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.

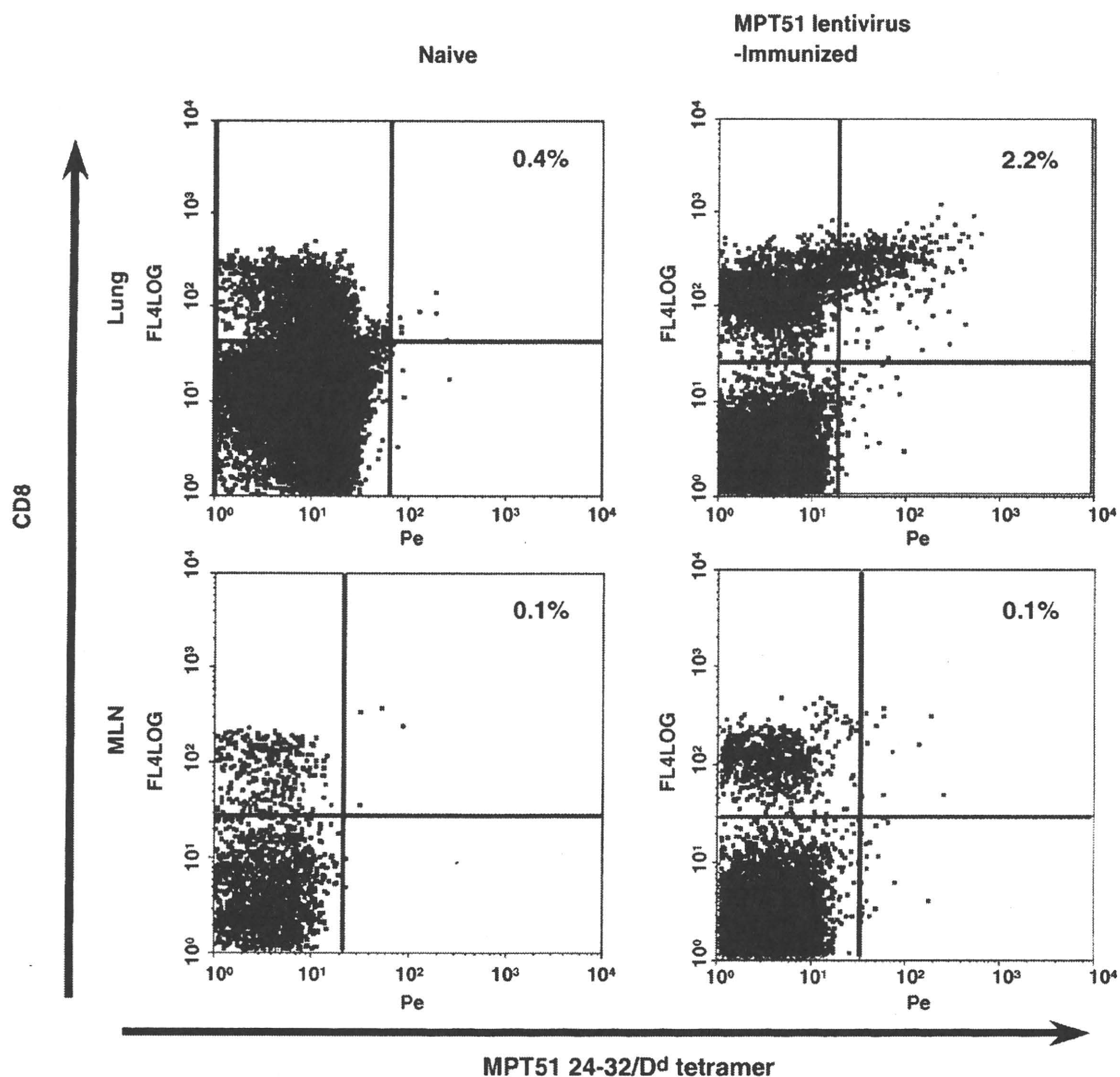

Fig. 3. Detection of MPT51 p24-32-specific memory CD8+ T cells in the lungs of mice intratracheally administered with MPT51 lentivirus. The lung and the MLN mononuclear cells were harvested from immunized mice 10 weeks after MPT51 lentivirus administration or from naïve mice and double-stained with anti-CD8 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.

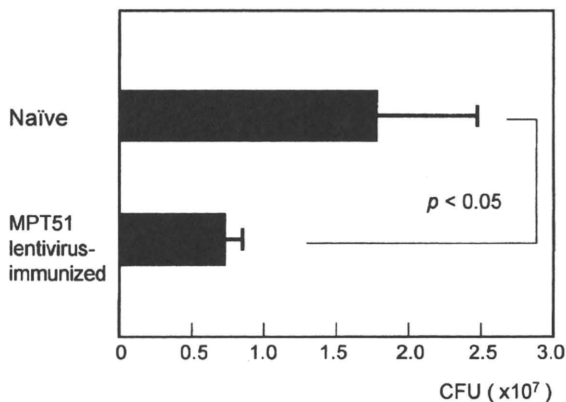

Fig. 4. Induction of protective immunity against virulent *M. tuberculosis* infection with MPT51 lentivirus vaccination. Mice were immunized with a single intratracheal administration of MPT51 lentivirus. The MPT51 lentivirus-immunized mice or naïve mice were challenged with intratracheal injection of 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

We thank the NIH Tetramer Facility for providing the MPT51 p24-32/H2-D^d tetramer complex, Dr. Isamu Sugawara (Research Institute of Tuberculosis, Tokyo, Japan) for providing *M. tuberculosis* H37Rv strain. This work was supported by Grants-in-Aid for Scientific Research and Centers of Excellence (COE) from the Ministry of Education, Culture, Sports, Science and Technology of Japan; by the United States-Japan Cooperative Medical Science Committee.

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INFECTION AND IMMUNITY, Apr. 2008, p. 1565–1571 Vol. 76, No. 4
0019-9567/08/$08.00+0 doi:10.1128/IAI.01381-07
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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[▽]

Taiki Aoshi,[1] Toshi Nagata,[2]* Mina Suzuki,[1] Masato Uchijima,[1] Dai Hashimoto,[3] Alireza Rafiei,[1] Takafumi Suda,[3] Kingo Chida,[3] and Yukio Koide[1]

Department of Infectious Diseases,[1] Department of Health Science,[2] and Department of Internal Medicine, Second Division,[3] Hamamatsu University School of Medicine, Hamamatsu 431-3192, Japan

Received 15 October 2007/Returned for modification 26 November 2007/Accepted 28 December 2007

$CD8^+$ 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.

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.

* Corresponding author. Mailing address: Department of Health Science, Hamamatsu University School of Medicine, 1-20-1 Handayama, Higashi-ku, Hamamatsu 431-3192, Japan. Phone and fax: 81-53-435-2332. E-mail: tnagata@hama-med.ac.jp.

▽ Published ahead of print on 22 January 2008.

Downloaded from iai.asm.org by on January 25, 2009

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 MPT5l 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% 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, 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 H_2SO_4, and then the absorbance at 450 nm was measured using an EZS-ABS microplate reader (Asahi Techno Glass, Tokyo, Japan).

