cleotides to the p65 subunit of NF-κB abrogates established experimental colitis in mice. Nat Med 1996;2:998–1004.
12. Hamamoto N, Maemura K, Hirata I, Murano M, Sasaki S, Katsu K. Inhibition of dextran sulphate sodium (DSS)-induced colitis in mice by intracolonically administered antibodies against adhesion molecules (endothelial leucocyte adhesion molecule-1 [ELAM-1] or intercellular adhesion molecule-1 [ICAM-1]). Clin Exp Immunol 1999;117:462–468.
13. Wahl C, Liptay S, Adler G, Schmid RM. Sulfasalazine: a potent and specific inhibitor of nuclear factor kappa B. J Clin Invest 1998;101:1163–1174.
14. Weber CK, Liptay S, Wirth T, Adler G, Schmid RM. Suppression of NF-κB activity by sulfasalazine is mediated by direct inhibition of IκB kinases α and β. Gastroenterology 2000;119:1209–1218.
15. Bantel H, Berg C, Vieth M, Stolte M, Kruis W, Schulze-Osthoff K. Mesalazine inhibits activation of transcription factor NF-κB in inflamed mucosa of patients with ulcerative colitis. Am J Gastroenterol 2000;95:3452–3457.
16. Scheinman RI, Gualberto A, Jewell CM, Cidlowski JA, Baldwin AS. Characterization of mechanisms involved in transrepression of NF-κB by activated glucocorticoid receptors. Mol Cell Biol 1995; 15:943–953.
17. Auphan N, DiDonato JA, Rosette C, Helmberg A, Karin M. Immunosuppression by glucocorticoids: inhibition of NF-κB activity through induction of IκB synthesis. Science 1995;270:286–290.
18. Hanauer SB. Inflammatory bowel disease. N Engl J Med 1996; 334:841–848.
19. Govindarajan VS. Turmeric-chemistry, technology, and quality. Crit Rev Food Sci Nutr 1980;12:199–301.
20. Jiang MC, Yang-Yen HF, Yen JJ, Lin JK. Curcumin induces apoptosis in immortalized NIH 3T3 and malignant cancer cell lines. Nutr Cancer 1996;26:111–120.
21. Singh AK, Sidhu GS, Deepa T, Maheshwari RK. Curcumin inhibits the proliferation and cell cycle progression of human umbilical vein endothelial cell. Cancer Lett 1996;107:109–115.
22. Huang TS, Lee SC, Lin JK. Suppression of c-Jun/AP-1 activation by an inhibitor of tumor promotion in mouse fibroblast cells. Proc Natl Acad Sci U S A 1991;88:5292–5296.
23. Xu YX, Pindolia KR, Janakiraman N, Noth CJ, Chapman RA, Gautam SC. Curcumin, a compound with anti-inflammatory and anti-oxidant properties, down-regulates chemokine expression in bone marrow stromal cells. Exp Hematol 1997;25:413–422.
24. Joe B, Rao UJ, Lokesh BR. Presence of an acidic glycoprotein in the serum of arthritic rats: modulation by capsaicin and curcumin. Mol Cell Biochem 1997;169:125–134.
25. Ruby AJ, Kuttan G, Babu KD, Rajasekharan KN, Kuttan R. Antitumour and antioxidant activity of natural curcuminoids. Cancer Lett 1995;94:79–83.
26. Singh S, Aggarwal BB. Activation of transcription factor NF-κB is suppressed by curcumin (diferuloylmethane). J Biol Chem 1995; 270:24995–25000.
27. Kumar A, Dhawan S, Hardegen NJ, Aggarwal BB. Curcumin (diferuloylmethane) inhibition of tumor necrosis factor (TNF)-mediated adhesion of monocytes to endothelial cells by suppression of cell surface expression of adhesion molecules and of nuclear factor-κB activation. Biochem Pharmacol 1998;55:775–783.
28. Bierhaus A, Zhang Y, Quehenberger P, Luther T, Haase M, Muller M, Mackman N, Ziegler R, Nawroth PP. The dietary pigment curcumin reduces endothelial tissue factor gene expression by inhibiting binding of AP-1 to the DNA and activation of NF-kappa B. Thromb Haemost 1997;77:772–782.
29. Jobin C, Bradham CA, Russo MP, Juma B, Narula AS, Brenner DA, Sartor RB. Curcumin blocks cytokine-mediated NF-κB activation and proinflammatory gene expression by inhibiting inhibitory factor I-κB kinase activity. J Immunol 1999;163:3474–3483.
30. Neurath MF, Fuss I, Kelsall BL, Stuber E, Strober W. Antibodies to interleukin 12 abrogate established experimental colitis in mice. J Exp Med 1995;182:1281–1290.
31. Yang F, de Villiers WJS, McClain CJ, Varilek GW. Green tea polyphenols block endotoxin-induced TNF production and lethality in a murine model. J Nutr 1998;128:2334–2340.
32. Yoshida A, Koide Y, Uchijima M, Yoshida TO. Dissection of strain difference in acquired protective immunity against *Mycobacterium bovis* Calmette-Guérin Bacillus (BCG). J Immunol 1995;155: 2057–2066.
33. Chomczynski P, Sacchi N. Single-step method of RNA isolation by acid guanidinium thiocyanate-phenol-chloroform extraction. Anal Biochem 1987;162:156–159.
34. Kang BY, Chung SW, Chung W, Im S, Hwang SY, Kim TS. Inhibition of interleukin-12 production in lipopolysaccharide-activated macrophages by curcumin. Eur J Pharmacol 1999;384:191–195.
35. Kanai T, Watanabe M, Okazawa A, Sato T, Yamazaki M, Okamoto S, Ishi H, Totsuka T, Iiyama R, Okamoto R, Ikeda M, Kurimoto M, Takeda K, Akira S, Hibi T. Macrophage-derived IL-18-mediated intestinal inflammation in the murine model of Crohn's disease. Gastroenterology 2001;121:875–888.
36. Fuss IJ, Neurath M, Boirivant M, Klein JS, de la Motte C, Strong SA, Fiocchi C, Strober W. Disparate $CD4^+$ lamina propria (LP) lymphokine secretion profiles in inflammatory bowel disease. Crohn's disease LP cells manifest increased secretion of IFN-gamma, whereas ulcerative colitis LP cells manifest increased secretion of IL-5. J Immunol 1996;157:1261–1270.
37. Parronchi P, Romagnani P, Annunziato F, Sampognaro S, Becchio A, Giannarini L, Maggi E, Pupilli C, Tonelli F, Romagnani S. Type 1 T-helper cell predominance and interleukin-12 expression in the gut of patients with Crohn's disease. Am J Pathol 1997;150: 823–832.
38. Bille N, Larsen JC, Hansen EV, Wurtzen G. Subchronic oral toxicity of turmeric oleoresin in pigs. Food Chem Toxicol 1985;23: 967–973.
39. Ammon HP, Wahl MA. Pharmacology of *Curcuma longa*. Planta Med 1991;57:1–7.
40. Commandeur JN, Vermeulen NP. Cytotoxicity and cytoprotective activities of natural compounds. The case of curcumin. Xenobiotica 1996;26:667–680.
41. Ravindranath V, Chandrasekhara N. Absorption and tissue distribution of curcumin in rats. Toxicology 1980;16:259–265.
42. Wahlstrom B, Blennow G. A study on the fate of curcumin in the rat. Acta Pharmacol Toxicol (Copenh) 1978;43:86–92.
43. Ravindranath V, Chandrasekhara N. Metabolism of curcumin — studies with [^{3}H]curcumin. Toxicology 1981-1982;22:337–344.
44. Rao CV, Simi B, Reddy BS. Inhibition by dietary curcumin of azoxymethane-induced ornithine decarboxylase, tyrosine protein kinase, arachidonic acid metabolism and aberrant crypt foci formation in the rat colon. Carcinogenesis 1993;14:2219–2225.
45. Pereira MA, Grubbs CJ, Barnes LH, Li H, Olson GR, Eto I, Juliana M, Whitaker LM, Kelloff GJ, Steele VE, Lubet RA. Effects of the phytochemicals, curcumin and quercetin, upon azoxymethane-induced colon cancer and 7,12-dimethylbenz[a]anthracene-induced mammary cancer in rats. Carcinogenesis 1996;17:1305–1311.
46. Plummer SM, Holloway KA, Manson MM, Munks RJ, Kaptein A, Farrow S, Howells L. Inhibition of cyclo-oxygenase 2 expression in colon cells by the chemopreventive agent curcumin involves inhibition of NF-κB activation via the NIK/IKK signalling complex. Oncogene 1999;18:6013–6020.

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Endosomal/lysosomal targeting of a single helper T-cell epitope of an intracellular bacterium by DNA immunisation induces a specific T-cell subset and partial protective immunity in vivo

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Abstract

We evaluated here the effect of the intracellular targeting of a helper T-cell (Th) epitope, literiolysin O 215–226 derived from *Listeria monocytogenes*, on induction of a specific Th by gene gun immunisation. Immunisation of C3H/He mice with pE215LAMP plasmid encoding the Th epitope fused with the endosomal/lysosomal targeting signal of lysosome-associated membrane protein (LAMP)-1 gave the epitope-specific proliferative responses of $CD4^+$ T lymphocytes. In addition, specific interferon-γ production from the splenocytes was observed. Concomitantly, pE215LAMP-immunised mice showed moderate, but significant protective immunity against listerial challenge. These results suggest that the intracellular targeting of a Th epitope to endosomal/lysosomal compartments by DNA immunisation is useful for eliciting a specific Th subset in vivo.

Keywords: DNA immunisation; LAMP-1; Th epitope; Listeriolysin O

1. Introduction

Inducing a specific T-cell subset by contriving a new immunisation method may be useful for designing effective vaccines for infectious disease, autoimmune disease, cancer, and also for clarifying the role of each T-cell subset in the immune responses. DNA vaccine is a promising immunising method for this purpose. DNA vaccine offers many advantages over other types of immunising methods: relatively easy design and construction by using recombinant DNA technique, chemical stability, relatively low cost, and a lack of infectious potential. More importantly, the procedure can induce cellular immunity (reviewed in [1,2]). Immunisation with gene gun bombardment has been reported to be capable of eliciting cellular immunity, especially cytotoxic T lymphocytes (CTL), because proteins or peptides produced from the plasmids are expressed in the cytoplasm of the plasmid-introduced antigen (Ag)-presenting cells and, therefore, tend to enter the major histocompatibility complex (MHC) class I Ag-processing pathway [3,4]. On the other hand, induction of $CD4^+$ T cells needs Ag presentation through MHC class II pathway. In general, endogenously expressed antigenic molecules are not supposed to enter MHC class II Ag-presentation pathway. However, the recent advance of knowledge concerning intracellular protein targeting makes it possible for even endogenously expressed molecules to enter MHC class II Ag-presentation pathway to induce $CD4^+$ T cells (reviewed in [5]). Here, we examined the effect of the intracellular targeting of a helper T-cell (Th) epitope in DNA immunisation on a specific $CD4^+$ T-cell subset induction.

Listeria monocytogenes is a facultative Gram-positive intracellular bacterium. Murine infection with *L. monocytogenes* is an excellent model system for studying cellular immunity against intracellular microorganisms (reviewed in [6,7]). For protection against the microorganism, the cellular immunity has been considered to play an essential role. Especially, both $CD8^+$ CTL and $CD4^+$ type 1 Th (Th1), which are specifically amplified at listerial infection,

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have been shown to play a critical role in the protective immunity by experiments of depletion and adoptive transfer of specific T-cell subsets [8–12], and by analyses of mutant mice with a genetic defect in the β2-microglobulin or the H2-A β chain gene [13,14]. $CD8^+$ CTL have been reported to play a superior role in protective immunity [13,15,16]. However, several papers demonstrated a significant role of the $CD4^+$ T-cell subset for protective immunity [17–19]. Virulent strains of *L. monocytogenes* secrete a sulfhydryl-activated pore-forming exotoxin, listeriolysin O (LLO), which has been identified as a major virulence factor [20]. LLO allows the bacterium to escape from the phagosome and replicate in the cytoplasm. LLO has also been known as a protective antigen, and T-cell epitopes in LLO have been identified. LLO 91–99 was identified as dominant $CD8^+$ CTL epitopes restricted to H2-K^d [21]. LLO 215–226 was confirmed as a dominant $CD4^+$ Th epitope restricted to the H2-E^k molecule [22,23]. We chose the latter epitope as a target peptide of minigene DNA immunisation in this study. Here we report that DNA vaccine of a listerial Th epitope is capable of eliciting a specific $CD4^+$ T cell in vivo, when the epitope is designed to localise in endosomal/lysosomal compartments by utilising the targeting signal of a lysosome-associated membrane protein (LAMP)-1 molecule.

2. Materials and methods

2.1. Animals

C3H/He mice (Japan SLC; Hamamatsu, Japan) were maintained in the animal facility of Hamamatsu University School of Medicine. Mice between 6 and 18 weeks of age were used for immunisation. All animal experiments were performed according to the Guidelines for Animal Experimentation, Hamamatsu University School of Medicine.

