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

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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 β2-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 β2-microglobulin-dependent systems other than the MHC class Ia system, such as the iron uptake system of the host macrophages that depends on β2-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 β2-microglobulin-dependent MHC class Ib molecules expressed almost exclusively on dendritic 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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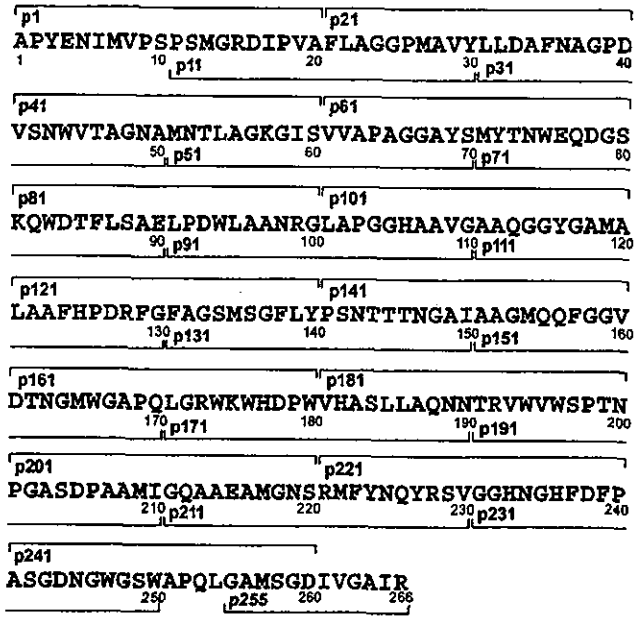

FIG. 1. Schematic representation of the 26 overlapping synthetic peptides from MPT51 of *M. tuberculosis*. All peptides were synthesized as 20-mer molecules overlapping by 10 aa with the neighboring peptides, with exception of a carboxyl-terminal 12-mer.

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 subodominat epitope) in C57BL/6 ($H2^b$) mice by employing gene gun immunization with plasmid DNA encoding MPT51, overlapping peptides spanning the entire mature sequence, and a computer-assisted algorithm.

MATERIALS AND METHODS

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

Construction of a plasmid DNA vaccine, pCI-MPT51. The DNA encoding the mature MPT51 molecule (Rv3803c) was amplified from a plasmid, pMB49 (29), by PCR with following primers: forward primer 5′-CCTCTAGAATGGCCCAT 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/in^2. Mice were *inoculated* with 2 μg of plasmid DNA four times at 1-week intervals. Mice were also vaccinated intravenously with 10^6 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 Davit H. Raulet, University of California, Berkeley) (9), and with the L^d gene (RMA-S-L^d [RL^d-E2]; provided by Keiko Udaka, 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%* CO_2 *in an* incubator.

Preparation of splenocyte culture supernatants for evaluation of IFN-γ production. Spleen cell suspensions (1×10^6 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% CO_2. 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 (4×10^6 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 (1×10^6 cells) were stimulated with 2×10^5 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 XMG1.2 (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 (1×10^7 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 μl) were incubated for 4 h at 37°C in the presence or absence of 5 μM 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% NaN_3 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 μM). 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 (Gilbertsville, Pa.) and a phycoerythrin-conjugated MAb specific for H2-L^d (28-14-8; eBiosceience, 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) × 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 μg 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 ^{51}Cr 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 μM 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)] × 100.

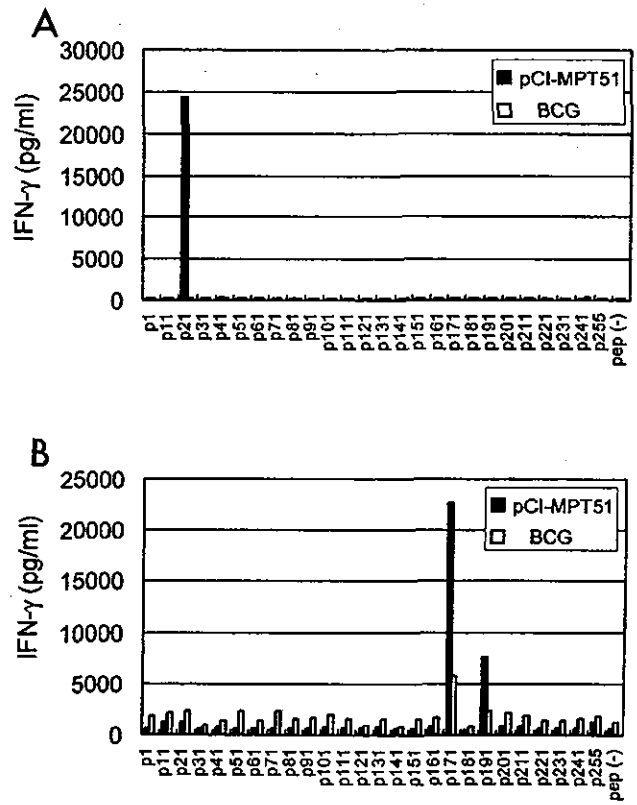

FIG. 2. IFN-γ production by spleen cells from BALB/c (A) or C57BL/6 mice (B) immunized with plasmid DNA encoding MPT51 (solid columns) or *M. bovis* BCG (open columns) in response to 1 of the 26 overlapping peptides (5 μM) covering the MPT51 molecule or medium alone [pep(−)]. Spleen cells from naïve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

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 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 cytomeric 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.dcrt.nih.gov/cgi-bin/molbio/ken_parker_comboform) (33) and SYFPEITHI Epitope Prediction (http://www.syfpeithi.de/) (35). We first synthesized three peptides, p22-32, p29-37, and p25-35 (Table 1), since p22-32 and p25-35 contain five H2 class Ia-binding candidate epitopes and two L^d-binding candidate epitopes, respectively, and p29-37 exhibits a high binding score with K^d. Cytometric analysis indicated that p22-32, but not p29-37 or p25-35, could significantly stimulate the immune $CD8^+$ T cells to synthesize IFN-γ (Fig. 3A). p25-35 exhibited poor IFN-γ inducer activity in the immune spleen cells, per-