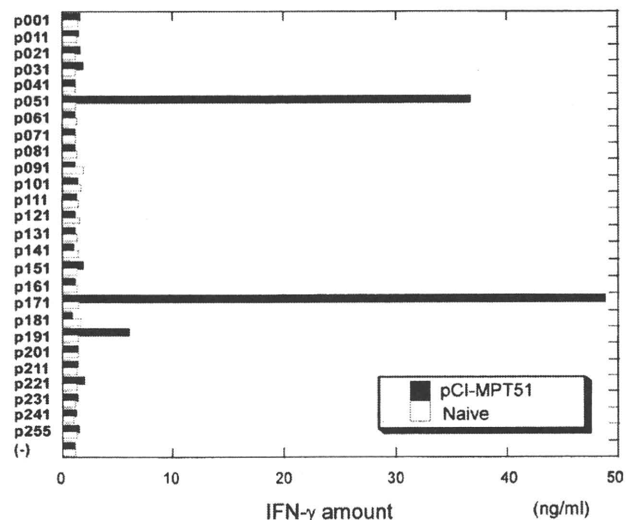

FIG. 1. IFN-γ production by spleen cells from HHD mice immunized with pCI-MPT51. The IFN-γ production by splenocytes from HHD mice immunized with the pCI-MPT51 plasmid in response to 1 of 26 overlapping peptides (5 μM) covering the MPT51 molecule or medium alone (−) was evaluated. Splenocytes from naïve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

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 (TLAG KGISVV), 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 (TL AGKGISV) 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

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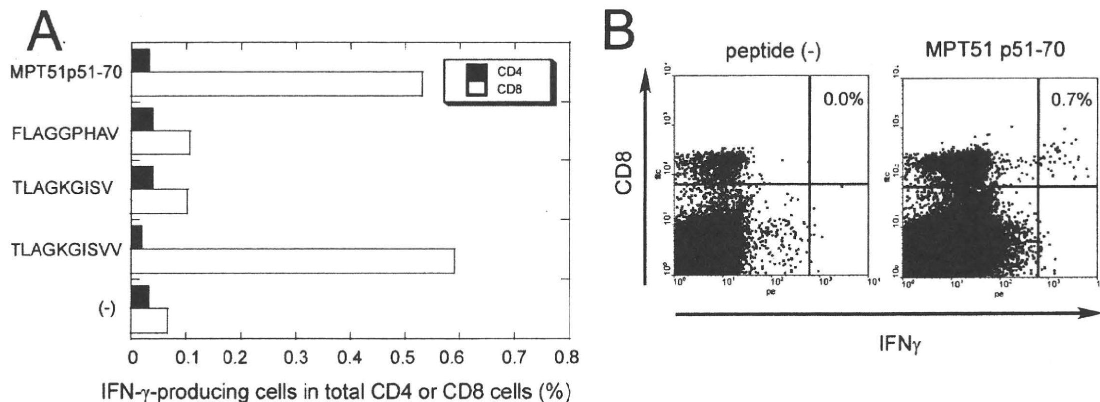

FIG. 2. Identification of a T-cell epitope in the MPT51 p53-62 peptide and the T-cell subset recognizing the epitope in HHD mice. (A) Levels of IFN-γ-producing T-cell subsets in spleens of HHD mice immunized with the pCI-MPT51 plasmid. Three-color flow cytometric analysis was performed for detection 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.

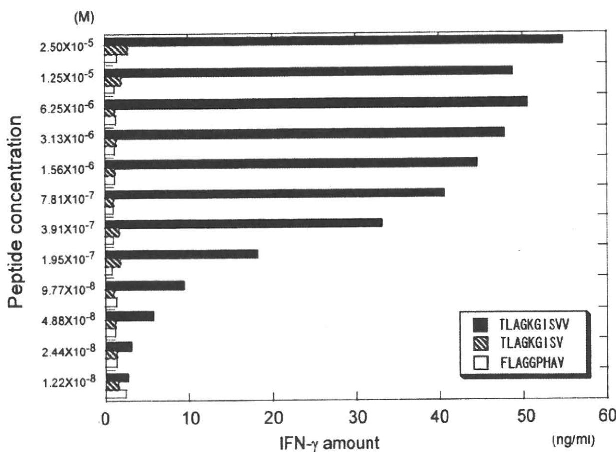

FIG. 3. MPT51 p53-62 is a dominant T-cell epitope in HHD mice. The IFN-γ production by splenocytes from HHD mice immunized with the pCI-MPT51 plasmid in response to twofold serially diluted doses of candidate peptides MPT51 p53-62 (TLAGKGISVV), p53-61 (TLAGKGISV), and p21-29 (FLAGGPHAV) was evaluated.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

presence of p53-62 (TLAGKGISVV) but not in the presence 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 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 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, 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-positive 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-positive individuals were tetramer positive. In parallel, the

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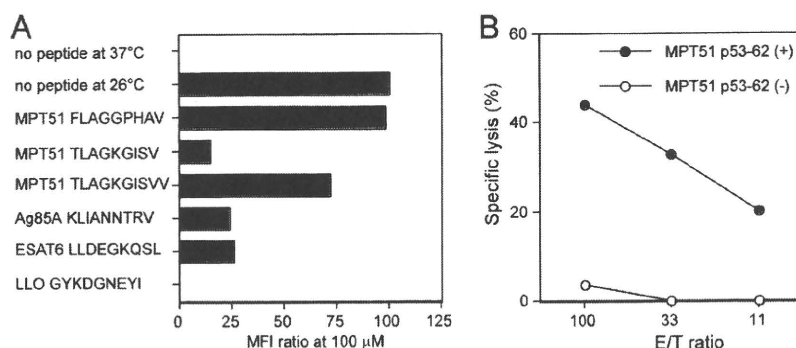

FIG. 4. MPT51 p53-62 peptide binds to cell surface HLA-A*0201 molecules and can be recognized by immune T cells in the context of HLA-A*0201. (A) HLA binding assay with T2 cells showing that MPT51 p21-29 (FLAGGPHAV) and MPT51 p53-62 (TLAGKGISVV) bound to HLA-A*0201 strongly, whereas MPT51 p53-61 (TLAGKGISV), the Ag85A-derived peptide KLIANNTRV, and the ESAT6-derived peptide 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.