2.2. Plasmid construction

The eukaryotic expression vector, pCI (Promega, Madison, WI, USA), was used as a backbone plasmid for the construction of plasmids for DNA immunisation. The oligonucleotides used for p215 plasmid are 5′-CCCGGG ATG AGC CAG CTG ATC GCC AAG TTT GGC ACC GCC TTT AAG TAG CCCGGG-3′ and the opposite strand of oligonucleotides which encode amino acid residues 215 to 226 of LLO, MSQLIAKFGTAFK, and a termination codon. These oligonucleotides were annealed and inserted into the *Sma*I site of pCI plasmid. The codon usage of the oligonucleotide for LLO 215–226 peptide was optimised to that of *Mus musculus* [24]. The oligonucleotides used for pE215 are, 5′-CCCAGCT ATG AGG TAC ATG ATT TTA GGC TTG CTC GCC CTT GCG GCA GCT GCA GGC AGC CAG CTG ATC GCC AAG TTT GGC ACC GCC TTT AAG TAG CCCGGG-3′ and the opposite strand oligonucleotide which encodes adenovirus E3 leader sequence [25] plus LLO 215–226. These oligonucleotides were annealed and inserted into *Sma* I site of pCI plasmid. For construction of the pE215LAMP plasmid, PCR was carried out using six overlapping oligonucleotides together (gene SOEing by overlap extension, [26]). Four oligonucleotides, having 15-nucleotide overlap regions at both ends, and two primers [5′-CCGGAATT-CACCATGAGGTACATGA-3′ (upper primer containing *Hin*dIII site) and 5′-CTAGTCTAGACTAGATGGTCT-GATA-3′ (lower primer containing *Nsp*I site)] were mixed in the PCR solution. The overlap extension PCR gave rise to a 213-bp DNA fragment encoding adenovirus E3 leader sequence/*Hin*dIII site/*Nsp*I site/murine LAMP-1 transmembrane and cytoplasmic regions [25,27]. The PCR product was digested with *Eco*RI and *Xba*I, sites of which were designed in these primers. The PCR product was purified and inserted into *Eco*RI and *Xba*I sites of pCI, resulting in pELAMP plasmid. Then, the plasmid was digested with *Hin*dIII and *Nsp*I and inserted with a codon-optimised double-stranded oligonucleotide encoding LLO 215–226 [5′-AG CTT AGC CAG CTG ATC GCC AAG TTT GGC ACC GCC TTT AAG ATG GAT AAC ATG-3′ (for coding strand) and 5′-TT ATC CAT CTT AAA GGC GGT GCC AAA CTT GGC GAT CAG CTG GCT A-3′ (for non-coding strand)].

During the cloning procedure, the DNA fragments were purified from agarose gels using a GeneClean II kit (Bio 101, La Jolla, CA, USA). The nucleotide sequences of the resultant plasmids were confirmed by dideoxy sequencing by using a ABI PRISM 310 Genetic Analyzer (Applied Biosystems, Foster City, CA, USA). Large-scale purification of plasmids was conducted with a Qiagen plasmid mega kit system (Qiagen, Valencia, CA, USA) and endotoxin was removed by Triton X-114 phase separation.

2.3. Mice immunisation

For DNA immunisation with Helios gene gun system (Bio-Rad Laboratories, Hercules, CA, USA), preparation of the cartridge of DNA-coated gold particle cartridge was followed according to the manufacturer's instruction manual. Finally, 0.5 mg of gold particles were coated with 1 μg of plasmid DNA and the injection was carried out with 0.5 mg gold per shot twice. To immunise C3H/He mice, the shaved abdominal skin was wiped with 70% ethanol. The spacer of the gene gun was held directly against the abdominal skin. Then, the device was discharged at a helium discharge pressure of 400 psi. Mice were injected with 2 μg of plasmid DNA four times at 1-week intervals.

2.4. Cell culture condition

Spleen cells from plasmid-immunised mice were maintained with RPMI-1640 medium supplemented with 10%

foetal bovine serum (FBS) at 37°C in a humidified 5% CO_2 atmosphere.

2.5. Lymphocyte proliferation assay

Spleen cells (5×10^5 per well) from the immunised mice were incubated for 48 h at 37°C in 96-well round-bottom tissue culture plates in the presence or absence of 1 μM of LLO 215–226 peptide. After 48 h in culture, de novo DNA synthesis was assessed by adding 0.5 μCi per well [methyl-^{3}H] thymidine (10 Ci mmol^{-1}; ICN Biochemicals, Irvine, CA, USA) for the last 12 h of culture. Triplicate cultures were harvested onto glass fibre filters, and the [methyl-^{3}H] thymidine incorporation was determined by counting the radioactivity (cpm) using a liquid scintillation counter.

2.6. Inhibition of lymphocyte proliferation with depletion of $CD4^+$ T-cell subset

CD4/CD8 specificity of proliferative T cells was tested by depletion studies with anti-murine CD4 mAb, GK1.5, anti-murine CD8α mAb, 53-6.7. The mAbs were purchased from PharMingen (San Diego, CA, USA). The immune spleen cells were added to the mAbs at 1 μg ml^{-1} and incubated for 1 h at 4°C. They were then centrifuged and the supernatant was discarded. The cells were resuspended in the cytotoxicity medium [RPMI-1640 medium with 25 mM HEPES buffer and 0.3% FBS containing rabbit complement (Cedarlane, Hornby, Canada)] and incubated for 1 h at 37°C. Then, the cells were washed with RPMI-1640 medium and used for the lymphocyte proliferation assay described in Section 2.5.

2.7. Enzyme-linked immunosorbent assay (ELISA) for IFN-γ

Spleen cells were harvested from the immunised mice. Recovered cells were plated in 24-well plates at 2×10^6 cells per well in the presence or absence of 1 μM of LLO 215–226 peptide for 5 days. Concentration of IFN-γ in the culture supernatants was determined by sandwich ELISA as described in our previous report [28].

2.8. Bacterial infection

L. monocytogenes EGD strain was kept virulent by in vivo passage. For the inoculation, a seed of *L. monocytogenes* was cultured overnight in trypticase soy broth (BBL, Sparks, MD, USA) at 37°C in a bacterial shaker and suitably diluted with phosphate-buffered saline. The exact infection dose was assessed retrospectively by plating. Mice were immunised four times with DNA vaccine plasmids as described above, or immunised by a single intraperitoneal injection with a sublethal dose of *L. monocytogenes* (1×10^4 CFU). One month later, the mice were challenged with 1×10^5 CFU of *L. monocytogenes*. Bacterial numbers of the spleens were determined 72 h after the challenge infection by plating 10-fold dilutions of tissue homogenates on trypticase soy agar plates (BBL).

2.9. Statistics

Data from multiple experiments were expressed as the means ± S.D. Statistical analyses were performed by using StatView-J 4.02 statistics program (Abacus Concepts, Berkeley, CA, USA). Data were analysed with one factor-analysis of variance followed by the Fisher's protected least significant difference test.

3. Results

3.1. Construction of plasmids for DNA immunisation

In order to evaluate the effect of different intracellular targeting of an MHC class II-restricted T-cell epitope for DNA immunisation, we constructed three different plasmids for expression of the amino acid residues 215 to 226 of LLO (Fig. 1). The plasmid p215 is a minigene plasmid

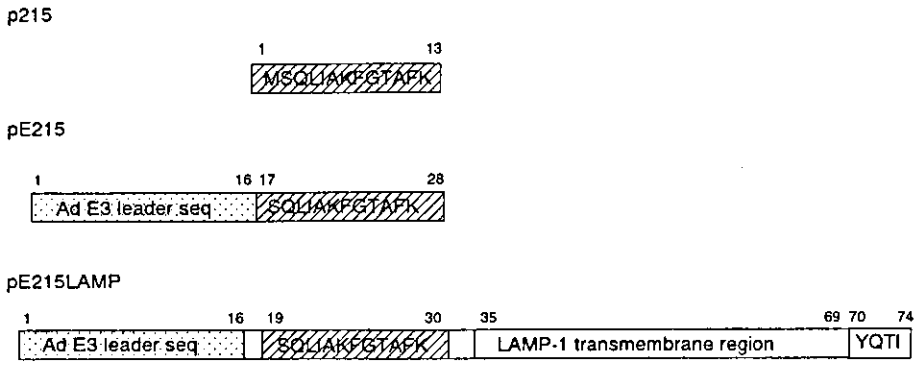

Fig. 1. The schema of gene products containing LLO 215–226 Th epitope deduced from the cDNA constructs prepared in this study (p215, pE215, and pE215LAMP). The hatched boxes indicate LLO 215–226 peptide and dotted boxes indicate adenovirus E3 leader sequence. The box depicted as YQTI in pE215LAMP is the cytoplasmic region of murine LAMP-1 molecule reported as the endosomal/lysosomal targeting signal. Amino acid numbers of each domain are shown above each schema.

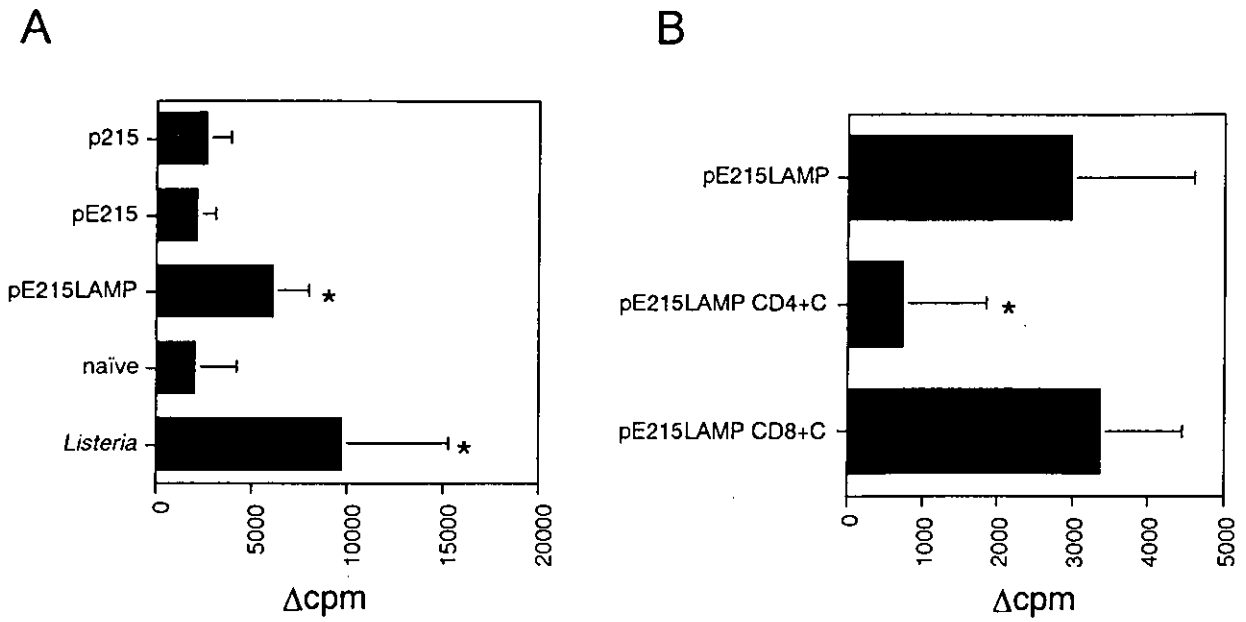

Fig. 2. Comparison of specific Th induction by immunisation with LLO 215–226 expression plasmids. A: Specific proliferative responses of spleen cells from p215, pE215, or pE215LAMP-immunised mice. C3H/He mice were immunised with each plasmid by using a gene gun four times at 1-week intervals. Spleen cells from the immunised mice were harvested three weeks after the last immunisation and cultured in vitro (5×10^5 per well) in the presence or absence of 1 μM of LLO 215–226 peptide for 2 days and pulsed with 0.5 μCi of [methyl-^{3}H] thymidine for the last 12 h. Results of naive and *L. monocytogenes*-immunised C3H/He mice are also shown as controls. The values indicate cpm per well. The means ± S.D. of Δcpm (cpm in the presence of the peptide minus cpm in the absence of the peptide) of four mice per group are shown. Asterisks indicate statistical significance ($P \leq 0.001$) compared with the value of naive mice. B: Inhibition of LLO 215–226-specific spleen cell proliferation by CD4$^+$ T-cell subset depletion. Spleen cells from pE215LAMP-immunised mice were treated with anti-CD4, or anti-CD8 mAb and rabbit complement, or remained untreated. Then, the lymphocyte proliferation assay was performed as in Fig. 2A. The means ± S.D. of Δcpm of quintuplicate determinations of a representative experiment are shown. Asterisks indicate statistical significance ($P \leq 0.02$) compared with the value of untreated control (pE215LAMP).

for expression of LLO 215–226 peptide alone. The peptide produced by this plasmid will be located in the cytoplasm of the plasmid-introduced cells. pE215 plasmid was designed for the expression of an LLO 215–226 peptide fused with an adenovirus E3 leader sequence at the N-terminus for localisation of the peptide in the endoplasmic reticulum. pE215LAMP was designed for the expression of an LLO 215–226 peptide fused with an adenovirus E3 leader sequence at the N-terminus and the transmembrane region and the endosomal/lysosomal targeting signal of murine LAMP-1 molecule at the C-terminus for localisation of the peptide in the endosomal/lysosomal compartments.

3.2. *Proliferative responses of spleen cells of mice immunised with expression plasmids for LLO 215–226 Th epitope and the epitope-specific IFN-γ production by the spleen cells*

In order to examine the effect of immunisation with different forms of LLO 215–226 expression plasmids, we performed lymphocyte proliferation assay after immunisation of C3H/He mice with the plasmids by using gene gun bombardment. We chose the immunisation method as it was a very reliable and reproducible method from our previous experience [29]. As shown in Fig. 2A, immunisation with pE215LAMP plasmid induced LLO 215–226-specific proliferative responses of spleen cells from the immunised mice, whose level was, however, weaker than those from mice immunised with a sublethal dose of *L. monocytogenes*. Immunisations with p215 and pE215 plasmids showed less proliferative responses to LLO 215–226 peptide stimulation, which were not significantly different from those observed in naive mice. Then we examined the subset of lymphocytes generated by priming with pE215LAMP plasmid DNA immunisation. As shown in Fig. 2B, the specific proliferation of spleen cells of mice immunised with pE215LAMP plasmid was reduced significantly by CD4$^+$ T-cell subset depletion, but not by CD8$^+$ T-cell subset depletion, indicating that lymphocytes proliferated by LLO 215–226 peptide stimulation are CD4$^+$ T cells.

Furthermore, we analysed IFN-γ amounts in the supernatants of spleen cell culture after 5 days of in vitro stimulation with LLO 215–226 peptide. Again, immunisation with pE215LAMP induced amounts of IFN-γ higher than those of mice immunised with other plasmid constructs examined (p215 and pE215) after the in vitro stimulation (Fig. 3). But the amounts were lower than those of mice immunised with a sublethal dose of *L. monocytogenes* (Fig. 3). We did not detect IL-4 production from spleen cells of the immunised mice with ELISA (the detection limit of IL-4 in our ELISA system was approximately 100 pg ml^{-1}; data not shown).

