Cell
PRESS

coefficients ($\mu m^2\ min^{-1}$) were calculated for individual tracks by linear regression of displacement[2] versus time plots with T Cell Analysis (John Dempster, University of Strathclyde). Clusters were analyzed in five randomly selected time points with PicViewer (John Dempster, University of Strathclyde) as previously described (Zinselmeyer et al., 2005).

Statistical Analysis

Non-normally distributed data were presented as medians and compared with the Mann-Whitney U-test (two groups) or with an ANOVA followed by a suitable multiple comparison procedure (Dunn's or Dunnett's test).

SUPPLEMENTAL DATA

Supplemental Data include eight figures and seven movies and can be found with this article online at http://www.immunity.com/cgi/content/full/29/3/476/DC1/.

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Contents lists available at ScienceDirect

Vaccine

Chemokine receptor-mediated delivery of mycobacterial MPT51 protein efficiently induces antigen-specific T-cell responses

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ARTICLE INFO

ABSTRACT

Here we evaluated the effects of immunization with a DNA vaccine encoding a fusion protein consisting of macrophage inflammatory protein-1α (MIP-1α) and MPT51 (a major secreted protein from *Mycobacterium tuberculosis*) on induction of specific CD8+ T cells. The DNA vaccine encoding the fusion protein could induce significantly higher number of the antigen-specific CD8+ T cells in mice than DNA vaccine encoding MPT51 alone. Also, splenocytes from mice immunized with the fusion DNA vaccine expressed higher level of IFN-γ mRNA and protein upon stimulation with an epitope peptide derived from MPT51 than those from mice immunized with a mixture of two DNA vaccines encoding either MPT51 or MIP-1α. These results suggest that DNA vaccine encoding MIP-1α-antigen fusion protein is able to be efficiently internalized into antigen-presenting cells via the chemokine receptor and induce higher level of antigen-specific CD8+ T-cell responses.

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1. Introduction

Mycobacterium tuberculosis, primary agent of tuberculosis (TB), is responsible for the three million deaths annually worldwide [1]. The only TB vaccine currently available is the attenuated *Mycobacterium bovis* strain bacillus Calmette-Guerin (BCG) which has been reported to have a variable protective efficiency [2]. The emergence of multi-drug-resistant strains of *M. tuberculosis* has given urgency to the need for novel agents and development of more effective vaccines.

Chemokines play an essential role in induction of inflammatory responses by trafficking of immune cells [3]. Chemokines bind to specific cell-surface receptors which are internalized after binding with ligands [4,5]. Chemokine receptors are differentially expressed on a variety of immune cells. Sentinel antigen-presenting cells (APCs), such as immature dendritic cells (DCs), express chemokine receptors such as CC chemokine receptor 5 (CCR5). CCR5 has been identified as the receptor for macrophage inflammatory protein-1α (MIP-1α), regulated on activation, normal T-cell expressed and secreted (RANTES), monocyte chemoattractant protein-1 (MCP-1), -2, -3, -4, and geotaxis [6]. CCR5 has been transported to early endosomes and subsequently recycled back to the cell surface or targeted for degradation [4]. There-

fore, it should be possible to harness the receptor binding and internalization of chemokine to increase the immunogenicity of vaccines. MIP-1α binds to two kinds of receptors, CCR1 and CCR5. In contrast, RANTES or MCPs bind to more than three receptors. In this study, we focused on the CCR5 and its ligand, MIP-1α as a simple molecular and cellular targeting model. The efficacy of MIP-1α-antigen fusion was examined by using DNA vaccine against *M. tuberculosis*. Antigen-specific T-cell responses appeared to be significantly enhanced by genetic fusion of MIP-1α to MPT51, one of major protective antigens of *M. tuberculosis* [7].

2. Materials and methods

2.1. Fusion gene cloning and plasmid constructions

The eukaryotic expression vector, pCI (Promega, Madison, WI, USA) containing a cytomegalovirus (CMV) immediate-early promoter, chimeric intron, and SV40 late polyadenilation signal, was used for construction for DNA vaccines. Murine MIP-1α gene was cloned by reverse transcription (RT)-PCR from total RNA of DCs. MIP-1α gene was fused with MPT51 gene via 14-amino acids (GTNDAQAPKSLEGT) spacer sequence and cloned into the EcoRI/XbaI sites of pCI vector (pCI-MIP-1α-MPT51). A plasmid expressing MIP-1α alone was constructed for control experiments. MIP-1α-fused GFP expression plasmid, pCI-MIP-1α-GFP, was constructed by the same strategy.

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2.2. Chemokine receptor binding assay

MIP-1α-fused GFP protein was prepared from the pCI-MIP-1α-GFP-transfected HEK293T cells. RAW264.7 cells, JAWS II cells or bone marrow-derrived DCs (BM-DCs) were incubated with the GFP fusion protein and phycoerythrin (PE)-labeled anti-CCR5 antibody (BD PharMingen, San Jose, CA, USA) for 30 min on ice and 15 min at room temperature. The samples were washed three times with phosphate-buffered saline (PBS) containing 1% fetal calf serum (FCS). Binding analysis was performed by using laser confocal microscopy (Olympus Fluoview, Tokyo, Japan).

2.3. Animals and immunization

BALB/c mice (between 8 and 10 weeks of age; Japan SLC, Hamamatsu, Japan) were maintained at the Institute for Experimental Animals, Hamamatsu University School of Medicine. All animal experiments were performed according to the Guideline for Animal Experimentation, Hamamatsu University School of Medicine.

For DNA immunization with Helios gene gun system (Bio-Rad Laboratories, Hercules, CA, USA), 0.5 mg of gold particles was coated with 1 μg of plasmid. Shaved skin of the abdomen was bombarded in vivo by two shots with the gene gun at a pressure of 400 psi. Each shot results in the delivery of 0.5 mg gold carrying 1 μg plasmid

DNA on approximately 1 cm^2 of skin. Four BALB/c mice for each group were injected with 2 μg of plasmid DNA three times at 1-week intervals.

2.4. Analysis of CD8$^+$ T cells by tetramer complexes

An MPT51 24–32 peptide/H2-D^d tetramer complex was kindly supplied by the NIH Tetramer Facility. MHC/peptide tetramer assay was performed as described previously [8]. In brief, 3 days after the last immunization, spleen cells were prepared and stained with PE-conjugated MPT51 24–32 peptide/H2-D^d tetramer complexes and fluorescein isothiocyanate (FITC)-conjugated anti-CD8 (BD PharMingen) monoclonal antibody for 30 min at 4 °C. After washing, cells were resuspended in PBS containing 1% bovin serum albumin, and then analyzed on an EPICS XL digital flow cytometer (Beckman Coulter, Miami, FL, USA).