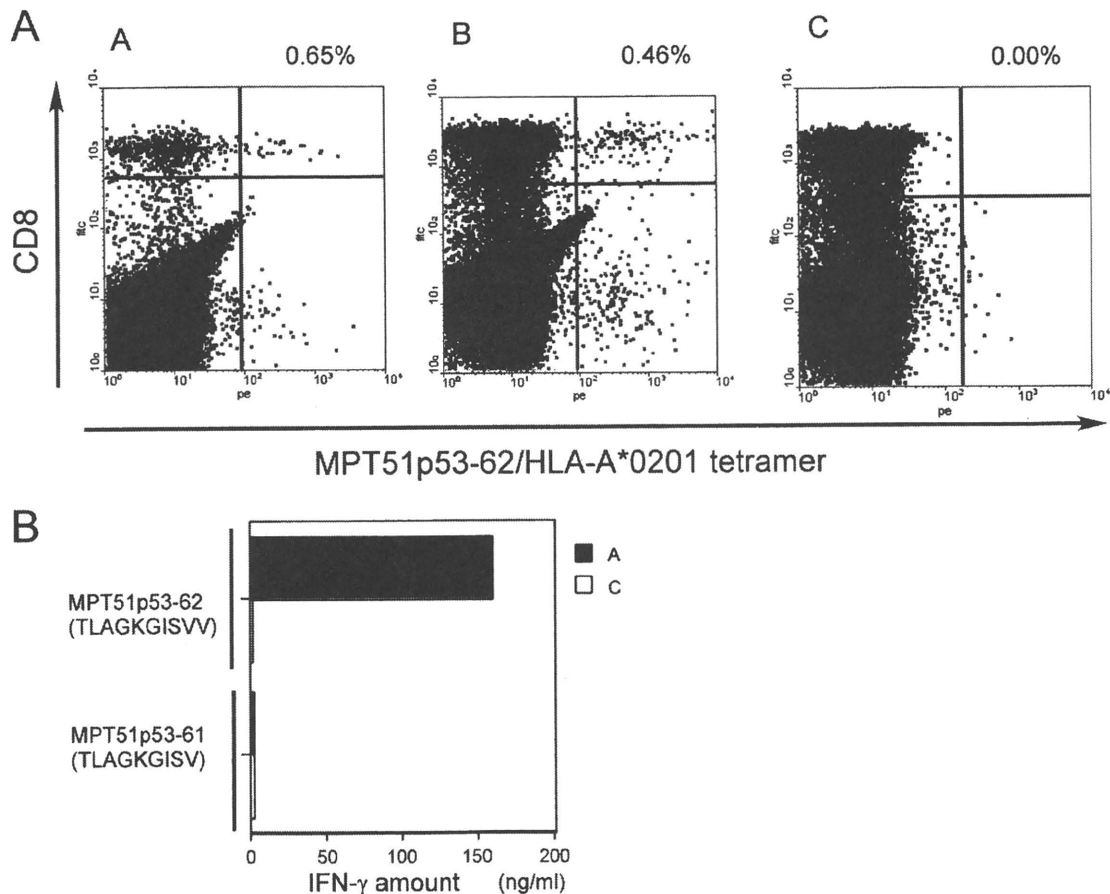

FIG. 5. Detection of MPT51 p53-62-specific memory T cells in PBMCs of HLA-A*0201$^+$ PPD-reactive healthy subjects. (A) Flow cytometric analyses to detect MPT51 p53-62-specific memory T cells in PBMCs of HLA-A*0201$^+$ PPD-reactive healthy subjects using MPT51 p53-62/HLA-A*0201 tetramer. PBMCs of the 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.

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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,

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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ACKNOWLEDGMENTS

This work was supported by grants-in-aid for scientific research from the Japanese Society for the Promotion of Science (grant 11670260 to T.N. and grant 13670268 to Y.K.), by a grant-in-aid for the Centers of Excellence research program from the Ministry of Education, Culture, Sports, Science and Technology of Japan, by a Health and Labor Science Research Grant for Research on Emerging and Re-emerging Infectious Diseases from the Ministry of Health, Labor and Welfare of Japan, and by a grant-in-aid from the United States-Japan Cooperative Medical Science Program.

We thank the NIH Tetramer Facility for providing the HLA-A*0201-peptide tetramer complex, P. Creswell (Yale University School of Medicine) for providing the TAP-deficient T2 cell line, and F. A. Lemonnier (Pasteur Institute) for providing the HLA-A*0201 transgenic HHD mice. The technical assistance of K. Shibata (Hamamatsu University School of Medicine) with the flow cytometric analysis is gratefully acknowledged.

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Editor: R. P. Morrison

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Cancer Letters 260 (2008) 137–145

CANCER Letters

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Effective induction of anti-tumor immune responses with oligomannose-coated liposome targeting to intraperitoneal phagocytic cells

Yuzuru Ikehara [a,b,*], Nobumitsu Shiuchi [c], Sanae Kabata-Ikehara [a,b], Hayao Nakanishi [b], Naoaki Yokoyama [d], Hideaki Takagi [c], Toshi Nagata [e], Yukio Koide [e], Kiyotaka Kuzushima [f], Toshitada Takahashi [g], Kunio Tsujimura [e], Naoya Kojima [c]

[a] *Molecular Medicine Team of Research Center for Medical Glycoscience, National Institute of Advanced Industrial Science and Technology, Centeral 2-12, Room 211, 1-1-1 Umezono, Tsukuba, Japan*

[b] *Division of Oncological Pathology, Aichi Cancer Center Research Institute, Nagoya, Japan*

[c] *Department of Applied Biochemistry, The Institute of Glycotechnology, Tokai University, Hiratsuka, Japan*

[d] *Research Unit for Molecular Diagnosis, National Research Center for Protozoan Diseases, Obihiro University of Agriculture and Veterinary Medicine, Obihiro, Japan*

[e] *Department of Infectious Diseases, Hamamatsu University School of Medicine, Hamamatsu, Japan*

[f] *Division of Immunology, Aichi Cancer Center Research Institute, Nagoya, Japan*

[g] *Aichi Comprehensive Health Science Center of the Aichi Health Promotion Foundation, Aichi, Japan*

Received 2 July 2007; received in revised form 22 October 2007; accepted 29 October 2007

Abstract

We recently established a novel drug delivery system (DDS) using oligomannose-coated liposomes (OMLs) which are probably taken up by macrophages (Mϕ) to carry anti-cancer drugs to milky spots known as preferential metastatic sites of gastric cancers [Y. Ikehara, T. Niwa, L. Biao, S.K. Ikehara, N. Ohashi, T. Kobayashi, Y. Shimizu, N. Kojima, H. Nakanishi, A carbohydrate recognition-based drug delivery and controlled release system using intraperitoneal macrophages as a cellular vehicle, Cancer Res. 66 (2006) 8740–8748]. In the present study, we applied this intraperitoneal DDS for systemic cancer immunotherapy employing ovalbumin (OVA) as a model antigen. The cells taking up the OMLs containing FITC-OVA injected into the peritoneal cavity were predominantly Mϕ, as they showed adhesive characteristics and expressed F4/80 and CD11b almost exclusively. The phagocytic cells also took up bare OVA directly to the same extent as OML-enclosed OVA (OML-OVA), as it is a highly mannosilated protein. The phagocytic cells taking up OML-OVA, however, could activate OVA-specific $CD8^+$ (from OT-I: $H\text{-}2K^b/OVA_{257-264}$-specific) and $CD4^+$ (from OT-II: $H\text{-}2A^b/OVA_{323-339}$-specific) T cells much more effectively *in vitro* than those taking up bare OVA. Furthermore, only the mice pre-immunized with OML-OVA rejected E.G7-OVA (OVA-transfected EL4) but not EL4. These results indicate that

* Corresponding author. Address: Molecular Medicine Team of Research Center for Medical Glycoscience, National Institute of Advanced Industrial Science and Technology, Tsukuba, Japan. Tel.: +81 29 861 5853; Fax: +81 29 861 3462.
E-mail address: yuzuru-ikehara@aist.go.jp (Y. Ikehara).

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doi:10.1016/j.canlet.2007.10.038

the OMLs can also be used as an effective antigen delivery system for cancer immunotherapy activating both CTL and Th subsets.
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Keywords: Drug delivery system; Cancer vaccine; Immune responses to cancer; Oligomannose liposome

1. Introduction

While recent advances in tumor immunology enable us to identify tumor antigens recognized by T cells and understand the molecular and cellular bases of T cell-mediated anti-tumor responses, the clinical realization of effective immunotherapy for solid tumors has not yet been convincingly achieved [1,2]. Many $CD8^+$ and $CD4^+$ T cells recognizing tumor antigen in the context of MHC class I and II, respectively, have been reported, and the former are known to be a major effector of the adaptive anti-tumor immune responses [3–5]. $CD4^+$ T cells play an important role for the expansion and persistence of $CD8^+$ T cells, while some of them are known to function as regulatory cells [5–7]. Optimal anti-tumor immune responses are therefore considered to require the concomitant activation of both $CD8^+$ and $CD4^+$ T cells and the selective activation of $CD4^+$ T cells with helper but not regulatory functions [8]. Endogenous and exogenous antigens are presented as peptides preferentially by MHC class I and II, respectively, and most tumor antigen peptides are derived from the proteins expressed endogenously. Novel methods to make tumor antigens presented simultaneously by both MHC class I and II molecules are therefore needed for the concomitant activation of antigen-specific $CD8^+$ and $CD4^+$ T cells, and many attempts have been made for this purpose [2,3,8].