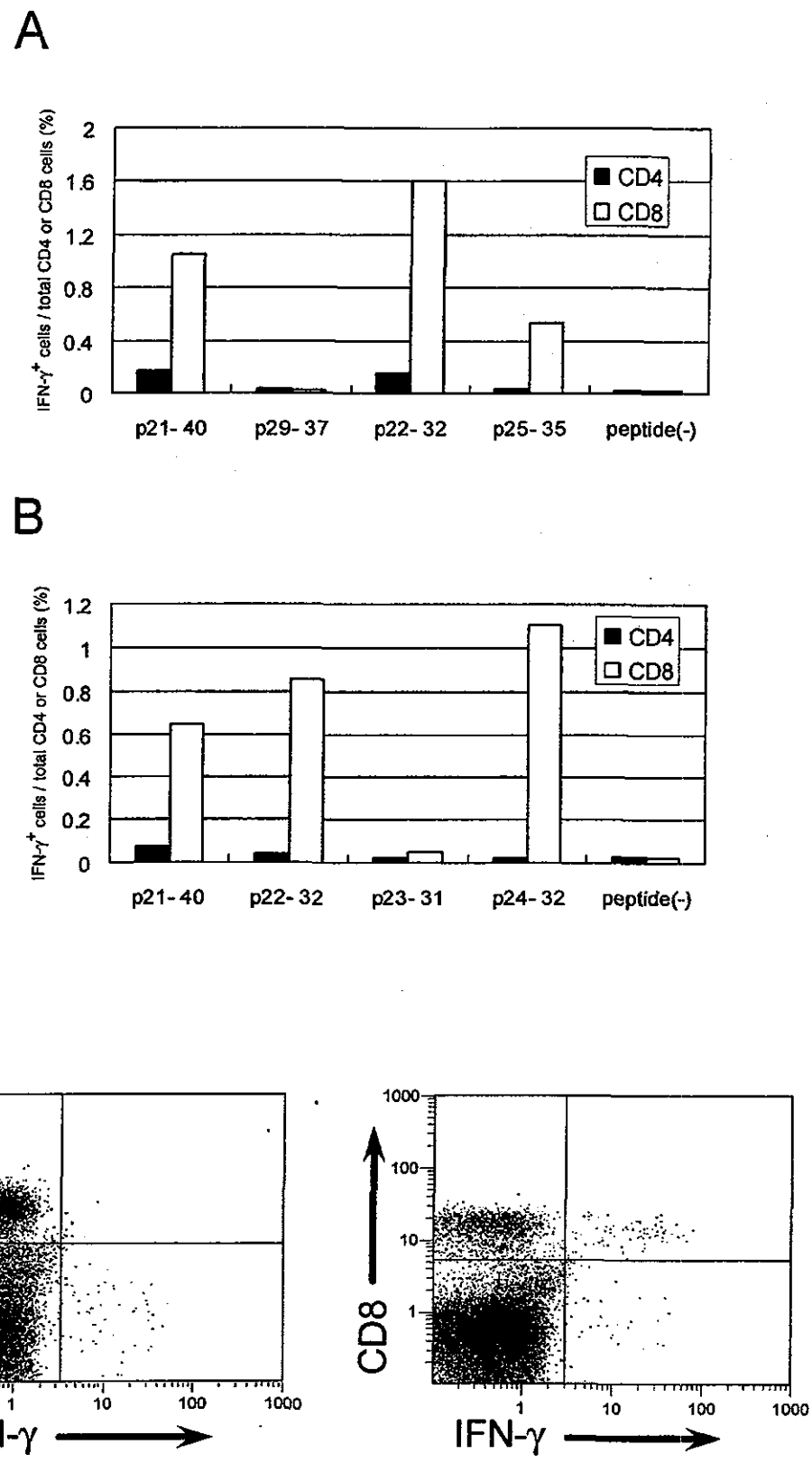

FIG. 3. *Identification of* a T-cell epitope on the MPT51 p21-40 peptide and a T-cell subset recognizing the epitope in BALB/c mice. Three-color flow cytometric analyses were performed to detect intracellular IFN-γ and T-cell subsets. (*A*) p22-32, but not p29-37 or p25-35, in the p21-40 peptide contained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

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	GPHAVYLL	57.6	—	—
p23–32	AGGPHAVYLL	11	—	—
p22–31	LAGGPHAVYL	40, 11	—	—
p23–31	AGGPHAVYL	57, 16	20	15
p24–32	GGPHAVYLL	48, 12	400	4.5, 12
p29–37	VYLLDAFNA	120	—	—
p25–35	GPHAVYLLDAF			
p27–35	HAVYLLDAF	—	—	10
p25–34	GPHAVYLLDA	—	—	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-

