

- [11] Chen L, Wang J, Zganiacz A, Xing Z. Single intranasal mucosal *Mycobacterium bovis* BCG vaccination confers improved protection compared to subcutaneous vaccination against pulmonary tuberculosis. *Infect Immun* 2004; 72(1):238-46.
- [12] Brossart P, Goldrath AW, Butz EA, Martin S, Bevan MJ. Virus-mediated delivery of antigenic epitopes into dendritic cells as a means to induce CTL. *J Immunol* 1997; 158:3270-6.
- [13] Nakamura Y, Suda T, Nagata T, Aoshi T, Uchijima M, Yoshida A, et al. Induction of protective immunity to *Listeria monocytogenes* with dendritic cells retrovirally transduced with a cytotoxic T lymphocyte epitope minigene. *Infect Immun* 2003; 71(4):1748-54.
- [14] Naldini L, Blömer U, Gallay P, Ory D, Mulligan R, Gage FH, et al. *In vivo* gene delivery and stable transduction of nondividing cells by a lentiviral vector. *Science* 1996; 272:263-7.
- [15] Dyllal J, Latouche J-B, Schnell S, Sadelain M. Lentivirus-transduced human monocyte-derived dendritic cells efficiently stimulate antigen-specific cytotoxic T lymphocytes, *Blood* 2001;97(1):114-21.
- [16] Rouas R, Uch R, Cleuter Y, Jordier F, Bagnis C, Mannoni P, et al. Lentiviral-mediated gene delivery in human monocyte-derived dendritic cells: Optimized design and procedures for highly efficient transduction compatible with clinical constrains. *Cancer Gene Ther* 2002; 9:715-24.
- [17] Zarei S, Abraham S, Arrighi J-F, Haller O, Calzascia T, Walker PR, et al. Lentiviral transduction of dendritic cells confers protective antiviral immunity *in vivo*. *J Virol* 2004; 78(14):7843-5.
- [18] Fe Medina M, Kobinger GP, Rux J, Gasmi M, Looney DJ, Bates P, et al. Lentiviral vectors pseudotyped with minimal filovirus envelopes increased gene transfer in murine lung. *Mol Ther* 2003; 8(5):777-89.
- [19] Miki K, Nagata T, Tanaka T, Kim YH, Uchijima M, Ohara N, et al. Induction of protective cellular immunity against *Mycobacterium tuberculosis* by recombinant attenuated

- self-destructing *Listeria monocytogenes* strains harboring eukaryotic expression plasmids for antigen 85 complex and MPB/MPT51. *Infect Immun* 2004; 72(4):2014-21.
- [20] Miyoshi H, Blömer U, Takahashi M, Gage FH, Verma IM. Development of a self-inactivating lentivirus vector. *J Virol* 1998; 72(10):8150-7.
- [21] Dull T, Zufferey R, Kelly M, Mandel RJ, Nguyen M, Trono D, et al. A third-generation lentivirus vector with a conditional packaging system. *J Virol* 1998; 72(11):8463-71.
- [22] Brown RH, Walters DM, Greenberg RS, Mitzner W. A method of endotracheal intubation and pulmonary functional assessment for repeated studies in mice. *J Appl Physiol* 1999; 87(6):2362-5.
- [23] Sato J, Schorey J, Ploplis VA, Haalboom E, Krahule L, Castellino FJ. The fibrinolytic system in dissemination and matrix protein deposition during a *Mycobacterium* infection. *Am J Pathol* 2003; 163(2):517-31.
- [24] Bukreyev A, Belyakov IM, Berozofsky JA, Murphy BR, Collins PL. Granulocyte-macrophage colony-stimulating factor expressed by recombinant respiratory syncytial virus attenuates viral replication and increases the level of pulmonary antigen-presenting cells. *J Virol* 2001; 75(24):12128-40.
- [25] Suzuki M, Aoshi T, Nagata T, Koide Y. Identification of murine H2-D^d- and H2-A^b-restricted T-cell epitopes on a novel protective antigen, MPT51, of *Mycobacterium tuberculosis*. *Infect Immun* 2004; 72(7):3829-37.
- [26] Zhu X, Venkataprasad N, Ivanyi J, Vordermeier HM. Vaccination with recombinant vaccinia viruses protects mice against *Mycobacterium tuberculosis* infection. *Immunolgy* 1997; 92(1):6-9.
- [27] McShane H, Pathan AA, Sander CR, Keating SM, Gilbert SC, Huygen K, et al. Recombinant modified vaccinia virus Ankara expressing antigen 85A boosts BCG-primed and naturally acquired antimycobacterial immunity in humans. *Nat Med* 2004; 10(11):1240-4.
- [28] Wang J, Thorson L, Stokes RW, Santosuosso M, Huygen K, Zganiacz A, et al. Single,