We recently developed a novel drug delivery system (DDS) using oligomannose-coated liposomes (OMLs) [9,10] which are effectively taken up by $F4/80^+$ intraperitoneal cells to carry anti-cancer drugs to milky spots known as a preferential metastatic site of gastric and ovarian cancers [9,11,12]. We demonstrated that this system could control the formation of overt metastasis of seeded gastric cancer cells at the extra-nodal lymphoid tissues such as the omentum [10].

In the present study, we applied this OML-based intraperitoneal DDS for cancer immunotherapy using ovalbumin (OVA) as a model antigen, aiming at the concomitant activation of antigen-specific $CD8^+$ and $CD4^+$ T cells. Peritoneal phagocytic cells took up OML containing OVA and then migrated into milky spots as previously reported. In addition, they activated both OVA-specific $CD8^+$ [13,14] and $CD4^+$ [15] T cells effectively *in vitro*. Spleen cells from OML-enclosed OVA (OML-OVA)-injected mice showed an effective killing activity against E.G7-OVA (OVA-transfected EL4) [16] but not EL4 [17] *in vitro*, and only the mice pre-immunized with OML-OVA rejected E.G7-OVA but not EL4 *in vivo*. In light of these results obtained *in vitro* and *in vivo*, the potential of our novel OML-based immunization method for the prevention of tumor metastasis is discussed.

2. Materials and methods

2.1. Mice

Female C57BL/6 (B6) mice ($H\text{-}2^b$) at 8–12 weeks of age were obtained from Charles River Japan Inc. (Yokohama, Japan) and kept under standard housing conditions. T cell receptor transgenic mice OT-I (specific for $H\text{-}2K^b/OVA_{257-264}$) [13,14] and OT-II ($H\text{-}2A^b/OVA_{323-339}$) [15] were obtained from the Jackson Laboratory (Bar Harbor, ME) and maintained under specific pathogen-free conditions. All animal experiments were performed under the experimental protocol approved by the Ethics Review Committee for Animal Experimentation of Aichi Cancer Center.

2.2. Cell lines

EL4 [17], a B6-derived thymoma cell line, was maintained in RPMI1640 medium (Invitrogen, Carlsbad, CA) supplemented with 8% fetal bovine serum, 0.2% L-glutamine, 100 U/ml penicillin, 100 μg/ml streptomycin, 0.1% Hepes, 0.1 mM non-essential amino acids, 1 mM sodium pyruvate, and 50 μM 2-ME (complete RPMI). EG.7-OVA (EL4 transfected with *OVA* gene) [16] was obtained from ATCC (Manassas, VA) and maintained in complete RPMI supplemented with 400 μg/ml G418 (Wako, Osaka, Japan) in a humidified 5% CO_2 incubator at 37 °C.

2.3. Man3-DPPE and liposome preparation

Dipalmitoylphosphatidylcholine (DPPC), cholesterol, and dipalmitoylphosphatidylethanolamine (DPPE) were purchased from Sigma–Aldrich (St. Louis, MO). Mannotriose (Man3: Manα1-6(Manα1-3)Man) was purchased from Funakoshi Co., Ltd. (Tokyo, Japan). Man3-DPPE was prepared by conjugation of the mannotriose with DPPE by reductive amination as described in previous papers [10,18]. The purity of Man3-DPPE was confirmed by high-performance thin-layer chromatography (Silica gel 60 HPTLC plate, MERCK, Darmstadt, Germany) and time-of-flight mass spectrometry (Auto FLEX, Bruker Daltonics, Bremen, Germany). The purified Man3-DPPE was quantified by determination of the phosphate contained.

Liposomes were prepared as described previously [10]. Briefly, a chloroform–methanol (2:1, v/v) solution containing 1.5 μmol of DPPC and 1.5 μmol of cholesterol was placed in a conical flask and dried by rotary evaporation. Subsequently, 2 ml ethanol containing 0.15 μmol of Man3-DPPE was added to the flask and evaporated to prepare a lipid film containing neoglycolipids. Procedures for protein-encasing of oligomannose-coated liposomes (OMLs) were performed as described previously [10]. The multilamellar vesicles were generated with either 200 μl of FITC-labelled or non-labelled OVA (5.0 mg/ml, Sigma–Aldrich), Alexa Fluor 680 (Molecular Probes, Eugene, OR)-labelled bovine serum albumin (BSA, 5 mg/ml, Sigma–Aldrich), or PBS in the dried lipid film by intense vortex dispersion. The multilamellar vesicles were extruded 10 times through polycarbonate membranes of 1 μm pore (Nucleopore, Pleasanton, CA). Liposomes entrapping proteins were separated from free untrapped proteins by four successive rounds of washing in PBS with centrifugation (20,000*g*, 30 min) at 4 °C. The amounts of entrapped proteins were measured using a modified Lowry protein assay reagent (Pierce, Rockford, IL) in the presence of 0.3% (w/v) sodium dodecyl sulfate using BSA as the standard.

2.4. Flow cytometry

One hour after intraperitoneal injection, peritoneal exudate cells (PEC) were recovered from B6 mice with 5 ml ice cold PBS. PEC were incubated on ice for 30 min with fluorescein-labelled antibodies against mouse hematopoietic cell lineage markers after blocking with mouse Fc Blocker (BD Biosciences, San Jose, CA) and then analysed on a FACS Calibur (BD Biosciences). The following monoclonal antibodies used in this study were purchased or kindly provided: anti-F4/80 (A3-1, Serotec Ltd., Oxford, UK), anti-MHC class II (M5/114.15.2, e-Bioscience, Boston, MA), anti-CD11b (M1/70.15, Caltag Laboratories, Burlingame, CA), anti-CD3ε (145-2C11, BD Biosciences), anti-CD19 (1D3, BD Biosciences), and anti-H-2K^{b}D^{b} (20-8-4S, Dr. E. Nakayama, Okayama University).

2.5. Macrophage depletion by plastic adhesion

PEC suspension (2×10^7 cells in 10 ml of complete RPMI) was poured into a 75 cm^2 tissue culture flask and incubated at 37 °C for 2 h in a humidified 5% CO_2 incubator. Non-adherent cells were collected with serum-free DMEM and subjected to FACS analysis.

2.6. In vitro activation of OVA-specific T cells

One hour after injection of either OML-encased OVA or bare OVA into the peritoneal cavity of B6 mice, PEC were recovered with 5 ml ice cold PBS. The PEC suspended in complete RPMI were seeded into a 96-well culture plate (5×10^5 cells in each well) and incubated at 37 °C for overnight in a humidified 5% CO_2 incubator. On the next day, non-adherent cells were washed out with complete RPMI, and co-cultured with 5×10^5 cells of either CD8$^+$ or CD4$^+$ T cells from the spleen of OT-I and OT-II mice, respectively. CD8$^+$ and CD4$^+$ T cells were prepared with the isolation kits for corresponding subsets (Miltenyi Biotec Inc., Auburn, CA). The supernatants were collected at 24, 48, and 72 h and assayed for IFN-γ production with Mouse IFN-γ ELISA kit (Pierce Biotechnology, Inc., Rockford, IL).