2.5. Quantitatification of IFN-γ mRNA with RT-PCR

Two weeks after the last immunization, spleen cells were prepared and plated at 1×10^7 cells/well in the presence of 1 μM of MPT51 24–32 peptide for 16 h. Total RNA was prepared by using ISO-GEN (Nippon gene, Tokyo, Japan), and then quantitative RT-PCR was performed as described previously [9]. The nucleotide sequences of primers used in this study are as follows:
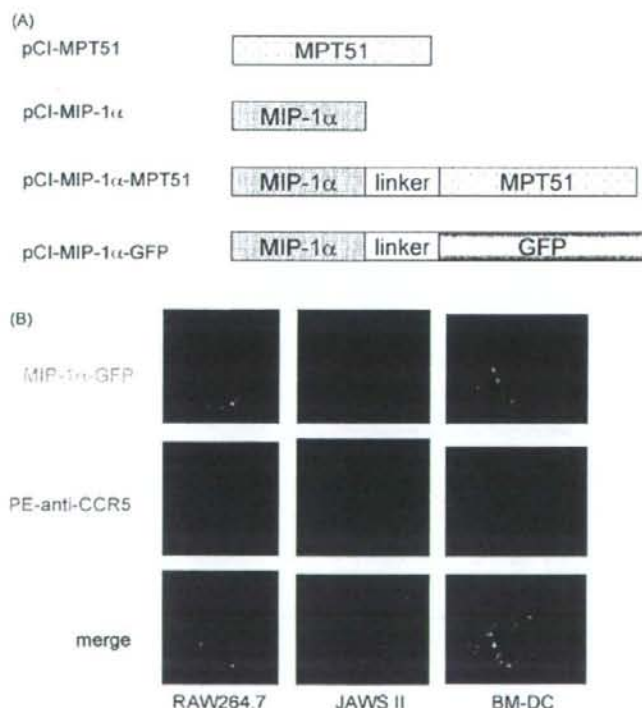
Fig. 1. (A) The schema of the gene products deduced from the expression vector plasmids prepared in this study. Mouse MIP-1α gene was cloned by RT-PCR from total RNA of dendritic cells. MIP-1α gene was fused with MPT51 gene via 14-amino acids spacer sequence (linker) and cloned into pCI vector. (B) Chemokine receptor binding assay. MIP-1α-fused GFP protein was prepared from the pCI-MIP-1α-GFP-transfected HEK293T cells. RAW264.7 cells, JAWS II cells, or BM-DCs were incubated with the GFP fusion protein and PE-labeled anti-CCR5 antibody for 30 min on ice and 15 min at room temperature. Binding analysis was performed by using laser confocal microscopy. (For interpretation of the references to color in the text, the reader is referred to the web version of the article.)

- IFN-γ:
  - 5'-TCTGAGACAATAAACGCTAC-3' (forward);
  - 5'-GAATCAGCAGCGACTCCTTT-3' (reverse).
- G3PDH:
  - 5'-ACCACAGTCCAT CCATCAC-3' (forward);
  - 5'-TCCACCACCCTGTTGCTGTA-3' (reverse).

2.6. Enzyme-linked immunosorbent assay (ELISA) of IFN-γ

Spleen cells were prepared from the immunized mice and plated in 96-well plates at 1×10^6 cells/well. Cells were stimulated with 1 μM of MPT51 24–32 peptide for 3 days. Concentration of IFN-γ in the culture supernatants was determined by a sandwich ELISA as described previously [10]. Data from multiple experiments were expressed in mean ± S.D. Statistical analyses were performed by using StatView-J 5.0 statistics program (SAS Institute Inc., Cary, NC, USA).

3. Results

3.1. Receptor binding and internalization of MIP-1α fusion protein

To investigate receptor binding and internalization of chemokine fusion protein, we constructed a MIP-1α-GFP expression plasmid (Fig. 1A). HEK293T cells were transiently transfected with pCI-MIP-1α-GFP plasmid and the cell lysates were used for receptor binding assay by using confocal microscopy. Most of the MIP-1α-GFP fusion proteins localized on the surface of murine macrophage-like RAW264.7 cells (Fig. 1B, left). Co-staining of the

cells with PE-labeled ant-CCR5 antibody showed co-localization of the MIP-1α-GFP protein and CCR5. In contrast, the MIP-1α-GFP proteins readily localized in the cytoplasm of JAWS II, a murine dendritic cell line, in the same experimental condition (Fig. 1B, center). CCR5 co-localized with the GFP-fused MIP-1α in the cytoplasm, which was shown in yellow. Similar results were obtained when BM-DCs were incubated with the MIP-1α-GFP protein (Fig. 1B, right). Taken together, these data suggested that MIP-1α fusion protein is capable of binding to CCR5 and is efficiently internalized especially into DCs.

3.2. Induction of MPT51-specific CD8+ T cells after immunization with fusion DNA vaccine

To construct a DNA vaccine against TB, we used MPT51, a major secreted protein of *M. tuberculosis*, since we demonstrated that the MPT51 could induce T-cell-mediated immune responses and protective immunity upon challenge with *M. tuberculosis* [7]. In order to evaluate the effect of immunization with MIP-1α-fused DNA vaccine, the epitope-specific CTL responses were monitored by quantitating MHC/peptide tetramer binding to CD8+ T cells following DNA immunization. A representative experiment is shown in Fig. 2. The antigen-specific CD8+ T cells were higher in number in spleen cells of mice immunized with the fusion DNA vaccine as compared to those of mice immunized with DNA vaccine encoding MPT51 alone or combination with the MIP-1α expression plasmid. These experiments demonstrate that MIP-1α fusion DNA vaccine efficiently induces antigen-specific CD8+ T cells.
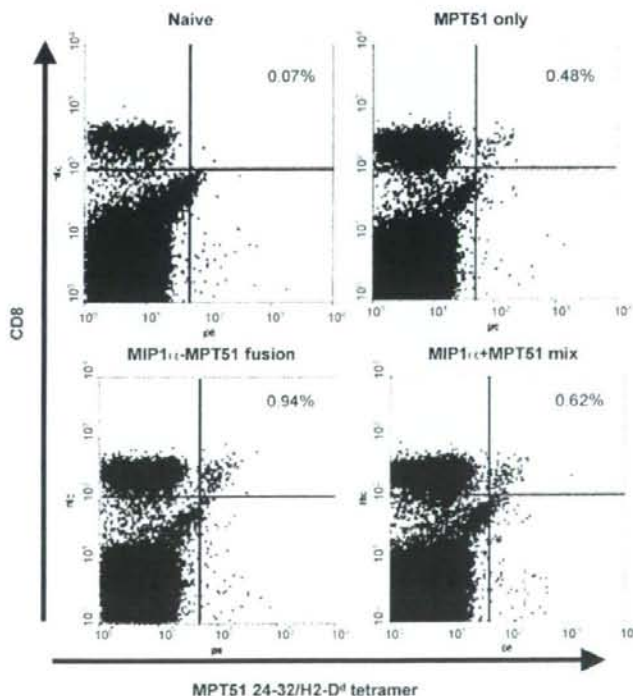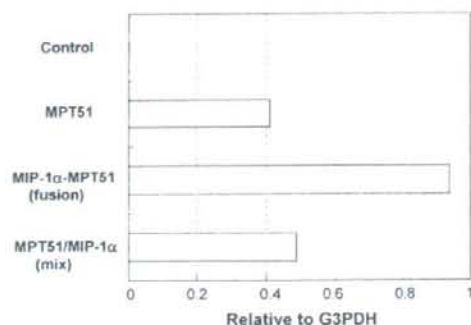

