAAMIGQAAEAMGNS
DRB1*0408       LGRWKWHDPWVHASLLAQNNTRVWVWSPTNPGASDPAAMIGQAAEAMGNS
DRB1*0410       LGRWKWHDPWVHASLLAQNNTRVWVWSPTNPGASDPAAMIGQAAEAMGNS
DRB1*0421       LGRWKWHDPWVHASLLAQNNTRVWVWSPTNPGASDPAAMIGQAAEAMGNS
DRB1*0423       LGRWKWHDPWVHASLLAQNNTRVWVWSPTNPGASDPAAMIGQAAEAMGNS
DRB1*0426       LGRWKWHDPWVHASLLAQNNTRVWVWSPTNPGASDPAAMIGQAAEAMGNS

DRB1*0305       LGRWKWHDPWVHASLLAQNNTRVWVWSPTNPGASDPAAMIGQAAEAMGNS
DRB1*0306       LGRWKWHDPWVHASLLAQNNTRVWVWSPTNPGASDPAAMIGQAAEAMGNS
DRB1*0307       LGRWKWHDPWVHASLLAQNNTRVWVWSPTNPGASDPAAMIGQAAEAMGNS
DRB1*0308       LGRWKWHDPWVHASLLAQNNTRVWVWSPTNPGASDPAAMIGQAAEAMGNS
DRB1*0309       LGRWKWHDPWVHASLLAQNNTRVWVWSPTNPGASDPAAMIGQAAEAMGNS
DRB1*0311       LGRWKWHDPWVHASLLAQNNTRVWVWSPTNPGASDPAAMIGQAAEAMGNS

DRB1*0701       LGRWKWHDPWVHASLLAQNNTRVWVWSPTNPGASDPAAMIGQAAEAMGNS
DRB1*0703       LGRWKWHDPWVHASLLAQNNTRVWVWSPTNPGASDPAAMIGQAAEAMGNS

DRB1*1114       LGRWKWHDPWVHASLLAQNNTRVWVWSPTNPGASDPAAMIGQAAEAMGNS
DRB1*1120       LGRWKWHDPWVHASLLAQNNTRVWVWSPTNPGASDPAAMIGQAAEAMGNS

DRB1*1302       LGRWKWHDPWVHASLLAQNNTRVWVWSPTNPGASDPAAMIGQAAEAMGNS
DRB1*1323       LGRWKWHDPWVHASLLAQNNTRVWVWSPTNPGASDPAAMIGQAAEAMGNS
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Fig. 3. Prediction of HLA-DR binding sites in MPT51 protein. Prediction of HLA-DR binding sites of MPT51 was performed with ProPred algorithm. The 20 HLA-DR alleles on which MPT51 p194–202 is predicted to be a core binding motif, are shown. Bold letters indicate predicted binding sites of MPT51 for each HLA-DR allele.

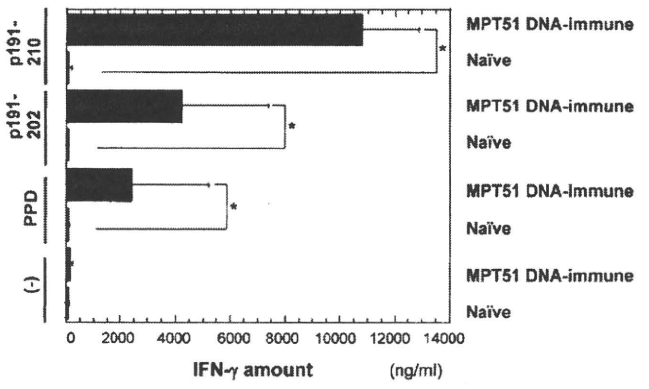

Fig. 4. IFN-γ production of MPT51 DNA-immune 004149-MM HLA-DRB1*0401-Tg mice splenocytes in response to MPT51 p191–210 or p191–202 peptides. IFN-γ production from splenocytes of MPT51 DNA-immune or naïve 004149-MM HLA-DRB1*0401-Tg mice was analyzed in the presence of MPT51 p191–210 (20-mer) or p191–202 (12-mer) peptides. Data are means and standard deviations from five to eight experiments. Asterisk indicates statistically significance with Student's *t* test ($p < 0.05$).

MPT51 p191–202-, or p191–210-specific T-cells. As shown in Fig. 4, the immune splenocytes, but not naïve splenocytes produced robust amounts of IFN-γ in response to MPT51 p191–202, suggesting that MPT51 p191–202 peptide is a *bona fide* HLA-DRB1*0401-restricted CD4+ T-cell epitope. Further, we examined whether CD4+ T-cells produce IFN-γ in response to MPT51 p191–202 or p191–210 peptide with intracellular IFN-γ staining assay. Again, MPT51 DNA-immune, but not naïve CD4+ T-cells produced IFN-γ in response to these MPT51 peptides (Fig. 5).

