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29) ヒト又は動物由来成分を原料として製造されている医薬品等の品質及び安全性確保について(医薬発1314号, H12.12.26)

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Identification of an HLA-A*0201-Restricted T-Cell Epitope on the MPT51 Protein, a Major Secreted Protein Derived from *Mycobacterium tuberculosis*, by MPT51 Overlapping Peptide Screening[▽]

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AQ: A

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CD8$^+$ T cells play a pivotal role in protection against *Mycobacterium tuberculosis* infection. We identified a novel HLA-A*0201-restricted CD8$^+$ T-cell epitope on a dominant secreted antigen of *M. tuberculosis*, MPT51, in HLA-A*0201 transgenic HHD mice. HHD mice were immunized with plasmid DNA encoding MPT51 with gene gun bombardment, and gamma interferon (IFN-γ) production by the immune splenocytes was analyzed. In response to overlapping synthetic peptides covering the mature MPT51 sequence, the splenocytes were stimulated to produce IFN-γ by only one peptide, p51-70. Three-color flow cytometric analysis of intracellular IFN-γ and cell surface CD4 and CD8 staining revealed that the MPT51 p51-70 peptide contains an immunodominant CD8$^+$ T-cell epitope. Further analysis using computer algorithms permitted identification of a bona fide T-cell epitope, p53-62. A major histocompatibility complex class I stabilization assay using T2 cells confirmed that this epitope binds to HLA-A*0201. The T cells were capable of lysing MPT51 p53-62 peptide-pulsed T2 cells. In addition, MPT51 p53-62-specific memory CD8$^+$ T cells were found in tuberculin skin test-positive HLA-A*0201$^+$ healthy individuals. Use of this HLA-A*0201-restricted CD8$^+$ T-cell epitope for analysis of the role of MPT51-specific T cells in *M. tuberculosis* infection and for design of vaccines against tuberculosis is feasible.

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Fn1 Tuberculosis (TB) is still a major cause of death due to infectious disease worldwide. There were an estimated 8.8 million new cases in 2005, and 1.6 million people died of TB (37). The problem of TB is increasing worldwide due to several factors, including the prevalence of multi-drug-resistant strains and coinfection with human immunodeficiency virus (23). The only TB vaccine currently available is the attenuated *Mycobacterium bovis* strain bacillus Calmette-Guérin (BCG), yet its efficacy against pulmonary TB in adults has been controversial (32). Therefore, there is an urgent need for an improved vaccine for TB (16).

Cell-mediated immunity plays a pivotal role in the control of *Mycobacterium tuberculosis* infection. There is mounting evidence that CD4$^+$ type 1 helper T lymphocytes (Th1) are involved in the development of resistance to this disease, primarily through the production of macrophage-activating cytokines, such as gamma interferon (IFN-γ) and tumor necrosis factor alpha. In addition, CD8$^+$ cytotoxic T lymphocytes (CTL) contribute to disease resistance since susceptibility to *M. tuberculosis* is increased in mice with a deficiency in CD8$^+$ T cells (17, 18, 31).

To design a new generation of vaccines, information on the antigenic make-up 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 (2, 4). 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- to 10-kDa proteins and the antigen 85 (Ag85) complex, a 30- to 32-kDa protein family (3).

The Ag85 complex (Ag85A, Ag85B, and Ag85C), which has mycolyltransferase activity in cell wall synthesis and in the biogenesis of cord factor (5) and the ability to bind to fibronectin (1), has been shown to be a major fraction of the secreted proteins of *M. tuberculosis* (35). Another major secreted protein, MPT51, was demonstrated to cross-react with the three components of the Ag85 complex at antibody levels and to exhibit primary protein structure similarity (37 to 43% at the amino acid level) with these components (22, 36). Using a DNA vaccine encoding MPT51, we found that MPT51 can induce specific cellular immune responses and protective immunity against challenge with *M. tuberculosis* (20), and we identified murine T-cell epitopes using C57BL/6 and BALB/c mouse strains (34).

Here, we identified an HLA-A*0201-restricted CD8$^+$ T-cell epitope on MPT51 by using a strategy that included HLA-A*0201 transgenic mice, gene gun immunization with expression plasmid DNA encoding MPT51, overlapping synthetic peptides spanning the entire mature MPT51 amino acid sequence, and computer algorithms.

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MATERIALS AND METHODS

Mice. HLA-A*0201 transgenic mice (HHD mice) (25) were kindly donated by F. A. Lemonnier (Pasteur Institute, France). HHD mice express a monochain in which the C terminus of human β2-microglobulin is covalently linked to the N terminus of the HLA-A2.1 heavy chain in a chimeric configuration (α3 domain of mouse origin) (25). In HHD mice, the HLA-A*0201 monochain is the only type of major histocompatibility complex (MHC) class I 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 3-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. We confirmed that HLA-A*0201 was expressed on spleen cells of the HHD mice that we used (data not shown).

Human subjects. HLA-A*0201$^+$ healthy donors who had previously been vaccinated with *M. bovis* BCG were recruited from the Hamamatsu University School of Medicine. Blood samples were taken after written permission was obtained from the individuals participating in this study.

Peptides. Peptides spanning the entire mature MPT51 amino acid sequence of *M. tuberculosis* (266 amino acid residues) were synthesized as 20-mers overlapping by 10 residues; the only exception was the carboxyl-terminal 12-mer from amino acid 255 to amino acid 266, which was described previously (34). Briefly, lyophilized peptides were purchased from Invitrogen Corporation (Carlsbad, CA), and the purity was confirmed by mass spectrometry. To identify the potential HLA-A*0201-restricted CD8$^+$ T-cell epitopes in a 20-mer peptide, computer-based T-cell epitope prediction algorithm programs were used, which were accessed through the websites of the National Institutes of Health BioInformatics and Molecular Analysis Section (BIMAS) HLA Peptide Binding Predictions (http://bimas.dcrt.nih.gov/cgi-bin/molbio/ken_parker_comboform) (24) and SYFPEITHI Epitope Prediction (http://www.syfpeithi.de/) (27). All peptides were dissolved in distilled water to obtain a concentration of 1 mM and stored at −80°C until use.

Immunization of mice. Mice were immunized with pCI-MPT51, a plasmid DNA vaccine encoding the mature MPT51 molecule (34), employing a gene gun bombardment system. For DNA immunization with the Helios gene gun system (Bio-Rad Laboratories, Hercules, CA), cartridges of DNA-coated gold particles were prepared according to the manufacturer's instructions. To immunize mice, the shaved abdominal skin was wiped with 70% ethanol. Mice were inoculated with 2 μg of the plasmid DNA four times at 1-week intervals.

Cell lines. The human transporter associated with peptide loading (TAP)-deficient T2 cell line (29) was kindly donated by Peter Creswell (Yale University School of Medicine). The cells were cultured in RPMI 1640 medium (Sigma-Aldrich, Inc., St. Louis, MO) supplemented with 10% heat-inactivated fetal calf serum (Thermo Electron, Melbourne, Australia) (RPMI/10FCS) in an incubator with a humidified atmosphere containing 5% CO_2.