Fig. 2. Detection of MPT51-specific CD8+ T cells with MPT51 24–32/H2-Dᵈ tetramer. Naïve and immune splenocytes were stained with PE-conjugated MPT51 24–32/H2-Dᵈ tetramer complexes and FITC-conjugated anti-CD8 antibodies for 30 min at 4 °C. Stained cells were analyzed by a digital flow cytometer. Similar results were obtained in three independent experiments.

Fig. 3. IFN-γ mRNA expression of immune splenocytes in the presence of MPT51 peptide. Splenocytes were prepared 2 weeks after the last immunization and incubated with MPT51 peptide for 16 h. After preparation of total RNA, quantitative RT-PCR analysis was performed. Expression levels of mRNAs are shown relative to that of G3PDH mRNA. Similar results were obtained in three independent experiments.

3.3. Induction of the epitope-specific IFN-γ expression by spleen cells

We next examined the ability of antigen-specific IFN-γ mRNA expression in the DNA vaccine-immunized spleen cells. Two weeks after the last immunization, spleen cells from immunized mice were stimulated with MPT51 24–32 peptide, CD8$^+$ T-cell epitope derived from MPT51, for 16 h and the IFN-γ mRNA expression level was determined by real-time quantitative RT-PCR. Amounts of antigen-specific IFN-γ mRNA considerably increased in spleen cells from the fusion DNA vaccine-immunized mice as compared with those of pCI-MPT51- and pCI-MPT51 + pCI-MIP-1α-immunized mice (Fig. 3). Furthermore, we evaluated the production of IFN-γ by spleen cells of immunized mice after 3 days in vitro stimulation with MPT51 24–32 peptide employing ELISA. As shown in Fig. 4, spleen cells of mice immunized with the fusion DNA vaccine produced the highest level of IFN-γ protein in response to MPT51 24–32 peptide among these three DNA vaccination patterns consistent with the mRNA induction data (Fig. 3). A plasmid encoding MIP-1α showed adjuvant effect to some extent.

4. Discussion

The potency of vaccine presumably relies on the ability to recruit APCs and deliver antigens to them, leading to efficient antigen pre-
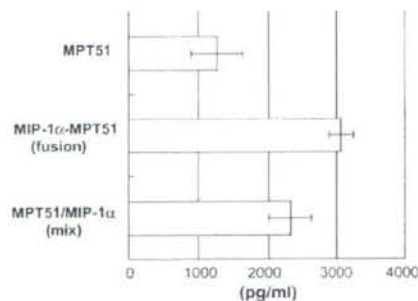
Fig. 4. IFN-γ production from immune splenocytes in response to MPT51 peptide stimulation. Immune spleen cells were cultured for 3 days at 1×10^7 ml^{-1} in the presence of MPT51 peptide. Concentration of IFN-γ in the culture supernatants was determined by ELISA. Results of four mice for each group are presented as mean ± S.D. Similar results were obtained in three independent experiments.

sentation to specific T cells. DCs are crucial in the activation of naïve T cells and induction of T cell-dependent immune responses. For this reason, experimental modification of vaccines, in particular genetic antigen delivery, has attracted much interest. Immature DCs, which are known as sentinel APCs, preferentially express CCR1, CCR2, CCR5, and CCR6 [11,12]. It has been reported that CCR1 or CCR5 internalizes into cells after ligand binding and recycles [4,5]. CCR5 also serves as a co-receptor for the entry of M-tropic human immunodeficiency into cells. Binding of viral gp120 to CCR5 reads to viral entry into the cells [13]. Therefore, it is possible to apply this phenomenon to vaccination. In this study, we evaluated the genetic fusion of MIP-1α to MPT51 to facilitate uptake and processing antigens, and to enhance DNA vaccine efficacy.

We here demonstrated that MIP-1α-GFP protein was quickly internalized and found in the cytosol, co-localized with CCR5 when a murine DC line, JAWS II cells or BM-DCs were incubated at room temperature (Fig. 1B). These data suggested that the fusion proteins not only retained its chemokine receptor binding properties of their nonfused chemokine counterparts, but also were efficiently internalized to the cytoplasm in immature DCs despite being linked to a relatively large antigen. The fate of the internalized MIP-1α fusion protein during receptor internalization remains unknown. Biragyn and his colleagues reported that MIP-3α fused melanoma-associated antigen are internalized via CCR6 to early/late endosomal and lysosomal compartment through a clathrin-dependent process and subsequently delivered to the cytosol for proteasomal processing, facilitating efficient cross-presentation to TAP-dependent MHC class I presentation pathway [14]. It is, therefore, possible that such cross-presentation is involved in the antigen-specific CD8$^+$ T cells induced with our MIP-1α-fused antigen.

Gene gun immunization is an efficient method for the administration of DNA vaccines [15]. Direct transfection of APCs or cross-presentation of exogenous antigen acquired from transfected nonimmune cells enables MHC class I-restricted activation of CD8$^+$ T cells [16,17]. Previously, we have reported that MPT51 possesses one CD8$^+$ T-cell epitope, p24–32, in BALB/c mice [18]. Therefore, we are able to examine the efficacy of MIP-1α-MPT51 DNA vaccine in inducing CD8$^+$ T cells using the epitope peptide. Using MPT51 24–32 peptide/H2-D^d tetramer, we demonstrated that gene gun immunizations into the skin of mice with plasmid DNA encoding MIP-1α-MPT51 protein induced high level of the epitope-specific CD8$^+$ T cells (Fig. 2). The efficient uptake of antigens by APCs may be particularly important for DNA vaccine, which typically expresses low amounts of antigen that are largely restricted to the local site of inoculation [19,20]. We also demonstrated that spleen cells from BALB/c mice immunized with DNA vaccine encoding MIP-1α-MPT51 secreted more IFN-γ in response to MPT51 24–32 peptide than those immunized with a DNA vaccine encoding MPT51 or with a mixture of two DNA vaccine encoding either MPT51 or MIP-1α. Several reports showed that co-immunization with DNA vaccines encoding antigens and chemokines enhanced the efficacy of vaccine by recruiting DCs to the inoculation sites [21]. In our hands, immunization with a mixture of pCI-MPT51 and pCI-MIP-1α also enhanced T-cell response although this activity was less than that induced by pCI-MIP-1α-MPT51 DNA vaccination. Furthermore, we also confirmed the effect of MIP-1α-MPT51 fusion protein vaccine. Mice immunized with MIP-1α-MPT51 fusion protein produced high level of MPT51-specific IFN-γ in response to MPT51 24–32 peptide (data not shown). This vaccine did not require the use of any additional adjuvants. In contrast, no antigen-specific IFN-γ production was induced in mice immunized with the mutant protein. Our data indicate that genetic fusion of chemokine to the antigen is more effective in terms of induction of T-cell responses than co-immunization methods.