4. Discussion

Understanding of the nature of protective immunity to *M. tuberculosis* would facilitate the development of the future vaccine. The cellular arm of the immune response mediated by CD4+ Th1 cells and CD8+ CTL has been established as a pivotal component of the protective immunity against *M. tuberculosis* [5,6]. CD4+ Th1 cells have been reported to contribute to protection against *M. tuberculosis* infection through inflammatory cytokine production such as IFN-γ. IFN-γ has been considered to contribute to the protective immunity through activating macrophages and increasing natural killer cell activity. However, IFN-γ production has been shown not to be the only correlate of protection against *M. tuberculosis* [22]. Production of IL-2 [23,24] or functions such as direct cytotoxic ability of CD4+ T-cells [25,26] may be important for the protective ability. Functions of MPT51 p191–202-specific CD4+ T-cells in this report except for IFN-γ production should be clarified in further study.

Reports concerning the involvement of CD4+ T-cells to contain *M. tuberculosis* infection have accumulated and an intense effort to identify *M. tuberculosis*-derived CD4+ T-cell epitopes that are presented by HLA class II molecules, has been done. HLA-DR-restricted T-cell epitopes in *M. tuberculosis* antigens have been identified, including epitopes in heat shock protein 65 [27], Ag 85B [28], ESAT-6 [29], Mce proteins [30], MPB70 [31], the 24 kDa secreted lipoprotein (LppX) [32], and PPE68 [33]. MPT51 is a dominant *M. tuberculosis*-derived secreted molecule which is related with Ag85 family molecules, Ag85A, Ag85B, and Ag85C. The molecules have been reported in a variety of mycobacteria. Functionally, the molecule has been implicated in fibronectin binding as in Ag85 family molecules [34]. However, MPT51 appears not to have mycolyltransferase activity that Ag85 family molecules share since MPT51 does not conserve the catalytic triad (Ser-His-Glu) in the aa sequence [35]. Therefore, MPT51 seems to have the particular function that remains to be clarified. Importantly, MPT51 has been reported as a potential marker for the diagnosis of TB, especially in AIDS patients. Ramalingam et al. [36] reported that early immune responses against 38 kDa and 27 kDa (MPT51) proteins were detected in pulmonary TB accompanied with HIV infection. In addition, we demonstrated that MPT51 plays a pivotal role in the protection against *M. tuberculosis* infection [15]. The identification of human T-cell epitopes in MPT51 protein would be very useful for early diagnosis of TB and development of TB vaccine.

HLA-Tg mice have been widely used for detection of HLA class II-restricted T-cell epitopes [37,38]. We used in this study 004149-MM HLA-DRB1*0401-Tg mice [19]. The Tg mice express the transgene-encoded proteins consisted of antigen-binding domains from HLA-DRA and HLA-DRB1*0401 molecules and the remaining domains from the H2-E^d-α and H2-E^d-β chains, which has the same antigen-binding specificity as HLA-DRB1*0401 molecules and function of presenting antigens to T-cells. In addition, the molecules are the only species of MHC class II molecule expressed in the mice due to endogenous H2-Aβ, H2-Eα deficiency [19].

We predicted HLA-DR-restricted T-cell epitope candidates in the 20-mer peptide by computer-based program, ProPred [20] in this study. The ProPred algorithm is a virtual matrix-based T-cell epitope prediction program based on TEPITOPE program [39]. TEPITOPE first extracts all possible 9-amino acid peptide frames from

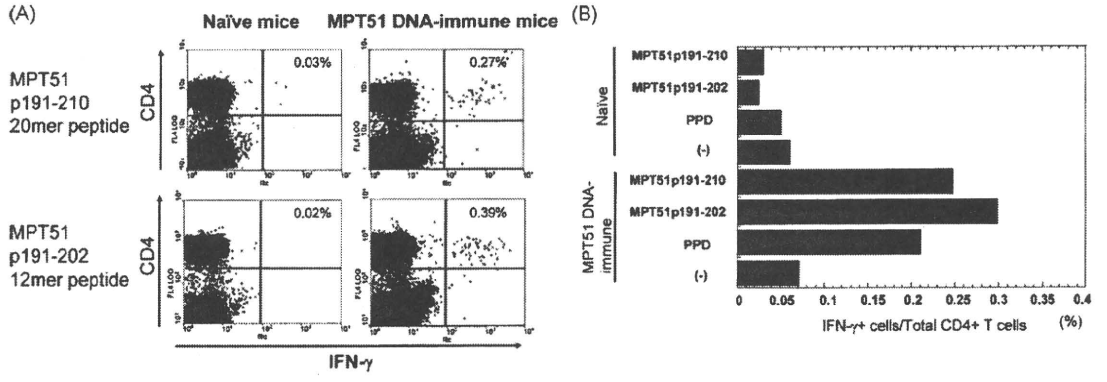

Fig. 5. Intracellular IFN-γ staining of spleen cells from MPT51 DNA-immune 004149-MM HLA-DRB1*0401-Tg mice. (A) The graph shows the percentages of IFN-γ-producing cells in total CD4+ T-cells after 4 h of stimulation with MPT51 p191–210 or p191–202 peptides. Intracellular IFN-γ and CD4 were detected by flow cytometry in or on the immune spleen cells after in vitro stimulation with the peptides. There were, however, no intracellular IFN-γ-positive CD8+ T-cells after in vitro stimulation with these peptides (data not shown). (B) Summary of frequency of IFN-γ-producing cells in CD4+ T-cells in spleens of MPT51 DNA-immune 004149-MM HLA-DRB1*0401-Tg mice. Data are means from two to four experiments.