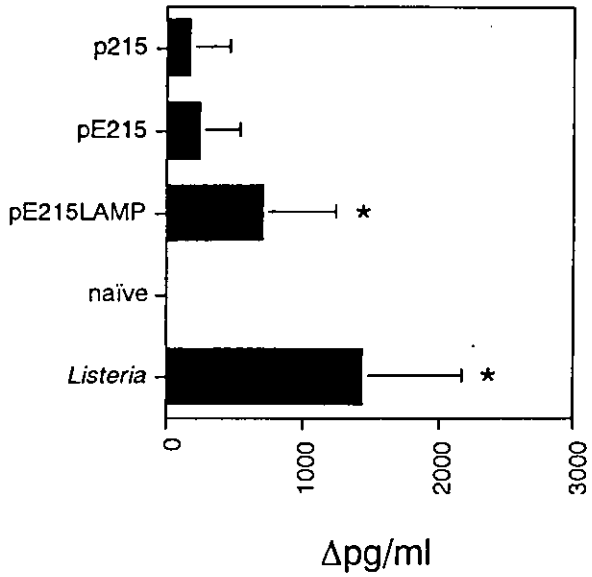

Fig. 3. IFN-γ production by spleen cells from p215, pE215, or pE215-LAMP-immunised mice. The spleen cells from the plasmid-immunised mice were cultured in vitro in the presence or absence of 1 μM of LLO 215–226 peptide for 5 days, and the culture supernatants were analysed by sandwich ELISA for IFN-γ. Results of naive and *L. monocytogenes*-immunised C3H/He mice are also shown as controls. The values represent the means ± S.D. of Δpg ml^{-1} (the value in the presence of the peptide minus the value in the absence of the peptide) of four to nine mice per group. Asterisks indicate statistically significant ($P < 0.005$) compared with the value of naive mice.

3.3. *Induction of protective immunity against listerial infection after immunisation with pE215LAMP plasmid*

In order to examine whether the immunity evoked by immunisation with these plasmids is associated with an increased resistance to infection of virulent *L. monocytogenes*, the in vivo protection experiment was carried out. Seventy-two hours after listerial challenge, mice immunised with different forms of LLO 215–226 expression plasmids were killed and CFU from the spleens were counted. As shown in Fig. 4, immunisation with p215 or pE215 did not show significant protective effects compared with naive mice. Immunisation with pE215LAMP conferred moderate but significant protective immunity against listerial challenge on the mice.

4. Discussion

In general, DNA immunisation with gene gun bombardment is thought to be able to introduce exogenous plasmid DNA into the cytoplasm of professional Ag-presenting cells located in the epidermis or dermis, especially, Langerhans' cells [3,4]. We examined here the endogenous presentation of MHC class II-restricted T-cell epitope derived from *L. monocytogenes* by DNA immunisation. In this study, we compared the immunisation effects due to the differential intracellular targeting of a single Th epitope of *L. monocytogenes* with gene gun bombardment. Immunisation of p215, which encodes LLO 215–226 peptide alone did not elicit specific Th induction. In our previous works, a similar minigene plasmid encoding LLO 91–99, a dominant MHC class I epitope, induced strong CTL activity by gene gun bombardment [29,30]. This result is not surprising, as proteins or peptides synthesised in the cytoplasm are usually processed through MHC class I Ag-presenting pathway. In addition, we also showed in a previous work that immunisation with an expression plasmid for a single Th epitope of ovalbumin (OVA 323–336) failed to induce specific lymphocyte proliferative responses [31]. Immunisation with pE215, which produces LLO 215–226 peptide connected with adenovirus E3 leader sequence at the N-terminus, did not elicit significant levels of proliferative T cells responsive to the epitope, either. In the case of MHC class I epitope, several papers demonstrated that addition of a leader sequence enhances CTL induction following gene gun bombardment [25,32,33], whereas, in the case of MHC class II epitope, targeting the peptide into the endoplasmic reticulum with adenovirus E3 leader sequence may not be sufficient for induction of Th. T cells responsive to a Th epitope, LLO 215–226, were able to be induced by immunisation of pE215LAMP plasmid encoding LLO 215-226-LAMP-1 fusion polypeptide. Furthermore, immunisation of the plasmid was capable of induc-

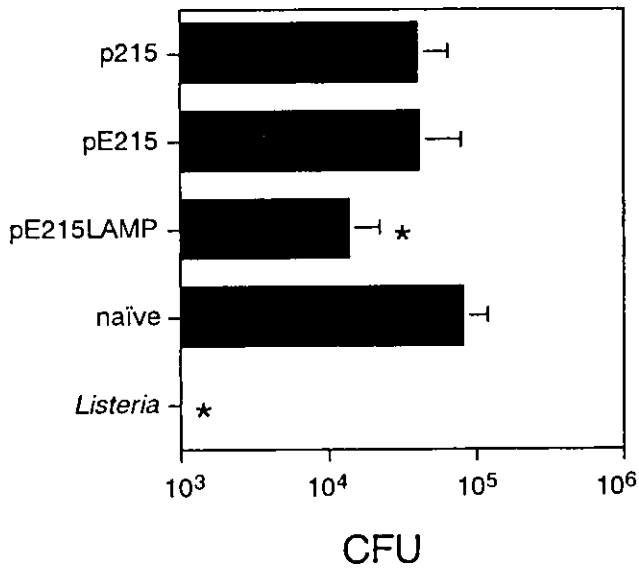

Fig. 4. Protective immunity induced by immunisation with LLO 215–226 expression plasmids. Mice were immunised with different forms of LLO 215–226 expression plasmids four times at 1-week intervals. One month after the last immunisation, the immunised mice were challenged with 1×10^5 CFU of *L. monocytogenes*. Bacterial numbers in the spleens were determined 72 h after the challenge infection by plating 10-fold dilutions of tissue homogenates on trypticase soy agar plates. Results of naive and *L. monocytogenes*-immunised C3H/He mice are also shown as controls. Results are expressed as the means ± S.D. for three mice for each group. Asterisks indicate statistical significance ($P < 0.05$) compared with the value of naive mice.

ing partial protection against *L. monocytogenes* challenge infection.

LAMP-1 molecule is a type 1 transmembrane protein located predominantly in lysosomes and late endosomes involving MHC class II Ag-processing pathway [34]. The cytoplasmic domain of LAMP-1 contains the amino acid sequence Tyr-Gln-Thr-Ile, which is important for endosomal/lysosomal protein targeting [35]. In vitro activation of Epstein–Barr virus- and influenza virus-specific $CD4^+$ memory CTLs was successfully demonstrated by using the LAMP-1 recombinant vaccinia virus expression system [36]. The similar in vivo system with naked DNA immunisation for induction of a specific $CD4^+$ T-cell subset has been examined. Ji et al. [37] showed that targeting human papillomavirus type 16 E7 molecule to the endosomal/lysosomal compartments by gene gun immunisation enhances the anti-tumour immunity enough to protect mice against a challenge with virus protein-expressing tumours and to eradicate preexisting tumour cells. As for the attempt to vaccinate against infectious diseases, Vidalin et al. [38] tried to induce $CD4^+$ CTL population against hepatitis C virus core protein by DNA immunisation with a plasmid for the LAMP-1 fusion protein. But they could not show proliferative responses of the spleen cells from the immunised mice. Their results might be caused by the nature of the hepatitis C virus core protein that does not allow eliciting specific immunity against the virus. Recently, Rodriguez et al. [39] reported successful $CD4^+$ T-cell induction by the immunisation of Th epitope minigene plasmid DNA by utilising the lysosomal targeting signal located in the C-terminal tail of lysosomal integral membrane protein (LIMP)-II. Interestingly, they showed that the immunological consequences varied depending on the Th epitopes examined. Our results showed not striking but yet significant effects of the targeting of a Th epitope on induction of T-cell population specific to the epitope.

So far, several reports have demonstrated a role of $CD4^+$ T cells on protective immunity against listerial challenge [17–19]. Verma et al. [40] demonstrated that the induction of $CD4^+$ T-cell population responsive to LLO 215–226 in vivo elicits partial protective immunity by using the *Salmonella* carrier system. They showed 1-log order reduction in numbers of the bacterium in the spleens and livers of immunised mice. As another approach, we recently showed that nice induction of protective immunity to *L. monocytogenes* by immunisation with plasmid DNA expressing LLO 215–226 Th epitope that replaces the class II-associated invariant chain (Ii) peptide (CLIP) of the Ii [41]. In that study, we showed an approximately 2-log order reduction in numbers of the bacterium in the spleens and livers of immunised mice. LAMP-1 DNA vaccine in this study showed less immunological effects compared to the Ii DNA vaccine [41] in terms of IFN-γ production by splenocytes and in vivo protection assay. LAMP-1 or LIMP-II DNA vaccine may be useful for the targeting of proteins as well as peptides to the endosomal/lysosomal compartments, although the CLIP-replaced type of Ii DNA vaccine can only afford to deliver small peptides, Th epitopes, in theory. However, immunological effects of LAMP-1 or LIMP-II DNA vaccine might be weaker than the CLIP-replaced type of Ii DNA vaccine, and will also depend on the target genes, as suggested in Rodriguez et al. [38].

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References

[1] Donnelley, J.J., Ulmer, J.B., Shiver, J.W. and Liu, M.A. (1997) DNA vaccines. Annu. Rev. Immunol. 15, 617–648.
[2] Alarcon, J.B., Waine, G.W. and McManus, D.P. (1999) DNA vaccines: technology and application as anti-parasite and anti-microbial agents. Adv. Parasitol. 42, 343–410.
[3] Condon, C., Watkins, S.C., Celluzzi, C.M., Thompson, K. and Falo Jr., L.D. (1996) DNA-based immunization by in vivo transfection of dendritic cells. Nature Med. 2, 1122–1128.
[4] Tüting, T., Storkus, W.J. and Falo Jr., L.D. (1998) DNA immunization targeting the skin: molecular control of adaptive immunity. J. Invest. Dermatol. 111, 183–188.
[5] van Bergen, J., Ossendorp, F., Jordens, R., Mommaas, A.M., Drijfhout, J.-W. and Koning, F. (1999) Get into the groove! Targeting antigens to MHC class II. Immunol. Rev. 172, 87–96.
[6] Cossart, P. and Mengaud, J. (1989) *Listeria monocytogenes* a model system for the molecular study of intracellular parasitism. Mol. Biol. Med. 6, 463–474.
[7] Kaufmann, S.H.E. (1993) Immunity to intracellular bacteria. Annu. Rev. Immunol. 11, 129–163.
[8] Kaufmann, S.H.E., Hug, E., Vath, U. and Muller, I. (1985) Effective protection against *Listeria monocytogenes* and delayed-type hypersensitivity to listerial antigens depend on cooperation between specific $L3T4^+$ and $Lyt2^+$ T cells. Infect. Immun. 48, 263–266.
[9] Czuprynski, C.J. and Brown, J.F. (1987) Dual regulation of anti-bacterial resistance and inflammatory neutrophil and macrophage accumulation by $L3T4^+$ and $Lyt2^+$ *Listeria*-immune T cells. Immunology 60, 287–293.
[10] Bishop, K. and Hinrichs, D.J. (1987) Adoptive transfer of immunity to *Listeria monocytogenes*: the influence of in vitro stimulation on lymphocyte subset requirement. J. Immunol. 139, 2005–2009.
[11] Czuprynski, C.J. and Brown, J.F. (1990) Effects of purified anti-Lyt-2 mAb treatment on murine listeriosis: comparative roles of $Lyt\text{-}2^+$ and $L3T4^+$ cells in resistance to primary and secondary infection, delayed-type hypersensitivity and adoptive transfer of resistance. Immunology 71, 107–112.
[12] Sasaki, T., Mieno, M., Udono, H., Yamaguchi, T., Usui, K., Hara, K., Shiku, H. and Nakayama, E. (1990) Roles of $CD4^+$ and $CD8^+$ cells, and the effect of administration of recombinant murine interferon γ in listerial infection. J. Exp. Med. 171, 1141–1154.
[13] Ladel, C.H., Flesch, I.E.A., Arnoldi, J. and Kaufmann, S.H.E. (1994) Studies with MHC-deficient knock-out mice reveal impact of both