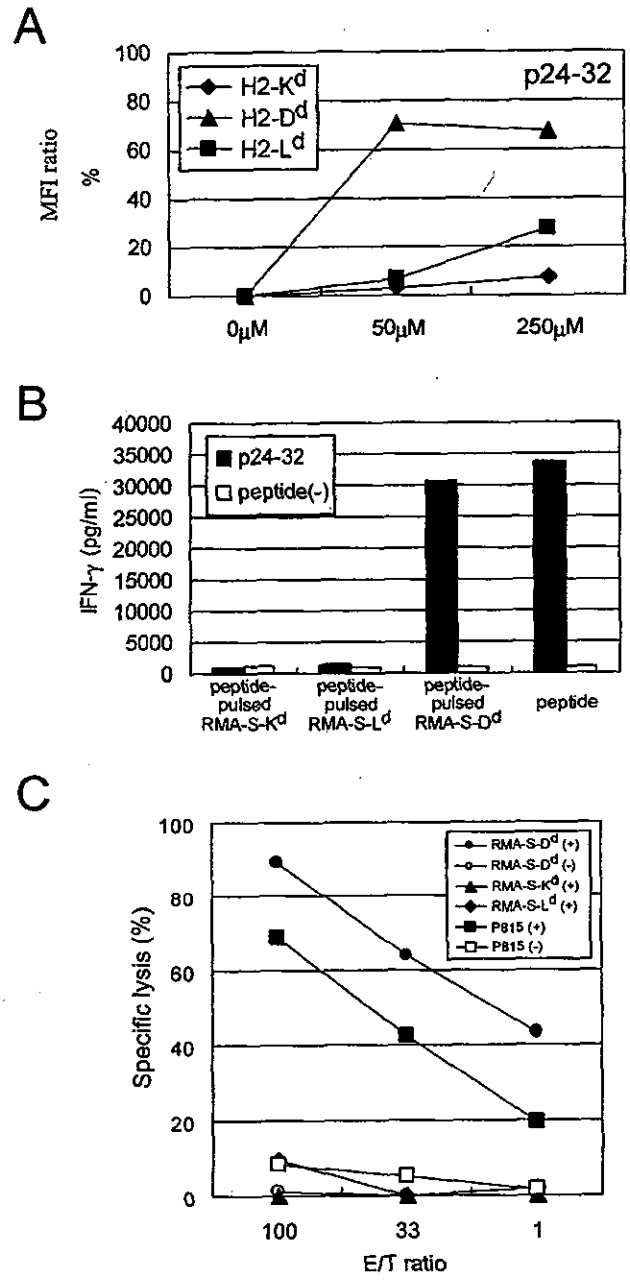

FIG. 4. p24-32 peptide binds to H2-D^d and can be recognized by immune T cells in the context of H2-D^d. (A) MHC class I stabilization assay indicated that the p24-32 peptide binds to H2-D^d. The p24-32 peptide was able to stabilize expression of D^d molecules. The MFI ratio was calculated as described 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.

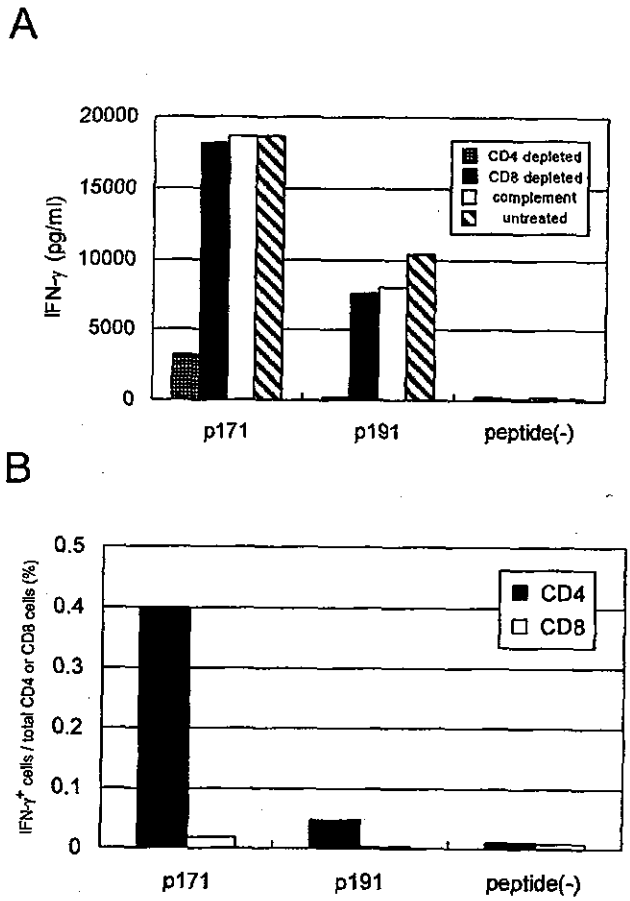

FIG. 5. p171 and p191 are $CD4^+$ Th1 epitopes in C57BL/6 mice. (A) Depletion of $CD4^+$ T cells with MAb plus complement, but not $CD8^+$ T cells, eliminated IFN-γ production in response to the p171 and p191 peptides. (B) Three-color flow cytometric analyses for staining of intracellular IFN-γ and T-cell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

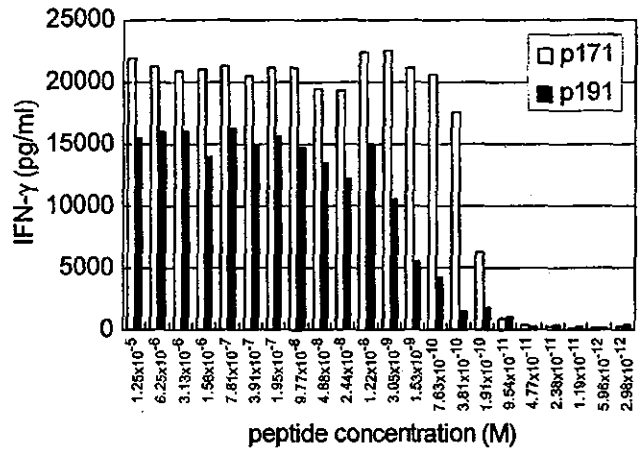

FIG. 6. p171 and p191 are dominant and subdominant Th1 epitopes in C57BL/6 mice. The immune spleen cells were stimulated with serially twofold diluted p171 and p191 peptides, and the IFN-γ concentrations in the supernatants were determined.

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 $V\beta\ 11^+$ 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 proteosome), (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 subdominat Th1 epitope, which are located in p171-190 and p191-210, respectively. Since C57BL/6 mice have a deletion in the Eα 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 $H2^b$ β2-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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免疫と疾患