the input protein sequence. Each amino acid in this 9-mer peptide is assigned to relative binding values using the position-specific, and peptide side chain-specific values from the virtual matrices. The first anchor position (p1) was shown to be a key factor for high affinity binding to HLA-DR molecules and only aliphatic or aromatic amino acid residues are considered as p1 anchor [40,41]. We found a core HLA-DRB1*0401 binding motif composed of 9-mer peptide, p194–202 (WVWSPTNPG) in MPT51 p191–210 region at the default threshold setting of ProPred algorithm. As described in Section 3, the same region was also predicted to bind to HLA-DR4 at the highest score in RANKPEP algorithm [21], which is programmed on position-specific scoring matrices.

MHC class II binding motifs are not so restricted compared with MHC class I binding motifs, but some rules of MHC class II binding motifs have been reported. The DRB1*0401 binding motif was reported to require an aromatic or aliphatic anchor residue in position 1 (Y, W, F, L, I, V, M), and another anchor residue in position 6, defined as either a hydroxyl (S or T) or hydrophobic (L, V, I, or M) residue [42]. MPT51 p194–202 amino acid residues, W in position 1 and T in position 6, fit the rule.

T-cell epitopes which are restricted to a variety of HLA alleles (promiscuous T-cell epitopes) are quite useful for development of epitope vaccines and diagnostic tools as human population is heterogeneous for HLA alleles. As described in Section 3, this region was predicted to bind 20 alleles among a total of 50 alleles of HLA-DR type in ProPred algorithm (40%). This result suggests that the region is a promiscuous T-cell epitope, although a report mentioned that the peptides predicted to bind over 50% HLA-DR alleles in ProPred were considered promiscuous for binding [43]. The frequency of the memory T-cells and the kinetics in human subjects after *M. tuberculosis* infection would be important issues to be clarified in future.

In conclusion, we identified one HLA-DRB1*0401-restricted CD4+ Th1 epitope on MPT51 in HLA-DRB1*0401-Tg mice, which may play a pivotal role in the protection against *M. tuberculosis* infection. The 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 and also for future TB vaccine design.

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Serodiagnosis of Pulmonary Disease Due to *Mycobacterium avium* Complex Proven by Bronchial Wash Culture

To the Editor:

The diagnosis of *Mycobacterium avium* complex (MAC) pulmonary disease (MAC-PD) is often complicated and time consuming. MAC-PD is diagnosed according to the guidelines set forth by the American Thoracic Society in 2007, which include clinical and microbiologic criteria.[1] Bronchoscopy to obtain bronchial wash for a bacterial culture is often considered in patients in whom MAC-PD is difficult to diagnose by routine sputum examination; however, it is difficult to perform bronchoscopy in all patients. A novel approach to help diagnose such cases has been needed.

We previously reported the usefulness of a serodiagnostic test to determine serum IgA antibodies against a mycobacterial glycopeptidolipid (GPL) core for diagnosing MAC-PD proven by sputum culture.[2-5] The present study was conducted to assess the accuracy of this test by comparing the results with bronchial wash cultures in patients with MAC-PD and negative sputum culture.

Bronchoscopy was performed in 56 patients suspected to have MAC-PD based on their symptoms and the presence of small nodular infiltrates with bronchiectasis on chest CT scans. None of the patients were known to be seropositive for HIV. The results of the bronchial wash cultures were positive for MAC in 28 patients (50%), who then received a diagnosis of MAC-PD. The culture results were negative for MAC in the remaining one-half, who were assigned to the non-MAC disease group. The levels of serum IgA antibody against the GPL core antigen of MAC were measured using an enzyme immunoassay kit (TAUNS Laboratory Inc; Shizuoka, Japan) before bronchoscopy, and the values were compared between the two groups.

Serum IgA antibody levels to GPL core antigen were significantly higher in the MAC-PD group (5.0 ± 4.7 U/mL) than in the non-MAC disease group (0.1 ± 0.3 U/mL) ($P < .0001$). With the cutoff value set at 0.7 U/mL according to a previous study,[5] the number of patients with seropositivity and seronegativity with or without MAC-PD is summarized in Table 1. The sensitivity, specificity, and positive and negative predictive values for diagnosing MAC-PD were 78.6%, 96.4%, 95.7%, and 81.8%, respectively.