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 a concentration of 1×10^6 cells per well in the presence or absence of 5 μM of each MPT51 peptide at 37°C with an atmosphere containing 5% CO_2. Supernatants were harvested 24 h later and stored at −20°C until they were assayed. The 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 previously (34), with some modifications. The following method was used. The 96-well ELISA plates (EIA/RIA A/2; Costar, Cambridge, MA) were coated with 2 μg ml^{-1} of capture antibody (anti-murine IFN-γ monoclonal antibody [MAb] R4-6A2; BD Biosciences, San Jose, CA) at 4°C overnight, washed with phosphate-buffered saline supplemented with 0.05% Tween 20 (PBS-Tween), and blocked with Block One blocking solution (Nakalai Tesque, Kyoto, Japan) at room temperature for 45 min. After washing with PBS-Tween, the culture supernatants were added to the plates and the plates were incubated at 4°C overnight. After washing 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 1 h at room temperature. After washing with PBS-Tween, horseradish peroxidase-conjugated streptavidin (eBioscience, San Diego, CA) was added, and the preparations were incubated for 30 min at room temperature. After washing, the 3,3′,5,5′-tetramethylbenzidine one-component horseradish peroxidase microwell substrate (BioFX Laboratories, Owings Mills, MD) was added to the plates to detect bound horseradish peroxidase-conjugated streptavidin. After 5 min, the enzyme reaction was stopped by adding 2 M H_2SO_4, and then the absorbance at 450 nm was measured using an E7S-ABS microplate reader (Asahi Techno Glass, Tokyo, Japan).

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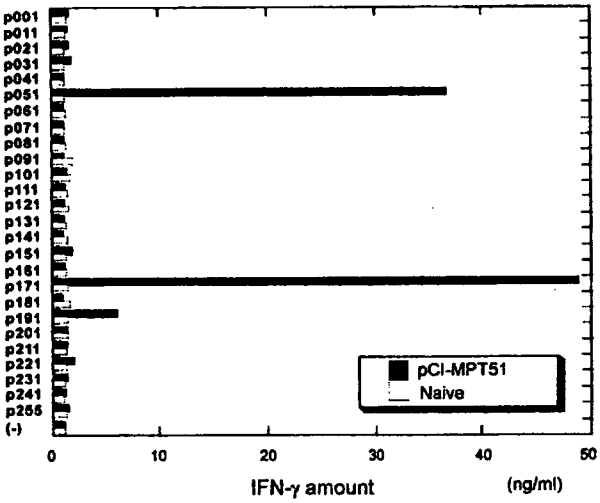

FIG. 1. IFN-γ production by spleen cells from HHD mice immunized with pCI-MPT51. The IFN-γ production by splenocytes from HHD mice immunized with the pCI-MPT51 plasmid in response to 1 of 26 overlapping peptides (5 μM) covering the MPT51 molecule or medium alone (−) was evaluated. Splenocytes from naïve HHD mice were also examined as a control. The data are representative of the results of three independent experiments.

MHC stabilization assay. The abilities of peptides to bind to HLA-A*0201 were measured by determining the stabilization of class I molecules on the surface of T2 cells (33). T2 cells (1×10^6 cells ml^{-1}) were cultured at 26°C overnight and then incubated for 1 h in the presence or absence of peptides (50 or 250 μM). Cells were then incubated at 37°C for 2 h and washed with FACS buffer [phosphate-buffered saline supplemented with 1% fetal calf serum], and the cell surface expression of HLA-A*0201 molecules was detected by flow cytometry (EPICS XL; Beckman Coulter, Fullerton, CA) using a mouse MAb specific for HLA class I molecules (34-1-2S; Cedarlane, Ontario, Canada), followed by treatment with fluorescein isothiocyanate (FITC)-labeled anti-mouse immunoglobulin antibodies (Rockland, Gilbertsville, PA). The results were expressed as the mean fluorescence intensity (MFI) ratio, determined as follows: [(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.

Intracellular IFN-γ staining. An antigen-specific T-cell *subset* was also identified by simultaneous flow cytometric assessment of the T-cell phenotype and intracellular IFN-γ synthesis.

The methods used for cell surface staining of CD4 and CD8 and intracellular IFN-γ staining have been described previously (34). Intracellular IFN-γ staining was performed using a Cytofix/Cytoperm Plus (with GolgiStop) kit (BD Biosciences, San Diego, CA) according to the manufacturer's instructions.

Cytotoxicity assay. One week after the last immunization, immune spleen cells (2×10^7 cells) were cocultured for 5 days with 2×10^7 syngeneic splenocytes treated with 100 μg ml^{-1} of mitomycin C (Kyowa Hakko, Tokyo, Japan) and pulsed with peptide for 2 h at 37°C. Each well also received 10 U ml^{-1} of human recombinant interleukin-2 (Hoffmann-La Roche, Nutley, NJ). Cell-mediated cytotoxicity was measured by using a conventional ^{51}Cr release assay as described previously (34). Briefly, the target cells used in this study were T2 cells pulsed with peptide at a concentration of 1 μM for 15 h at 37°C. Target cells (1×10^4 cells/well) were incubated for 5 h in triplicate 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.

Tetramer staining. A phycoerythrin (PE)-labeled HLA-A*0201/MPT51 p53-62 tetramer complex was kindly supplied by the NIH Tetramer Facility. After 10 days of in vitro stimulation with the MPT51 p53-62 peptide, spleen cells of immune HHD mice were treated with ammonium chloride and potassium chloride lysis buffer for 5 min at room temperature to remove erythrocytes,

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TABLE 1. Candidate HLA-A*0201-restricted T-cell epitopes in the p51-70 peptide of the MPT51 molecule

Peptide[a]	Amino acid sequence[b]	Estimated scores for restriction molecules	
		BIMAS	SYFPEITHI
p51-70	MNTLAGKGISVVAPAG GAYS		
Nonamers			
p53-61	**TLAGKGISV**	69.552	27
p54-62	LAGKGISVV	1.549	22
Decamers			
p53-62	**TLAGKGISVV**	65.588	28
p50-59	AMNTLAGKGI	7.535	19
p52-61	NTLAGKGISV	3.574	18

[a] Data for peptides ranked in the top 20 in the BIMAS or SYFPEITHI algorithms are shown.
[b] Boldface type indicates peptide sequences that were synthesized and used for experiments. Underlining indicates anchor residues. The G residues in p53-62 are residues that are associated with good binding to A*0201, as suggested by Ruppert et al. (28).

washed twice with RPMI 1640 medium, and resuspended in RPMI/10FCS. For some experiments, peripheral blood mononuclear cells (PBMCs) from purified protein derivative (PPD)-reactive HLA-A*0201$^+$ human healthy subjects were prepared by LeucoSep (Greiner Bio-One, Frickenhausen, Germany) according to the manufacturer's instructions. These cells (1×10^6 cells) were washed twice with FACS buffer and stained with the PE-labeled HLA-A*0201/MPT51 p53-62 tetramer and FITC-labeled anti-mouse or -human CD8 MAb for 30 min at 4°C. The cells were then washed with FACS buffer twice and analyzed with a digital flow cytometer (EPICS XL; Beckman Coulter).