In summary, our data suggest that MIP-1α-antigen fusion proteins encoded by DNA vaccine vector are efficiently internalized into APCs and induce higher level of antigen-specific T-cell responses. The immunization strategies shown here would be applicable to not only DNA vaccine but also to pre-monitoring the design of protein vaccination for induction of CTL.

Acknowledgements

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In vivo hierarchy of individual T-cell epitope-specific helper T-cell subset against an intracellular bacterium

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ARTICLE INFO

ABSTRACT

Cellular immunity is indispensable for efficient protection against intracellular bacterial infection. CD4+ and CD8+ T cells specific for a variety of antigenic peptides derived from particular bacteria are induced after the infection. T cells recognizing different antigenic peptides have been speculated to have different functions in terms of the protective immunity. We here induced individual CD4+ T cells specific for each antigenic peptide derived from Listeria monocytogenes independently with DNA vaccines using gene gun bombardment and compared the CD4+ T-cell populations for their ability on the specific protective immunity against lethal listerial challenge and analyzed their characteristics.

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1. Introduction

Listeria monocytogenes is a facultative Gram-positive intracellular bacterium. Murine infection with L. monocytogenes is an excellent model system for studying cellular immunity against intracellular microorganisms [1]. For the protection against the microorganism, CD4+ helper T-lymphocytes (Th), in addition to CD8+ cytotoxic T-lymphocytes (CTL), which are specifically amplified at listerial infection, have been shown to play a critical role in the protective immunity against challenge by lethal dose of L. monocytogenes [2–5].

CD4+ T-cell epitopes as well as CD8+ T-cell epitopes, derived from L. monocytogenes have been identified so far. Especially, T-cell epitopes derived from two critical virulent factors of L. monocytogenes, listeriolysin O (LLO) and p60 protein have been intensively studied. LLO is a sulfhydryl-activated pore-forming exotoxin. It allows the bacterium to escape from the phagosome and to replicate in the cytoplasm [1]. LLO 215–226 was first identified as a dominant CD4+ Th epitope restricted to H2-E^k molecule [6,7]. p60 protein is a murein hydrolase and has been shown to be involved in the invasion of mammalian cells [1]. Following LLO 215–226 peptide, p60 301–312 was then reported as an H2-A^d-restricted CD4+ Th epitope [8]. Further, Geginat et al. [9] reported several T-cell epitopes of LLO and p60 protein in BALB/c and C57BL/6 mice using an approach for the direct ex vivo identification and characterization of T-cell epitopes based on the screening of peptide spot libraries with ex vivo isolated spleen cells in a highly sensitive enzyme-linked immunospot (ELISPOT) assay. They found in their system, four CD8+ T-cell epitopes and six CD4+ T-cell epitopes in BALB/c mice and two CD8+ T-cell epitopes and five CD4+ T-cell epitopes in C57BL/6 mice including previously identified ones [9].

In our previous works, we have investigated individual T-cell epitope-specific T-cell responses against L. monocytogenes using minigene DNA vaccine system [10–12]. For CD8+ T-cell epitope-specific responses, we showed that interaction between T cells against dominant and subdominant epitopes does not operate in the generation of the hierarchy among individual CD8+ T-cell epitopes with minigene DNA vaccination [11]. For CD4+ T-cell epitope-specific responses, we have reported that LLO 215–226-specific T-cell response evoked by DNA vaccination was capable of inducing protective immunity against lethal listerial challenge in C3H mice [13].

Here, we compared four different H2-A^d-, or H2-E^d-restricted CD4+ T-cell epitope-specific responses in terms of the protective immunity in BALB/c mice by immunization of invariant chain (Ii) cDNA whose major histocompatibility complex (MHC) class II-associated Ii peptide (CLIP) region was replaced by the antigenic peptides. The antigenic peptide/CLIP-replaced Ii gene immunization would be an efficient method for presenting antigenic peptides of interest to Th in vivo [13–16]. Using this system, each individual epitope peptide has been expected to be expressed in similar amounts in vivo [11] and would be feasible for evaluation of each CD4+ T-cell epitope peptide for induction of the specific T-cell

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responses including the protective ability against following listerial challenge.

2. Materials and methods

2.1. Animals

BALB/c mice (Japan SLC, Hamamatsu, Japan) were maintained in the Animal Facility of Hamamatsu University School of Medicine. Mice between 6 and 18 weeks of age were used for immunization. All animal experiments were performed according to the Guidelines for Animal Experimentation, Hamamatsu University School of Medicine.

2.2. Plasmid construction

pCI-mli p41-LLO215m, a DNA vaccine plasmid for LLO 215–226 (SQLIAKFGTAFK), has been reported previously [13]. DNA vaccine plasmids for LLO 189–200 (WNEKYAQAYPNV; pCI-mli p41-LLO 189m), p60 367–378 (SSASAIIAEAAQK; pCI-mli p41–p60 367m), and p60 301–312 (EAAKPAPAPSTN; pCI-mli p41–p60 301m) were constructed similarly (Fig. 1). In brief, double-stranded oligonucleotides coding for each T-cell epitope peptide replaced HindIII-NspI DNA fragment coding for CLIP region of murine Ii p41 molecule in pCI-mli p41 plasmid [13,15]. The oligonucleotides for T-cell epitopes derived from L. monocytogenes were codon-optimized to mouse. The nucleotide sequences of the resultant plasmids were confirmed by dideoxy sequencing, using ABI PRISM 310 Genetic Analyzer (Applied Biosystems, Foster City, CA, USA). During the cloning procedure, the DNA fragments were purified from agarose gels using GeneClean II kit (Bio 101, La Jolla, CA, USA). Large-scale purification of plasmids was conducted with Qiagen plasmid mega kit system (Qiagen, Valencia, CA, USA) and endotoxin was removed by Triton X-114 phase separation.