In conclusion, the serodiagnostic test can accurately predict MAC positivity when compared with the results of bronchial wash cultures and may be safe and useful as an adjunct to diagnose MAC-PD. In particular, we consider that this approach may be useful in elderly patients for whom bronchoscopy cannot be performed because of other underlying conditions or in patients who are reluctant to undergo such an invasive procedure for very mild signs and symptoms.

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Table 1—*Results of the Serodiagnostic Test for* Mycobacterium avium *Complex Pulmonary Disease*

Patient Characteristics	Age, y	Sex, M (F)	Seropositive	Seronegative
MAC-PD, n = 28	65.8 ± 8.8	0 (28)	22	6
Non-MAC disease, n = 28	62.5 ± 13.7	10 (18)	1	27

The levels of serum IgA antibody to GPL core antigen were significantly higher in the MAC-PD group than in the non-MAC disease group ($P < .0001$). The sensitivity and specificity for diagnosing MAC-PD were 78.6% and 96.4%, respectively. GPL = glycopeptidolipid; MAC = *Mycobacterium avium* complex; MAC-PD = *M avium* complex pulmonary disease.

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High Prevalence of *Pseudomonas aeruginosa* From Oropharyngeal Biofilm in Patients With Cerebrovascular Infarction and Dysphagia

To the Editor:

Aspiration pneumonia develops after the aspiration of colonized oropharyngeal contents.[1] The elderly or patients with cerebrovascular disease (CVD) are often subjected to aspiration pneumonia because bacteria colonized in the oral cavity and oropharynx easily enter the lung during sleep and usually undergo repeated silent aspiration.[2] Aspiration pneumonia is increasing in patients with dysphagia, and aspiration pneumonia-associated mortality is a most serious problem in elderly patients. Interestingly, it has been reported that oral health care for elderly patients in nursing homes reduces bacterial pneumonia.[3,4] Therefore, it is very important to determine the characteristics of oropharyngeal microflora in patients with CVD to plan the optimum oral care to prevent aspiration pneumonia. From this standpoint, we investigated initial pharyngeal microflora in patients with CVD and dysphagia requiring daily nursing (Table 1). This study protocol was approved by the Ethics Committee of Chikamori Rehabilitation Hospital. We collected swab samples from the oropharynx of 55 patients with CVD (26 with dysphagia and 29 without dysphagia). To count the colony-forming units, the swabs, which were diffused into sterile medium, were inoculated onto agar plate using the spiral system as described previously.[5] In addition to bacterial culture, polymerase chain reaction with bacterial-specific primers was used for bacterial identification. A higher prevalence (38.5%; 10/26) of *Pseudomonas aeruginosa* was observed in patients with CVD and dysphagia than in patients with CVD and without dysphagia (3.4%; 1/29; $P < .01$). The prevalence of *Staphylococcus* spp (30.7% and 24.1%, respectively) and *Candida* spp (46.2% and 31.0%, respectively) in both groups was similar. Moreover, the bacterial number of *P aeruginosa* in patients with CVD and dysphagia was significantly higher than in the group without dysphagia. Ten (47.6%) of 21 patients with CVD and dysphagia who needed complete or some assistance in daily living had *P aeruginosa* in their oropharyngeal microflora. Regarding the mode of nutritional intake, seven (63.6%) of 11 patients with CVD and dysphagia who were administered nutrition through a catheter had *P aeruginosa* in their oropharyngeal microflora. Interestingly, four (25%) of 16 patients with CVD and dysphagia whose test results were negative for *P aeruginosa* in oropharyngeal microflora were also administered nutrition through a catheter, and only one (3.4%) of 29 patients with CVD and without dysphagia had positive test results for *P aeruginosa* in oropharyngeal microflora. More importantly, these observations suggest that the high detection rate of *P aeruginosa* from oropharyngeal microflora in patients with CVD is increased by the status of dysphagia, not catheter use. Our data highlight that the care of oropharyngeal microflora, especially *P aeruginosa*, may be important to prevent aspiration pneumonia in patients with CVD and dysphagia.

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総　説

結核菌に対するT細胞誘導ワクチンの試み

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結核菌は通性細胞内寄生細菌であり，その感染防御には細胞性免疫の誘導が必須である。主なエフェクターはCD4$^+$ 1型ヘルパーT細胞（Th1）とCD8$^+$細胞傷害性T細胞（CTL）である。結核菌抗原のうち，適切な感染防御抗原に特異的なT細胞を効率よく誘導することが，新規の結核ワクチンに求められている。

種々の免疫方法が，結核菌に対する細胞性免疫誘導のために検討されてきた。DNAワクチンは安全にかつ効率よく細胞性免疫，特にCD8$^+$ CTLを誘導できる。我々は，遺伝子銃法によるDNAワクチンの手法を用いて，結核菌抗原であるMPT51等に対する特異的T細胞エピトープを明らかにしてきた。弱毒リステリアをキャリアとしたAg85複合体DNAワクチンが結核菌感染防御免疫の誘導に有効であった。また組換えレトロウイルス導入樹状細胞ワクチンがCD8$^+$およびCD4$^+$ T細胞の誘導に有効であった。さらに組換えレンチウイルスを用いた経気道免疫が，肺局所におけるCD8$^+$ CTLの誘導に有効であることを示した。本稿ではこれらの我々の研究の知見を軸に，結核免疫研究の動向を概説したい。