RESULTS

IFN-γ production in response to overlapping synthetic peptides from MPT51 in HHD mice. Splenocytes from HHD mice immunized with a DNA vaccine encoding mature MPT51 (pCI-MPT51) were stimulated with the overlapping MPT51 peptides for 24 h, and the IFN-γ concentrations in culture supernatants were determined by ELISA. As shown in Fig. 1, robust IFN-γ production was observed in splenocytes from MPT51 DNA-vaccinated HHD mice after stimulation with peptide 51 (p51) (amino acid residues 51 to 70) and peptide 171 (p171) (amino acid residues 171 to 190). In addition, weak IFN-γ production was observed in the splenocytes in the presence of peptide 191 (p191) (amino acid residues 191 to 210). Since the HHD mice that we used in this study had a C57BL/6 background (25) and we observed that only CD4$^+$ T cells produced IFN-γ in response to p171 and p191, we concluded that CD4$^+$ T cells responded to these peptides presented on H2-A^b molecules and produced IFN-γ (34). As expected, spleen cells from naïve HHD mice showed no significant IFN-γ production in response to any MPT51 peptide. F1

Identification of a 10-mer CD8$^+$ T-cell epitope in peptide p51-70 of MPT51. Since CD8$^+$ T-cell epitopes presented by MHC class I molecules comprise 8 to 10 amino acids and are generally 9 amino acids long, we pursued a line of inquiry to identify the fine HLA-A*0201-restricted CD8$^+$ T-cell epitope. We predicted candidate peptides in the 20-mer peptide by using the computer-based programs BIMAS HLA Peptide Binding Predictions and SYFPEITHI Epitope Prediction. Using the BIMAS program, we found that a 9-mer peptide, p53-61 (TLAGKGISV), and a 10-mer peptide, p53-62 (TLAGKGISVV), showed high scores for binding to the HLA-A*0201 molecule in the region containing amino acid residues 51 to 70 (the binding scores were 69.552 for p53-61 and 65.588 for p53-62) (Table 1). In addition, the SYFPEITHI program also produced high scores for these peptides (27 for p53-61 and 28 for p53-62) (Table 1). Therefore, we synthesized p53-61 (TLAGKGISV) and p53-62 (TLAGKGISVV). In addition, we synthesized the p21-29 peptide (FLAGGPHAV) since this peptide had the highest HLA-A*0201 binding scores with the BIMAS and SYFPEITHI programs (319.939 and 29, respectively). Three-color flow cytometric analysis showed that the number of IFN-γ-producing CD8$^+$ T cells increased in the T1

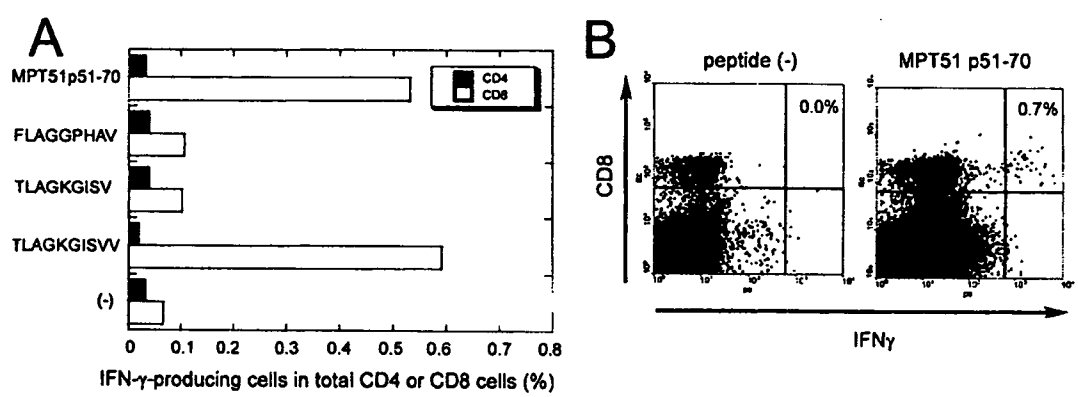

FIG. 2. Identification of a T-cell epitope in the MPT51 p53-62 peptide and the T-cell subset recognizing the epitope in HHD mice. (A) Levels of IFN-γ-producing T-cell subsets in spleens of HHD mice immunized with the pCI-MPT51 plasmid. Three-color flow cytometric analysis was performed for detection of intracellular IFN-γ and cell surface CD4 and CD8 molecules after immune splenocytes were cultured in the presence of the MPT51-derived peptides p51-70 (20-mer peptide), p21-29 (FLAGGPHAV), p53-61 (TLAGKGISV), and p53-62 (TLAGKGISVV). The data are the percentages of IFN-γ-producing CD4$^+$ or CD8$^+$ cells in the total CD4$^+$ or CD8$^+$ cells after 4 h of stimulation with peptides. The results of a representative experiment are shown. (B) Representative flow cytometry data for intracellular IFN-γ and cell surface CD8 staining of spleen cells of HHD mice immunized with the pCI-MPT51 plasmid after 4 h of stimulation with the MPT51 p51-70 peptide. The percentages of IFN-γ-producing cells in the total CD8$^+$ cells are shown.

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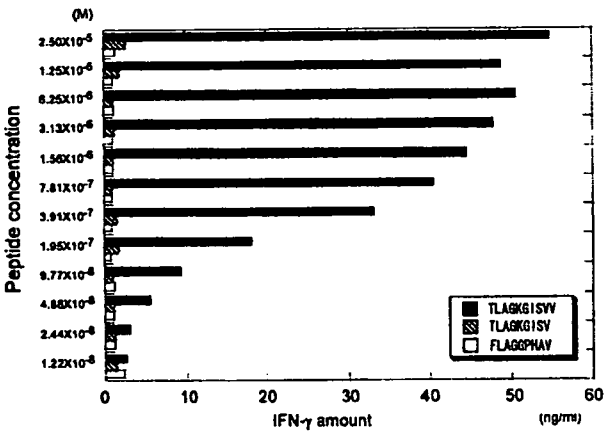

FIG. 3. MPT51 p53-62 is a dominant T-cell epitope in HHD mice. The IFN-γ production by splenocytes from HHD mice immunized with the pCI-MPT51 plasmid in response to twofold serially diluted doses of candidate peptides MPT51 p53-62 (TLAGKGISVV), p53-61 (TLAGKGISV), and p21-29 (FLAGGPHAV) was evaluated. The data are representative of the results of three independent experiments.

presence of p53-62 (TLAGKGISVV) but not in the presence F2 of p53-61 (TLAGKGISV) or p21-29 (FLAGGPHAV) (Fig. 2). The MPT51 p53-62 peptide was confirmed to stimulate splenocytes derived from MPT51 DNA-immune HHD mice in a dose-dependent manner. The minimum concentration of this peptide for inducing IFN-γ production by the splenocytes was F3 approximately 5×10^{-8} M (50 nM) (Fig. 3).