MHC I- and MHC-II-dependent T cell responses on *Listeria monocytogenes* infection. J. Immunol. 153, 3116–3122.
[14] Roberts, A.D., Ordway, D.J. and Orme, I.M. (1993) *Listeria monocytogenes* infection in β2 microglobulin-deficient mice. Infect. Immun. 61, 1113–1116.
[15] Mielke, M.A., Ehlers, S. and Hahn, H. (1988) T-cell subsets in delayed-type hypersensitivity, protection, and granuloma formation in primary and secondary *Listeria* infection in mice: superior role of Lyt-2$^+$ cells in acquired immunity. Infect. Immun. 56, 1920–1925.
[16] Lukacs, K. and Kurlander, R. (1989) Lyt-2$^+$ T cell-mediated protection against listeriosis: protection correlates with phagocyte depletion but not with IFN-γ production. J. Immunol. 142, 2879–2886.
[17] Kaufmann, S.H.E., Hug, E., Vath, U. and De Libero, G. (1987) Specific lysis of *Listeria monocytogenes*-infected macrophages by class II-restricted L3T4$^+$ T cells. Eur. J. Immunol. 17, 237–246.
[18] Magee, D.M. and Wing, E.J. (1988) Cloned L3T4$^+$ T lymphocytes protect mice against *Listeria monocytogenes* by secreting IFN-γ. J. Immunol. 141, 3203–3207.
[19] Rakhmilevich, A.L. (1993) Evidence for a significant role of CD4$^+$ T cells in adoptive immunity to *Listeria monocytogenes* in the liver. Immunology 82, 249–254.
[20] Bouwer, H.G., Nelson, C.S., Gibbons, B.L., Portnoy, D.A. and Hinrichs, D.J. (1992) Listeriolysin O is a target of the immune response to *Listeria monocytogenes*. J. Exp. Med. 175, 1467–1471.
[21] Pamer, E.G., Harty, J.T. and Bevan, M.J. (1991) Precise prediction of a dominant class I MHC-restricted epitope of *Listeria monocytogenes*. Nature 353, 852–855.
[22] Safley, S.A., Jensen, P.E., Reay, P.A. and Ziegler, H.K. (1995) Mechanisms of T cell epitope immunodominance analyzed in murine listeriosis. J. Immunol. 155, 4355–4366.
[23] Ziegler, H.K., Safley, S.A. and Hiltbold, E. (1994) Definition of T cell epitopes of *Listeria monocytogenes* and regulation of antigen processing by the bacterial exotoxin listeriolysin-O (LLO). In: Antigen Processing and Presentation (Humphreys, R.E. and Pierce, S.K., Eds.), pp. 295–307. Academic, San Diego, CA.
[24] Nakamura, Y., Gojobori, T. and Ikemura, T. (1999) Codon usage tabulated from the international DNA sequence databases: its status 1999. Nucleic Acids Res. 27, 292. (access via: http://www.dna.affrc.go.jp/~nakamura/CUTG.html).
[25] Ciernik, I.F., Berzofsky, J.A. and Carbone, D.P. (1996) Induction of cytotoxic T lymphocytes and antitumor immunity with DNA vaccines expressing single T cell epitopes. J. Immunol. 156, 2369–2375.
[26] Horton, R.M. and Pease, L.R. (1991) Recombination and mutagenesis of DNA sequences using PCR. In: Directed Mutagenesis: A Practical Approach (McPherson, R.M., Ed.), pp. 217–247. IRL, Oxford, UK.
[27] Wu, T.-C., Guarnieri, F.G., Staveley-O'Carroll, K.F., Viscidi, R.P., Levitsky, H.I., Hedrick, L., Cho, K.R., August, J.T. and Pardoll, D.M. (1995) Engineering an intracellular pathway for major histocompatibility complex class II presentation of antigens. Proc. Natl. Acad. Sci. USA 92, 11671–11675.
[28] Yoshida, A., Koide, Y., Uchijima, M. and Yoshida, T.O. (1995) Dissection of strain difference in acquired protective immunity against *Mycobacterium bovis* Calmette-Guérin bacillus (BCG). J. Immunol. 155, 2057–2066.
[29] Yoshida, A., Nagata, T., Uchijima, M., Higashi, T. and Koide, Y. (2000) Advantage of gene gun-mediated over intramuscular inoculation of plasmid DNA vaccine in reproducible induction of specific immune responses. Vaccine 18, 1725–1729.
[30] Nagata, T., Uchijima, M., Yoshida, A., Kawashima, M. and Koide, Y. (1999) Codon optimization effect on translational efficiency of DNA vaccine in mammalian cells: analysis of plasmid DNA encoding a CTL epitope derived from microorganisms. Biochem. Biophys. Res. Commun. 261, 445–451.
[31] Nagata, T., Higashi, T., Aoshi, T., Suzuki, M., Uchijima, M. and Koide, Y. (2001) Immunization with plasmid DNA encoding MHC class II binding peptide/CLIP-replaced invariant chain (Ii) induces specific helper T cells in vivo: the assessment of Ii p31 and p41 isoforms as vehicles for immunization. Vaccine 20, 105–114.
[32] Anderson, K., Creswell, P., Gammon, M., Hermes, J., Williamson, A. and Zweerink, H. (1991) Endogenously synthesized peptide with an endoplasmic reticulum signal sequence sensitizes antigen processing mutant cells to class I-restricted cell-mediated lysis. J. Exp. Med. 174, 489–492.
[33] Iwasaki, A., Dela Curz, C.S., Young, A.R. and Barber, B.H. (1999) Epitope-specific cytotoxic T lymphocyte induction by minigene DNA immunization. Vaccine 17, 2081–2088.
[34] Chen, J.W., Cha, Y., Yuksel, K.U., Gracy, R.W. and August, J.T. (1988) Isolation and sequencing of a cDNA clone encoding lysosomal membrane glycoprotein mouse LAMP-1. J. Biol. Chem. 263, 8754–8758.
[35] Guarnieri, F.G., Arterburn, L.M., Penno, M.B., Cha, Y. and August, J.T. (1993) J. Biol. Chem. 268, 1941–1946.
[36] Thomson, S.A., Burrows, S.R., Misko, I.S., Moss, D.J., Coupar, B.E.H. and Khanna, R. (1998) Targeting a polyepitope protein incorporating multiple class II-restricted viral epitopes to the secretory/endocytic pathway facilitates immune recognition by CD4$^+$ cytotoxic T lymphocytes: a novel approach to vaccine design. J. Virol. 72, 2246–2252.
[37] Ji, H., Wang, T.-L., Chen, C.-H., Pai, S.I., Hung, C.-F., Lin, K.-Y., Kurman, R.J., Pardoll, D.M. and Wu, T.-C. (1999) Targeting human papillomavirus type 16 E7 to the endosomal/lysosomal compartment enhances the antitumor immunity of DNA vaccines against murine human papillomavirus type 16 E7-expressing tumors. Hum. Gene Ther. 10, 2727–2740.
[38] Vidalin, O., Tanaka, E., Spengler, U., Trepo, C. and Inchauspe, G. (1999) Targeting of hepatitis C virus core protein for MHC I or MHC II presentation does not enhance induction of immune responses to DNA vaccination. DNA Cell Biol. 18, 611–621.
[39] Rodriguez, F., Harkins, S., Redwine, J.M., De Pereda, J.M. and Whitton, J.L. (2001) CD4$^+$ T cells induced by a DNA vaccine: immunological consequences of epitope-specific lysosomal targeting. J. Virol. 75, 10421–10430.
[40] Verma, N.K., Ziegler, H.K., Wilson, M., Khan, M., Safley, S., Stocker, B.A.D. and Schoolnik, G.K. (1995) Delivery of class I and class II MHC-restricted T-cell epitopes of listeriolysin of *Listeria monocytogenes* by attenuated *Salmonella*. Vaccine 13, 142–150.
[41] Nagata, T., Aoshi, T., Suzuki, M., Uchijima, M., Kim, Y.-H., Yang, Z. and Koide, Y. (2002) Induction of protective immunity to *Listeria monocytogenes* by immunization with plasmid DNA expressing a helper T-cell epitope that replaces the class II-associated invariant chain peptide of the invariant chain. Infect. Immun. 70, 2676–2680.

ELSEVIER

Pretreatment of Recipients With Mitomycin-C–Treated Dendritic Cells Induces Significant Prolongation of Cardiac Allograft Survival in Mice

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INJECTION of mitomycin-C (MMC)-treated spleen cells is known to induce donor-specific unresponsiveness to cardiac allografts in rats.[1] However, the mechanisms by which the unresponsiveness is induced after MMC treatment are uncertain. Because dendritic cells (DCs) are highly efficient initiators and regulators of immune responses as bone marrow (BM)-derived professional antigen-presenting cells (APCs), we hypothesized that, after MMC treatment, DCs might play a key role in the induction of unresponsiveness. In the current study, we focused on the effect of MMC-treated DCs on murine heart allograft survival.

MATERIALS AND METHODS

Animals

Male BALB/c (*H-2*^{d}) mice were used as donors, male C57BL/6 (*H-2*^{b}) mice as recipients, and male C3H/HeJ (*H-2*^{k}) mice as the third party. The mice were obtained from Japan SLC (Hamamatsu, Japan), and maintained in clean cages under pathogen-free conditions in the animal facility of Hamamatsu University School of Medicine.

Propagation and Purification of DCs

The method for in vitro propagation of DC progenitors was basically that described by Inaba et al.[2] Cells (2×10^6) were cultured in plates in DMEM supplemented with 10% (v/v) heat-inactivated fetal calf serum (Sigma Co, St Louis, Mo) and 0.1 ng/mL recombinant mouse GM-CSF (Pepro Tech, Inc) either alone or together with 0.1 ng/mL recombinant mouse IL-3 or IL-4 (Pepro Tech).

MMC Treatment

For treatment of MMC (Kyowa Hakko Co, Osaka, Japan), a suspension of 8×10^6 DCs in 2 mL of basal medium containing 50 μg/mL of MMC was incubated for 30 minutes at 37°C. After treatment, the cell suspension was washed three times with basal medium. Cell viability, as assessed by the trypan blue dye exclusion test, was >90% after MMC treatment. C57BL/6 mice were preinjected with 8×10^6 MMC-treated, or untreated, BALB/c DCs in 0.5 mL of DMEM through the penile vein under ether anesthesia. The control group was injected with 0.5 mL of DMEM alone.

Heart Grafting

Ten days after DC injection, hearts from either BALB/c or C3H/HeJ mice were transplanted heterotopically into C57BL/6 recipients according to Corry's method,[3] with some modifications. Heart graft survival was monitored daily by palpation, and rejection as detected by cessation of the heart beat was confirmed by direct inspection and histology.

Histologic Examination

On posttransplant day 5, the grafts were removed and fixed with 4% paraformaldehyde in PBS (pH 7.4). Paraffin-embedded sections were stained with hematoxylin-eosin.

Reverse Transcriptase (RT)-PCR

Five days after grafting, RT-PCR was performed to quantitate cytokine mRNA in the grafts. The total RNA was extracted as previously described.[4] One microgram of RNA was reverse-transcribed into cDNA in a 20-μL total reaction volume.[5] Two microliters of each cDNA synthesis product was amplified in a thermal cycler (Model TSR-300 Thermal Sequencer, Iwaki Glass Co) for 35 cycles of denaturation (96°C for 1 minute), annealing (55°C for 1 minute), and extension (72°C for 1 minute). Sense/antisense oligonucleotide primers for β-actin, IFN-γ, and IL-4 were constructed as 5′-GCA CCA CAC CTT CTA CAA TGA G-3′/5′-AAA TAG CACAGC CGT GAT AGC AAC-3′, 5′-TTA CTG CCA CGG CAC AGT CAT A-3′/5′-TCG GAT GGC TCA TTG AAT GCT TGG-3′, and 5′-ACA GAC CAT RRG ARG GGT CT-3′/5′-GTG ATG.TGG ACT TGG ACT CA-3′, respectively. The amplified DNA was electrophoresed through 6% acrylamide gels and visualized by ethidium-bromide staining with ultraviolet rays.

Statistical Methods

Mean survival times (MSTs) were expressed as mean ± SD. Statistical comparisons between groups were performed with Student's *t* test.

RESULTS

As shown in Table 1, BALB/c cardiac allograft survival in C57BL/6 recipients was prolonged significantly by intrave-

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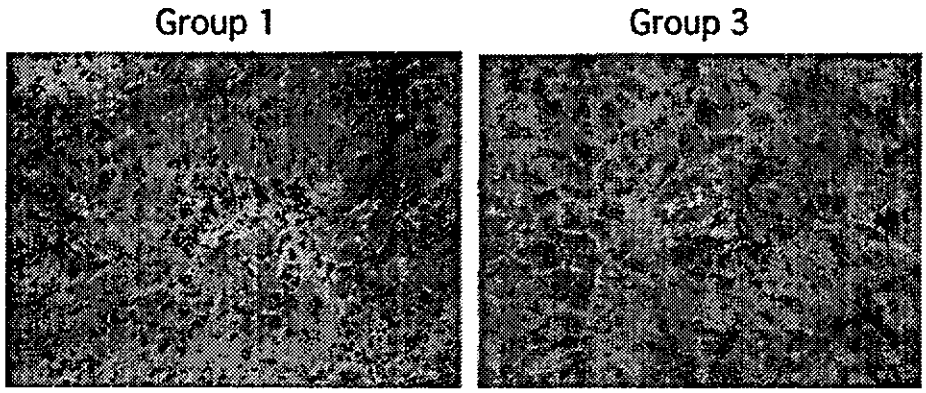

Fig 1. Histologic findings in cardiac grafts at 5 days after grafting. There was marked infiltration of mononuclear cells around the vessels in the control grafts (group 1), whereas little cell infiltration was observed in group 3.

nous administration of BALB/c DCs, which had been stimulated by GM-CSF plus IL-4 prior to treatment with MMC (25.7 ± 1.7 days) (group 3) when compared with MMC-untreated DCs that had been cultured with GM-CSF plus IL-4 (group 2), MMC-treated DCs that had been precultured with GM-CSF plus IL-3 (10.1 ± 2.1 days) (group 4), or untreated controls (6.7 ± 0.4 days) (group 1). Interestingly, survival was also prolonged by intravenous administration of MMC-treated or untreated DCs that had been cultured with GM-CSF alone. However, C57BL/6 mice given MMC-treated BALB/c DCs, which had been cultured with GM-CSF plus IL-4, rejected third party C3H/HeJ cardiac allografts in normal fashion (group 7), indicating that the prolongation of graft survival observed with MMC-treated DCs was donor-specific.

The histologic findings of the cardiac transplants at 5 days after grafting revealed a marked infiltration of mononuclear cells around the vessels of control grafts (group 1) (Fig 1). Similar histologic findings were observed in grafts from animals in groups 2, 4, and 7 (data not shown), whereas little cell infiltration was observed in animals of group 3 (Fig 1), or of groups 5 and 6 (data not shown).

IFN-γ mRNA expression (Fig 2) was detected in control BALB/c grafts (group 1) and in those of group 4 (data not shown). Whereas IFN-γ mRNA was not detectable in group 3, IL-4 mRNA was faintly detected.

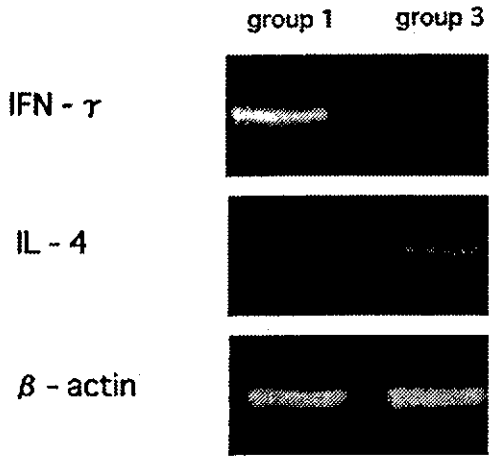

Fig 2. RT-PCR of cytokine mRNA in the graft 5 days after grafting. IFN-γ mRNA was clearly detected in the control BALB/c graft (group 1). Although group 3 showed no IFN-γ mRNA, IL-4 mRNA was faintly detected in each group.