1. はじめに

結核は，結核菌（*Mycobacterium tuberculosis*）による感染症である。1882年にRobert Kochが結核菌を発見してからすでに130年近くになるが，世界的にみると依然として最も重要な感染症のひとつである。世界では全人口の3分の1にあたる約20億人が感染しており，2006年の統計では約920万人の結核患者が発生し，約170万人が結核で死亡し，有病者は2,200万人である（118）。罹患者の多くは，HIVとの共感染が深刻な社会問題となっているサハラ砂漠以南のアフリカや多剤耐性結核菌が拡がっている東欧，旧ソ連の地域で発生している。これらの地域では，結核対策が緊急の課題である。

現在使われているワクチンは約90年前（1921年）に導入されたウシ結核菌（*Mycobacterium bovis*）の弱毒株であるBCG（bacille Calmette-Guérin）のみである。BCGの結核防御効果は，小児の結核性髄膜炎や粟粒結核には効果があるものの（87），調査および使用したBCG株の違いにより0～85％と異なり一定の評価は得られていない（23）。そこでBCGに代わる新世代のワクチン開発が求められている。

結核菌に暴露したヒトの20～50％が感染し，感染者のうち約5％が2～5年のうちに結核を発症するが，残りの95％のツベルクリン反応陽性感染者は結核菌の潜伏感染に移行すると考えられている。そのうち約5％に後に内在する結核菌が再燃し結核を発症すると考えられている（94）。すなわち結核菌感染者の多くでは，結核菌を排除しないものの再燃しないのには十分な感染防御免疫が働いているともいえる。新世代のワクチンは，このような感染防御免疫をより有効に機能させる必要がある。

2. 結核菌の感染防御免疫

1）結核菌に有効なエフェクター

結核菌は，主に肺胞マクロファージの食胞内で生き延びる細胞内寄生細菌である。このような細胞内寄生細菌に対する感染防御には，細胞性免疫が必須であることが知られている（46）。細胞性免疫のエフェクター細胞であるT細胞のうち，インターフェロン（IFN）-γの産生等のいわゆる1型免疫応答を示すCD4$^+$ 1型ヘルパーT細胞（CD4$^+$ Th1）とCD8$^+$細胞傷害性T細胞（CTL）が，結核菌の主要な感染防御エフェクター細胞である（46）。原則的には，食胞内寄生細菌に対しては，主要組織適合性複合体（major histocom-

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patibility complex; MHC）クラスII経路の抗原提示が行われCD4+ T細胞が誘導されるはずであり，CD8+ T細胞の誘導の機構は不明であった。しかし結核菌などの食胞内寄生細菌は，マクロファージに取り込まれた後，食胞内でクロス・プレゼンテーションによってMHCクラスI分子を介した抗原提示が行われることが示されている(37)。Kaufmannらのグループは，結核菌感染細胞ではアポトーシスが誘導され，それによって感染細胞から遊離したアポトーシス小胞（apototic vesicles）を介したクロス・プレゼンテーションが起こり，効率よくCD8+ T細胞が誘導されるのではないかと推測している(117)。なおCD8+ T細胞は，特に潜伏期の結核の制御に重要であるとの報告もある(108)。またCD4+ T細胞のサブタイプであるTh17細胞は，感染防御に重要なTh1細胞を肺局所に誘導することにより感染防御に役立っていると考えられている(47)。いずれにしても，これらのT細胞が認識する結核菌抗原の同定が，結核菌に対する有効な感染防御免疫の誘導にとって第1のステップである。

これらのエフェクター細胞が産生するサイトカインが，重要な役割を果たしていることが知られている。特にこれらの細胞が結核菌抗原に反応して産生するIFN-γは感染防御に重要であることが，IFN-γあるいはIFN-γ受容体の欠損マウスやヒトの遺伝疾患の解析から明らかになっている(25)。そのため，結核菌特異的T細胞から産生されるIFN-γ量やIFN-γ分泌T細胞数は，疑問も指摘されてはいるものの(1)，結核菌に対する感染防御の指標として考えられている。

なお，細胞内寄生細菌に対する感染防御において，抗体は効果がないというのが一般的な考え方であるが，結核菌表面のヘパリン結合性赤血球凝集素付着因子（heparin-binding haemagglutinin; HBHA）(81)やアラビノマンナン(103)に対する特異抗体の投与が，結核菌の経気道感染に対して感染防御に働くことも報告されている。このような細菌表面抗原に対する抗体は，結核菌が宿主細胞に侵入するのを阻害する効果があるのかもしれない。