Binding affinity of the p53-62 peptide to the HLA-A*0201 molecule. We then examined the binding affinity of the MPT51 p53-62 peptide to the HLA-A*0201 molecule by measuring the binding stability with T2 cells, and we compared this peptide with several other *M. tuberculosis*-derived epitopes in terms of binding stability. T2 cells are defective for endogenous class I presentation due to the TAP deficiency, but peptide loading on AQ: D MHC molecules stabilizes the expression of MHC on the cell surface (33). The MHC molecules stabilized with the appropriate peptides could be detected by flow cytometry with an MAb to the HLA-A*0201 molecule. As shown in Fig. 4A, F4 MPT51 p21-29 (FLAGGPHAV) and MPT51 p53-62 (TLAGKGISVV) were strongly bound to the HLA-A*0201 molecule on T2 cells, whereas MPT51 p53-61 (TLAGKGISV), a known *M. tuberculosis* Ag85A-derived HLA-A*0201-binding peptide (KLIANNTRV) (30), and an *M. tuberculosis* ESAT6-derived HLA-A*0201-binding peptide (LLDEGKQSL) (19) were relatively weakly bound to the HLA-A*0201 molecule.

To obtain insight into T-cell recognition of the MPT51 p53-62/HLA-A*0201 complex on T2 cells, we examined the cytotoxic T-cell response of immune mice to the peptide-MHC complex. As shown in Fig. 4B, immune splenocytes of MPT51 DNA-immune HHD mice after in vitro stimulation with MPT51 p53-62 peptide-pulsed autologous splenocytes lysed the peptide-pulsed T2 cells substantially. However, the immune splenocytes did not lyse MPT51 p21-29 peptide-pulsed T2 cells after in vitro stimulation with the peptide-pulsed autologous splenocytes (Fig. 4A), although the peptide bound relatively strongly to HLA-A*0201 on T2 cells (data not shown).

Detection of MPT51 p53-62-specific $CD8^+$ T cells in PBMCs of HLA-A*0201$^+$ PPD-reactive healthy subjects. Finally, we examined whether HLA-A*0201$^+$ PPD-reactive healthy subjects do have MPT51 p53-62-specific memory T cells. We screened PBMCs of HLA-A*0201$^+$ individuals for the presence of the memory T cells. HLA-A*0201$^+$ PPD-reactive PBMCs were subjected to MPT51 p53-62/HLA-A*0201 tetramer staining after in vitro stimulation with mitomycin C-treated MPT51 p53-62-pulsed autologous PBMCs for 10 days. As shown in Fig. 5A, PBMCs from some HLA-A*0201-posi- F5 tive PPD-reactive individuals showed larger amounts of MPT51 p53-62/HLA-A*0201 tetramer-positive $CD8^+$ T cells by flow cytometric analysis than PBMCs from HLA-A*0201-negative individuals. The PBMCs of two of five HLA-A*0201- AQ: E positive individuals were tetramer positive. In parallel, the

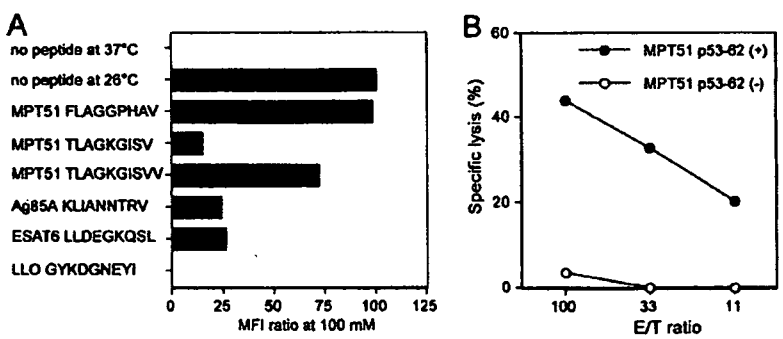

FIG. 4. MPT51 p53-62 peptide binds to cell surface HLA-A*0201 molecules and can be recognized by immune T cells in the context of HLA-A*0201. (A) HLA binding assay with T2 cells showing that MPT51 p21-29 (FLAGGPHAV) and MPT51 p53-62 (TLAGKGISVV) bound to HLA-A*0201 strongly, whereas MPT51 p53-61 (TLAGKGISV), the Ag85A-derived peptide KLIANNTRV, and the ESAT6-derived peptide AQ: G LLDEGKQSL bound to HLA-A*0201 relatively weakly. The MFI ratios in the presence of the indicated peptides at a concentration of 100 μM are shown. The listeriolysin O (LLO)-derived peptide GYKDGNEYI was used as a negative control. The expression of HLA-A*0201 on T2 cells cultured in the absence of any peptide at 37 or 26°C is also shown. Representative data from three independent experiments are shown. (B) Lysis of MPT51 p53-62 peptide-pulsed T2 cells by splenocytes from MPT51 DNA-immune HHD mice. Immune splenocytes (effectors) were incubated with target cells using the effector/target cell ratios (E/T ratio) indicated on the *x* axis. Representative data from three independent experiments are shown.