DISCUSSION

Type 1 T-helper cells (Th1) and type 2 T-helper cells (Th2) may cross-regulate each other in vivo through the release of IFN-γ and IL-4, respectively.[6] Binder and associates[7] reported that, after nondepleting CD4-targeted therapy in rats, Th1-type cytokines were diminished, whereas Th2-type cytokines were unchanged. In the current study, we observed marked infiltration of mononuclear cells and upregulation of INF-γ mRNA with little expression of IL-4 mRNA in heart grafts of the control group. In contrast, little cellular infiltration and downregulation of INF-γ mRNA and slight expression of IL-4 mRNA were noted in the BALB/c grafts in C57BL/6 recipients preinjected with BALB/c DCs that had been treated with MMC after preincubation with GM-CSF and IL-4 DCs (group 3). Therefore, the mononuclear cells infiltrating the grafts of the control group hosts seemed to be mainly Th1 cells, whereas the infiltrating cells in the grafts of group 3 may

Table 1. Cardiac Allograft Survival Time of BALB/c and C3H/HeJ in C57BL/6 Mice

Treatment of BALB/c DC Groups	Survival Time (Days)	MST ± SD
1. Media control	6, 6, 7, 7, 7, 7, 7	6.7 ± 0.4
2. IL-4 + GM-CSF	7, 7, 7, 8, 8	7.4 ± 0.5
3. IL-4 + GM-CSF + MMC	22, 25, 25, 26, 27, 29	25.7 ± 1.7†
4. IL-3 + GM-CSF + MMC	6, 6, 11, 11, 11, 12, 12, 12	10.1 ± 2.1*
5. GM-CSF + MMC	19, 25, 25, 28, 33	26.0 ± 3.6†
6. GM-CSF	20, 23, 28, 28, 32	26.2 ± 3.7†
7. IL-4 + GM-CSF + MMC(C3H)	7, 7, 8, 8, 8	7.6 ± 0.5

*$P < .05$ compared with group 1.
†$P < .01$ compared with groups 1 and 4.

have been mainly Th2 cells, although they were small in number. These results suggest that suppression of the Th1 response might be responsible for prolongation of BALB/c ($H\text{-}2^d$) allograft survivals in the complete MHC-disparate C57BL/6 ($H\text{-}2^b$) recipients.

The functional maturation of DCs and other APCs, such as macrophages or activated B cells, has been shown to depend on cytokine-induced upregulation of cell surface major histocompatibility complex (MHC) class II and T-cell costimulatory molecules, particularly the CD28 ligands, B7-1 (CD80) and B7-2 (CD86).[8,9] After culturing for 48 hours in the presence of GM-CSF plus IL-4, MHC class II, B7-1, and B7-2 markers were upregulated on freshly harvested bone marrow DC progenitors.[10] In contrast, cells stimulated with GM-CSF alone propagate into DC progenitors that are deficient in costimulatory molecules (MHC class II, B7-1, and B7-2), fail to stimulate primary mixed leukocyte reactions (MLR), and induce donor-specific T-cell anergy.[11] These progenitors prolong graft survival when injected systemically into recipients before grafting.[12] As expected, the results of the current study demonstrate that BALB/c DCs cultured with GM-CSF alone induce donor-specific unresponsiveness across a complete MHC-disparate C57BL/6 recipient, regardless of MMC treatment (groups 5 and 6).

Supplementation of cultures with IL-4 in addition to GM-CSF caused functional maturation of DCs with marked upregulation of MHC class II and costimulatory molecules.[10,11] Consistent with these observations, DCs pretreated with IL-4 plus GM-CSF failed to prolong graft survival (group 2), however, MMC treatment induced the mature DCs to prolong graft survival (group 3). MMC treatment inhibits not only proliferation of cells but also some degree of protein and mRNA synthesis in cell cultures.[13] Tanigawa et al[14] reported that expression of MHC class I and class II antigens was not changed by MMC treatment, although they observed that this treatment significantly impaired upregulation of intracellular adhesion molecule-1 (ICAM-1) on cultured spleen cells.[15] Thus, MMC treatment might affect the expression of adhesion molecules and costimulatory molecules on mature DCs, resulting in a donor-specific immunosuppressive effect. The mechanisms involved in this immunosuppression, may be distinct from those induced by DCs precultured with GM-CSF alone.

DCs cultured with GM-CSF plus IL-3 have been reported to express less MHC class II and costimulatory molecules compared with those cultured with GM-CSF plus IL-4 and show intermediate stimulatory activity in MLR.[16] Our results also show that MMC-treated DCs precultured with GM-CSF plus IL-3 exhibit an intermediate effect on prolongation of allograft survival.

Taken together, these results indicate that MMC-treated mature DCs induce donor-specific prolongation of cardiac allograft in mice by suppressing Th1 responses. Further study is necessary to clarify the mechanisms of this effect.

REFERENCES

1. Tanigawa T, Gotoh M, Nagano H, et al: Transplantation 67:653, 1999
2. Inaba K, Inaba M, Romani N, et al: J Exp Med 176:1693, 1992
3. Corry RJ, Winn HJ, Russell PS: Transplantation 16:343, 1973
4. Kita Y, Li XK, Ohba M, et al: Transplantation 68:758, 1999
5. Uchijima M, Yoshida A, Nagata T, et al: J Immunol 161:5594, 1998
6. Fitch FW, McKisic MD, Lancki DW, et al: Annu Rev Immunol 11:29, 1993
7. Binder J, Lehmann M, Graser E, et al: Transplantation 61:804, 1996
8. Larsen CP, Ritchie SC, Hendrix R, et al: J Immunol 152:5208, 1994
9. Inaba K, Witmer-Pack M, Inaba M, et al: J Exp Med 180:1849, 1994
10. Masli S, De Fazio SR, Gozzo JJ: Transplant Proc 29:1147, 1997
11. Lu L, McCaslin D, Starzel TE, et al: Transplantation 60: 1539, 1995
12. Fu F, Li Y, Qian S, et al: Transplantation 62:659, 1996
13. Ishioka C, Audisio RA, Kanishi Y, et al: Cancer Chemother Pharmacol 26:117, 1990
14. Tanigawa T, Monden M, Gotoh M, et al: Transplant Proc 26:3359, 1994
15. Tanigawa T, Gotoh M, Nagano H, et al: Transplant Proc 29:1149, 1997
16. Sato M, Iwakabe K, Ohta A, et al: Int Immunol 12:335, 2000

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Induction of Protective Immunity to *Listeria monocytogenes* by Immunization with Plasmid DNA Expressing a Helper T-Cell Epitope That Replaces the Class II-Associated Invariant Chain Peptide of the Invariant Chain

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***Listeria* epitope-specific helper T (Th) cells were able to be primed and induced in vivo by immunization with a plasmid carrying an invariant chain (Ii) gene whose class II-associated invariant chain peptide (CLIP) region was replaced by a *Listeria* Th epitope. Immunization of C3H/He mice with an Ii-LLO 215–226 plasmid induced specific interferon-γ- and interleukin 2-producing Th cells and conferred significant protective immunity against listerial infection.**

Listeria monocytogenes is a gram-positive intracellular bacterium. A murine model of *L. monocytogenes* infection has been well studied and is considered a good model for exploring immunity against intracellular bacteria (reviewed in references 2 and 12). Cellular immunity has been considered to play a pivotal role in protection against intracellular bacteria (15). A variety of effector cells have been reported or suggested to resolve infection. These include neutrophils, macrophages, NK cells, and γδ T cells, as well as αβ T cells (9, 15, 19, 24). Among these, αβ $CD8^+$ and $CD4^+$ T cells have been shown to play critical roles in protective immunity through experiments with the depletion and adoptive transfer of specific T-cell subsets (1, 3, 4, 10, 27) and analyses of mutant mice with a genetic defect in β2-microglobulin or the H2-Aβ chain gene (13, 25). $CD8^+$ cytotoxic T lymphocytes (CTL) have been reported to play a superior role in protective immunity (14, 18, 25). However, several papers have demonstrated a significant role for the $CD4^+$-T-cell subset in protective immunity (11, 16, 23). Helper T (Th) cells play an important role in many aspects of immunity, especially for modulating immune responses by producing special sets of cytokines. For protection against intracellular bacteria, the activation of macrophages is indispensable, and antigen (Ag)-specific type 1 Th (Th1) cells have been reported to play a pivotal role in the activation (reviewed in reference 12). To investigate the roles of Th cells in protective immunity, we attempted to induce Th cells by immunization with an expression plasmid for a single Th epitope of *L. monocytogenes*, amino acid residues 215 to 226 of listeriolysin O (LLO 215–226; SQLIAKFGTAFK), an H2-E^k-restricted Th epitope (26, 37). The attempt, however, was unsuccessful (see Fig. 2A, p215), although immunization with plasmids encoding a single CTL epitope was able to induce specific CTL (20, 28, 34). In support of this result, we also showed in a previous work that immunization with an expression plasmid for a single Th epitope of ovalbumin (OVA 323–336) failed to induce specific lymphocyte proliferation (21).

The invariant chain (Ii) molecule plays a central role in major histocompatibility complex (MHC) class II-mediated Ag presentation. It associates with MHC class II molecules in the endoplasmic reticulum so as to block premature loading of peptides on the molecules there. The Ii molecule works as a molecular chaperone for MHC class II transport to the endosomal compartment, where antigenic peptides are replaced with the class II-associated Ii peptide (CLIP) region of the molecule (reviewed in reference 8). Several groups have reported that MHC class II-positive cultured cells transfected with Ii cDNA whose CLIP region was replaced with a Th epitope of interest efficiently stimulate specific Th lines in vitro (5, 17, 29). In the present study, we investigated the effect of a single epitope-specific Th on protective immunity against *L. monocytogenes*, using immunization by gene gun bombardment with Ii plasmid DNA expressing a Th epitope that replaces the CLIP region.

We constructed the plasmid for genetic immunization based on murine Ii p41 isoform cDNA. The *Eco*RI fragment containing Ii p41 cDNA was inserted into the *Eco*RI site in the pCI eukaryotic expression plasmid (Promega, Madison, Wis.). The CLIP region in the Ii cDNA was removed and replaced by a synthetic double-stranded oligonucleotide coding for LLO 215–226, resulting in pCI-mIi p41-LLO215m (Fig. 1). The oligonucleotide was designed so as to be adapted to the codon usage most frequent in mouse and human (22).

In order to examine whether pCI-mIi p41-LLO215m induces specific T cells in vivo, we immunized C3H/He mice ($H2^k$; Japan SLC, Hamamatsu, Japan) with the plasmid by gene gun bombardment. We chose this immunization method because, based on our previous experience, it is a very reliable and reproducible method (36). All animal experiments were performed according to the animal care guidelines of our uni-

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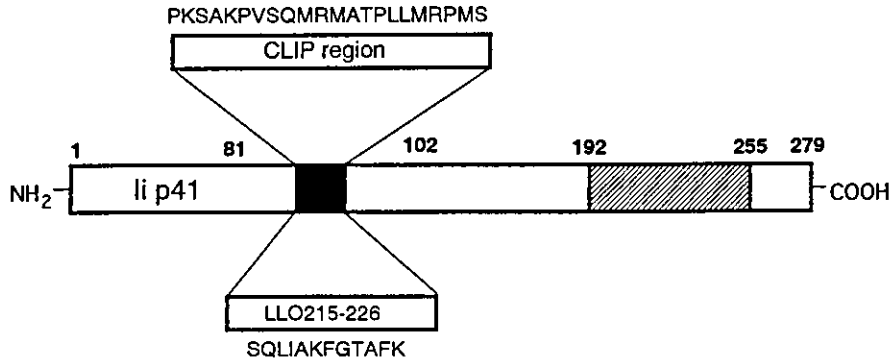

FIG. 1. Schema of murine Ii molecule whose CLIP is replaced by LLO 215–226 (mIi p41-LLO215) deduced from the cDNA construct. The nucleotides encoding the CLIP region in the murine Ii p41 cDNA were replaced with the oligonucleotide coding for LLO 215–226 H2-E^k binding peptide. The cDNA was subcloned into the *Eco*RI site of pCI. The deduced amino acid sequences of the replaced CLIP region and the antigenic peptide LLO 215–226 are shown.

versity. The plasmid DNA immunization was performed with the Helios gene gun system (Bio-Rad Laboratories, Hercules, Calif.). The preparation of a DNA-coated gold particle cartridge was performed following the manufacturer's instruction manual. Finally, 0.5 mg of 1.0-μm-diameter gold particles were coated with 1 μg of plasmid DNA, and the injection was carried out with 0.5 mg of gold/shot. Then, the mice were injected in the abdomen with 1 μg of plasmids at a helium discharge pressure of 400 lb/in^2 four times at weekly intervals.

Three weeks after the last immunization, a lymphocyte proliferation assay was performed with splenocytes from the immunized mice. After treatment with Tris-buffered 0.83% ammonium chloride to lyse erythrocytes, splenocytes (5×10^5/well) from pCI-mIi p41-LLO215m-immunized mice were incubated for 48 h at 37°C in 96-well round-bottom tissue culture plates in RPMI-1640 medium supplemented with 10% fetal calf serum (FCS) in the presence or absence of 1 μM LLO 215–226 peptide. Then, DNA synthesis was assessed by adding 0.5 μCi of [*methyl*-^{3}H]thymidine (6.7 Ci/mmol; ICN Biochemicals, Irvine, Calif.)/well for the last 14 h of culture. The cultures were harvested onto glass fiber filters, and the radioactivity was counted by liquid scintillation. As shown in Fig. 2A, immunization with the plasmid allowed splenocytes to proliferate after incubation in the presence, but not in the absence, of LLO 215–226 peptide at a level comparable to that of viable *Listeria* immunization. We could not detect any significant LLO 215–226-specific lymphocyte proliferation by immunization with p215 (LLO 215–226 expression plasmid) or pCI-mIi p41 (wild-type Ii p41 expression plasmid). We could not detect nonspecific proliferative responses with pCI-mIi p41-LLO215m when accompanied by incubation with an irrelevant peptide (LLO 189–200; Fig. 2A).

Furthermore, the CD4-CD8 specificity of proliferative lymphocytes was tested by depletion studies with the anti-murine CD4 monoclonal antibody (MAb) GK1.5 or the anti-murine CD8α MAb 53–6.7 (PharMingen, San Diego, Calif.). These MAbs were added to the immune splenocytes, at 1 μg/ml, and the splenocytes were incubated for 1 h at 4°C. They were then centrifuged, and the supernatants were discarded. The cells were resuspended in cytotoxicity medium (RPMI-1640 medium with 25 mM HEPES buffer and 0.3% FCS) containing rabbit complement (Cedarlane, Hornby, Ontario, Canada) and incubated for 1 h at 37°C. The dead cells were removed by Lympholite-M reagent (Cedarlane). The recovered cells were used for the lymphocyte proliferation assay described above. The LLO 215–226-specific proliferative responses of splenocytes from the immunized mice was reduced significantly by $CD4^+$-T-cell subset depletion but not by $CD8^+$-T-cell subset depletion, indicating that LLO 215–226-specific T cells generated by pCI-mIi p41-LLO215m plasmid DNA immunization belong to the $CD4^+$-T-cell subset (Fig. 2B).