2）結核菌の感染防御抗原

結核菌は，抗酸菌に特有な脂質に富む細胞壁を有するため，それら脂質が，抗原提示細胞上のCD1分子に結合し特異的免疫を誘導することが知られているが，主要な感染防御抗原はやはりタンパクだと考えられている。1998年にColeらの結核菌H37Rv株のゲノム塩基配列の報告(9)により，すべての結核菌遺伝子によるDNAワクチンは原則的に作製可能となったが，どのタンパク抗原を選択するかが重要な問題である。結核菌の死菌免疫ではなく生菌による免疫が，感染防御に有効であることから(77)，主に分泌タンパクが感染防御抗原となると考えられている。実際，結核の感染防御に有効な細胞性免疫は細胞質内のタンパクよりも分泌タンパクまたは細胞表面タンパクによって誘導されることがマウス，モルモットなどによる研究から判明している(2, 35)。記憶T細胞の，結核菌の電気泳動によるタンパク分画への応答性を解析することにより，主に約20-kDa以下の低分子量の分泌タンパク（culture fluid proteins; CFPs）および分子量30 kDa付近のAntigen（Ag）85複合体に属するタンパク，そして分泌タンパクではないが熱ショックタンパク（heat shock proteins; Hsp）が，免疫反応性が高いことがわかってきた。次に挙げる代表的な感染防御抗原が知られている。

Ag85複合体タンパク：Ag85複合体タンパクは，30～32-kDaの主要な分泌タンパクであり，結核菌の細胞壁の脂質成分を合成するミコリルトランスフェラーゼ活性，またフィブロネクチン結合活性を有する一群のタンパクである(6, 115)。Ag85A（p32A, 32-kDa），Ag85B（p30, MPT59, α抗原；30-kDa），Ag85Cからなる。これらのタンパクは，*Mycobacterium*属でよく保存されており，またこれらのタンパクに相同性のあるタンパクとしてMPT51がある(66, 73)。

低分子量分泌タンパク：20-kDa以下の低分子量分泌タンパク抗原の中で特に研究されているのがESAT6（early secreted antigenic target 6-kDa protein）とCFP10（culture filtrate protein 10）である。ESAT6とCFP10の遺伝子は，すべてのBCGで欠損しているゲノムRD1（region of deletion-1）領域にマップされている。従って，これらの抗原をツベルクリン液（purified protein derivative; PPD）の代わりに診断に用いることにより，結核菌感染による免疫応答とBCGによる免疫応答とを識別できるため，近年，特異度・敏感度の高い結核診断薬として用いられている（QuantiFERON-TB；Cellstis社）。

熱ショックタンパク：種々の分子量を持つ熱ショックタンパクは，マウスやヒトの抗体やT細胞の認識抗原であることが判明している。このうちワクチン候補としてよく研究されているHsp65（heat shock protein 65-kDa）は肺胞マクロファージ内の結核菌が多量に産生するストレスタンパクであり(52)，大腸菌GroEL，ヒトHsp60とアミノ酸レベルで50%以上の相同性が報告されている。これ以外にHsp70と呼ばれているタンパクもあるが，これは大腸菌DnaK，ヒトHsp70とアミノ酸レベルで50%以上の相同性が報告されている。これらの熱ショックタンパク特異的T細胞が結核菌感染したヒトやマウスで強力に誘導され，それが感染防御に有効であることが報告されている(52)。

休眠期タンパク：上記の分泌タンパクは，主に感染の急性期（acute stage）に結核菌が分泌するタンパクであるが，それ以外に感染の比較的後期あるいは休眠期（dormant stage）に発現し，かつT細胞によって認識される抗原タンパクがわかってきた。休眠期に発現するタンパクとしては，DosRレギュロン内のタンパク(44)，あるいはDNA結合タンパクであるMDP1（mycobacterial DNA-binding protein-1）(55)等がある。

3）結核菌の感染防御抗原T細胞エピトープ

結核菌抗原のT細胞エピトープは，結核菌抗原の中で，実際に抗原提示細胞上のMHC分子に結合し，特異的T細胞が認識するペプチド部分のことである。MHC分子に結合できるペプチドの中で，T細胞上のT細胞受容体（T-cell

recepotor; TCR）分子が認識してT細胞を誘導できる抗原ペプチドのことをいう。T 細胞エピトープであるためには，MHC 分子に結合できることは必要条件であるが十分条件ではなく，通常，抗原タンパクの中でTCRが認識しうるT細胞エピトープペプチド部分は数箇所に限定される（エピトープ・セレクション）。

遺伝子銃法によるDNAワクチンは，結核菌抗原のT細胞エピトープの同定にたいへん有効である。Huygen らのグループは，この方法により Ag85 複合体抗原（Ag85A, Ag85B, Ag85C）のマウスT細胞エピトープを同定している（11，18）。それによると，遺伝子銃法によるDNAワクチンにより結核菌感染に比べ，より多くの $CD4^+$ および $CD8^+$ T細胞エピトープが同定された。すなわちより多くの抗原特異的T細胞集団が誘導できたと報告している。我々も遺伝子銃法による DNA ワクチンは，再現性よく安定に免疫応答が起こることを確認している（123）。DNA ワクチンにより，宿主細胞，特に抗原提示細胞内で目的遺伝子からのタンパク合成が起こるため，MHC クラスI分子を介した抗原提示機構が働くことが推測され，特に $CD8^+$ T 細胞エピトープは非常に効率よく検出することができると考えられる（49）。