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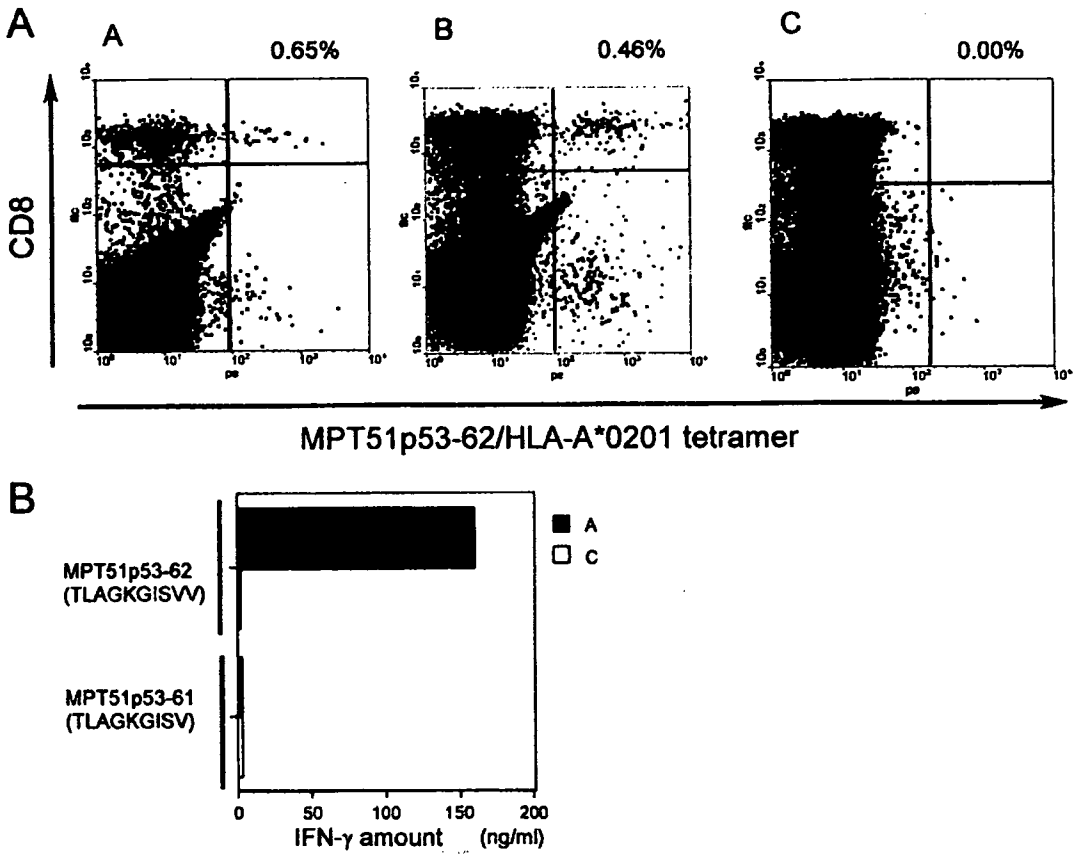

FIG. 5. Detection of MPT51 p53-62-specific memory T cells in PBMCs of HLA-A*0201$^+$ PPD-reactive healthy subjects. (A) Flow cytometric analyses to detect MPT51 p53-62-specific memory T cells in PBMCs of HLA-A*0201$^+$ PPD-reactive healthy subjects using MPT51 p53-62/HLA-A*0201 tetramer. PBMCs of the healthy subjects were prepared and cultured for 10 days together with mitomycin C-treated, MPT51 p53-62-pulsed autologous PBMCs and then subjected to flow cytometric analysis after treatment with PE-conjugated MPT51 p53-62/HLA-A*0201 tetramer and FITC-conjugated anti-human CD8 MAb staining (graphs A and B). HLA-unmatched PBMCs were used as a negative control (graph C). Representative flow cytometry patterns are shown. The percentages of tetramer-positive cells in the total CD8$^+$ cells are indicated. (B) IFN-γ production by PBMCs of HLA-A*0201$^+$ PPD-reactive healthy subjects stimulated with MPT51 p53-62 (TLAGKGISVV)- or p53-61 (TLAGKG ISV)-pulsed autologous PBMCs for 10 days as evaluated by an IFN-γ ELISA. Samples A and C correspond to graphs A and C in panel A.

tetramer-positive PBMCs produced large amounts of IFN-γ after in vitro stimulation (Fig. 5B).

DISCUSSION

Here we identified induction of an MPT51 p53-62/HLA-A*0201-specific T-cell population by using HLA-A*0201 transgenic mice (HHD mice) and the MPT51 expression plasmid pCI-MPT51. From the data described above, we were able to draw the following conclusions about a T-cell epitope on the mature MPT51 molecule of *M. tuberculosis*: (i) MPT51 p53-62 peptide is a bona fide HLA-A*0201-restricted CD8$^+$ T-cell epitope and (ii) epitope-specific memory T cells were detected in PBMCs of HLA-A*0201-positive PPD-reactive healthy subjects.

A greater understanding of the nature of protective immunity to *M. tuberculosis* would facilitate development of a vaccine. The cellular arm of the immune response mediated by CD4$^+$ Th1 and CD8$^+$ CTL has been determined to be a pivotal component of protective immunity against *M. tuberculosis* (17). IFN-γ secretion, cytotoxic ability, and direct killing of *M. tuberculosis* by CD8$^+$ T cells have been speculated to be involved in protection (18). We report here that an MPT51 p53-62 peptide/HLA-A*0201 complex can be recognized by CD8$^+$ T cells producing IFN-γ and exhibiting CTL activity.

Reports concerning the involvement of CD8$^+$ T cells in containing *M. tuberculosis* infection in human have been accumulating, and intense efforts have been made to identify *M. tuberculosis*-derived CD8$^+$ T-cell epitopes that can be presented by HLA class I molecules. *M. tuberculosis*-derived HLA-A*0201-restricted T-cell epitopes have been identified, including epitopes in Ag85A (30), ESAT-6 (19), Ag85B (14), heat shock protein 65 (7), the 16-kDa protein (6), the 28-kDa protein (8), the 38-kDa protein (8), superoxide dismutase (9), alanine dehydrogenase (9), glutamine synthetase (9), the 19-kDa protein (21), and Rv0341 (12).

MPT51 is a dominant *M. tuberculosis*-derived secreted molecule which is related to the Ag85 family molecules Ag85A,

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Ag85B, and Ag85C. Such molecules have been found in a variety of mycobacteria (22). Functionally, these molecules have been implicated in fibronectin binding, like Ag85 family molecules (1). However, MPT51 appears not to have mycolyltransferase activity, which Ag85 family molecules have, since MPT51 does not have the catalytic triad (Ser-His-Glu) in its amino acid sequence (36). Therefore, MPT51 seems to have a function that remains to be clarified. Importantly, MPT51 has been reported to be a potential marker for the diagnosis of TB, especially in AIDS patients. Ramalingam and colleagues (26) reported that early immune responses against 38- and 27-kDa (MPT51) proteins were detected in pulmonary TB patients, accompanied by human immunodeficiency virus coinfection. In addition, we demonstrated that MPT51 DNA vaccination using an attenuated *Listeria* carrier vaccination system induced protection against *M. tuberculosis* infection in mice (20).

HLA transgenic mice have been widely used for detection of HLA-restricted T-cell epitopes. In this study we used HHD mice. In HHD mice, the HLA-A*0201 monochain is the only type of MHC class I molecule expressed (25). Firat and colleagues (11) reported that not only the size but also the diversity of the $CD8^+$ T-cell receptor repertoire is substantially larger in HHD mice than in A*0201/K^b transgenic mice, which still express mouse $H2^b$ class I molecules. In addition, we used the computer algorithm programs BIMAS and SYFPEITHI for epitope prediction. These programs were helpful for narrowing down the amino acid region of the bona fide T-cell epitope.