Next, we examined specific gamma interferon (IFN-γ), interleukin-2 (IL-2), and IL-4 production by splenocytes from mice immunized with the pCI-mIi p41-LLO215m plasmid. Splenocytes from the immunized mice were plated in 24-well plates at 2×10^6/well in RPMI 1640 medium supplemented with 10% FCS in the presence or absence of 1 μM LLO 215–226 peptide for 4 days in the case of IFN-γ and IL-4 and for 1 day in the case of IL-2. The concentrations of cytokines in the culture supernatants were determined by sandwich enzyme-linked immunosorbent assay, as described elsewhere (35). All of the MAbs used were purchased from PharMingen. As shown in Table 1, we observed the production of significant amounts of IFN-γ and IL-2 by splenocytes from mice immunized with pCI-mIi p41-LLO215m, but not with pCI-mIi p41, at a level comparable with that from mice immunized with viable *Listeria* after in vitro culture in the presence of LLO 215–226 peptide. In addition, splenocytes of pCI-mIi p41-LLO215m-immunized mice after incubation with an irrelevant MHC class II binding peptide did not produce significant amounts of IFN-γ and IL-2. We could not detect significant levels of IL-4 by using the same culture supernatants of splenocytes from all mice examined (Table 1).

To ascertain if Th cell effectors evoked by plasmid immunization are associated with an increased resistance to infection by the virulent *L. monocytogenes* EGD strain, in vivo protection experiments were carried out. The bacterium was kept virulent by in vivo passage. For inoculation, a seed of *L. monocytogenes* was cultured overnight in trypticase soy broth (BBL, Sparks, Md.) at 37°C in a bacterial shaker and suitably diluted with phosphate-buffered saline. The exact infection dose was assessed retrospectively by plating. Mice were immunized with pCI-mIi p41-LLO215m four times at weekly intervals or were immunized by a single intraperitoneal injection with a sublethal dose of *L. monocytogenes* (10^4 CFU) as a positive control.

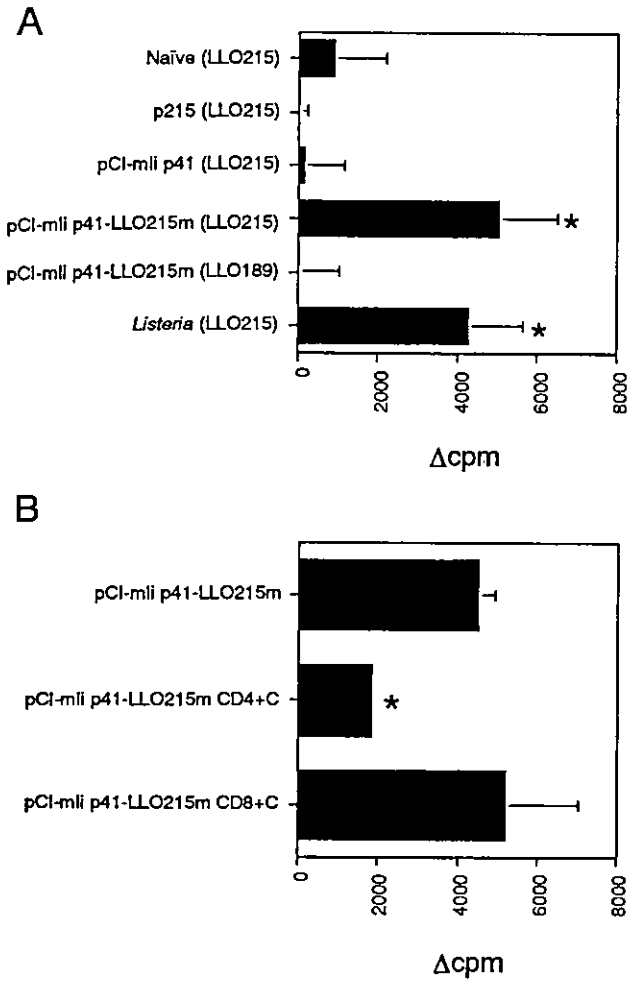

FIG. 2. Ag-specific proliferation of splenocytes from mice immunized with an Ii plasmid expressing LLO 215–226, which replaces CLIP. Mice were immunized with p215, pCI-mIi p41, or pCI-mIi p41-LLO215m by gene gun bombardment four times at weekly intervals. (A) Splenocytes of the immunized mice were harvested 3 weeks after the last immunization and cultured in vitro in the presence or absence of 1 μM LLO 215–226 peptide (LLO215) or LLO 189–200 control peptide (LLO189) for 2 days and pulsed with 0.5 μCi of [*methyl*-^{3}H]thymidine for the last 12 h. The values represent the mean and standard deviation of Δcpm (counts per minute in the presence of peptide minus counts per minute in the absence of the peptide) of quintuplicate determinations in a representative experiment. The asterisks indicate statistical significance ($P < 0.001$) compared with the values in naive mice. One-factor analysis of variance followed by Fischer's protected least-significant difference test was used in all statistical analyses. (B) Inhibition of LLO 215–226-specific splenocyte proliferation by CD4$^+$-T-cell subset depletion. The splenocytes from pCI-mIi p41-LLO215m-immunized mice were treated with an anti-CD4 or anti-CD8 MAb and rabbit complement. Then, the lymphocyte proliferation assay was performed. The mean and standard deviation of Δcpm of quintuplicate determinations of a representative experiment are shown. The asterisks indicate statistical significance ($P < 0.005$) compared with the value for untreated splenocytes (pCI-mIi p41-LLO215m).

TABLE 1. Cytokine production by splenocytes from C3H/He mice immunized with pCI-mIip41-LLO 215m plasmid

Immunization	Stimulation[a]	Cytokine production (pg/ml)[b]		
		IFN-γ	IL-2	IL-4
pCI-mIi p41	–	163	416	62
	LLO215	140	638	19
pCI-mIi p41-LLO 215m	–	266	32	49
	LLO215	2,535	2,046	35
	LLO189	66	226	40
Listeria	–	287	377	73
	LLO215	2,034	1,053	49

[a] Spleen cells of immunized mice (2×10^6 per well) were cultured in the presence of 1 μM LLO 215-226 peptide (LLO215) or control peptide LLO 189–200 (LLO189) or in the absence of any peptides (–).

[b] After 4 (IFN-γ and IL-4) or 1 (IL-2) day, cytokine concentrations in culture supernatants were quantified by sandwich enzyme-linked immunosorbent assay. The mean of duplicate wells of representative data is shown.

The immunized mice were challenged intraperitoneally with 2×10^5 CFU of *Listeria* 3 weeks after the last immunization. Bacterial numbers in the spleens and livers were determined 72 h after the challenge infection by plating 10-fold dilutions of tissue homogenates on trypticase soy agar. As shown in Fig. 3, immunization with pCI-mIi p41-LLO215m dramatically decreased the bacterial numbers in the spleens and livers of the immunized mice. Verma et al. (32) also demonstrated by using a *Salmonella* carrier system that induction of a CD4$^+$-T-cell population responsive to LLO 215–226 elicits partial protective immunity. In their system, reduction in the number of *Listeria* cells was more significant in the livers of LLO 215–226-immunized mice than in the spleens. Our data also show that LLO 215–226-immunized mice were somewhat better protected against *Listeria* challenge in the liver than in the spleen compared with *Listeria*-immunized mice (Fig. 3). On the other hand, Geginat et al. (6) reported enhanced protection by p60-specific CD4$^+$-T-cell clones in the spleen compared with the liver in their adoptive-transfer system of the CD4$^+$-T-cell clones. This discrepancy might be attributable to differences in the experimental design, including intraperitoneal listerial challenge (this work and Verma et al. [32]) versus intravenous listerial challenge (Geginat et al. [6]). In addition, other reports also demonstrated a role for CD4$^+$ T cells in protective immunity against listerial challenge (11, 16, 23). The mechanisms of the protective immunity elicited by CD4$^+$ T cells have been speculated upon. *Listeria*-specific CD4$^+$ T cells may act by direct lysis of the infected target cells (11). Alternatively, the cells may show the bystander effect by secretion of cytokines, especially IFN-γ. IFN-γ will enhance the killing activity of macrophages or augment induction of CD8$^+$ CTL (33).

We report here that DNA immunization with an Ii expression plasmid whose encoded CLIP region has been replaced by a *Listeria*-derived Th epitope successfully induces T cells specific to the epitope in vivo. Attempts to induce specific Th cells by using Ii plasmids in the cell line system (5, 17, 29; reviewed in reference 30) or, recently, in vivo (21, 31) have been reported. Here, we showed that a similar system can be applied successfully for DNA vaccination against infectious diseases. This is the first report showing that a single immunization with Ii plasmid DNA whose encoded CLIP region has been replaced by a Th epitope induces effective protective immunity against a microorganism. One of the advantages of gene immunization with T-cell epitope minigene plasmids is that we can compare the immunogenicities of all of the T-cell epitopes at the same expression level in vivo. We have analyzed the hierarchy of the magnitudes of immunogenicity of three *List-*

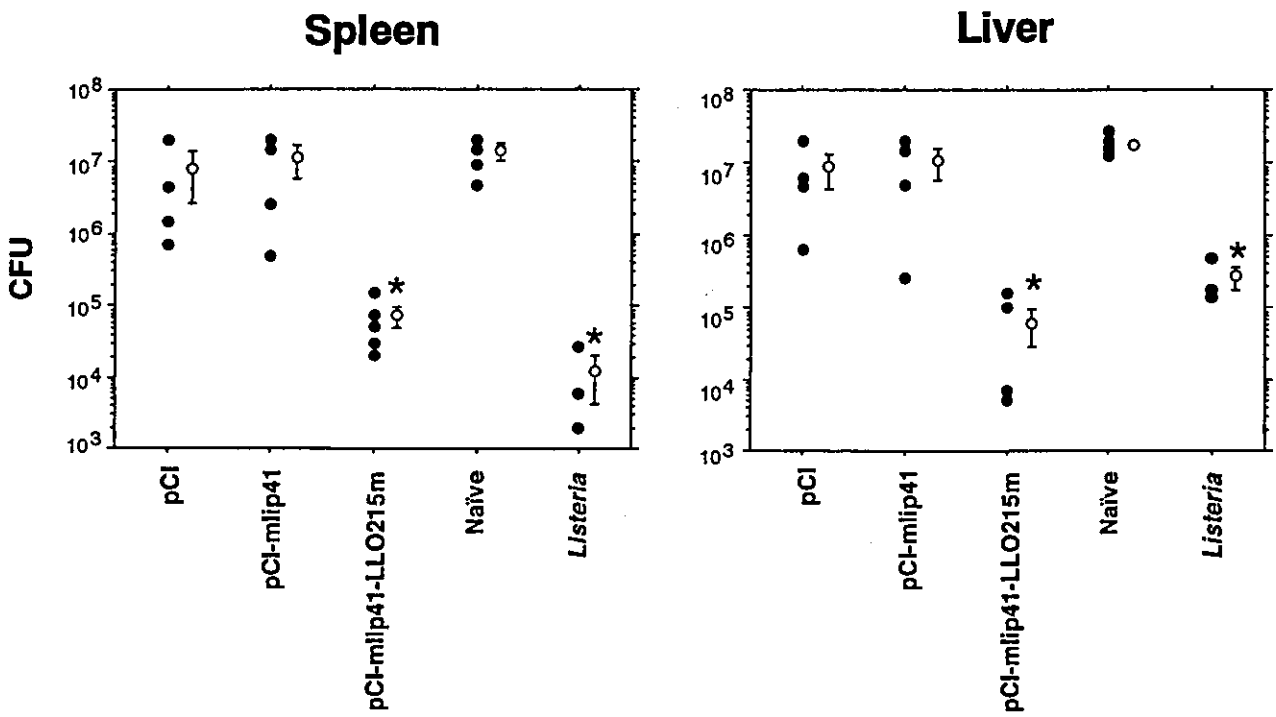

FIG. 3. Protective immunity induced by immunization with pCI-Ii p41-LLO215m. C3H/He mice were immunized with pCI-Ii p41-LLO215m four times at weekly intervals. Three weeks after the last immunization, the mice were challenged with 2×10^5 CFU of *L. monocytogenes*. Three to five mice were used for each group. The bacterial numbers in the spleens and livers were determined 72 h after challenge infection by plating 10-fold dilutions of tissue homogenates on trypticase soy agar plates. The results for pCI-immunized, pCI-mli p41-immunized, naïve, and *Listeria*-immunized mice are also shown as controls. The numbers of bacteria recovered from the spleen and liver of each immunized mouse are shown. The mean and standard deviation of each group are also shown. The asterisks indicate statistical significance ($P < 0.03$) compared with the values in naïve mice.

eria-derived CTL epitopes by using a gene immunization system with minigenes for three listerial CTL epitopes (34). Using the system discussed here, it is interesting to compare the immunodominance of several Th epitopes, including those recently identified from LLO and p60, in the induction of protective immunity (7). Furthermore, we plan to examine the effect of combinatorial induction of both CTL and Th cell subsets in order to induce more effective protective immunity against the bacterium by using the system discussed here.