- mucosal, but not parenteral, immunization with recombinant adenoviral-based vaccine provides potent protection from pulmonary tuberculosis. *J Immunol* 2004; 173:6357-65.
- [29] Santosuosso M, Zhang X, McCormick S, Wang J, Hitt M, Xing Z. Mechanisms of mucosal and parenteral tuberculosis vaccinations: Adenoviral-based mucosal immunization preferentially elicits sustained accumulation of immune protective CD4 and CD8 T cells within the airway lumen. *J Immunol* 2005; 174:7986-94.
- [30] Esslinger C, Chapatte L, Finke D, Miconnet I, Guillaume P, Lévy F, et al. *In vivo* administration of a lentiviral vaccine targets DCs and induces efficient CD8⁺ T cell responses. *J Clin Invest* 2003; 111(11):1673-81.
- [31] Gallichan WS, Rosenthal KL. Long-lived cytotoxic T lymphocyte memory in mucosal tissues after mucosal but not systemic immunization. *J Exp Med* 1996; 184:1879-90.
- [32] Giri PK, Verma I, Khuller GK. Enhanced immunoprotective potential of *Mycobacterium tuberculosis* Ag85 complex protein based vaccine against airway *Mycobacterium tuberculosis* challenge following intranasal administration. *FEMS Immunol Med Microbiol* 2006; 47:233-41.
- [33] Kyd JM, Foxwell AR, Cripps AW. Mucosal immunity in the lung and upper airway. *Vaccine* 2001; 19:2527-33.
- [34] Aguilar D, Infante E, Martin C, Gormley E, Gicquel B, Hernandez Pando R. Immunological responses and protective immunity against tuberculosis conferred by vaccination of Balb/C mice with the attenuated *Mycobacterium tuberculosis* (*phoP*) SO2 strain. *Clin Exp Immunol* 2006; 147:330-8.
- [35] Kamath AB, Woodworth J, Xiong X, Taylor C, Weng Y, Behar SM. Cytolytic CD8⁺ T cells recognizing CFP10 are recruited to the lung after *Mycobacterium tuberculosis* infection. *J Exp Med* 2004; 200(11):1479-89.
- [36] Serbina NY, Flynn JL. CD8⁺ T cells participate in the memory immune response to *Mycobacterium tuberculosis*. *Infect Immun* 2001; 69(7):4320-8.
- [37] Sallusto F, Lenig D, Forster R, Lipp M, Lanzavecchia A. Two subsets of memory T

lymphocytes with distinct homing potentials and effector functions. *Nature* 1999; 401:708-12.

- [38] Hogan RJ, Zhong W, Usherwood EJ, Cookenham T, Roberts AD, Woodland DL. Protection from respiratory virus infections can be mediated by antigen-specific CD4⁺ T cells that persist in the lungs. *J Exp Med* 2001; 193(8):981-6.

Figure legends

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. 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.

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 to 6 weeks after MPT51 lentivirus administration are shown. Percentages in the figure indicate those of tetramer-positive cells in CD8⁺ cells.

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 \pm SD from 6 mice per each group are shown.