HLA-A*0201-restricted $CD8^+$ T-cell epitopes have been identified in a variety of antigens, including antigens derived from cancers, viruses, bacteria, and protozoans. The main anchor amino acid positions are position 2 (Leu) and position 9 (Val), which were conserved in MPT51 p53-62 (TLAGKGIS VV). Most HLA-A*0201-restricted T-cell epitopes were nonamer peptides (10, 24), but some epitopes were decamer peptides, such as influenza virus matrix protein p59-68 (15). It is shown here that the MPT51 p53-62 decamer peptide was capable of binding to HLA-A*0201 and stimulating $CD8^+$ T cells of immune HHD mice, but the MPT51 p53-61 nonamer was not able to do these things. The conformational and electrostatic differences between the nonamer and the decamer should affect their binding affinity to the HLA-A*0201 molecule and subsequent T-cell responses. Ruppert and colleagues (28) studied in detail the roles of different amino acid residues at each position of nonamer or decamer peptides for binding to the HLA-A*0201 molecule. They suggested that the nonamer and decamer peptides have different preferences for amino acid residues for binding to the HLA-A*0201 molecule. For example, they showed that Tyr, Phe, and Trp residues at positions 1, 3, and 5 in nonamer peptides and Gly residues at positions 4 and 6 in decamer peptides are preferred for binding to HLA-A*0201. According to the speculation of these workers, the MPT51 p53-62 peptide seems to have better A*0201 binding features than the MPT51 p53-61 peptide (Gly residues at positions 4 and 6 in the MPT51 p53-62 peptide are suggested to be associated with good A*0201 binding) (Table 1). Interestingly, the MPT51 p21-29 peptide (FLAGGPHAV) was not immunogenic in terms of IFN-γ production and CTL ability, although this peptide showed high affinity to HLA-A*0201 (Fig. 4A), as predicted by MHC binding algorithms. Previous reports showed that there is a strong association between immunodominance and HLA binding affinity (13). But the results described here suggest that binding of peptides to the restricted MHC molecules is a prerequisite for T-cell epitopes; however, not all the peptides which show high-affinity binding for MHC molecules are necessarily immunodominant epitopes.

When we examined HLA-A*0201$^+$ PPD-reactive PBMCs for the response against MPT51 p53-62, we observed the specific $CD8^+$ T-cell response in some individuals. However, we could not detect $CD8^+$ T-cell responses in HLA-matched subjects without in vitro stimulation with the peptide. Therefore, we cannot rule out the possibility that these T cells were primed in vitro during stimulation with the peptide. The frequency of the memory T cells and the kinetics after *M. tuberculosis* infection are important issues to be clarified in the future.

In conclusion, we identified one HLA-A*0201-restricted $CD8^+$ CTL epitope on MPT51 in HHD mice, which may play a pivotal role in protection against *M. tuberculosis* infection. The identification of T-cell epitopes should be very useful for further elucidating the role of MPT51-specific T cells in protective immunity using tetramer staining or intracellular cytokine staining and also for future vaccine design.

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Intratracheal administration of third-generation lentivirus vector encoding MPT51 from *Mycobacterium tuberculosis* induces specific CD8+ T-cell responses in the lung

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Abstract

The present study evaluates the potential of improved third-generation lentivirus vector with respect to their use as an *in vivo*-administered T-cell vaccine against tuberculosis. Intratracheal administration of the lentivirus vector encoding MPT51 of *Mycobacterium tuberculosis* could induce MPT51-specific CD8+ T cells in the mediastinal lymph nodes 2 weeks after the administration. The vaccination could generate MPT51-specific memory CD8+ T cells in the lung, but not in the lymph nodes. Further, a single intratracheal immunization of MPT51 lentiviral vaccine decreased significantly the number of virulent *M. tuberculosis* in the lung after intratracheal challenge of the bacillus. These findings suggest that intratracheal immunization of the third-generation lentiviral vaccines is a promising vaccination strategy against pulmonary tuberculosis.

Key words: Intratracheal immunization, Lentivirus, MPT51, *Mycobacterium tuberculosis*

1. Introduction

Tuberculosis (TB) has been a major cause of death by infectious diseases worldwide. There were an estimated 8.8 million new TB cases in 2005, and 1.6 million people died of TB [1]. An attenuated strain of *Mycobacterium bovis* Bacillus Calmette-Guérin (BCG) is only currently available anti-TB vaccine which is effective against the severe child forms of TB, yet its efficacy against pulmonary TB in adult is controversial [2]. It is evident that there is an urgent need for a novel and more reliable anti-TB vaccine [3].

Although the mechanisms of protection against TB have not been completely determined, cell-mediated immunity plays an important role in the control of *M. tuberculosis* infection. There is mounting evidence that type 1 helper T cells are involved in the development of resistance to the disease, primarily through the production of macrophage-activating cytokines, such as interferon-γ (IFN-γ) [4]. 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 [5].

Dendritic cells (DC) are the most potent antigen-presenting cells. DC capture bacteria and other pathogens. Then, they migrate to regional lymphoid organs, where they present antigens (Ag) to naïve T cells [6]. DC are also known to confer T cells the ability to home to non-lymphoid sites. Activated effector/memory T cells migrate preferentially to tissues that are connected to the secondary lymphoid organs where Ag first encountered [7]. In this context, intratracheal vaccination is an attractive option to induce protective immunity against TB at the lung. In fact, *M. bovis* BCG administered via the respiratory route has been shown to be more effective than when it was given subcutaneously [8-11]. However, intratracheal administration of *M. bovis* BCG may cause severe inflammation in the trachea. For the intratracheal vaccination, such risk of adverse reactions should be avoided. The development of recombinant viral vector systems for gene therapy has prompted examination of their efficacy in gene delivery

to DC and in direct immunization. Adenovirus vectors were shown to deliver Ag genes to DC. However, pre-existing immunity to viral proteins expressed by the vector prevented effective immunization [12]. Retroviral vectors based on murine leukemia virus have been employed to express Ag in DC [13]. However, the retroviral vectors only infect dividing cells.

Lentiviral vectors have been shown to efficiently transduce a variety of nondividing cells, including DC [14]. Successful transduction of DC with lentiviral vectors has been reported [15-17]. In addition, lentiviral vectors pseudotyped with minimal filovirus envelopes have been reported to increase gene transfer in murine lung [18]. Third-generation self-inactivating (SIN) lentiviral vector was chosen in this study because of its advanced safety profile, allowing its administration *in vivo*, and because of the presumed absence of pre-existing anti-vector immunity.

Our aim was to develop third-generation lentivirus vectors that express an *M. tuberculosis* Ag and efficiently induce cell-mediated immunity against pulmonary TB by the intratracheal instillation. As a target Ag, we employed MPT51, the protective character of which we have shown in our previous report [19].

2. Materials and methods

2.1. Mice

BALB/c mice (8 to14 weeks of age; Japan SLC; Hamamatsu, Japan) were maintained in the Animal Facility of Hamamatsu University School of Medicine. All animal experiments were performed according to the Guidelines for Animal Experimentation, Hamamatsu University School of Medicine.