2.3. DNA immunization

For DNA immunization with Helios gene gun system (Bio-Rad Laboratories, Hercules, CA, USA), preparation of the cartridge of DNA-coated gold particle cartridge was followed to the manufacturer's instruction manual. Finally, 0.5 mg of gold particles was coated with 1 μg of plasmid DNA and the injection was carried out with 0.5 mg gold per shot twice. Mice were injected with 2 μg of plasmid DNA four times at 1-week intervals.

2.4. Lymphocyte proliferation assay

Spleen cells (5×10^5 per well) from the immunized mice were maintained with RPMI-1640 medium supplemented with 10% fetal bovine serum (FBS) at 37 °C in a humidified 5% CO_2 atmosphere. They were incubated for 48 h at 37 °C in 96-well round-bottom tissue culture plates in the presence or absence of 1 μM of individual Th-epitope peptide. After 48 h in culture, de novo DNA synthesis was assessed by adding 0.5 μCi per well [methyl-^{3}H] thymidine (10 Ci mmol^{-1}; ICN Biochemicals, Irvine, CA, USA) for the last 12 h of culture. Triplicate cultures were harvested onto glass fiber filters, and the [methyl-^{3}H] thymidine incorporation was determined by counting the radioactivity (cpm) using liquid scintillation counter.

2.5. Bacterial infection

L. monocytogenes EGD strain was kept virulent by in vivo passage. For the inoculation, a seed of L. monocytogenes was cultured overnight in trypticase soy broth (BBL, Sparks, MD, USA) at 37 °C in a bacterial shaker and suitably diluted with phosphate-buffered saline. The exact infection dose was assessed retrospectively by plating. Mice were immunized four times with DNA vaccine plasmids as described above. One month later, the mice were challenged with 2×10^4 CFU of L. monocytogenes by intravenous injection. Bacterial numbers of the spleens were determined 72 h after the challenge infection by plating 10-fold dilutions of tissue homogenates on trypticase soy agar plates (BBL).

2.6. Cytokine assay for spleen cells from immunized mice

One month after the last immunization, 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 individual T-cell epitope peptide for 5 days. Concentration of cytokines (interferon (IFN)-γ, tumor necrosis factor (TNF)-α, interleukin (IL)-5, IL-4, IL-2) in culture supernatants were determined by cytometric bead assay (CBA). Fifty micro-liter of culture supernatants were assayed for these cytokines using mouse Th1/Th2 cytokine CBA kit (BD Biosciences Pharmingen, San Diego, CA, USA) according to the manufacturer's instruction.

2.7. Statistics

Data from multiple experiments were expressed as the means ± S.E. Statistical analyses were performed by using StatView-J 5.0 statistics program (Abacus Concepts, Berkeley, CA, USA). Data were analyzed with one factor-analysis of variance followed by the Fisher's protected least significant difference test.

3. Results

3.1. Proliferative responses of spleen cells of mice immunized with expression plasmids for Th epitopes

In order to examine CD4$^+$ T-cell responses against four different Th epitopes of L. monocytogenes in BALB/c mice, we constructed four DNA vaccine plasmids for in vivo antigen presentation of LLO 215–226, LLO 189–200, p60 367–378, and p60 301–312 (Fig. 1). The DNA vaccine constructs are expression plasmids for recombinant murine Ii cDNA whose CLIP region were replaced by an oligonucleotide encoding CD4$^+$ T-cell epitope peptide [13,15]. The plasmid DNAs were injected by gene gun bombardment into BALB/c mice
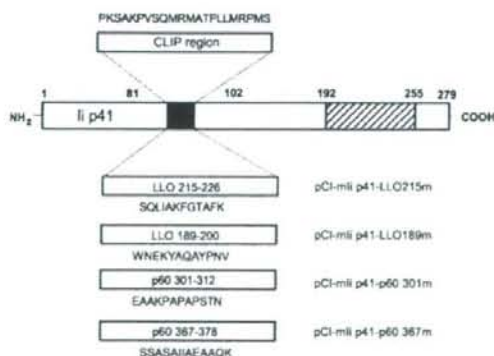
Fig. 1. The schema of murine Ii p41 molecule whose CLIP is replaced by LLO 215–226, LLO 189–200, p60 367–378, or p60 301–312 deduced from the cDNA construct (pmli p41-LLO215 m, pmli p41-LLO189 m, pmli p41–p60 367 m, or pmli p41–p60 301 m, respectively). The deduced amino acid sequences of the replaced CLIP region and the Th-epitope peptides are shown. Amino acid numbers of each domain of murine Ii p41 molecule are also shown.
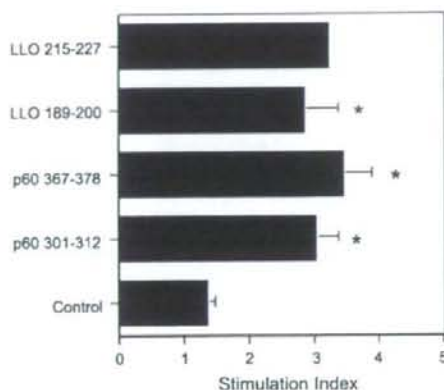
Fig. 2. Individual Th-epitope-specific proliferative responses of spleen cells from mice immunized with Th-epitope expression plasmids. BALB/c mice were immunized with each plasmid by using gene gun four times at 1-week intervals. Spleen cells from the immunized mice were harvested 1 month after the last immunization and cultured in vitro (5×10^5 per well) in the presence or absence of 1 μM of each Th-epitope peptide for 2 days and pulsed with 0.5 μCi of [methyl-^{3}H] thymidine for last 12 h. Results of control wild-type li p41 expression plasmid-immunized mice are also shown as a control. The means ± S.E. of stimulation index (cpm in the presence of the peptide divided by cpm in the absence of the peptide) of three mice per group are shown except for two mice for LLO 215–227 group. Asterisks indicate statistical significance ($p < 0.05$) compared with the value of control mice.

Fig. 3. Evaluation of protective immunity induced by immunization with Th-epitope expression plasmids. Mice were immunized with each Th-epitope expression plasmid four times at 1-week intervals. One month after the last immunization, the immunized mice were challenged i.v. with 2×10^3 CFU of *L. monocytogenes*. Bacterial numbers in the spleens were determined 72 h after the challenge infection by plating 10-fold dilutions of tissue homogenates on trypticase soy agar plates. Results of naive mice are also shown as a control (Naive). Results are expressed as the mean ± S.E. for three to four mice for each group except for two mice for LLO 189–200 group. Asterisk indicates statistical significance ($p < 0.05$) compared with the value of mice immunized with p60 301–312 DNA vaccine.

as shown in Materials and methods section. We chose the immunization method as it was a very reliable and reproducible method from our previous experience [17].
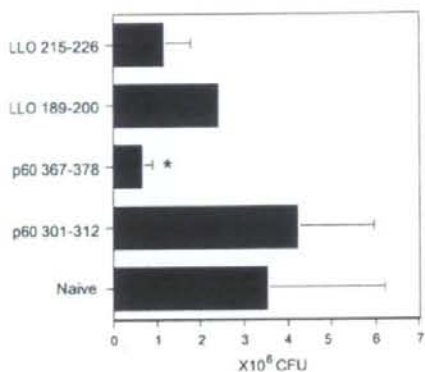
First, we performed lymphocyte proliferation assay one month after the last immunization. As shown in Fig. 2, immunization with each Th-epitope peptide expression plasmid induced individual peptide-specific proliferative responses of the spleen cells. The stimulation indexes were similar for four T-cell epitope peptides. These results indicated that the DNA vaccination successfully induced individual epitope-specific T-cell responses.