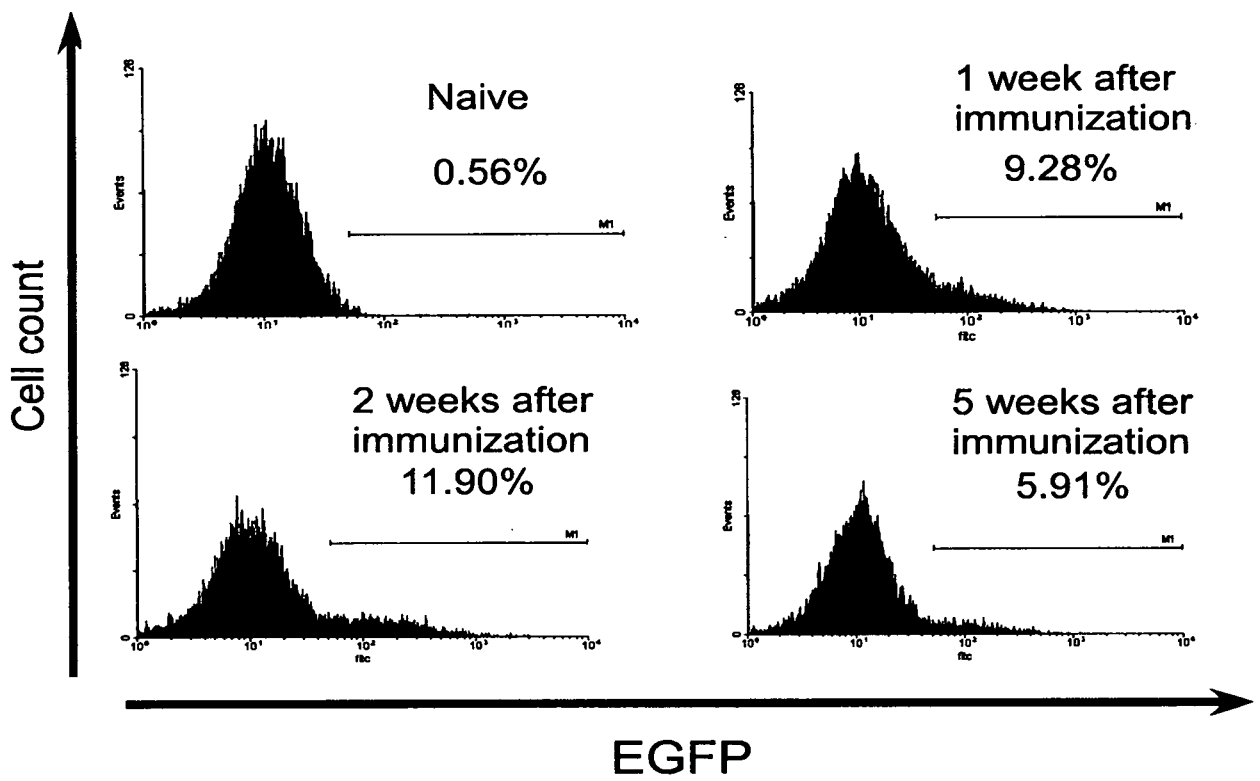


Fig. 1, Hashimoto et al.