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REFERENCES

1. **Bishop, K., and D. J. Hinrichs.** 1987. Adoptive transfer of immunity to *Listeria monocytogenes*. The influence of in vitro stimulation on lymphocyte subset requirements. J. Immunol. **139**:2005–2009.
2. **Cossart, P., and J. Mengaud.** 1989. *Listeria monocytogenes*. A model system for the molecular study of intracellular parasitism. Mol. Biol. Med. **6**:463–474.
3. **Czuprynski, C. J., and J. F. Brown.** 1987. Dual regulation of anti-bacterial resistance and inflammatory neutrophil and macrophage accumulation by L3T4$^+$ and Lyt 2$^+$ *Listeria*-immune T cells. Immunology **60**:287–293.
4. **Czuprynski, C. J., and J. F. Brown.** 1990. Effects of purified anti-Lyt-2 MAb treatment on murine listeriosis: comparative roles of Lyt-2$^+$ and L3T4$^+$ cells in resistance to primary and secondary infection, delayed-type hypersensitivity and adoptive transfer of resistance. Immunology **71**:107–112.
5. **Fujii, S., S. Senju, Y.-Z. Chen, M. Ando, S. Matsushita, and Y. Nishimura.** 1998. The CLIP-substituted invariant chain efficiently targets an antigenic peptide to HLA class II pathway in L cells. Hum. Immunol. **59**:607–614.
6. **Geginat, G., M. Lalic, M. Kretschmar, W. Goebel, H. Hof, D. Palm, and A. Bubert.** 1998. Th1 cells specific for a secreted protein of *Listeria monocytogenes* are protective *in vivo*. J. Immnol. **160**:6046–6055.
7. **Geginat, G., S. Schenk, M. Skoberne, W. Goebel, and H. Hof.** 2001. A novel approach of direct *ex vivo* epitope mapping identifies dominant and subdominant CD4 and CD8 T cell epitopes from *Listeria monocytogenes*. J. Imunol. **166**:1877–1884.
8. **Germain, R. N.** 1999. Antigen processing and presentation, p. 287–340. *In* W. E. Paul (ed.), Fundamental immunology, 4th ed. Lippincott-Raven Publishers, Philadelphia, Pa.
9. **Hiromatsu, K., Y. Yoshikai, G. Matsuzaki, S. Ohga, K. Muramori, K. Matsumoto, J. A. Bluestone, and K. Nomoto.** 1992. A protective role of γ/δ T cells in primary infection with *Listeria monocytogenes* in mice. J. Exp. Med. **175**:49–56.
10. **Kaufmann, S. H. E., E. Hug, U. Vath, and I. Muller.** 1985. Effective protection against *Listeria monocytogenes* and delayed-type hypersensitivity to listerial antigens depend on cooperation between specific L3T4$^+$ and Lyt 2$^+$ T cells. Infect. Immun. **48**:263–266.
11. **Kaufmann, S. H. E., E. Hug, U. Väth, and G. De Libero.** 1987. Specific lysis of *Listeria monocytogenes*-infected macrophages by class II-restricted L3T4$^+$ T cells. Eur. J. Immunol. **17**:237–246.
12. **Kaufmann, S. H. E.** 1993. Immunity to intracellular bacteria. Annu. Rev. Immunol. **11**:129–163.
13. **Ladel, C. H., I. E. A. Flesch, J. Arnoldi, and S. H. E. Kaufmann.** 1994. Studies with MHC-deficient knock-out mice reveal impact of both MHC-I- and MHC-II-dependent T cell responses on *Listeria monocytogenes* infection. J. Immunol. **153**:3116–3122.
14. **Lukacs, K., and R. Kurlander.** 1989. Lyt-2$^+$ T cell-mediated protection against listeriosis. Protection correlates with phagocyte depletion but not with IFN-γ production. J. Immunol. **142**:2879–2886.
15. **Mackaness, G. B.** 1962. Cellular resistance to infection. J. Exp. Med. **116**: 381–417.

16. **Magee, D. M., and E. J. Wing.** 1988. Cloned L3T4$^+$ T lymphocytes protect mice against *Listeria monocytogenes* by secreting IFN-γ. J. Immunol. **141:** 3203–3207.
17. **Malcherek, G., C. Wirblich, N. Willcox, H.-G. Rammensee, J. Trowsdale, and A. Melms.** 1998. MHC class II-associated invariant chain peptide replacement by T cell epitopes: engineered invariant chain as a vehicle for directed and enhanced MHC class II antigen processing and presentation. Eur. J. Immunol. **28:**1524–1533.
18. **Mielke, M. A., S. Ehlers, and H. Hahn.** 1988. T-cell subsets in delayed-type hypersensitivity, protection, and granuloma formation in primary and secondary *Listeria* infection in mice: superior role of Lyt-2$^+$ cells in acquired immunity. Infect. Immunol. **56:**1920–1925.
19. **Mombaerts, P., J. Arnoldi, F. Russ, S. Tonegawa, and S. H. E. Kaufmann.** 1993. Different roles of αβ and γδ T cells in immunity against an intracellular bacterial pathogen. Nature **365:**53–56.
20. **Nagata, T., M. Uchijima, A. Yoshida, M. Kawashima, and Y. Koide.** 1999. Codon optimization effect on translational efficiency of DNA vaccine in mammalian cells: analysis of plasmid DNA encoding a CTL epitope derived from microorganisms. Biochem. Biophys. Res. Commun. **261:**445–451.
21. **Nagata, T., T. Higashi, T. Aoshi, M. Suzuki, M. Uchijima, and Y. Koide.** 2001. Immunization with plasmid DNA encoding MHC class II binding peptide/CLIP-replaced invariant chain (Ii) induces specific helper T cells *in vivo*: the assessment of Ii p31 and p41 isoforms as vehicles for immunization. Vaccine **20:**105–114.
22. **Nakamura, Y., T. Gojobori, and T. Ikemura.** 1999. Codon usage tabulated from the international DNA sequence databases; its status 1999. Nucleic Acids Res. **27:**292.
23. **Rakhmilevich, A. L.** 1993. Evidence for a significant role of CD4$^+$ T cells in adoptive immunity to *Listeria monocytogenes* in the liver. Immunology **82:** 249–254.
24. **Rakhmilevich, A. L.** 1995. Neutrophils are essential for resolution of primary and secondary infection with *Listeria monocytogenes*. J. Leukoc. Biol. **57:**827–831.
25. **Roberts, A. D., D. J. Ordway, and I. M. Orme.** 1993. *Listeria monocytogenes* infection in β2 microglobulin-deficient mice. Infect. Immunol. **61:**1113–1116.
26. **Safley, S. A., P. E. Jensen, P. A. Reay, and H. K. Ziegler.** 1995. Mechanisms of T cell epitope immunodominance analyzed in murine listeriosis. J. Immunol. **155:**4355–4366.
27. **Sasaki, T., M. Mieno, H. Udono, T. Yamaguchi, K. Usui, K. Hara, H. Shiku, and E. Nakayama.** 1990. Roles of CD4$^+$ and CD8$^+$ cells, and the effect of administration of recombinant murine interferon γ in listerial infection. J. Exp. Med. **171:**1141–1154.
28. **Uchijima, M., A. Yoshida, T. Nagata, and Y. Koide.** 1998. Optimization of codon usage of plasmid DNA vaccine is required for the effective MHC class I-restricted T cell responses against an intracellular bacterium. J. Immunol. **161:**5594–5599.
29. **van Bergen, J., S. P. Schoenbeger, F. Verreck, R. Amons, R. Offringa, and F. Koning.** 1997. Efficient loading of HLA-DR with a T helper epitope by genetic exchange of CLIP. Proc. Natl. Acad. Sci. USA **94:**7499–7502.
30. **van Bergen, J., F. Ossendorp, R. Jordens, A. M. Mommaas, J.-W. Drijfhout, and F. Koning.** 1999. Get into the groove! Targeting antigens to MHC class II. Immunol. Rev. **172:**87–96.
31. **van Tienhoven, E. A. E., C. T. B. ten Brink, J. van Bergen, F. Koning, W. van Eden, and C. P. M. Broeren.** 2001. Induction of antigen specific CD4+ T cell responses by invariant chain based DNA vaccines. Vaccine **19:**1515–1519.
32. **Verma, N. K., H. K. Ziegler, M. Wilson, M. Khan, S. Safley, B. A. D. Stocker, and G. K. Schoolnik.** 1995. Delivery of class I and class II MHC-restricted T-cell epitopes of listeriolysin of *Listeria monocytogenes* by attenuated *Salmonella*. Vaccine **13:**142–150.
33. **Widmann, C., P. Romero, J. L. Maryanski, G. Corradin, and D. Valmori.** 1992. T helper epitopes enhance the cytotoxic response of mice immunized with MHC class I-restricted malaria peptides. J. Immunol. Methods **155:**95–99.
34. **Yamada, T., H. Uchiyama, T. Nagata, K. Chida, M. Uchijima, H. Nakamura, and Y. Koide.** 2001. Protective cytotoxic T lymphocyte responses induced by DNA immunization against immunodominant and subdominant epitopes of *Listeria monocytogenes* are noncompetitive. Infect. Immun. **69:**3427–3430.
35. **Yoshida, A., Y. Koide, M. Uchijima, and T. O. Yoshida.** 1995. Dissection of strain difference in acquired protective immunity against *Mycobacterium bovis* Calmette-Guérin Bacillus (BCG). Macrophages regulate susceptibility through the cytokine network and the induction of nitric oxide synthase. J. Immunol. **155:**2057–2066.
36. **Yoshida, A., T. Nagata, M. Uchijima, T. Higashi, and Y. Koide.** 2000. Advantage of gene gun-mediated over intramuscular inoculation of plasmid DNA vaccine in reproducible induction of specific immune responses. Vaccine **18:**1725–1729.
37. **Ziegler, H. K., S. A. Safley, and E. Hiltbold.** 1994. Definition of T cell epitopes of *Listeria monocytogenes* and regulation of antigen processing by the bacterial exotoxin listeriolysin-O (LLO), p. 295–307. *In* R. E. Humphreys and S. K. Pierce (ed.), Antigen processing and presentation. Academic Press, Inc., San Diego, Calif.

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Immunization with plasmid DNA encoding MHC class II binding peptide/CLIP-replaced invariant chain (Ii) induces specific helper T cells in vivo: the assessment of Ii p31 and p41 isoforms as vehicles for immunization

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Abstract

A single helper T cell (Th) epitope-specific T cell subset was successfully induced in vivo by immunization with plasmid DNA encoding MHC class II binding peptide/class II-associated invariant chain peptide (CLIP)-replaced murine Ii molecules. Spleen cells from mice immunized by gene gun bombardment with plasmid DNA for Ii p31 and p41 molecules, whose CLIP regions were replaced with an I-A^d-restricted Th epitope, ovalbumin (OVA) 323–336, showed the specific proliferation and interferon-γ (IFN-γ) production. A20-2J B cell lines having these plasmids were capable of stimulating spleen cells from the immunized mice and naïve DO10-transgenic mice bearing the epitope-specific T cell receptor (TCR) transgenes by examining the specific proliferative response and IFN-γ production. Some mice immunized with the Ii p41-OVA323, but not with the Ii p31-OVA323 plasmid, produced the peptide-specific antibodies, suggesting the functional difference between Ii isoforms. © 2001 Elsevier Science Ltd. All rights reserved.

Keywords: DNA immunization; Invariant chain; Th epitope

1. Introduction

Naked plasmid DNA immunization has been considered to be a promising new vaccine strategy which offers new approaches for prevention and therapy for a variety of diseases, including infectious diseases, cancers, allergic and autoimmune diseases (reviewed in [1,2]). Theoretically, this procedure has potential advantages over other types of vaccines in that the procedure can induce cell-mediated immunity, especially, cytotoxic T lymphocytes (CTL). In DNA immunization, especially by gene gun bombardment, plasmid DNA injected is considered to be transfected into antigen-presenting cells (APC) directly in vivo and elicits de novo protein synthesis in the cytoplasm [3]. Many papers described the satisfactory CTL induction by using DNA immunization (reviewed in [1,2]). In our previous works, we also have demonstrated that DNA immunizations with the minigene plasmid encoding a single dominant CTL epitope against listeriolysin O, a major virulent and also protective antigen (Ag) of *Listeria monocytogenes*, do induce strong CTL activity in vivo [4,5].

Helper T cells (Th) play important roles in many aspects of immunity, especially for modulating immune responses by producing special sets of cytokines. For protection against intracellular microorganisms, activation of macrophages is indispensable and Ag-specific type 1-helper T cells (Th1) have been reported to play important roles for the activation (reviewed in [6]). Similarly, allergen-specific Th1 induction may alleviate allergic reactions caused by enhanced type 2-helper T cell (Th2) response to allergens [7,8]. On the contrary, specific Th2 have been speculated to be useful for the control of organ-specific autoimmune diseases including insulin-dependent diabetes mellitus and some inflammatory bowel diseases (reviewed in [9]). In general, the induction of specific Th requires Ag processing in the context with MHC class II pathway as exogenously acquired Ags. In the past few years, we have learned a considerable amounts about the cell biology of MHC class II pathway and the understanding has enabled even endogenously synthesized Ags to be localized in MHC class II compartments (reviewed in [10]). DNA immunization method which induces only particular

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Th population without antibody (Ab) production may be advantageous as Ab evoked against them could, in some cases, give undesirable consequences. For example, such Abs could potentially cross-react with self proteins. Therefore, it may be useful to develop the immunization method, with which Ag presentation process takes place in the APC without egressing the gene product out of transgene-transfected cells in vivo. Several studies have been reported focusing on efficient specific Th induction by using the cultured cells as mentioned before (reviewed in [10]). Several groups have reported that MHC class II-positive cultured cells transfected with invariant chain (Ii) cDNA, in which class II-associated Ii-derived peptide (CLIP) region was replaced with a Th epitope of interest, efficiently stimulate specific T cell lines [11–13]. The Ii molecules play a pivotal role in MHC class II-mediated Ag presentation. They associate with MHC class II molecules in the endoplasmic reticulum so as to block premature loading of peptides on MHC class II molecules there. The Ii molecules work as a molecular chaperone for MHC class II transport to the endosomal compartment, where Ii molecules are proteolytically degraded and antigenic peptides are replaced with the CLIP region of the molecules (reviewed in [14,15]). Therefore, the antigenic peptide/CLIP-replaced Ii gene immunization should be a very efficient method for presenting antigenic peptides of interest to Th in vivo. Especially, it may be the case for cutaneous gene bombardment, in which, Ag presentation by directly-transfected dendritic cells play a predominant role for immune induction [16,17]. In this study, we report that immunization with expression plasmids for murine Ii p31 and p41 isoforms, whose CLIP regions were replaced by an ovalbumin (OVA) Th epitope, efficiently induces specific Th in vivo by gene gun bombardment. Furthermore, we evaluated the difference between effects of immunization by CLIP-replaced Ii p31 and p41 isoform expression plasmids and the functional difference was discussed.