2.7. CTLs assay

B6 mice were immunized biweekly three times by intraperitoneal injection of 1 μg OVA in liposome/mouse with or without oligomannose coating. Spleen cells were isolated from the mice 1 week after the last challenge, and 1×10^6 spleen cells were stimulated with 10 μg OVA in 1 ml for 72 h. The effector cells thus prepared were co-cultured with target cells (E.G7-OVA or EL4) at various effector/target ratios for 8 h at 37 °C, and the cytotoxicity was measured with CytoTox96 Non-Radioactive Cytotoxicity assay kit (Promega, Madison, WI).

2.8. Tumor assay

Tumor cells (in 0.2 ml) were injected intradermally into the backs of mice with a 27 gauge needle. The diameter of the tumors was measured with Vernier calipers twice at right angles to calculate the mean diameter, and the survival time after tumor challenges was followed.

3. Results

3.1. OMLs are taken up preferentially by intraperitoneal macrophages

We showed that neoglycolipid-coated liposomes are ingested by intraperitoneal cells much more effectively than non-coated liposomes [10]. Of those, OMLs are incorporated most effectively, and the cells ingesting

OMLs are preferentially F4/80^{+} and migrate into extranodal lymphoid tissues in the omentum after the uptake. We have also shown that the OML-ingesting cells are very useful drug delivery vehicles for cancer chemotherapy in the previous study [9,10]. To verify whether the OMLs are applicable also for cancer immunotherapy, we first analyzed in detail the peritoneal cells incorporating OMLs. Bovine serum albumin (BSA, Sigma–Aldrich) was labelled with Alexa Fluor 680, encased in OML and then injected into the peritoneal cavity of B6 mice. One hour after the injection of OMLs containing Alexa Fluor 680-labelled BSA, PEC were collected and analyzed. As shown in Fig. 1A, PEC were divided into three groups based on the incorporation of OMLs. When adherent cells were removed by plastic dish adherence, only the population with higher OML uptake (R1) disappeared (Fig. 1B). In addition, most cells of R1 express F4/80 and CD11b but not CD3 and CD19 (Fig. 1C), suggesting that R1 population preferentially consists of macrophages (Mϕ). The PEC with lower OML uptake (R2) did not express F4/80, and nearly 2/3 of them were considered to be B cells because of their CD19 expression. These results together confirmed that OMLs injected into the peritoneal cavity were ingested preferentially by Mϕ, and also indicate that OML is a good vehicle for the phagocytosis of non-glycosylated proteins.

3.2. Phagocytic cells ingesting OMLs activate both CD8 and CD4 T cells in vitro in an antigen-specific manner

We next analyzed the antigen-presenting capacity of the phagocytic cells ingesting OMLs containing ovalbumin (OVA) as an antigen. CD8^{+} T cells from OT-I (a transgenic strain of T cell receptor (TCR) recognizing $OVA_{257\text{–}264}$ peptide presented by H-2K^{b}) and CD4^{+} T cells from OT-II (a transgenic strain of TCR recognizing $OVA_{323\text{–}339}$ peptide presented by H-2A^{b}) were used as responder cells. When these T cells were co-cultured with adherent cells enriched from PEC of the mouse intraperitoneally injected with OML-encased OVA (OML-OVA), both CD8^{+} and CD4^{+} T cells produced large amounts of IFN-γ (Fig. 2). Though adherent cells from the mice injected with soluble OVA also stimulated both CD8^{+} and CD4^{+} T cells, much higher amounts of OVA were needed compared to those from the mice injected with OML-OVA. Mϕ ingesting OML-OVA are supposed to

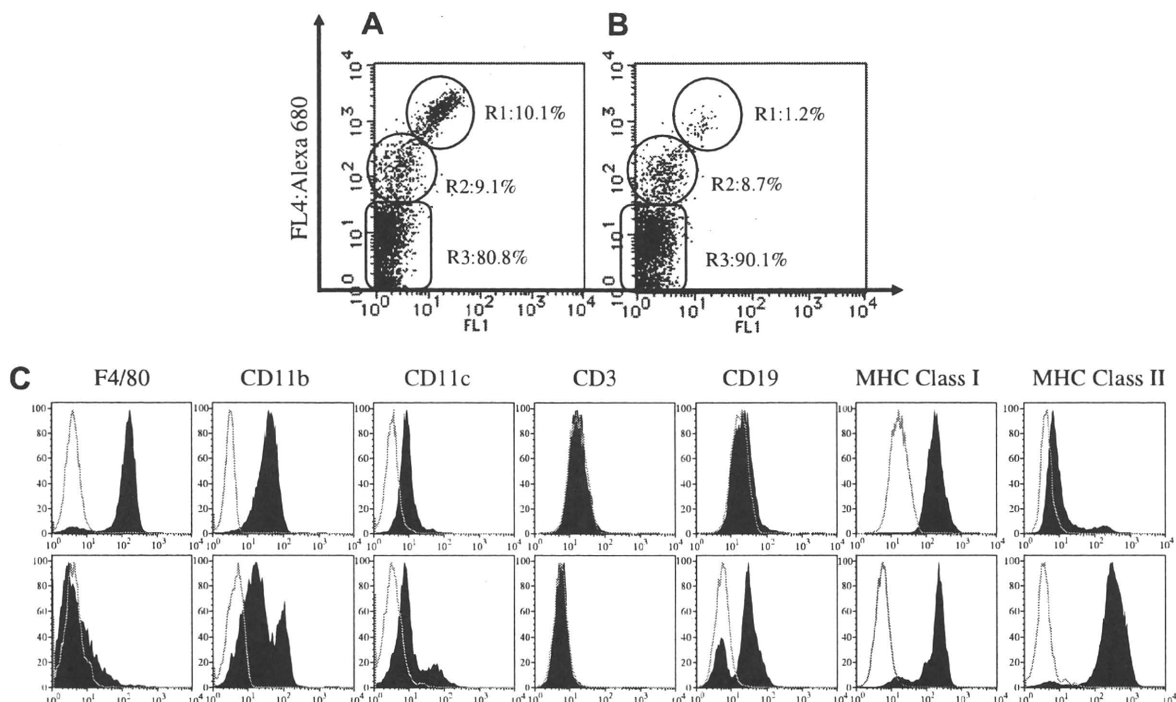

Fig. 1. OMLs injected into the peritoneal cavity were ingested preferentially by adhesive cells. One hour after injection of OMLs containing Alexa Fluor 680-labelled BSA, PEC were collected and their fluorescence was analyzed by flow cytometry (A). Non-adherent PEC were further isolated by plastic adherence for 2 h and analyzed (B). (C) Phenotypic analysis of PEC derived from OML-injected mice. One hour after injection of OMLs containing Alexa Fluor 680-labelled OVA, PEC were collected and stained with mAbs indicated. As shown in (A) and (B), PEC were divided into three groups based on their fluorescence intensity of Alexa Fluor 680, and the surface phenotypes of R1 and R2 were further analyzed.