3.2. Induction of protective immunity against listerial infection after immunization with Th epitope expression plasmids

In order to examine whether the immunity evoked by immunization with each Th-epitope peptide expression plasmid is associated with an increased resistance to infection of virulent *L. monocytogenes*, the in vivo protection experiment was carried out. Seventy-two hours after listerial challenge, mice were sacrificed and CFU from the spleens were counted. As shown in Fig. 3, bacterial number in spleens of mice immunized with plasmids for p60 367–378 and LLO 215–226 tended to be reduced compared with that of naïve mice. On the contrary, bacterial number in spleens of mice immunized with plasmids for p60 301–312 and LLO 189–200 tended to be similar with that of naïve mice. Immunization with plasmid for p60 301–312 even increased the bacterial number in spleens compared with that of naive mice.

3.3. Cytokine production from spleen cells of mice immunized with expression plasmids for individual Th epitopes

We are interested in what caused different protective effects by immunization with these different Th-epitope peptide expression plasmids. One of the reasons may be cytokine profiles produced by specific Th. We therefore analyzed cytokine profiles in the

supernatants of immune spleen cell culture after 5-day in vitro stimulation with individual Th-epitope peptides.

As shown in Fig. 4, spleen cells from mice immunized with plasmids for LLO 215–226 and p60 367–378 tended to express TNF-α and IFN-γ after the Th-epitope peptide stimulation. Whereas, immune spleen cells with plasmids for LLO 189–200 and p60 301–312 tended to withdraw the expression of IFN-γ, although these tendencies were rather moderate.

4. Discussion

Following infection from a variety of pathogenic microorganisms, specific CD4$^+$ and CD8$^+$ T-cell responses are induced against various antigens of the microorganisms. CD8$^+$ T-cell responses are evoked mainly by microorganisms located in the cytoplasm
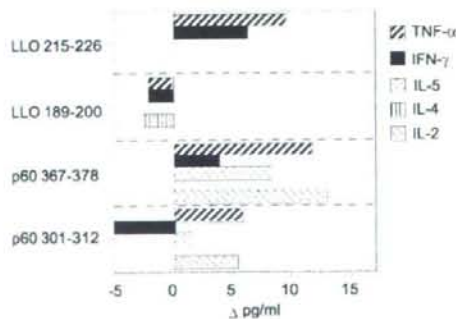
Fig. 4. Cytokine productions by spleen cells from Th-epitope expression plasmid-immunized mice. The spleen cells of mice immunized with each Th-epitope expression plasmid were harvested 1 month after the last immunization and cultured in vitro in the presence or absence of 1 μM of each Th-epitope peptide for 5 days, and the culture supernatants were analyzed by CBA. The values represent the means of Δpg/ml (the value in the presence of the peptide minus the value in the absence of the peptide) of two mice per each group.

of host cells, e.g., in the case of infection of viruses, as well as *L. monocytogenes*. CD4+ T-cell responses are induced mainly by infection of extracellularly located microorganisms or microorganisms located in the phagosome, e.g., in the case of infection of *Salmonella*, *Legionella*, and *Mycobacterium* spp. [18]. CD4+ T-cell responses have been also shown to be induced by *L. monocytogenes* and play an important role in the protective immunity against listerial challenge. In murine *Listeria* infection model, CD4+ T-cell responses have been studied. MHC class II gene-deficient mice were reported to be more sensitive to lethal listerial challenge compared with their control heterozygous littermates [19]. Geginat et al. [8] reported that adoptive transfer of p60 301–312-specific IFN-γ-producing CD4+ T-cell line into BALB/c mice induced protective immunity against lethal listerial challenge, suggesting that CD4+ T cells are positively involved in protective immunity. On the contrary, Kursar et al. [20] reported that depletion of CD4+ T cells during immunization with nonviable *L. monocytogenes* enhanced CD8+ T cell-mediated protection against listeriosis, suggesting involvement of regulatory T-cell population in failure of induction of protective immunity by nonviable *Listeria* vaccination.

There has been reported functional diversity of helper T cells [21]. Especially, so-called T helper 1 (Th1)/T helper 2 (Th2) dichotomy has been reported to be important for determining the following immunological outcome [22]. Th1-type CD4+ T cells produce abundant IFN-γ, TNF-α, or IL-2. These cytokines were known to be involved mainly in cell-mediated immunity. Preexisting memory CD4+ Th1, but not Th2 T-cell subset at the time of CD8+ T-cell priming resulted in increased CD8+ T-cell responses to bacterial and viral pathogens [23]. On the contrary, Th2-type CD4+ T-cells produce IL-4, IL-5, or IL-10. These cytokines were known to be involved in humoral immunity. Induction of Th1 or Th2 CD4+ T cells would be affected by many variable factors. They include, immunization method used, type of antigen-presenting cells and/or density of costimulatory molecules on the cells, factors evoking innate immunity such as adjuvants and infectious agents. Further, antigenic peptide dose may be critical for determining Th1/Th2 balance. Hosken et al. [24] investigated the relationship of antigenic peptide dose and Th1/Th2 selection using an in vitro system with naive T cells from ovalbumin-specific T-cell receptor transgenic mice. In the system, they showed that low peptide dose (0.01–0.04 μM) induced Th2 responses (dominant IL-4 production and less IFN-γ production) and high peptide dose (3.7–100 μM) induced Th1 responses (dominant IFN-γ production).

In addition to them, several reports suggested antigenic peptide affinity to MHC may also be involved in determining Th1/Th2 selection [25–27]. The peptides of higher affinity for a given MHC class II molecule elicited a shift towards the Th1 subset. In our work, exact affinity of peptides to MHC was not clear, but RANKPEP MHC-binding peptide prediction program (http://bio.dfci.harvard.edu/Tools/rankpep.html) predicted the affinity by calculating MHC-binding scores for each peptide. The scores for antigenic peptides studied here were as follows. LLO 215–226, 11.829 for H2-E^d; LLO 189–200, 7.104 for H2-A^d; p60 367–378, 11.261 for H2-A^d; p60 301–312, 11.44 for H2-A^d. The scores seemed not so different from each other except for LLO 189–200, whose score showed somewhat lower than those of other peptides. T-cell receptor affinity for the peptide-MHC and T-cell receptor repertoire (which may affect T-cell receptor Vβ chain usage) may also affect the Th1/Th2 selection.