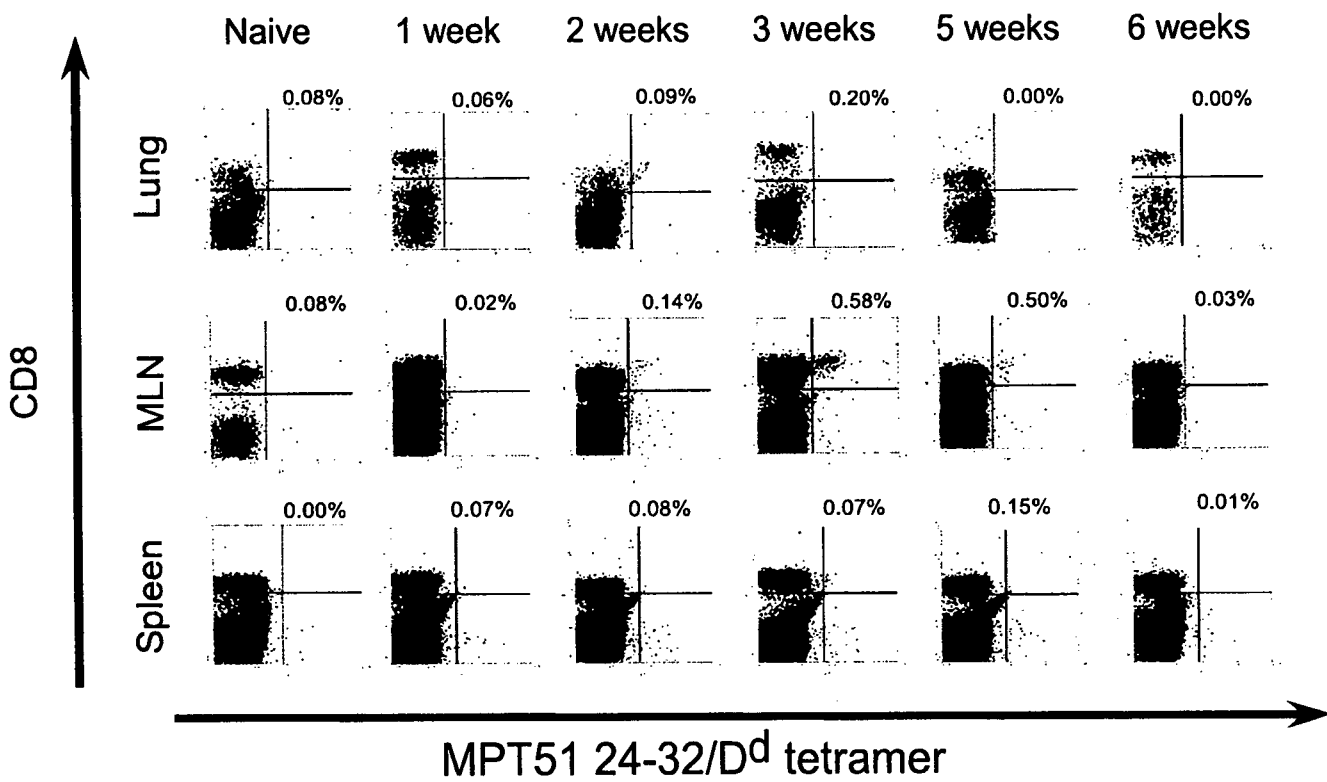


Fig. 2, Hashimoto et al.