（前篇）

—自然・獲得免疫と疾患—

宮坂信之 監修

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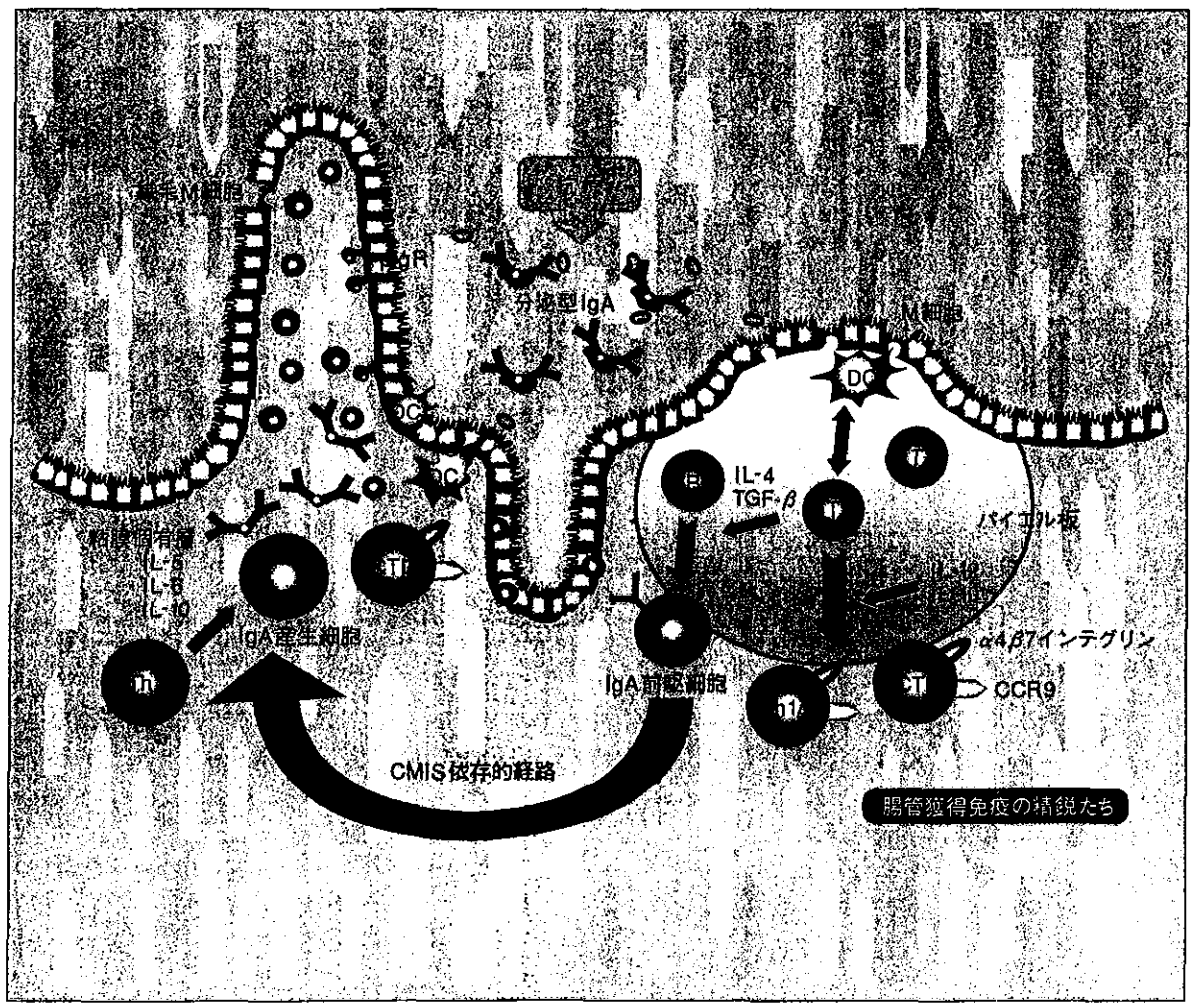

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● 自然・獲得免疫と疾患

結　核

＊国立病院機構近畿中央胸部疾患センター 臨床研究センター 結核研究部長
岡 田 全 司＊

要 旨

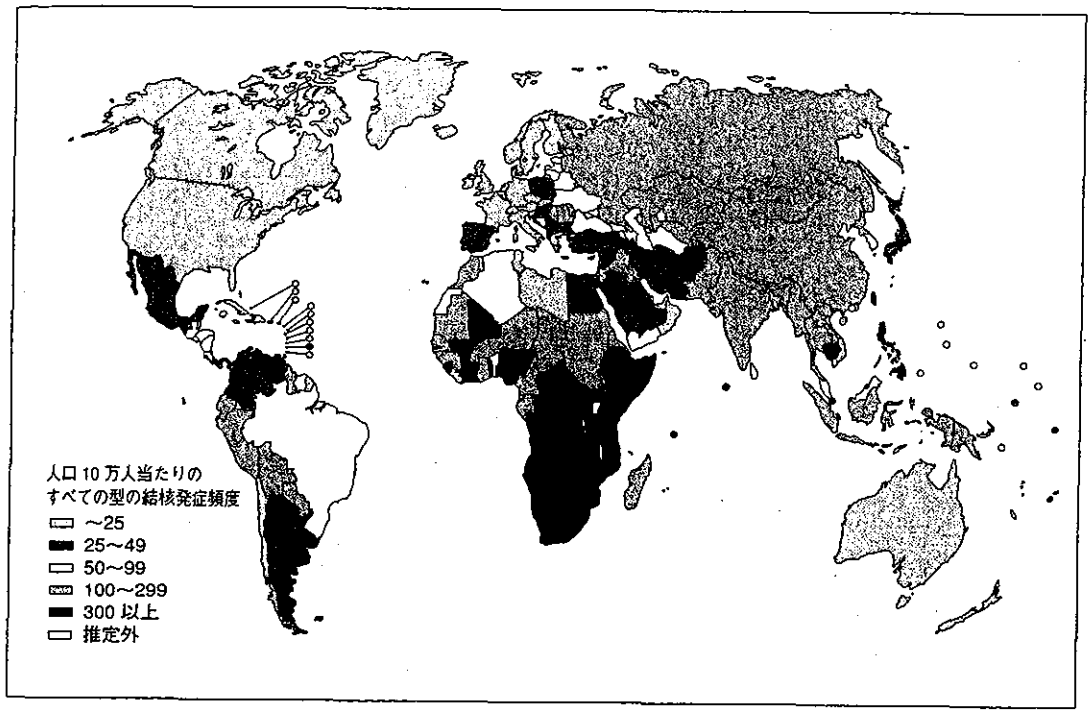
結核症は毎年800万人発症，200万人が死亡の世界最大の感染症である．結核に対する宿主抵抗性は獲得免疫のT細胞免疫，特にキラーT，Th1細胞，我々が示した結核菌殺傷タンパク質granulysinが重要である．一方，TLRなどを介する結核自然免疫も明らかとなりつつある．我々は世界に先駆けてBCGより100倍強力なHsp65＋IL－12 DNAワクチンを開発した．T細胞免疫を介する結核予防ワクチン効果を示した．

はじめに

結核は，いまだに世界の1／3の20億人が結核菌に感染しており，その中から毎年800万人の結核患者が発症し，200万人が毎年結核で死亡している最大の感染症の1つである（図1 WHOレポート2002年）[1~4]．本邦でも1998年から結核罹患率の増加・横ばいが認められ，1999年“結核緊急事態宣言”が厚生省より出された．結核症に対する宿主の抵抗性イコール細胞性免疫といって過言ではない．特に獲得免疫（キラーT細胞とTh1ヘルパーT細胞）が重要であり，最近では自然免疫の結核への関与が再び重要視されている．1998年，米国CDCは結核に対し，政府・学術機関・企業が一体となって新世代の