2.2. Lentivirus vector production

The improved third generation lentivirus system had been developed [14, 20, 21]. The system comprised of following plasmids. pCAG-HIVgp is a packaging plasmid in which all accessory genes (*vif*, *vpr*, *vpu*, and *nef*) and regulatory genes (*tat* and *rev*) are deleted.

pCMV-VSV-G-RSV-Rev is an expression plasmid for vesicular stomatitis virus G glycoprotein and Rev protein. The SIN plasmid, pCSII-CMV-MCS-IRES-EGFP contains a multiple cloning site and the gene encoding enhanced green fluorescent protein (EGFP). MPT51 DNA fragment was inserted into the vector, resulted in pCSII-CMV-MPT51-EGFP. The MPT51 recombinant lentivirus vector was generated by transient transfection of 293T cells with pCAG-HIVgp (10 μg), pCMV-VSV-G-RSV-Rev (10 μg), and pCSII-CMV-MPT51-EGFP (17 μg) plasmids using 10-cm dishes with DoFect-GT1 (Dojindo, Kumamoto, Japan) transfection reagent. 293T cells were cultured in Dulbecco's modified Eagle medium (DMEM; Sigma-Aldrich, St. Louis, MO, USA) containing 10% heat-inactivated fetal calf serum (FCS; Invitrogen, Carlsbad, NM, USA). Culture supernatants were collected every 24 h for 3 days, filtered through a 0.45-mm pore size filter, and concentrated by two-times ultracentrifugation at 50000× *g* at 20°C for 120 min. The viral supernatants were concentrated 1000 times with the ultracentrifugation, finally resuspended in sterile phosphate-buffered saline (PBS), and stored at -80°C until use. The virus titers were determined on 293T cells by measurement of EGFP expression using flow cytometry. Titers of 1 to 2 × 10^8 infectious units (IU) ml^{-1} were usually obtained through the experiments.

2.3. Intratracheal administration

Mice were anesthetized with an intraperitoneal administration of 0.075 mg ketamine/0.015 mg xylazine per gram weight of mouse. Intratracheal administration of 5 × 10^6 IU of MPT51 lentivirus in 50 μl of sterile PBS was performed by infusion through the vocal cords using a fiber optic light source (LG-PS2, Olympus Optical, Tokyo, Japan) for illuminating the entrance into the trachea [22, 23].

2.4. Lung tissue lymphocyte isolation

Lungs were removed from mice, transported in RPMI 1640 medium (5 ml per lung; Sigma-Aldrich), and cut into small pieces (1-2 mm^2) with a forceps. Tissue pieces were digested with 3500 dornase units ml^{-1} of DNase I (Calbiochem, Darmstadt, Germany) and 75 units ml^{-1} of

collagenase type II (Invitrogen) at 37°C for 2 h. The digest was filtrated through a 70-μm nucleopore filter and centrifuged (300× *g*, 10 min). The cell pellets were resuspended in PBS containing 0.01 M EDTA and chilled on ice for 5 min, and then subjected to centrifugation in Ficoll-Paque Plus solution (Amersham Pharmacia Biotech, Uppsala, Sweden) at 400× *g* and 20°C for 30 min. The pulmonary mononuclear cell interface was collected, washed twice, and resuspended in 5 ml of RPMI 1640 medium containing 10% FCS (RPMI/10FCS) [24].

2.5. Analysis of CD8+ T cells using MPT51 p24-32 peptide/H2-D^d tetramer complex

An MPT51 p24-32 peptide/H2-D^d tetramer complex was kindly supplied by the NIH Tetramer Facility. Cells were treated ammonium chloride and potassium chloride (ACK) lysis buffer for 5 min at room temperature to remove erythrocytes and washed twice with RPMI 1640 medium and resuspended in RPMI/10FCS. The 1×10^6 cells were stained with phycoerythrin (PE)-conjugated MPT51 p24-32 peptide/H2-D^d tetramer complex, fluorescein isothiocyanate (FITC)-conjugated anti-CD8 (53-6.7; BD PharMingen, San Diego, CA, USA), and PE-Cy5-conjugated anti-CD4 (RM4-5; BD PharMingen) monoclonal antibodies (mAb) at 4°C for 30 min. After washing, the cells were resuspended in PBS containing 0.1% sodium azide and 1% bovine serum albumin, and then analyzed on an EPICS digital flow cytometer (EPICS XL; Beckman Coulter, Miami, FL, USA).

2.6. Quantification of IFN-γ with cytokine enzyme-linked immunosorbent assay (ELISA)

Spleen cells were harvested from the immunized mice. Recovered cells were plated in 24-well plates at 2×10^6 cells per well in the presence or absence of 1 μM of MPT51 p24-32 peptide for 5 days. Concentration of IFN-γ in the culture supernatants was determined by a sandwich ELISA as described in our previous report [25].

2.7. Protection assay against M. tuberculosis *infection*

Immunized mice were subjected with intratracheal injection of 1×10^4 CFU of *M. tuberculosis* H37Rv 10 weeks after MPT51 lentivirus immunization. Mice were sacrificed 5 weeks later and

the bacterial numbers in the lung were counted in CFU on Middlebrook 7H10 medium (Becton Dickinson, Sparks, MD, USA). *M. tuberculosis* H37Rv was kindly donated by Dr. Isamu Sugawara (Research Institute of Tuberculosis, Tokyo, Japan).

2.8. Statistics

Data from multiple experiments were expressed as the means ± SD. Statistical analyses were performed by using StatView-J 5.0 statistics program (SAS Institute, Inc., Cary, NC, USA). Data were analyzed with one factor-analysis of variance followed by the Fisher's protected least significant difference (PLSD) test.

3. Results

3.1. EGFP expression of cells in bronchoalveolar lavage fluid (BALF) of mice intratracheally-immunized with MPT51 lentivirus

The lentivirus vector used in this study was pseudotyped with vesicular stomatitis virus glycoprotein and thus was taken up through the normal endocytotic pathway. Therefore, it is able to transduce a wide variety of cells. We first examined EGFP expression of cells in BALF after intratracheal administration of MPT51 lentivirus vector vaccine. As shown in Fig. 1, EGFP expression was observed 1 week after lentivirus administration and the peak of expression was reached around two weeks after the administration. This observation indicates that the cells in BALF, most of which are macrophages, are transduced by the lentiviral vector and that protein expression of transduced vector requires at least 1 week after the administration. We also examined EGFP expression in the mediastinal lymph nodes (MLN). Preferential EGFP expression in $CD11c^+$ cells in the MLN was observed 2 weeks after the administration (data not shown).