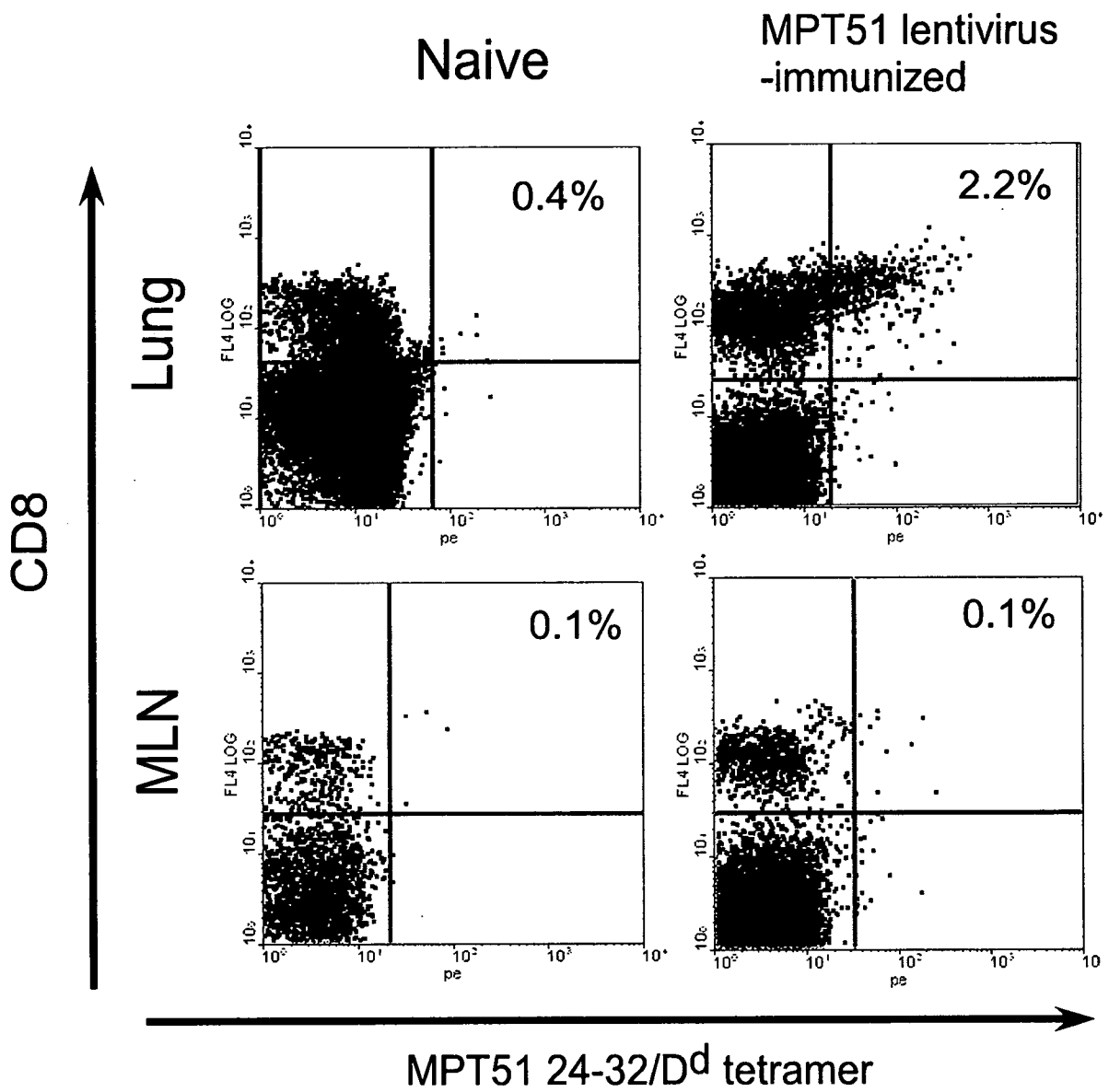


Fig. 3, Hashimoto et al.

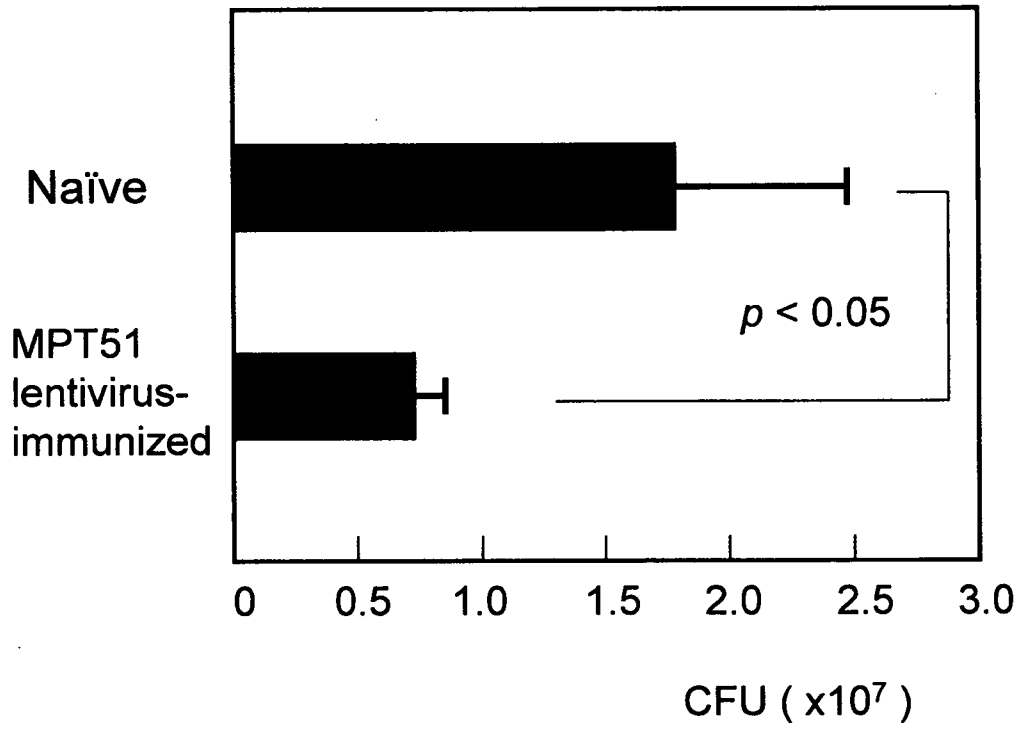


Fig. 4, Hashimoto et al.

1
2
3 **In vivo hierarchy of individual T-cell epitope-specific helper T-cell subset**
4 **against an intracellular bacterium**

5
6 Toshi Nagata^{a,*}, Taiki Aoshi^b, Masato Uchijima^b, Yukio Koide^b

7 ^a*Department of Health Science*, ^b*Department of Infectious Diseases*,

8 *Hamamatsu University School of Medicine*,

9 *1-20-1 Higashi-ku, Handa-yama, Hamamatsu 431-3192, Japan*

10

11

12 *Corresponding author. Tel. and fax: +81 53 435 2332.