キーワード：キラーT細胞，granulysin，結核ワクチン，結核症，
Hsp65 DNA＋IL－12 DNAワクッン

図1　2002年度推定結核罹患率頻度



結核ワクチン開発の必要性を強く主張する発表をした．また，ACETは国民の健康に対する大敵である結核撲滅のためには，BCGに代わる有効なワクチンが必要であることを示した．しかしながら，BCGに代わる結核ワクチンは欧米でも臨床応用には至っていない．我々はBCGよりもはるかに強力なDNAワクチンやリコンビナントBCGワクチンの開発に成功した（図2）[5~8]．新しい抗結核ワクチン開発と結核感染免疫におけるキラーT細胞の機能解明および自然免疫のToll－like受容体，マクロファージ（Mφ）と結核についても述べる[9)10)]．

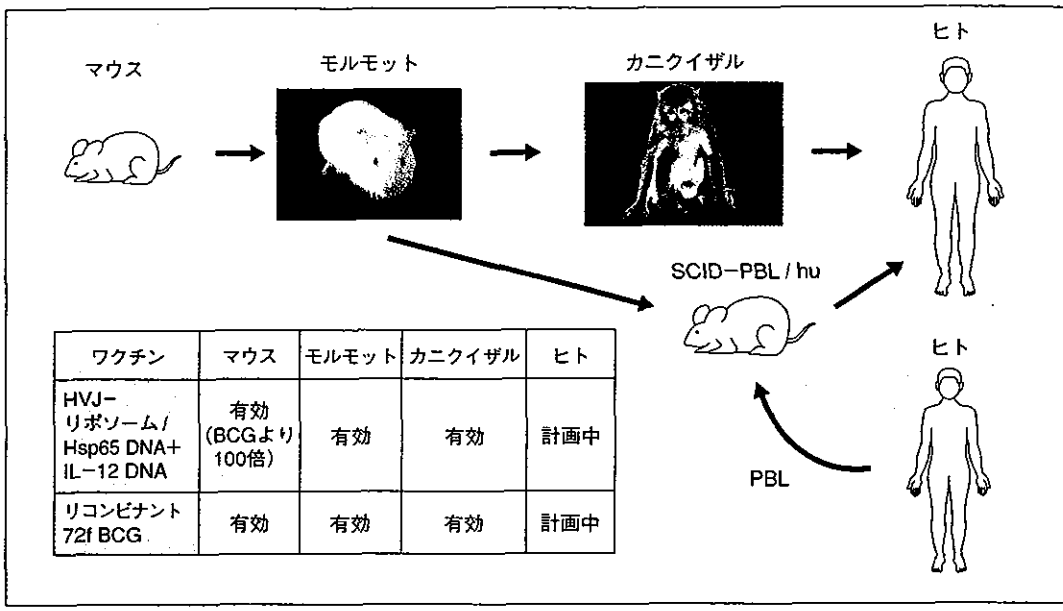
結　核　症

1．結核症の現状

結核症は最大の再興感染症で，HIV感染に伴う結核合併症や多剤耐性結核が大きな問題である．

2000年の本邦結核死亡率は10万人に2.1，罹患率は31.0である．日本の結核罹患率は，欧米の約5倍も高く，アジア（中国，インドな

図2 新しい結核ワクチンの開発



ワクチン	マウス	モルモット	カニクイザル	ヒト
HVJ−リポソーム/Hsp65 DNA+IL−12 DNA	有効（BCGより100倍）	有効	有効	計画中
リコンビナント72f BCG	有効	有効	有効	計画中

略語：巻末の「今号の略語」参照

ど）やアフリカ地域に多い．

感染した人の5～10％の人が発病し，発病は免れた人でも1/3以上の人は結核菌を身体の中に抱えたまま高齢に達している．結核菌は身体の抵抗力（免疫力）によって抑え込まれ冬眠状態（dormacy）になっている．高齢，糖尿病，エイズ，副腎皮質ホルモンによる治療，慢性腎不全（人工透析），抗関節リウマチ薬　抗 TNFα 抗体などで免疫力が低下すると，冬眠していた結核菌が暴れ出す．

2．BCG ワクチン

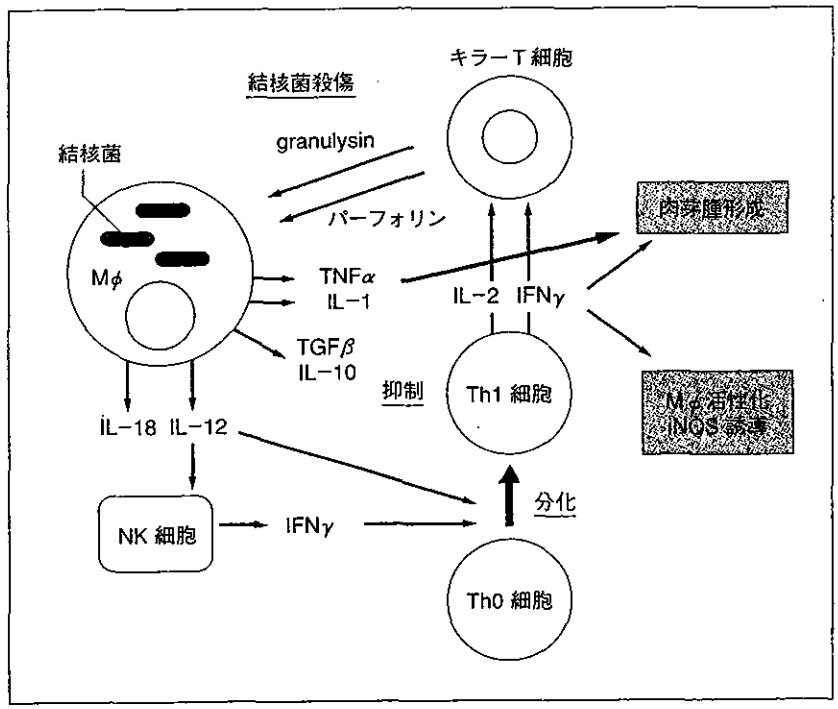
BCG は結核予防ワクチンとして，本邦では 2003 年3月まで小児，小，中学生の予防ワクチンとして使用されてきた．しかし，小児結核（特に結核性髄膜炎）には有効であるが成人に対してのワクチン効果は，ばらつきが大きく賛否両論であり，本邦でも BCG 接種は乳幼児のみと法改正がなされた[10]．