In this study, we compared four CD4+ T-cell epitope-specific T-cell responses against *L. monocytogenes* using gene gun DNA vaccine system. We used DNA vaccines of CD4+ T-cell epitope peptide-Ii chain cDNA chimeric DNA constructs for that purpose as described in our previous work [13,15]. The results in this study showed that individual epitope-specific CD4+ T-cell responses are different in terms of protective immunity and cytokine production profiles although the same DNA vaccination system was performed for each epitope-specific CD4+ T cells. In results of the in vivo protection experiment, p60 367–378-specific and LLO 215–226-specific T-cells tended to have protective ability against listeriosis, but p60 301–312-specific and LLO 189–200-specific T cells did not (Fig. 3). Related with the result, p60 367–378 and LLO 215–226 peptides had tendency to induce IFN-γ production, whereas, p60 301–312 and LLO 189–200 peptides did not in the cytokine assay (Fig. 4). These apparent results suggest that the level of IFN-γ production may affect the protective ability of each peptide-specific T cells.

We tried to evaluate cytokine profiles of each T-cell epitope-specific T cells using CBA system, but the cytokine expression levels were rather moderate. This may be caused by several reasons. BALB/c mice may induce relatively low CD4+ T-cell responses when compared with CD8+ T-cell responses to LLO and p60 molecules of *L. monocytogenes* [9]. Further evaluation would be definitely necessary.

In conclusion, selection of CD4+ T-cell epitopes would be critical for construction of multi-epitope vaccination. Minigene DNA vaccination would serve a feasible system for evaluation of each T-cell epitope for induction of protective immunity against various pathogens.

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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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ARTICLE INFO

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.

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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 *Mycobacterium 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 (DCs) are the most potent antigen-presenting cells. DCs capture bacteria and other pathogens. Then, they migrate to regional lymphoid organs, where they present antigens (Ag) to naïve T cells [6]. DCs 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

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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 (8–14 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-μm pore size filter, and concentrated two times with ultracentrifugation at 50,000 × 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 $1–2 \times 10^8$ 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. Bronchoalveolar lavage (BAL)

Mice were killed and a midline incision was made to expose the trachea. An 18-G catheter was inserted into the trachea, and the

lungs were lavaged with 5 ml of ice-cold sterile PBS. Lavage cells were collected by centrifugation at 300 × g for 10 min at 4 °C and washed with PBS.

2.5. 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].

2.6. 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 with 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).

2.7. 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].

2.8. 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).

2.9. Statistics

Data from multiple experiments were expressed as the means ± S.D. 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.
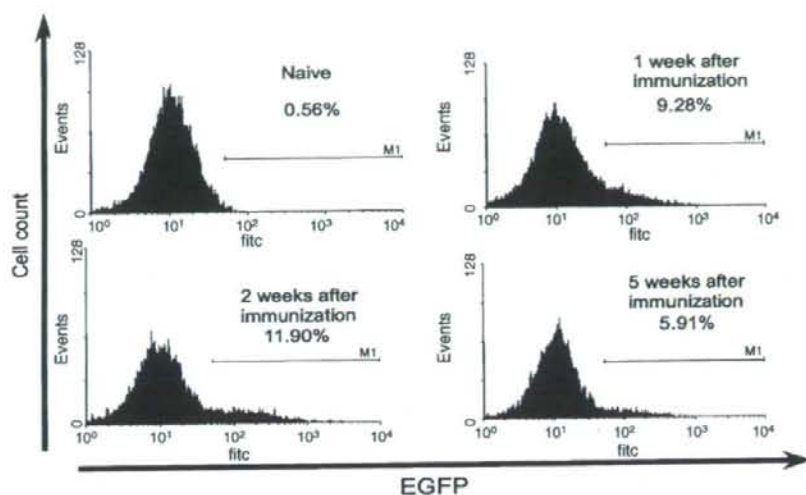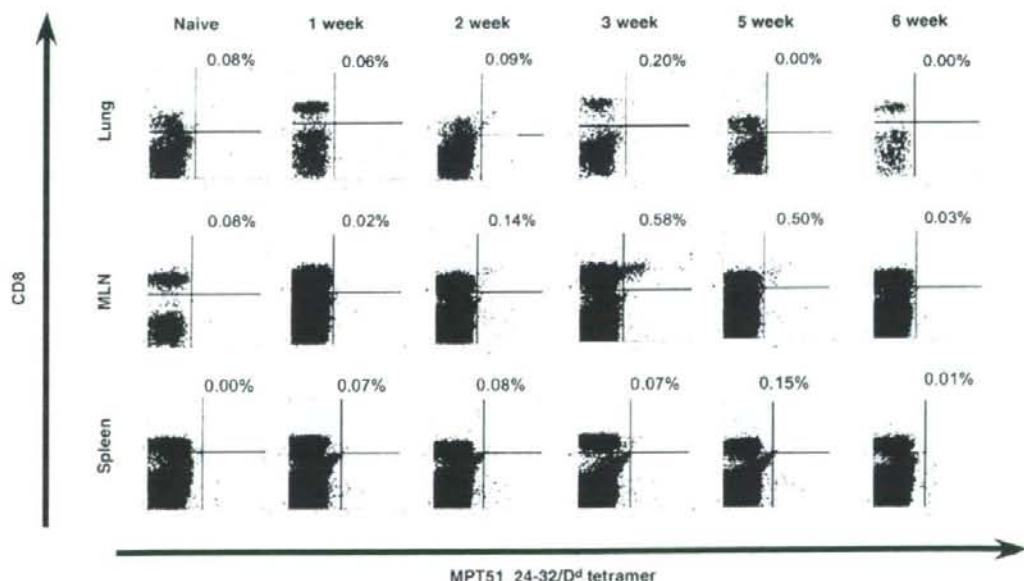
Fig. 1. EGFP expression of cells in BALF of MPT51 lentivirus-administered mice. Mice were intratracheally administered with MPT51 lentivirus and EGFP expression was measured by a flow cytometry every 1 week for 6 weeks after MPT51 lentivirus administration. Representative data are shown from three independent data which showed similar results. Percentages in the figure indicate those of EGFP-positive cells in total cells in BALF.

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 2 weeks after the administration. This observation indicates that the cells in BALF, most of which

Fig. 2. MPT51 p24-32-specific CD8+ T cells in the lungs, MLN, and spleens of mice intratracheally administered with MPT51 lentivirus. Mononuclear cells were harvested from the lungs, MLN, and spleens of immunized mice and double-stained with anti-CD8 mAb and MPT51 p24-32/H2-D^d tetramer and measured by a flow cytometry. Representative data of 1–6 weeks after MPT51 lentivirus administration are shown. Percentages in the figure indicate those of tetramer-positive cells in CD8+ cells.