我々のT細胞エピトープ同定の方法は，結核菌抗原遺伝子を組み込んだ DNA ワクチンを純系マウスあるいは，ヒト HLA のトランスジェニックマウスに免疫した後，脾臓を取り出し，脾細胞の結核菌抗原全体を網羅する約 20 アミノ酸からなるペプチドとの反応性を指標にしてT細胞エピトープを含むペプチド領域を限定する。その後，BIMAS（BioInformatics and Molecular Analysis Section）や SYFPEITHI 等の MHC 結合タンパクの予測アルゴリズム（80, 85）を併用し，$CD8^+$ T細胞エピトープなら8～10アミノ酸からなるペプチドに絞り込む方法である（図1）。

主要な結核菌抗原のT細胞エピトープの一部を表1にまとめた。感染防御抗原のT細胞エピトープを同定することには次のような意義があると考えている。まず第1に，感染防御抗原に対するT細胞応答を正確に解析できる。T細胞エピトープがわかれば，MHC テトラマー法（主に $CD8^+$ T 細胞の場合）や細胞内サイトカイン染色法を用いれば簡便に特定のエピトープ特異的T細胞の数を計測することが可能である。たとえばAg85A分子はC57BL/6マウス（$H2^b$）では $CD8^+$ T細胞エピトープが存在しないため，$CD4^+$ T細胞ノックアウト（KO）マウスではAg85A DNAワクチンが結核菌感染に無効であるが $CD8^+$ T 細胞 KO マウスでは有効であるとの報告がある（16）。我々も MPT51 T 細胞エピトープの解析により，MPT51 に対して BALB/c マウスでは $CD8^+$ T細胞，C57BL/6マウスでは $CD4^+$ T細胞のみが応答することを示した（97）。T 細胞エピトープによっては，感染防御に非常に有効なものとそうでないもの，あるいは逆に悪影響を及ぼすもの等，ヒエラルキーが存在する（69, 113, 120）。そこでT細胞エピトープが明らかになれば，感染防御に有効なT細胞エピトープに特異的T細胞のみを誘導することが可能となる。たとえば $Ag85B_{240–254}$（Ag85B の 240～254 番目のアミノ酸部分；peptide-25）は強力な IFN-γ を誘導し，感染防御に有効であることが報告されている（45）。さらに，T 細胞エピトープワクチンを用いれば，細胞内寄生菌に対する感染防御に悪影響を及ぼす可能性のある抗体の誘導を避けることができる。

これまで我々は先に述べた結核菌感染防御抗原のうち，MPT51 のマウスおよびヒト T 細胞エピトープ（3，4，97，110），MDP1 のマウス T 細胞エピトープ（96），および低分子量分泌タンパク（論文投稿中）のマウス T 細胞エピトープを同定してきた。そのうち，$MPT51_{24–32}$ は $H2\text{-}D^d$ 拘束性の優勢（ドミナント）マウス $CD8^+$ T 細胞エピトープであり，種々のワクチンの解析に利用している（33，107）。

ここに挙げたT細胞エピトープのうち，特に $CFP10_{32–39}$ ペプチド特異的 $CD8^+$ T 細胞は，C3H 系マウスの結核菌噴霧感染3週後，肺内 $CD8^+$ T 細胞の30%を占めるほど増幅し（43），かつこのエピトープ特異的T細胞は感染防御能があることが示されている（119）。興味あることに，ESAT6

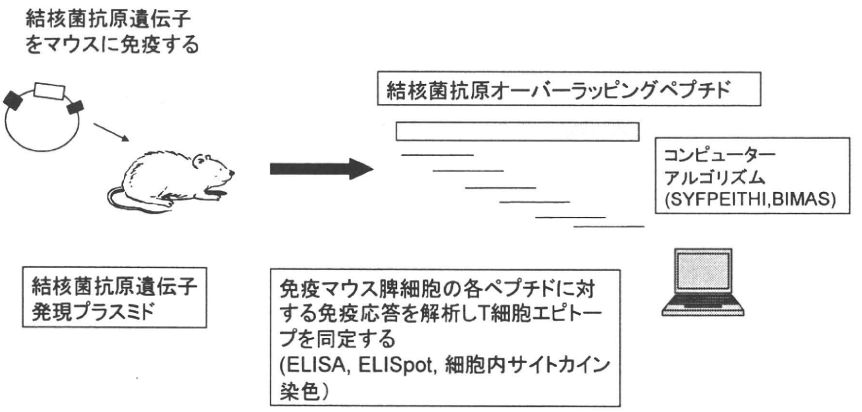

図1. DNAワクチンを用いた結核菌抗原T細胞エピトープの同定
遺伝子銃を用いたDNAワクチンは，再現性よく特異的T細胞を誘導するため，抗原のT細胞エピトープの同定に最適である。抗原全体をカバーするペプチドライブラリーとMHC結合ペプチド推測アルゴリズム等を用いることにより効率よくT細胞エピトープを同定できる（4，96，97，110）。