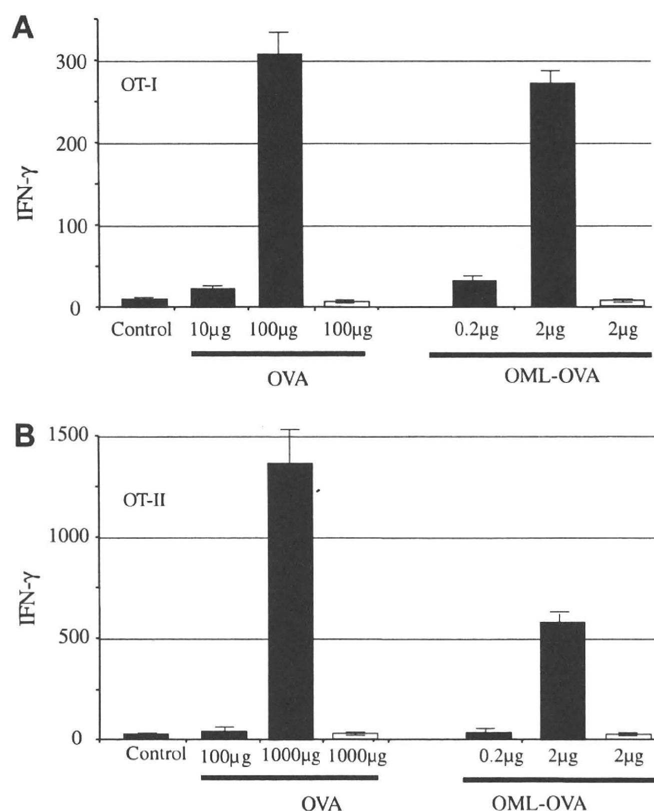

Fig. 2. Mϕ ingesting OVA encased in OML activate OVA-specific $CD8^+$ and $CD4^+$ T cells much more effectively than those ingesting soluble OVA. One hour after intraperitoneal injection of antigens, PEC were prepared from mice, and adherent cells were enriched by plastic adherence. $CD8^+$ and $CD4^+$ T cells were purified from spleen of OT-I (A) and OT-II (B), respectively, co-cultured (closed bar) with adherent PEC or cultured adherent PEC alone (open bar) for 24 h, and then production of IFN-γ in supernatants was tested by ELISA. PEC recovered from mice without any treatment was used as control. OML-OVA, OVA encased in OML; OVA, OVA; control, OML containing PBS. The graph shows the average and standard error from three independent experiments.

present antigen effectively also *in vivo*, as they effectively induced proliferation responses of OVA-specific $CD8^+$ T cells in the spleens of OT-I mice (Supplement Figure 1).

We next analyzed the uptake efficiency of OML-encased and soluble OVA and found that peritoneal phagocytic cells effectively uptake OVA irrespective of sugar encapsulation (Fig. 3). The uptake of soluble OVA is probably mediated by mannose receptors, as it is known as a highly mannosylated protein [19]. These results together indicate that OML-mediated ingestion promotes the presentation of OVA peptides by both MHC class I and II molecules by enhancing the antigen processing but not the uptake efficiency.

3.3. *Induction of antigen-specific cytotoxic T lymphocytes (CTL) in vitro by OML-mediated immunization*

We next performed CTL assay to detect OVA-specific T cells in the spleen. Only the spleen cells from mice immunized with OML-OVA but not bare liposome (BL)-encased OVA showed cytotoxicity against E.G7-OVA. The spleen cells from neither group showed cytotoxicity against EL4, confirming that OVA-specific CTL can be effectively induced *in vivo* by OML-OVA immunization (Fig. 4).

3.4. *OML-mediated immunization induces antigen-specific anti-tumor immunity in vivo*

We finally examined whether intraperitoneal immunization with OMLs also induces antigen-specific anti-tumor immunity *in vivo*. Mice were immunized intraperitoneally with OVA with or without OML encasing and then challenged with E.G7-OVA or EL4. As shown in Fig. 5, only the mice immunized with OML-OVA survived for more than 70 days when challenged with E.G7-OVA, while naïve and bare OVA-immunized mice died within 55 days. All the mice including those immunized with OML-OVA died within 30 days when challenged with EL4, indicating that the rejection of

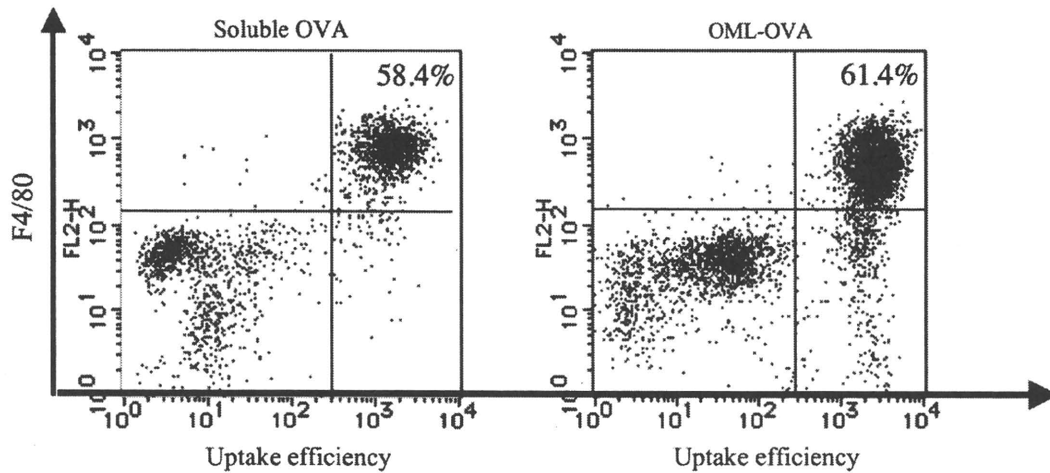

Fig. 3. Peritoneal F4/80^{+} cells uptake OVA effectively irrespective of carbohydrate encapsulation. One hour after injection of either soluble FITC-OVA (20 μg) or OML-encapsulated FITC-OVA (20 μg) into the peritoneal cavity of B6 mice, uptake efficiency of FITC-OVA by peritoneal cells was analyzed by flow cytometry together with F4/80 expression.

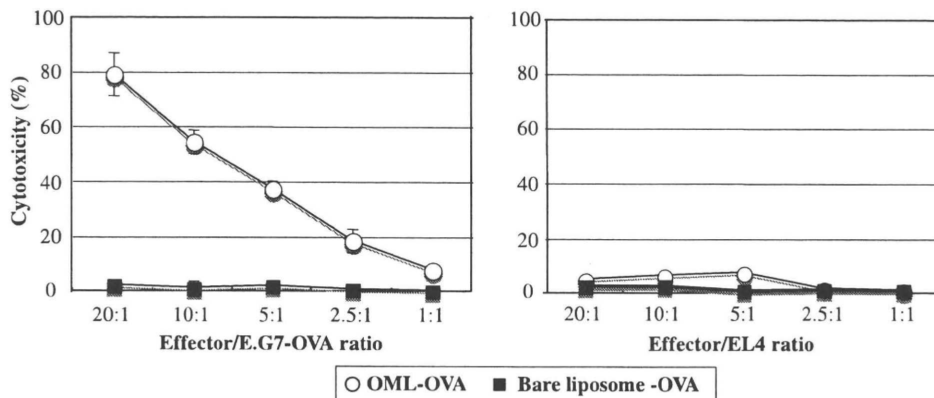

Fig. 4. OML-OVA-generated OVA-specific cytotoxicity. B6 mice were immunized biweekly three times by intraperitoneal injection of 1 μg OVA encased in oligomannose-coated (OML-OVA, open symbols) or bare liposomes (bare liposome-OVA, closed symbols). Spleen cells were isolated from mice one week after the last challenge, and 1×10^{6} cells were stimulated with 10 μg OVA in 1 ml for 72 h. The graph shows the average and standard error from three independent experiments.

E.G7-OVA is OVA-specific. These results together showed that OML-mediated immunization can induce systemic immune response robust enough to protect mice from tumor challenge in an antigen-specific manner.