13 *E-mail address:* tnagata@hama-med.ac.jp (T. Nagata)

1 **Abstract**

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

10

11 *Key words:* DNA immunization; Th epitope; *Listeria monocytogenes*

12

1 1. Introduction

2

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

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

24 In our previous works, we have investigated individual T-cell epitope-specific T-cell responses
25 against *L. monocytogenes* using minigene DNA vaccine system [10-12]. For CD8⁺ T-cell

1 epitope-specific responses, we showed that interaction between T cells against dominant and
2 subdominant epitopes does not operate in the generation of the hierarchy among individual CD8⁺
3 T-cell epitopes with minigene DNA vaccination [11]. For CD4⁺ T-cell epitope-specific responses,
4 we have reported that LLO 215-226-specific T-cell response evoked by DNA vaccination was
5 capable of inducing protective immunity against lethal listerial challenge in C3H mice [13].

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

15

16 **2. Materials and methods**

17 *2.1. Animals*

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

22 *2.2. Plasmid construction*

23 pCI-mIi p41-LLO215m, a DNA vaccine plasmid for LLO 215-226 (SqliAKFGTAFK), has been
24 reported previously [13]. DNA vaccine plasmids for LLO 189-200 (WNEKYAQAYPNV;
25 pCI-mIi p41-LLO 189m), p60 367-378 (SSASAIIEAAQK; pCI-mIi p41-p60 367m), and p60

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

11 2.3. DNA immunization

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

17 2.4. Lymphocyte proliferation assay

18 Spleen cells (5×10^5 per well) from the immunized mice were maintained with RPMI-1640
19 medium supplemented with 10% fetal bovine serum (FBS) at 37°C in a humidified 5% CO₂
20 atmosphere. They were incubated for 48 h at 37°C in 96-well round-bottom tissue culture plates
21 in the presence or absence of 1 µM of individual Th-epitope peptide. After 48 h in culture, *de*
22 *novo* DNA synthesis was assessed by adding 0.5 µCi per well [methyl-³H] thymidine (10 Ci
23 mmol⁻¹; ICN Biochemicals, Irvine, CA, USA) for the last 12 h of culture. Triplicate cultures
24 were harvested onto glass fiber filters, and the [methyl-³H] thymidine incorporation was
25 determined by counting the radioactivity (cpm) using liquid scintillation counter.

1 **2.5. Bacterial infection**

2 *L. monocytogenes* EGD strain was kept virulent by in vivo passage. For the inoculation, a seed
3 of *L. monocytogenes* was cultured overnight in trypticase soy broth (BBL, Sparks, MD, USA) at
4 37°C in a bacterial shaker and suitably diluted with phosphate-buffered saline. The exact
5 infection dose was assessed retrospectively by plating. Mice were immunized four times with
6 DNA vaccine plasmids as described above. One month later, the mice were challenged with $2 \times$
7 10^4 CFU of *L. monocytogenes* by intravenous injection. Bacterial numbers of the spleens were
8 determined 72 h after the challenge infection by plating 10-fold dilutions of tissue homogenates
9 on trypticase soy agar plates (BBL).

10 **2.6. Cytokine assay for spleen cells from immunized mice**

11 One month after the last immunization, spleen cells were harvested from the immunized mice.
12 Recovered cells were plated in 24-well plates at 2×10^6 cells per well in the presence or absence
13 of 1 μ M of individual T-cell epitope peptide for 5 days. Concentration of cytokines (interferon
14 (IFN)- γ , tumor necrosis factor (TNF)- α , interleukin (IL)-5, IL-4, IL-2) in culture supernatants was
15 determined by cytometric bead assay (CBA). Fifty micro liter of culture supernatants were
16 assayed for these cytokines using mouse Th1/Th2 cytokine CBA kit (BD Biosciences Pharmingen,
17 San Diego, CA, USA) according to the manufacturer's instruction.