したがって成人にも有効な新しい結核ワクチンの開発が切望されている．

3．WHO STOP TB Partnership

新しい結核ワクチンの開発研究が高く評価され WHO STOP TB

図3 抗結核免疫とマクロファージ，ヘルパーT細胞，キラーT細胞活性化



Mφ：マクロファージ
略語：巻末の「今号の略語」参照

Partnership および WHO STOP TB VACCIENE GROUP MEETING メンバーに選出された．

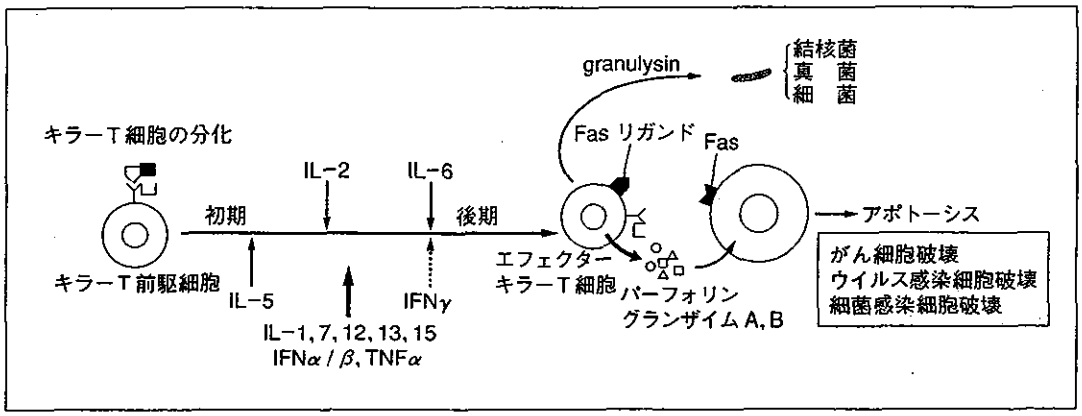
獲得免疫と結核

結核感染に対する免疫力は Mφ，$CD4^+$T細胞，NK 細胞，γδT細胞，キラーT細胞（$CD8^+$T と $CD8^-$T）および肉芽腫形成の総合的な抵抗力である（図3）．また，1998 年 Nature に結核菌 H37Rv ゲノム全塩基が掲載され，遺伝子レベルで結核免疫を解析しうることになった[11]．

1．キラーT細胞（$CD8^+$T細胞）

CD8 あるいは β_2 ミクログロブリン遺伝子や TAP 遺伝子ノックアウトマウスでは抗結核免疫が十分でなく，動物は死亡する．すなわち，結核における $CD8^+$T細胞はマウスで抗結核免疫に重要である（図4）．

図4 キラーT細胞活性化と細胞傷害機構

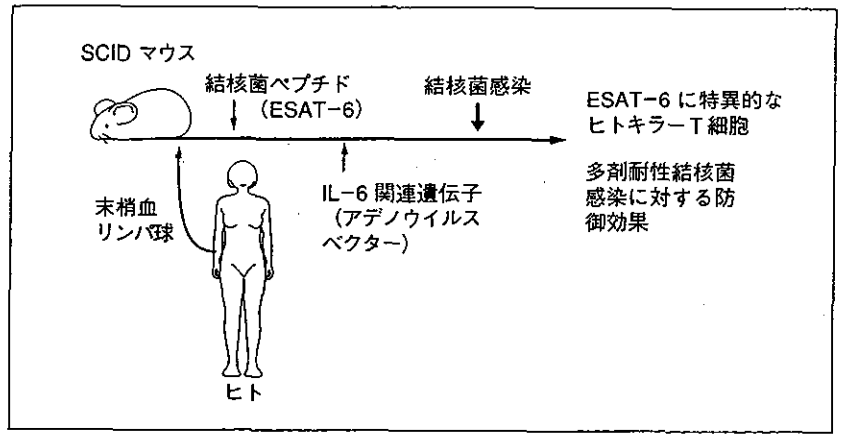


略語：巻末の「今号の略語」参照

キラーT細胞の1つの役割として IFNγ を分泌して抗結核免疫に寄与するが，次に述べる結核感染 Mφ を殺して，結核菌の増殖の場をなくし結核菌を殺す役割の方が重要である．$CD8^+$T細胞が結核菌で感染した Mφ を Fas 依存性，顆粒依存性の機構で溶かし，最終的には結核菌を殺すことが報告されている[12)13)]．このT細胞は CD1 拘束性でミコール酸，LAM，ホスファチジルイノシトール，グルコースモノミコール酸，イソプレノイド糖脂質（Cd1c と結合）などの結核菌脂質とリポグリカンを認識する．このキラーT細胞の顆粒内のタンパク質である granulysin は直接細胞外の結核菌を殺す．この機序は結核菌細胞膜を不完全な状態にすることによる．granulysin は病原細菌，真菌，寄生虫の生存を減少させる．さらにパーフォリンとの共存下で Mφ 内の結核菌も殺すと考えられている．これはパーフォリンにより Mφ に穴が開き，Mφ 内の結核菌に直接 granulysin が作用するためと思われる．我々は結核患者，特に多剤耐性結核患者ではキラーT細胞の mRNA の発現およびタンパク質の発現が低下していることを明らかにした[5)7)]．すなわち，我々はキラーT細胞の granulysin（分子量 9,000）産生低下が多剤耐性結核発症と大きな関連があるのではないかと考えている．一方，キラーT細胞の TRAIL（TNF－related apoptosis inducing ligand）とパーフォリンが抗結核免疫に重要である興味深い結果を得た．

一方，MHC クラスI拘束性の結核菌の 38 kD タンパク質，Hsp65

図5 SCID-PBL/huマウスを用いた結核菌ペプチドに特異的なヒトキラーT細胞の *in vivo* における誘導



略語：巻末の「今号の略語」参照

タンパク質を認識するマウス $CD8^+$ キラーT細胞や 19 kD タンパク質，Ag85，CFP10（Mtb11）を認識するヒト $CD8^+$ キラーT細胞が報告されている[3]．ESAT-6 抗原に対するキラーT細胞で HLA-A2 とは 82～90 位の 9 個のアミノ酸 AMASTEGNV，が結合してキラーT細胞がこれらを認識する．我々は世界に先駆けて確立した，ヒト生体内結核免疫応答解析モデル SCID-PBL/hu に，この ESAT-6 ペプチドを投与し，これに特異的で HLA-A2 拘束性を示すヒトキラーT細胞を生体内で誘導することに初めて成功した[2)7)13)]（図5）．