3.2. Induction of MPT51-specific CD8+ T cells in the MLN

To address whether intratracheal administration of lentiviral vector vaccine results in the

induction of MPT51-specific CD8+ T cells in tissues, CD8+ T cells were monitored in the lung, MLN, and the spleen by staining with an MPT51 p24-32 peptide/H2-D^d tetramer. As shown in Fig. 2, kinetic analysis revealed that the Ag-specific CD8+ T cells appeared 2 weeks after the administration in the MLN and the peak of response was reached around 3 weeks after that. In contrast, there appeared no detectable Ag-specific CD8+ T cells in the lung and the spleen until 6 weeks after the administration.

3.3. Detection of MPT51-specific memory CD8+ T cells in the lung

We then examined the lung cells derived from mice intratracheally-immunized with MPT51 lentivirus for detection of MPT51-specific memory CD8+ T cells. We isolated mononuclear cells from lungs of the immunized mice 10 weeks after the administration and stimulated them by MPT51 p24-32 peptide for 5 days. Then, expansion of MPT51-specific CD8+ T cells in the lung and the MLN was evaluated in flow cytometry with MPT51 p24-32/H2-D^d tetramer and anti-CD8 mAb staining. As shown in Fig. 3, expansion of MPT51-specific CD8+ T cells was observed in the lung of MPT51 lentivirus-immunized mice, but not in the MLN of the immunized mice and in naïve mice, indicating that MPT51 lentivirus intratracheal administration was able to induce MPT51-specific CD8+ memory cells in the lung.

3.4. Induction of protective immunity against M. tuberculosis *by intratracheal MPT51 lentivirus administration*

We finally evaluated the effects of intratracheal administration of MPT51 lentiviral vector on induction of protective immunity against *M. tuberculosis* infection. We intratracheally administered 1×10^4 CFU of *M. tuberculosis* H37Rv to mice 10 weeks after MPT51 lentivirus immunization. Five weeks after *M. tuberculosis* challenge, lungs were prepared from the mice and the CFU of *M. tuberculosis* were evaluated. The CFU in MPT51 lentivirus-immunized mice were significantly lower than those of naïve mice as shown in Fig. 4.

4. Discussion

The present study evaluated the potential of third-generation lentivirus vector with respect to the use as mucosal anti-TB T-cell vaccine. From data described above, we were able to draw the following conclusions. (1) The intratracheal administration of the lentivirus vector encoding MPT51 from *M. tuberculosis* is capable of inducing specific CD8+ T cells in the MLN. (2) MPT51-specific memory CD8+ T cells appear in the lung, but not in the MLN. (3) A single intratracheal immunization of MPT51 lentiviral vaccine decreased significantly the number of virulent *M. tuberculosis* in the lung after intratracheal challenge of the bacillus.

The development of a variety of TB vaccine systems has been reported in order to obtain more effective TB vaccines over BCG vaccine, which is a gold standard of TB vaccine for the time being. Virus-based vaccine strategy is one of them. Vaccinia virus-mediated TB vaccines have been reported. Zhu and colleagues [26] showed that immunization with 19kDa- and 38kDa-glycol-lipoproteins of *M. tuberculosis* reduced the bacterial numbers of virulent *M. tuberculosis* bacillus in the lungs of immunized mice. Vaccinia virus-based vaccines have been successfully used as a boosting vaccine following BCG- or DNA-based priming vaccination [27]. Adenoviral TB vaccines have been also examined in Dr. Xing's group [28, 29]. Wang and colleagues [28] reported that a single mucosal, but not parenteral, immunization with recombinant adenoviral-based TB vaccine encoding antigen 85A of *M. tuberculosis* provides potent protection from pulmonary tuberculosis.

We here showed that a single intratracheal MPT51 lentivirus administration was effective for inducing antigen-specific CD8+ T-cell responses in the lung. To our knowledge, it is the first report of lentivirus-based vaccine trial for TB. Esslinger and colleagues [30] showed that lentiviral vector injection into the footpad of mice transduced DC that appears in the draining lymph node and in the spleen. They showed that *in vivo* administration of lentivector was superior to transfer of transduced DC or peptide/adjuvant vaccination in terms of both amplitude

and longevity of the CTL response. The results in this present study further showed the effectiveness of lentiviral vector system for mucosal T cell-based vaccination.

Mucosal immunization studies have been reported against airway *M. tuberculosis* infection. Gallichan and Rosenthal [31] showed that long-term mucosal CTL memory was observed only with mucosal, but not systemic, immunization with an adenoviral herpes vaccine. Among a variety of mucosal immunization routes, intranasal vaccination is one of the promising immunization routes for various TB vaccines [9-11, 28, 29]. Giri and colleagues [32] showed that intranasal vaccination with antigen 85A and antigen 85B of *M. tuberculosis* induced a significantly higher level of IFN-γ, interleukin (IL)-12, and IL-4 in cervical lymph nodes over subcutaneous vaccination. Further, intranasal vaccination with these Ag imparted protection against *M. tuberculosis* comparable with that obtained from intranasal or subcutaneous *M. bovis* BCG immunization. Another route for eliciting mucosal immunity in the lung is intratracheal administration of vaccines [33]. The method of intratracheal injection used in this study is a simple and rapid method without any invasive procedure [22, 23], compared with conventional intratracheal infection method with tracheal incision step (e.g., [34]). The present study showed the feasibility of the intratracheal intubation method of mucosal immunization for airway infections.

We showed here that MPT51-specific memory CD8+ T cells generated in the lung after a single intratracheal instillation. Kamath and colleagues [35] showed that CFP10-specific and TB10.3/4-specific CD8+ T cells are greatly enriched in the lung compared with other sites of infection such as spleen or lymph nodes. They showed that CD8+ T cells are cytolytic *in vivo* and their cytolytic activity could be detected even as late as 260 days after infection. Servina and Flynn [36] also observed that at 1 week post challenge with *M.tuberculosis* via aerosol, over 30% of CD8+ and CD4+ T cells in the lungs of immune mice expressed the activation marker CD69 and could be restimulated to produce IFN-γ, showing a rapid response of CD8+ and CD4+ T cells

in the lungs following challenge. These rapidly expanded CD8+ T cells in the lung are derived from lung-resident memory CD8+ T cells because we showed that lung mononuclear cells 10 weeks after MPT51 lentivirus administration responded to MPT51 p24-32 peptide *in vitro* and produced IFN-γ. The result indicates that memory CD8+ T cells do exists in the lung. But, some of them may be derived from central memory cells in parabronchial lymph nodes, which are recruited to the lung immediately after *M. tuberculosis* challenge, although we could not detect memory CD8+ T cells in the MLN [37].

Our vaccine was capable of reducing the number of CFU challenged by about 50%, which seems to be not enough in terms of clinical relevance. Since we employed a single administration of the vaccine expressing a single protective antigen, MPT51, booster vaccines and the vaccine expressing several protective antigens could be required for the development of clinically effective vaccine.

As memory T cells present in the lung have been reported to contribute mainly to protection of the host from secondary airway infection [38], the results in the present study suggest that lentivirus TB vaccine represents a promising novel TB vaccine capable of potent mucosal T-cell immune responses.

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