18 **2.7. Statistics**

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

23

24 **3. Results**

25 **3.1. Proliferative responses of spleen cells of mice immunized with expression plasmids for Th**

1 *epitopes*

2 In order to examine CD4⁺ T-cell responses against four different Th epitopes of *L.*
3 *monocytogenes* in BALB/c mice, we constructed four DNA vaccine plasmids for in vivo antigen
4 presentation of LLO 215-226, LLO 189-200, p60 367-378, and p60 301-312 (Fig. 1). The DNA
5 vaccine constructs are expression plasmids for recombinant murine Ii cDNA whose CLIP region
6 were replaced by an oligonucleotide encoding CD4⁺ T-cell epitope peptide [13, 15]. The plasmid
7 DNAs were injected by gene gun bombardment into BALB/c mice as shown in Materials and
8 methods section. We chose the immunization method as it was a very reliable and reproducible
9 method from our previous experience [17].

10 First, we performed lymphocyte proliferation assay one month after the last immunization.
11 As shown in Fig. 2, immunization with each Th-epitope peptide expression plasmid induced
12 individual peptide-specific proliferative responses of the spleen cells. The stimulation indexes
13 were similar for four T-cell epitope peptides. These results indicated that the DNA vaccination
14 successfully induced individual epitope-specific T-cell responses.

15 *3.2. Induction of protective immunity against listerial infection after immunization with Th epitope*
16 *expression plasmids*

17 In order to examine whether the immunity evoked by immunization with each Th-epitope
18 peptide expression plasmid is associated with an increased resistance to infection of virulent *L.*
19 *monocytogenes*, the *in vivo* protection experiment was carried out. Seventy-two hours after
20 listerial challenge, mice were sacrificed and CFU from the spleens were counted. As shown in
21 Fig. 3, bacterial number in spleens of mice immunized with plasmids for p60 367-378 and LLO
22 215-226 tended to be reduced compared with that of naïve mice. On the contrary, bacterial
23 number in spleens of mice immunized with plasmids for p60 301-312 and LLO 189-200 tended to
24 be similar with that of naïve mice. Immunization with plasmid for p60 301-312 even increased
25 the bacterial number in spleens compared with that of naive mice.

1 3.3. Cytokine production from spleen cells of mice immunized with expression plasmids for
2 individual Th epitopes

3 We are interested in what caused different protective effects by immunization with these
4 different Th-epitope peptide expression plasmids. One of the reasons may be cytokine profiles
5 produced by specific Th. We therefore analyzed cytokine profiles in the supernatants of immune
6 spleen cell culture after 5-day *in vitro* stimulation with individual Th-epitope peptides.
7 As shown in Fig. 4, spleen cells from mice immunized with plasmids for LLO 215-226 and p60
8 367-378 tended to express TNF- α and IFN- γ after the Th-epitope peptide stimulation. Whereas,
9 immune spleen cells with plasmids for LLO 189-200 and p60 301-312 tended to withdraw the
10 expression of IFN- γ , although these tendencies were rather moderate.

11

12 **4. Discussion**

13 Following infection from a variety of pathogenic microorganisms, specific CD4⁺ and CD8⁺
14 T-cell responses are induced against various antigens of the microorganisms. CD8⁺ T-cell
15 responses are evoked mainly by microorganisms located in the cytoplasm of host cells, e.g., in the
16 case of infection of viruses, as well as *L. monocytogenes*. CD4⁺ T-cell responses are induced
17 mainly by infection of extracellularly-located microorganisms or microorganisms located in the
18 phagosome, e.g., in the case of infection of *Salmonella*, *Legionella*, and *Mycobacterium* spp. [18].
19 CD4⁺ T-cell responses have been also shown to be induced by *L. monocytogenes* and play an
20 important role in the protective immunity against listerial challenge. In murine *Listeria* infection
21 model, CD4⁺ T-cell responses have been studied. MHC class II gene-deficient mice were
22 reported to be more sensitive to lethal listerial challenge compared with their control heterozygous
23 littermates [19]. Geginat and colleagues [8] reported that adoptive transfer of p60
24 301-312-specific IFN- γ -producing CD4⁺ T-cell line into BALB/c mice induced protective
25 immunity against lethal listerial challenge, suggesting that CD4⁺ T cells are positively involved in

1 protective immunity. On the contrary, Kursar and colleagues [20] reported that depletion of
2 CD4⁺ T cells during immunization with nonviable *L. monocytogenes* enhanced CD8⁺ T
3 cell-mediated protection against listeriosis, suggesting involvement of regulatory T cell population
4 in failure of induction of protective immunity by nonviable *Listeria* vaccination.