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 (MLNs). Preferential EGFP expression in CD11c$^+$ cells in the MLN was observed 2 weeks after the administration (data not shown).
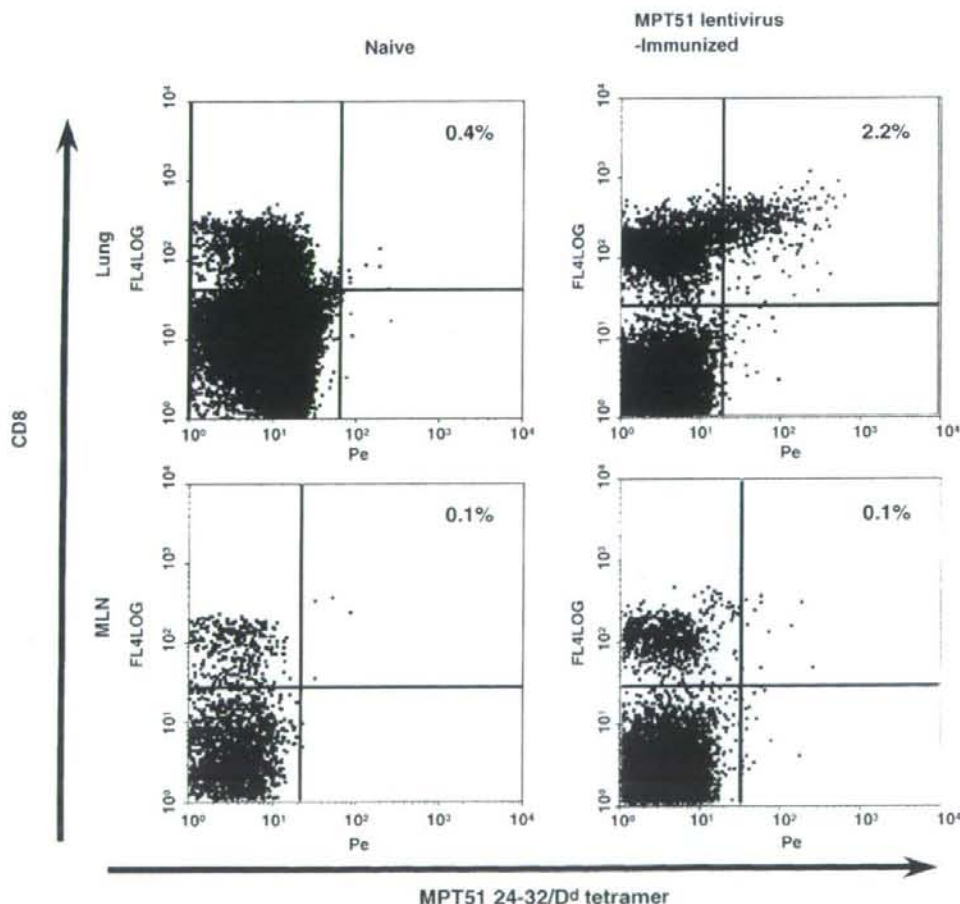
3.2. Induction of MPT51-specific CD8+ T cells in the MLN

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.

Fig. 3. Detection of MPT51 p24-32-specific memory CD8+ T cells in the lungs of mice intratracheally administered with MPT51 lentivirus. The lung and the MLN mononuclear cells were harvested from immunized mice 10 weeks after MPT51 lentivirus administration or from naïve mice and double-stained with anti-CD8 mAb and MPT51 p24-32/H2-D^d tetramer and measured by a flow cytometry. Representative data are shown from three independent data which showed similar results. Percentages in the figure indicate those of the tetramer-positive cells in CD8+ cells.
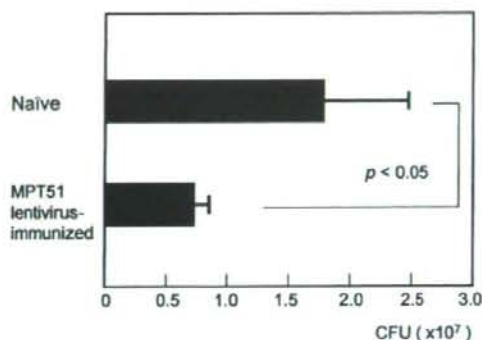
Fig. 4. Induction of protective immunity against virulent *M. tuberculosis* infection with MPT51 lentivirus vaccination. Mice were immunized with a single intratracheal administration of MPT51 lentivirus. The MPT51 lentivirus-immunized mice or naïve mice were challenged with intratracheal injection of 1×10^4 CFU of *M. tuberculosis* H37Rv. Five weeks after the challenge, the numbers of the challenged bacillus in the lungs were counted. The means ± S.D. from six mice per each group are shown.

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 et al. [26] showed that immunization with 19-kDa- and 38-kDa-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 et al. [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 et al. [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 et al. [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 et al. [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. Serbina 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 application. Since we employed a single administration of the vaccine expressing a single protective antigen, MPT51, booster vaccines and the vaccine expressing several protective antigens should 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.

Acknowledgements

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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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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 immuno-dominant 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.

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 SYF PEITHI 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% CO₂.

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% CO₂. 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, TMB (3,3',5,5'-tetramethylbenzidine) one-component horseradish peroxidase amino hydrogen peroxide 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 H₂SO₄, and then the absorbance at 450 nm was measured using an EZS-ABS microplate reader (Asahi Techno Glass, Tokyo, Japan).

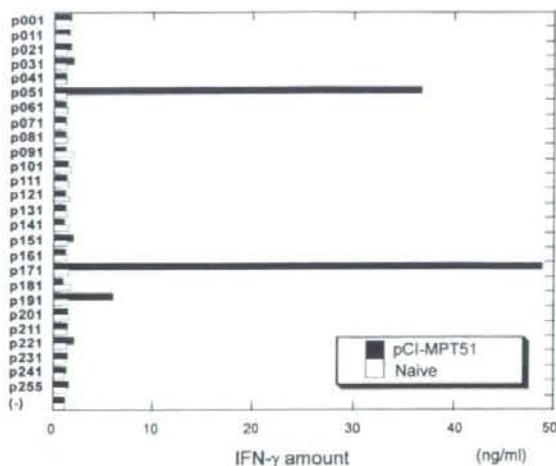FIG. 1. IFN-γ production by spleen cells from HHD mice immunized with pCI-MPT51. The IFN-γ production by splenocytes from HHD mice immunized with the pCI-MPT51 plasmid in response to 1 of 26 overlapping peptides (5 μM) covering the MPT51 molecule or medium alone (−) was evaluated. Splenocytes from naïve 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 (34-1-25; 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 ⁵¹Cr 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,