4. Discussion

In this study, we demonstrated that our novel OML-based drug delivery system (DDS) targeted to intraperitoneal phagocytic cells can also be used for the induction of systemic immune responses. After ingesting OML-encased OVA (OML-OVA), intraperitoneal phagocytic cells to extra-nodal lymphoid tissues in abdominal cavity and presented OVA-derived peptides in the context of both MHC class I and II molecules. Only the mice pre-immunized with OML-OVA rejected E.G7-OVA but not EL4 challenged subcutaneously. These results together indicate that the OMLs can be used as an effective antigen delivery system for immunotherapy activating both CTL and Th subsets. Fig. 6 shows the plausible induction process of anti-tumor immunity starting from phagocytic cells triggered by OML injection.

OMLs are very useful not only for the promotion of non-glycosylated protein uptake by antigen-presenting cells but also for the enhancement of antigen-processing of encased antigens. Endogenous

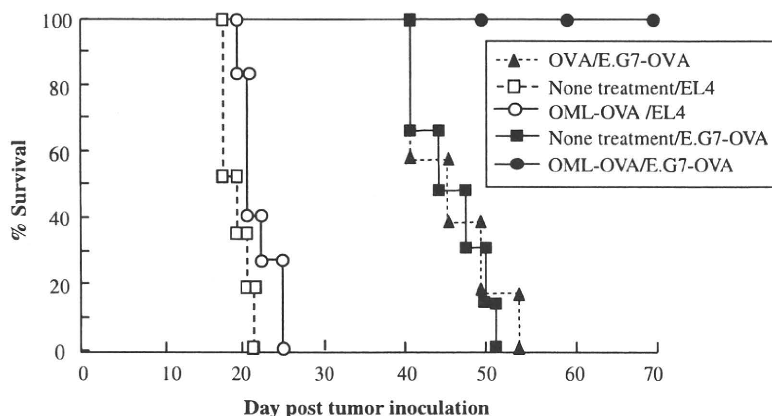

Fig. 5. OML-mediated immunization induces antigen-specific anti-tumor immunity *in vivo*. OML-OVA-immunized (circles) and naïve (squares) mice were challenged with E.G7-OVA (closed circles and squares) or EL4 cells (open circles and squares). As a control, mice were immunized with soluble OVA and challenged with E.G7-OVA (closed triangles).

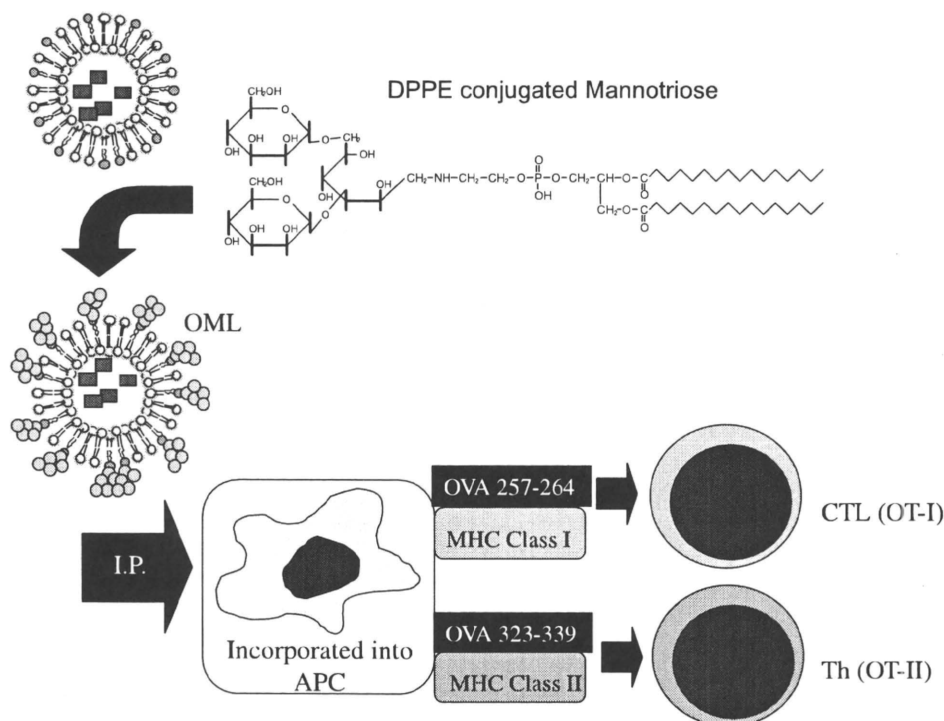

Fig. 6. Possible mechanism of OML-based vaccine delivery. Coating of bare liposomes with DPPE-conjugated mannotriose facilitates functions of intraperitoneal macrophages, resulting in antigen-specific activation of both CTL and Th populations.

and exogenous antigens are presented preferentially by MHC class I and II, respectively. OML-OVA, however, were effectively directed to both pathways, even when added exogenously. This advantage of OML-mediated immunization will hopefully facilitate the simultaneous activation of tumor antigen-specific $CD4^+$ and $CD8^+$ T cells as shown here with OVA. It is also very interesting to study the mechanism by which OML-mediated ingestion of antigens enhances the antigen presentation by both MHC molecules [20–22]. So far, we observed the up-regulation of CD80 and CD86 on OML-ingesting cells (in press on Cytokine, H. Takagi et al.), but it seems very important to know the additional signals to

make antigen presenting cells more immunogenic [23]. Additional adjuvant effects of various cytokines and/or toll-like receptor ligands on OML-mediated immunization are now being investigated.

Another advantage of OML-mediated immunization is Th1-skewing of the cytokine profiles. Indeed, OT-I and OT-II T cells stimulated with antigen-presenting cells ingesting OML-OVA produced IFN-γ but not IL-4 or IL-10 (unpublished observation). Moreover, our previous study demonstrated that the OML-mediated immunization protects BALB/c mice against *Leishmania major* infection, possibly due to the Th1-skewing of immune responses [24]. We observed that phagocytic cells ingesting OML preferentially produce IL-12 (unpublished observation), suggesting this cytokine is a key of Th1-skewing as reported previously [25]. Further investigation of the mechanism of this Th1-skewing of immune responses induced by OML-mediated immunization is currently underway.

Cells belonging to the monocyte-Mϕ lineage have been known to be heterogeneous, reflecting the plasticity and versatility of these cells in response to various microenvironmental signals [26]. Mϕ are now roughly categorized into M1 and M2 based on their functional properties, and several studies revealed that M1 and M2 promote type I and type II Th responses, respectively [27–29]. It is also reported that M1 and M2 are prone to induce inflammatory and immunoregulatory responses, respectively [29]. A possible concern of our DDS system is therefore the protumoral effects by M2 with antigen-encased OMLs, as they are supposed to express macrophage mannose receptors induced by IL-4 [30]. Although at least our *in vitro* study clearly showed OML-mediated skewing to type I immune responses, more precise investigation including the conditions for M1 polarization should be done especially in tumor-bearing mice. In addition, characteristics of the small population of non-Mϕ cells ingesting OMLs should be investigated as well.

In order to use our DDS in clinical study, the best administration routes should be determined to pursue repetitive vaccination while avoiding possible side effects. As generally acknowledged, intraperitoneal administration is accompanied with a high risk of side effects such as catheter-related complications, and abdominal pain [31]. In this connection, we have already obtained anti-tumor effects by subcutaneous injection of OML-OVA similar to those by intraperitoneal injection. However, side effects induced by subcutaneous injection of OMLs should be further investigated to assure their safe clinical application.