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

22 In addition to them, several reports suggested antigenic peptide affinity to MHC may also be
23 involved in determining Th1/Th2 selection [25-27]. The peptides of higher affinity for a given
24 MHC class II molecule elicited a shift towards the Th1 subset. In our work, exact affinity of
25 peptides to MHC was not clear, but RANKPEP MHC-binding peptide prediction program

1 (<http://bio.dfci.harvard.edu/Tools/rankpep.html>) predicted the affinity by calculating
2 MHC-binding scores for each peptide. The scores for antigenic peptides studied here were as
3 follows. LLO 215-226, 11.829 for H2-E^d; LLO 189-200, 7.104 for H2-A^d; p60 367-378, 11.261
4 for H2-A^d; p60 301-312, 11.44 for H2-A^d. The scores seemed not so different from each other
5 except for LLO 189-200, whose score showed somewhat lower than those of other peptides.
6 T-cell receptor affinity for the peptide-MHC and T-cell receptor repertoire (which may affect
7 T-cell receptor V β chain usage) may also affect the Th1/Th2 selection.

8 In this study, we compared four CD4⁺ T-cell epitope-specific T-cell responses against *L.*
9 *monocytogenes* using gene gun DNA vaccine system. We used DNA vaccines of CD4⁺ T-cell
10 epitope peptide-Ii chain cDNA chimeric DNA constructs for that purpose as described in our
11 previous work [13, 15]. The results in this study showed that individual epitope-specific CD4⁺
12 T-cell responses are different in terms of protective immunity and cytokine production profiles
13 although the same DNA vaccination system was performed for each epitope-specific CD4⁺ T cells.
14 In results of the in vivo protection experiment, p60 367-378-specific and LLO 215-226-specific T
15 cells tended to have protective ability against listeriosis, but p60 301-312-specific and LLO
16 189-200-specific T cells did not (Fig. 3). Related with the result, p60 367-378 and LLO 215-226
17 peptides had tendency to induce IFN- γ production, whereas, p60 301-312 and LLO 189-200
18 peptides did not in the cytokine assay (Fig. 4). These apparent results suggest that the level of
19 IFN- γ production may affect the protective ability of each peptide-specific T cells.

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

25 In conclusion, selection of CD4⁺ T-cell epitopes would be critical for construction of

1 multi-epitope vaccination. Minigene DNA vaccination would serve a feasible system for
2 evaluation of each T-cell epitope for induction of protective immunity against various pathogens.

3

4 **Acknowledgements**

5 We thank Dr. M. Mitsuyama (Kyoto University, Japan) for generously providing the *L.*
6 *monocytogenes* EGD strain. This work was supported by Grants-in-Aid for Scientific Research
7 and Centers of Excellence (COE) from the Ministry of Education, Culture, Sports, Science and
8 Technology of Japan.

9

10 **References**

- 11 [1] Pamer EG. Immune responses to *Listeria monocytogenes*. Nat Rev Immunol
12 2004;4:812-23.
- 13 [2] Kaufmann SHE, Hug E, Vath U, Muller I. Effective protection against *Listeria*
14 *monocytogenes* and delayed-type hypersensitivity to listerial antigens depend on
15 cooperation between specific L3T4⁺ and Lyt2⁺ T cells. Infect Immun 1985;48:263-6.
- 16 [3] Kaufmann SHE., Hug E, Vath U, De Libero G. Specific lysis of *Listeria*
17 *monocytogenes*-infected macrophages by class II-restricted L3T4⁺ T cells. Eur J Immunol
18 1987;17:237-46.
- 19 [4] Magee DM, Wing EJ. Cloned L3T4⁺ T lymphocytes protect mice against *Listeria*
20 *monocytogenes* by secreting IFN- γ . J Immunol 1988;141:3203-7.
- 21 [5] Rakhmilevich AL. Evidence for a significant role of CD4⁺ T cells in adoptive immunity to
22 *Listeria monocytogenes* in the liver. Immunology 1993;82:249-54.
- 23 [6] Safley SA, Jensen PE, Reay PA, Ziegler HK. Mechanisms of T cell epitope
24 immunodominance analyzed in murine listeriosis. J Immunol 1995;155:4355-66.
- 25 [7] Ziegler HK, Safley SA, Hiltbold E. Definition of T cell epitopes of *Listeria*