表1. 結核菌抗原のT細胞エピトープ（一部）

抗原	ペプチド	MHC拘束性	反応性T細胞	参考文献
Ag85A	p60–68 (9-mer)	K^d	マウスCD8	11
	p144–152 (9-mer)	K^d	マウスCD8	11
	p101–120 (20-mer)	E^d	マウスCD4	39
	p241–260 (20-mer)	A^b	マウスCD4	39
	p261–280 (20-mer)	A^b	マウスCD4	18
Ag85B	p240–254 (15-mer)	A^b	マウスCD4	45, 122
	p262–279 (18-mer)	A^b	マウスCD4	18
	p143–152 (10-mer)	A*0201	ヒトCD8	27
	p199–207 (9-mer)	A*0201	ヒトCD8	27
	p10–27 (18-mer)	DR3, 52, 53	ヒトCD4	64
	p19–36 (18-mer)	Promiscuous	ヒトCD4	64
	p91–108 (18-mer)	Promiscuous	ヒトCD4	64
MPT51	p24–32 (9-mer)	D^d	マウスCD8	97
	p171–190 (20-mer)	A^b	マウスCD4	97
	p51–70 (10-mer)	A*0201	ヒトCD8	4
	p191–202 (12-mer)	Promiscuous	ヒトCD4	110
Hsp65	p489–503 (15-mer)	A^d	マウスCD4	65
	p369–377 (19-mer)	A*0201	ヒトCD8	8
	p3–13 (11-mer)	DR3	ヒトCD4	26
ESAT6	p1–20 (20-mer)	$H2^{b,d}$	マウスCD4	7
	p51–70 (20-mer)	$H2^{a,k}$	マウスCD4	7
	p72–95 (24-mer)	DR52, DQ2	ヒトCD4	63
CFP10	p32–39 (8-mer)	K^k	マウスCD8	43
	p11–25 (15-mer)	A^k	マウスCD4	43
MDP1	p23–31 (9-mer)	D^b	マウスCD8	96
	p41–60 (20-mer)	A^d, E^k	マウスCD4	96
	p111–130 (20-mer)	E^k	マウスCD4	96
	p141–160 (20-mer)	E^k	マウスCD4	96

のT細胞エピトープである$ESAT6_{1-20}$と$ESAT6_{51-70}$ペプチドの感染防御誘導能を，C57BL/6とCBAのF1マウス（$H2^{b/k}$）のペプチド免疫で比較したところ，強い免疫応答性を示す$ESAT6_{1-20}$ペプチド（ドミナント・エピトープ）ではなく，より弱い免疫応答性を示す$ESAT6_{51-70}$ペプチド（サブドミナント・エピトープ）の免疫でのみ，感染防御効果が認められている(75)。感染防御能の強さと免疫応答性の強さは必ずしも一致しない可能性もあり，この点に関しては今後の研究が必要である。

基本的に抗原提示分子であるMHC分子の種類によってT細胞エピトープも異なるが，表1のAg85BおよびMPT51抗原のHLA-DR拘束性T細胞エピトープで認められるように多くのタイプのHLA-DRによって抗原提示されうる（promiscuous）T細胞エピトープもある。多くのMHC分子型の中で互いに似通ったペプチドを結合しうる，いわゆるMHC分子のスーパータイプがあり，同一のスーパータイプに属するMHC分子では結合ペプチドも共通である可能性が高い。特に日本人の約60%が持つHLA-A24に結合する抗原ペプチドとマウスH2-K^dに結合するペプチドが類似していることから，マウスH2-K^d拘束性のT細胞エピトープは，HLA-A24拘束性のT細胞エピトープでもある可能性が高いことが指摘されている(74)。なおマウスで感染防御抗原として機能するタンパクは基本的にはヒトにおいても感染防御抗原であることが多い。したがってマウスのT細胞エピトープの同定は，マウスで感染実験を行なうときに有益であるのみならずヒトの研究の基盤になると考えている。

3. 組換えBCGの試み

遺伝子操作を利用してBCGを改良する試みが行なわれている(95)。BCGによる$CD8^+$T細胞応答の報告はあるものの，BCG免疫においては，MHCクラスI経路を介した抗原提示は，結核菌に比べ弱いと考えられている。結核菌と対照的に，BCGの増殖はβ2-ミクログロブリン欠損マウスにおいて影響を受けない(24, 50)。我々もBCG免疫により$CD4^+$T細胞の応答は強いが$CD8^+$T細胞の応答は弱い