In the previous study, we reported that the formation of intraperitoneal metastasis of seeded gastric cancer cells in milky spots can be controlled with OMLs containing anti-cancer drugs [10]. In the present study, we have further extended the possibility of OMLs for the immunotherapy of systemic metastasis and existing tumor cells aside from milky spots. Oligomannose coating of liposomes showed the best uptake efficiency by intraperitoneal Mϕ among the neoglycolipids so far tested, and the encased antigen was effectively presented by both MHC class I and II molecules. However, the additional effects for immune responses by other neoglicolipids (shown here) have not been studied at all so far. We have a great interest in their effects on immune responses and are seeking sugar materials with immunoregulatory properties. If such materials are found, we believe that further study of our sugar-coated liposome technology will find it also to be applicable for antigen-specific regulation of autoimmune diseases and allergy.

Acknowledgments

We thank Ms. Y. Matsudaira and Ms. K. Nishida for their excellent technical assistance. This work was supported by the Industrial Technology Research Grant Program in 04' from the New Energy and Industrial Technology Development Organization (NEDO) of Japan, and in part by a grant for Hi-tech research program from Tokai University and by the Program for Promotion of Basic Research Activities for Innovative Biosciences (PROBRAIN). The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked advertisement in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.canlet.2007.10.038.

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Serodiagnosis of *Mycobacterium avium*–Complex Pulmonary Disease Using an Enzyme Immunoassay Kit

Seigo Kitada[1], Kazuo Kobayashi[2], Satoshi Ichiyama[3], Shunji Takakura[3], Mitsunori Sakatani[4], Katsuhiro Suzuki[4], Tetsuya Takashima[5], Takayuki Nagai[5], Ikunosuke Sakurabayashi[6], Masami Ito[7], Ryoji Maekura[1], for the MAC Serodiagnosis Study Group

[1]Department of Internal Medicine, National Hospital Organization (NHO) National Toneyama Hospital, Toyonaka-shi, Osaka, Japan; [2]Department of Immunology, National Institute of Infectious Diseases, Shinjuku-ku, Tokyo, Japan; [3]Department of Clinical Laboratory Medicine, Graduate School of Medicine, Kyoto University, Kyoto-shi, Kyoto, Japan; [4]Department of Internal Medicine, NHO Kinki-chuo Chest Medical Center, Sakai-shi, Osaka, Japan; [5]Department of Medicine, Osaka Prefectural Medical Center for Respiratory and Allergic Diseases, Habikino-shi, Osaka, Japan; [6]Department of Laboratory Medicine, Saitama Medical Center, Jichi Medical University, Saitama-shi, Saitama, Japan; and [7]Department of Internal Medicine, Sakamoto Hospital, Toyonaka-shi, Osaka, Japan

***Rationale*: The diagnosis of *Mycobacterium avium*–complex pulmonary disease (MAC-PD) and/or its discrimination from pulmonary tuberculosis (TB) is sometimes complicated and time consuming.**
***Objectives*: We investigated in a six-institution multicenter study whether a serologic test based on an enzyme immunoassay (EIA) kit was useful for diagnosing MAC-PD and for distinguishing it from other lung diseases.**
***Methods*: An EIA kit detecting serum IgA antibody to glycopeptidolipid core antigen specific for MAC was developed. Antibody levels were measured in sera from 70 patients with MAC-PD, 18 with MAC contamination, 37 with pulmonary TB, 45 with other lung diseases, and 76 healthy subjects.**
***Measurements and Main Results*: Significantly higher serum IgA antibody levels were detected in patients with MAC-PD than in the other groups ($P < 0.0001$). Setting the cutoff point at 0.7 U/ml resulted in a sensitivity and specificity of the kit for diagnosing MAC-PD of 84.3 and 100%, respectively. Significantly higher antibody levels were also found in patients with nodular-bronchiectatic disease compared with fibrocavitary disease in MAC-PD ($P < 0.05$). There was a positive correlation between the extent of disease on chest computed tomography scans and the levels of antibody ($r = 0.43$, $P < 0.05$) in patients with MAC-PD.**
***Conclusions:* The EIA kit is useful for the rapid diagnosis of MAC-PD and for differentiating MAC-PD from pulmonary TB and, if validated by studies in other populations, could find wide application in clinical practice.**

Keywords: nontuberculous mycobacteria; immunocompetence; sensitivity and specificity

The prevalence of disease due to nontuberculous mycobacteria has been increasing recently (1–5). In Japan, *Mycobacterium avium* complex (MAC) accounts for approximately 70% of nontuberculous mycobacterial disease (6). MAC is now widely recognized as an important pathogen that causes chronic and progressive pulmonary disease even in immunocompetent patients and not only in those who are immunosuppressed. The diagnosis of MAC-PD is complicated because, in contrast to *Mycobacterium tuberculosis*, MAC contamination of clinical specimens can come from environmental sources such as water, dust, and soil, and because this organism may colonize the respiratory tract without any accompanying invasive disease (4). Thus, isolation of MAC from sputa is often of no clinical significance. Diagnosis of pulmonary disease due to MAC is complicated and time consuming when made according to the guidelines of the American Thoracic Society (ATS) (1), because MAC is ubiquitous in nature and the diagnosis requires clinical findings and its repeated isolation from sputum. In addition, it is also difficult to discriminate MAC-PD from infection due to other mycobacteria in the absence of culture results, because clinical features, such as symptomatic or radiographic findings, are very similar in mycobacterial diseases. In the context of infection control, it is particularly important to distinguish between MAC-PD and pulmonary tuberculosis (TB).

To overcome these difficulties, we have developed a serologic test for the glycopeptidolipid (GPL) antigen specific for MAC, and have reported its clinical usefulness (7–9). The levels of antibody to GPL core were measured by an enzyme immunoassay (EIA) using sera of immunocompetent patients with MAC-PD. MAC-PD could be discriminated from pulmonary TB, *Mycobacterium kansasii* pulmonary disease and MAC colonization/contamination using this serologic test. Healthy subjects were seronegative. Of the different immunoglobulin (Ig) subclasses, best results were obtained by the measurement of IgA, with a sensitivity of 92.5% and specificity of 95.1%. These results suggest that the test is useful as a diagnostic aid. In the present study, to apply this test widely in clinical practice, we

AT A GLANCE COMMENTARY

Scientific Knowledge on the Subject

The diagnosis of pulmonary disease due to ubiquitous *Mycobacterium avium* complex (MAC) is complicated, and requires clinical findings together with repeatedly positive sputum culture.

What This Study Adds to the Field

An enzyme immunoassay kit for measuring human serum antibody to glycopeptidolipid core antigen specific for MAC was developed. The kit is useful for the serodiagnosis of MAC pulmonary disease and could find wide application in clinical practice.

(Received in original form May 25, 2007; accepted in final form December 13, 2007)

Supported by grants from the Ministry of Health, Labor, and Welfare (Research on Emerging and Re-emerging Infectious Diseases, Health Sciences research grants); the Ministry of Education, Culture, Sports, Science, and Technology; Tauns Laboratory, Inc.; and the Osaka Tuberculosis Research Foundation.

Correspondence and requests for reprints should be addressed to Seigo Kitada, M.D., Department of Internal Medicine, National Hospital Organization National Toneyama Hospital, 5-1-1 Toneyama, Toyonaka-shi, Osaka 560-8552, Japan. E-mail: kitadas@toneyama.hosp.go.jp

This article contains an online supplement, which is accessible from this issue's table of contents at www.atsjournals.org

Am J Respir Crit Care Med Vol 177. pp 793–797, 2008
Originally Published in Press as DOI: 10.1164/rccm.200705-771OC on December 13, 2007
Internet address: www.atsjournals.org