2. Materials and methods

2.1. Mice

BALB/c mice (Japan SLC; Hamamatsu, Japan) and DO10 T cell receptor (TCR) transgenic mice (BALB/c genetic background) expressing the TCR in T-cell hybridoma DO11.10, reactive to chicken OVA ([18]; kindly provided by Dr. Kenneth M Murphy (Washington University, St. Louis, MO) via Dr. Masato Kubo (Science University of Tokyo, Japan) were maintained in the animal facility of Hamamatsu University School of Medicine. DO10 TCR-transgenic mice were selected by staining peripheral blood leukocytes with the anti-clonotype monoclonal Ab (mAb), KJ1-26 [19]. All transgenic mice used were heterozygous for the TCR transgene. Mice between 6 and 18 weeks of age were used for immunization and kept in filter-cap cages during experiments.

2.2. Plasmid construction

The cDNAs encoding for murine Ii p31 and p41 molecules (pcEXV-mIi p31 and pcEXV-mIi p41) were kindly donated by Dr. Ronald N. Germain (National Institutes of Health, Bethesda, MD). The *Eco*RI fragment containing Ii p31 or p41 cDNA was inserted into *Eco*RI site in pCI eukaryotic expression plasmid (Promega, Madison, WI), resulting in pCI-mIi p31 or pCI-mIi p41, respectively. The unique *Hin*dIII and *Nsp*I sites in pCI were removed by restriction enzyme digestion and blunted with T4 DNA polymerase before starting the plasmid construction. After insertion of Ii cDNAs into pCI, the part of 3′-untranslated region of Ii cDNAs, which contains an additional *Hin*dIII site, was removed by *Avr* II and *Xba* I digestion and the digested plasmids were self-ligated subsequently. The regions covering CLIP-encoding sequence (*Hin*dIII–*Nsp*I DNA fragment encoding LPKSAKPVSQMRMATPLLMRPMSMDNM) were replaced with a double-stranded oligonucleotide encoding for OVA 323–336 peptide, resulting in pCI-mIi p31-OVA323 and pCI-mIi p41-OVA323. Nucleotide sequences of oligonucleotides used in this study were as follows: the coding strand oligonuleotide; 5′-AGCTTATCAGCCAGGCCGTGCACGCCGCCCACGCCGAGATCAACGAGATGGATAACATG-3′ and the non-coding strand oligonuleotide; 5′-TTATCCATCTCGTTGATCTCGGCGTGGGCGGCGTGCACGGCCTGGCTGATA-3′, encoding OVA 323–336 peptide, ISQAVHAAHAEINE, flanked with a leucine at the N-terminus and MDNM at the C-terminus.

The full-length chicken OVA cDNA expression plasmid, pCI-OVA was described previously [20]. For OVA 323–336 expression plasmid, pCI-OVA323, the oligonucleotides encoding OVA 323–336 peptide (ISQAVHAAHAEINE) flanked with a starting codon, ATG at 5′-end and a stop codon, TAA at 3′-end, i.e. 5′-GGGATGATATCTCAAGCTGTCCATGCAGCACATGCAGAAATCAATGAATAACCC-3′ and 5′-GGGTTATTCATTGATTTCTGCATGTGCTGCATGGACAGCTTGAGATATCATCCC-3′, were annealed and inserted into *Sma* I site in pCI. The nucleotide sequences of the resultant plasmids were confirmed by dideoxy sequencing, using ABI PRISM 310 Genetic Analyzer (Applied Biosystems; Foster City, CA). Large-scale purification of plasmids was conducted with Qiagen plasmid mega kit system (Qiagen, Valencia, CA) and endotoxin was removed by Triton X-114 phase separation.

2.3. Mice immunization

For plasmid DNA immunization with Helios gene gun system (Bio-Rad Laboratories, Hercules, CA), preparation of the cartridge of DNA-coated gold particles was followed to the manufacturer's instruction manual. Finally, 0.5 mg of gold particles was coated with 1 μg plasmid DNA and the injection was carried out with 0.5 mg gold/shot. To immunize mice, the shaved abdominal skin was wiped with 70% ethanol. The spacer of the gene gun was held directly against

the abdominal skin. Then, the device was discharged at a helium discharge pressure of 400 psi. Mice were injected with 1 μg of plasmids four times at a week interval in the abdomen. Control animals were received 100 μg of OVA protein emulsified in complete Freund's adjuvant (CFA) or in alum, intradermally two times at 2-week interval.

2.4. Cells and culture condition

Spleen cells from plasmid-immunized and DO10-transgenic mice and A20-2J cells (BALB/c-derived B lymphoblastoid cell line; [21]) were maintained with RPMI-1640 medium supplemented with 10% fetal calf serum (FCS) in a humidified atmosphere of 5% CO_2 incubator.

2.5. Transfection of A20-2J cells

A20-2J cells were transfected with 20 μg of pCI-mIi p31-OVA323 or pCI-mIi p41-OVA323 with 2 μg of pPGK-Neo, to allow drug selection, by electroporation at 250 V and 960 μF in K-PBS buffer (30.8 mM NaCl, 120.7 mM KCl, 8.1 mM Na_2HPO_4, 1.46 mM KH_2PO_4, and 10 mM $MgCl_2$) by a Gene Pulser electroporation apparatus (Bio-Rad Laboratories). Transfectants were selected and maintained in media containing 0.5 mg/ml G418 (Gibco/BRL, Rockville, MD).

2.6. Cell fixation

After washing with PBS, A20-2J cell pools having pCI-mIi p31-OVA323 or pCI-mIi p41-OVA323, were treated with 4% paraformaldehyde (Sigma, St. Louis, MO) freshly prepared in PBS for 15 min at 37°C and subsequently washed with PBS to remove remaining paraformaldehyde.

2.7. Lymphocyte proliferation assay

Whole spleen cell suspensions were treated with Tris-buffered 0.83% ammonium chloride to lyse erythrocytes. Then, the spleen cells (5×10^5 per well) from the immunized and DO10-transgenic mice were incubated for 48 h at 37°C in 96-well round-bottom tissue culture plates in the presence or absence of 100 μg/ml of OVA (Sigma), or in some cases, with 2.5×10^4 fixed A20-2J cell pools having pCI-mIi p41, pCI-mIi p31-OVA323, or pCI-mIi p41-OVA323. Then, DNA synthesis was assessed by adding 0.5 μCi per well [methyl-^{3}H] thymidine (6.7 Ci/mmol; ICN Biochemicals, Irvine, CA) for the last 12 h of culture. Cultures were harvested onto glass fiber filters, and the radioactivity was counted by liquid scintillation.

2.8. Inhibition of Ag-specific T cell proliferation with depletion of CD4 T cell subset

CD4/CD8 specificity of proliferative T cells was tested by depletion studies with anti-murine CD4 mAb, GK1.5, anti-murine CD8a mAb, 53-6.7, and also anti-murine CD45R/B220 mAb, RA3-6B2 as a control. The mAbs used were purchased from PharMingen (San Diego, CA). The immune spleen cells were added with the mAbs at 1 μg/ml and incubated for 1 h at 4°C. Then, they were centrifuged and the supernatant was discarded. The cells were resuspended in cytotoxicity medium (RPMI-1640 medium with 25 mM Hepes buffer and 0.3% FCS) containing rabbit complement (Cedarlane; Hornby, Ont., Canada) and incubated for 1 h at 37°C. Then, the cells were washed with RPMI-1640 medium and used for the lymphocyte proliferation assay described in 2.7.

2.9. Inhibition of Ag-specific T cell proliferation with MHC class II mAb treatment

The inhibitory activity of anti-I-A^d/E^d mAb, 2G9 (PharMingen) for Ag-specific T cell proliferation was tested by addition of the mAb at 1 μg/ml to the immune spleen cell culture at the initiation of cultivation as described by Tsuji et al. [22]. Data were expressed as net cpm (Δcpm: cpm after in vitro stimulation in the presence of OVA protein minus cpm in the absence of OVA protein). As a control, the isotype-matched anti-murine CD45R/B220 mAb, RA3-6B2 (PharMingen) was also used.

2.10. Cytokine enzyme-linked immunosorbent assay (ELISA)

Spleen cells were harvested from the immunized mice or DO10-transgenic mice. Recovered cells were incubated for 5 days in 24-well plates at 2×10^6 cells per well in RPMI-1640 medium supplemented with 10% FCS in the presence or absence of 100 μg/ml of OVA (Sigma), or in some cases, with 1×10^5 fixed A20-2J cell pools having pCI-mIi p41, pCI-mIi p31-OVA323, or pCI-mIi p41-OVA323. Concentration of interferon-γ (IFN-γ), interleukin (IL)-2, IL-4, and IL-5 in the culture supernatant was determined by sandwich ELISA as described elsewhere [23]. For the sandwich ELISA, following combinations of coating and biotinylated mAbs were used; R4-6A2 and XMG1.2 for IFN-γ, JES6-1A12 and JES6-5H4 for IL-2, 11B11 and BVD6-24G2 for IL-4, TRFK5 and TRFK4 for IL-5. All mAbs were purchased from PharMingen. The amount of cytokines was calculated by using standard murine recombinant cytokine curves run on the same immunoplate. The background level measured with the medium only has been subtracted.

2.11. Anti-OVA ELISA

Animals were bled from the retro-orbital plexus 1 month or 2 weeks after the last immunization and sera were prepared. Microtiter plates (Corning Glass Works, Corning, NY) were coated with OVA (Sigma; 20 μg/ml) in phosphate

buffer (pH 9) by overnight incubation at 4°C and washed three times with PBS containing 0.05% Tween-20 (PBS/Tween) to remove the unbound Ag. Plates were incubated with diluted sera in blocking buffer (30% Block Ace (Dainippon seiyaku, Osaka, Japan) in PBS/Tween) at 4°C overnight. After washing five times with PBS/Tween, plates were incubated with alkaline phosphatase-conjugated anti-mouse IgG Ab (Cosmo-Bio, Tokyo, Japan) 1000X diluted in Ab dilution solution (10% Block Ace in PBS/Tween). After washing five times with PBS/Tween, the amount of the bound Ab was determined by addition of *p*-nitrophenyl phosphatase substrate solution (Sigma). After 1 h incubation at room temperature, the OD at 405 nm was measured by EL340 I automatic plate reader (Bio-tek instruments, Winooski, VT). The background level measured with the medium only has been subtracted. For the blocking experiment, sera 100× diluted by Ab dilution solution were incubated with 50 μM of OVA 323–339 peptide (ISQAVHAAHAEINEAGR), or LLO 319–327 peptide (IKNSSFKAV) as a negative control, at room temperature for 1 h before ELISA.

2.12. Statistics

Data from multiple experiments were expressed as mean± S.D. Statistical analyses were performed by using the Stat View-J 4.02 statistics program (Abacus Concepts, Berkeley, CA). Data were analyzed by Student's two-tailed *t*-test for two groups (Fig. 3B), or by one-factor ANOVA followed by the Fisher's protected least significant difference (PLSD) test for three or more groups (Figs. 2, 3A and 4).

3. Results

3.1. Construction of CLIP-replaced Ii expression plasmids

Two types of murine Ii molecules, Ii p31 and p41, which are generated by alternative splicing from a single gene, have been reported [24]. Based on these Ii isoform cDNAs, we constructed two CLIP-replaced murine Ii cDNA expression plasmids whose CLIP regions were substituted to an OVA Th epitope (Fig. 1). The nucleotides covering CLIP region in the Ii cDNAs were removed and replaced by a synthetic double-stranded oligonucleotide encoding OVA 323–336 peptide (ISQAVHAAHAEINE), an I-A^d-restricted Th epitope [25]. We used *Hind*III and *Nsp*I sites located close to CLIP regions of Ii cDNAs to replace the synthetic oligonucleotide. The *Hind*III–*Nsp*I DNA fragment covering CLIP region contains the nucleotides encoding LPKSAKPVSQMRMATPLLMRPMSMDNM, where CLIP core sequences are the peptide corresponding to murine Ii 85–101 (KPVSQMRMATPLLMRPM) [26] and nucleotides coding for a leucine at the N-terminus and MDNM at the C-terminus were added because *Hind* III and *Nsp*I digestion of the Ii cDNAs removes the portion. The resultant plasmids are pCI-mIi p31-OVA323 and pCI-mIi p41-OVA323.

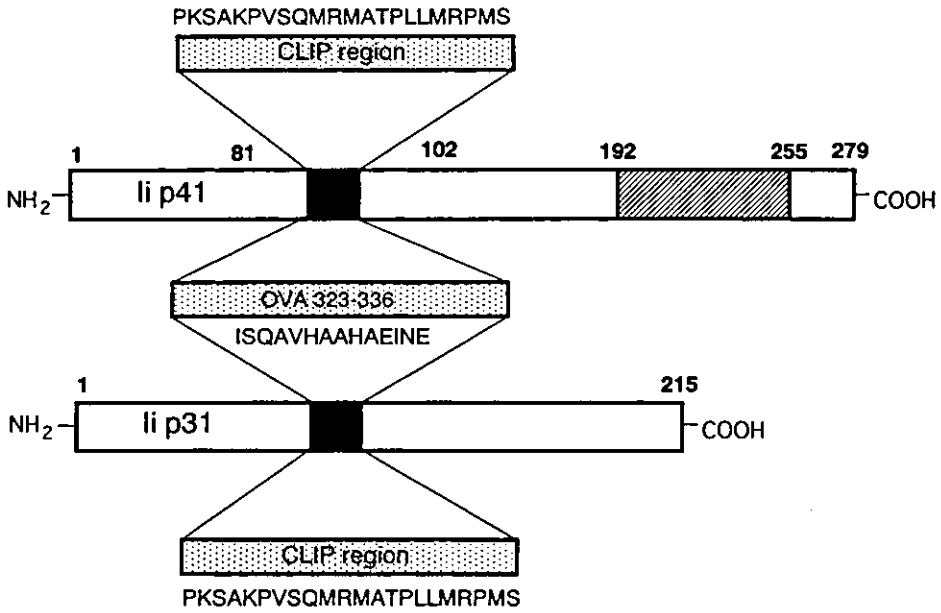

Fig. 1. The schema of CLIP-replaced murine Ii molecules deduced from the cDNA constructs prepared in this study. The double-stranded oligonucleotide coding for OVA 323–336 I-A^d binding peptide was replaced with CLIP regions of murine Ii isoform cDNAs, mIi p41 and p31. The amino acid sequences of replaced CLIP region and OVA 323–336 peptide are shown. The numbers indicate the amino acid numbers of Ii molecules. The region shown by hashed line in Ii p41 is an additional segment derived from alternative splicing, unique to Ii p41.