Reed S，Alderson M R らは結核菌に対するヒト CD8 陽性キラーT細胞クローンを確立したが，HLA-A，B，C，DR，DQ，CD1 に拘束性を示さない非古典的拘束性キラーT細胞と古典的な HLA に拘束性を示すキラーT細胞クローンの2種を確立した．またI-E領域に拘束性の結核特異的キラーT細胞も報告された．

2．キラーT細胞分化とサイトカイン（キラーT細胞分化因子）

我々は $CD8^+$ キラーT細胞（Tc）の誘導にはヘルパーT細胞（Th 細胞）から産生されるサイトカインが必要であることを初めて明らかにした．MHC クラスII抗原を認識しキラーT細胞分化因子を産生する Th 細胞は $CD4^+CD8^-$ であり，MHC クラスI抗原を認識しキラーT細胞分化因子を産生する Th 細胞は $CD8^+$ である．また，モノクローナル抗 IL-2 抗体を用いて，IL-2 はキラーT細胞誘導に必須な

因子の1つであることを示した[14]（図4）.

さらに，IL－2 とは異なるサイトカインもT細胞分化誘導に必要であることをキラーT細胞分化因子を産生するヒトT細胞ハイブリドーマ，および IL－2 依存性ヒト Th クローンを世界に先駆けて確立し明らかにした．その解析の結果，IL－6，IFNγ がキラーT細胞分化因子として強力なキラーT細胞分化を誘導することを明らかにした[15][16]．我々は IL－6 が Tc 誘導の後期の分化段階に作用することを解明した[16]（図4）．多剤耐性結核患者 PBL において，これらのキラーT細胞分化因子すなわち IL－2，IFNγ，IL－6 の著明な低下を認めた[5][6][8~10]．また，糖尿病合併難治性結核患者では PPD 特異的キラーT細胞の分化誘導の著しい低下を明らかにした[5][6][8~10]．

3．サイトカインと結核免疫

抗結核免疫に IFNγ，TNFα，IL－6，IL－12 が重要であることは解析されている．（文献[9]参照）

4．Th1 リンパ球，Th2 リンパ球

$CD4^+$ T細胞が結核免疫に重要であることは MHC クラス $II^{-/-}$ マウスや $CD4^{-/-}$ マウス抗 CD4 抗体投与マウスで明らかとなっている（Th1 細胞と結核免疫については総説[10]参照）.

自然免疫と結核

1．マクロファージ（Mφ）

結核菌の増殖場所は Mφ 内である．一方，Mφ は異物貪食能と細胞内殺菌能および抗原提示能を持つ．したがって結核菌が優位に立つか，ヒト（生体）が優位に立つかの戦争でもある．（詳細は文献[2][3]参照）

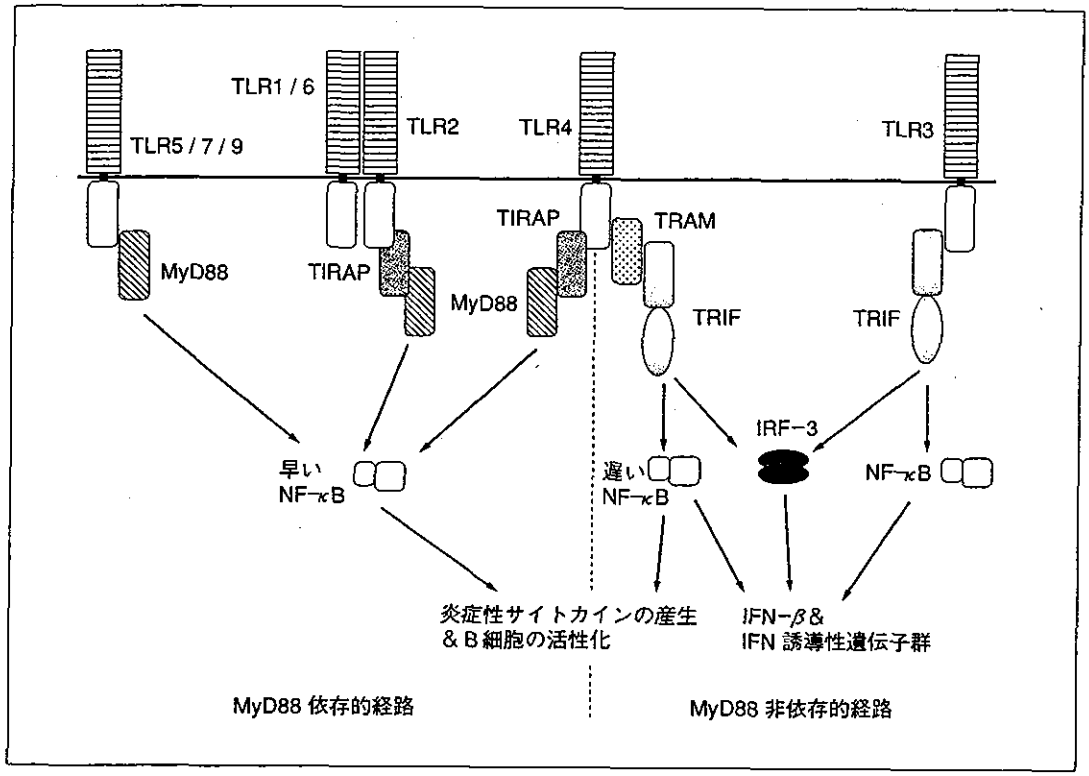
2．Toll－like 受容体および Pathogen Recognition Receptor とマクロファージ・樹状細胞活性化

最近発見された Toll－like 受容体（TLR）ファミリーが自然免疫の重要な役割を果たしている[17]．

TLR（TLR1～TLR10）はそのリガンドによって大きく3つに分類される（図6）.

このうち菌体膜由来の糖脂質を認識する TLR としては，TLR1,

図6 TIR ドメインを含むアダプター分子群による TLR シグナル伝達経路の制御（文献[23]より引用改変）



略語：巻末の「今号の略語」参照

TLR2, TLR4, TLR6, TLR9 である.

結核菌の細胞壁（LAM, mAGP, total lipid）による応答は TLR2 を介する（表1）. 一方, 結核生菌に対する反応には TLR2 と TLR4 が必要である. 病原株の *M. tuberculosis* 由来の Man LAM は Mϕ を活性化しないが, 非病原性の抗酸菌は異なる glycolipid Ara LAM よりなり, これは TLR2 を介して Mϕ を活性化する. この差が発病の差となる可能性もある. 結核菌体成分 19 kD のリポタンパク質が TLR2 を介して Mϕ を活性化する. また, 抗酸菌 DNA から見いだされた CpG モチーフ（パリンドローム配列）は感染防御免疫能を増強することが示されていたが, CpG 受容体に対する TLR9 が審良らによりクローニングされた.

TLR2 の場合, 細胞内領域の 2 つの変異（Arg753Gln と Arg677Trp）が認められ, Arg753Gln は敗血症にかかりやすく, Arg677Trp はア

表1 TLR と結核菌体成分

結核菌体成分	受容体
LAM	TLR2
CWS	TLR2 / 4
ペプチドグリカン	TLR2 / 4
19－kDa リポタンパク質	TLR2
CpG リピート	TLR9

略語：巻末の「今号の略語」参照

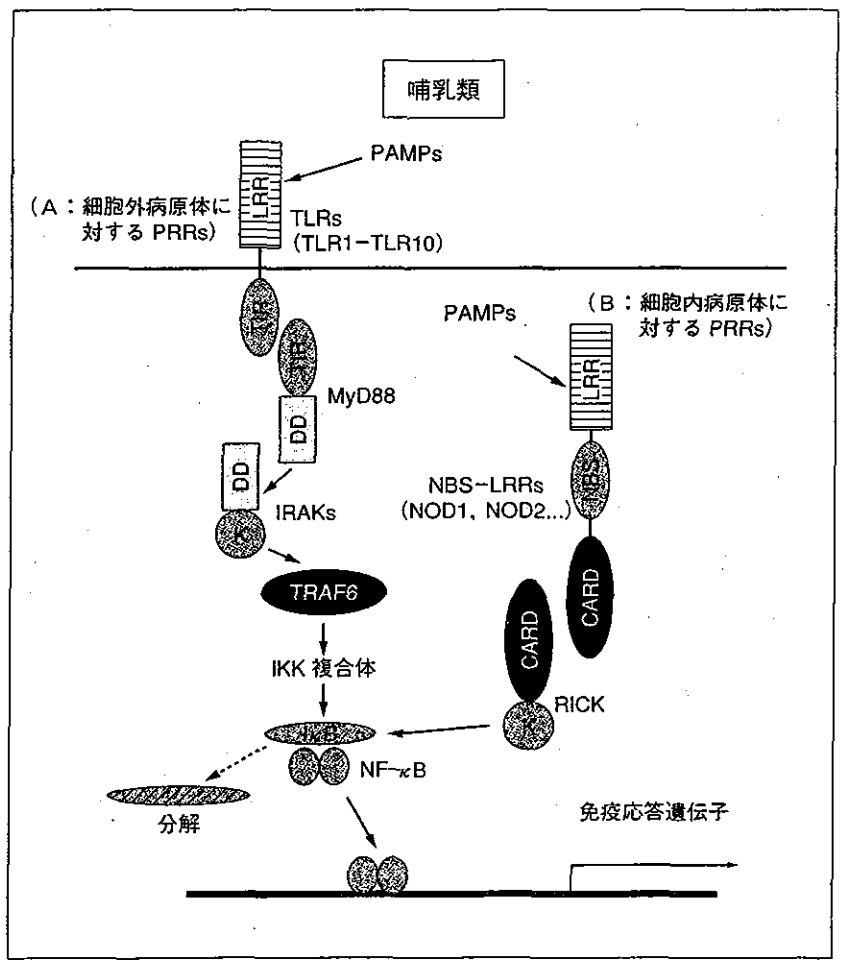
ジア人において *M. leprae* による結節性ハンセン症と関連している．

TLR はそれぞれ病原微生物由来の構成成分を認識する．TLR シグナルを介するシグナル伝達経路には MyD88 を介する MyD88 依存的経路と MyD88 を介さない MyD88 非依存的経路の2つが存在する．主に前者はすべての TLR を介した炎症性サイトカインの産生を，後者は主に TLR3・TLR4 を介したインターフェロン（IFN）および IFN 誘導性遺伝子群の産生を担う．

この MyD88 非依存的経路を担うアダプター分子が TRIF である．TRIF が TLR3 と TLR4 の MyD88 非依存的経路に共有されているのに対し，TRAM は MyD88 非依存的（TRIF 依存的）経路を TLR4 シグナルに特異的に与えるアダプター分子である．また，TIRAP はすべての TLR に共有された MyD88 依存的経路を，TLR1，2，6 と TLR4 にシグナル特異的に与える役割をもつ．我々は竹田との共同研究で $TRIF^{-/-} \times MyD88^{-/-}$ ダブルノックアウトマウスを用い，結核菌に対する易感染性を解析しつつある．

TLR 以外にも PRR（pathogen recognition receptor）として DC－SIGN，NOD ファミリー，マンノース受容体，スカベンジャー受容体，dectin－1 が挙げられる．HIV や *M. tuberculosis* は DC－SIGN に結合して樹状細胞に入り込むが，その際，その TLR による自然免疫機構の活性化を抑制し，これらの病原体の生存を有利にする機構が働いていることが示された．NOD1，NOD2 を中心とする CARD ファミリーの分子は，膜貫通領域を持たず，細胞質タンパク質として存在する（図7）．NOD2 は，古くより菌体由来の免疫調整物質として知られていた PGN の構成成分であるムラミルジペプチド

図 7　Pattern recognition receptors による病原体認識



A：Toll ファミリーによる細胞外病原体認識機構
B：NBS–LRRs ファミリーによる細胞内病原体認識機構
PRRs (pattern–recognition receptor)
PAMPs (pathogen associated moloculara patterns)
CARD：カスパーゼ再生ドメイン，DD：致死ドメイン，K：キナーゼドメイン，
LRR：ロイシンリッチリピート，LZ：ロイシンジッパー，NBS：核酸結合部位，
TIR：Toll / IL–1 受容体ドメイン
略語：巻末の「今号の略語」参照

(MDP) を認識することが，示された．

結核免疫における自然免疫と獲得免疫の関連機構

1．Hsp タンパク質

Hsp は細胞外では自然免疫と獲得免疫をつなぐという immunity