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Figure legends

Fig. 1. A. The schema of the gene products deduced from the expression vector plasmids prepared in this study. Mouse MIP-1α gene was cloned by RT-PCR from total RNA of dendritic cells. MIP-1α gene was fused with MPT-51 gene via 14-amino acids spacer sequence and cloned into pCI vector.

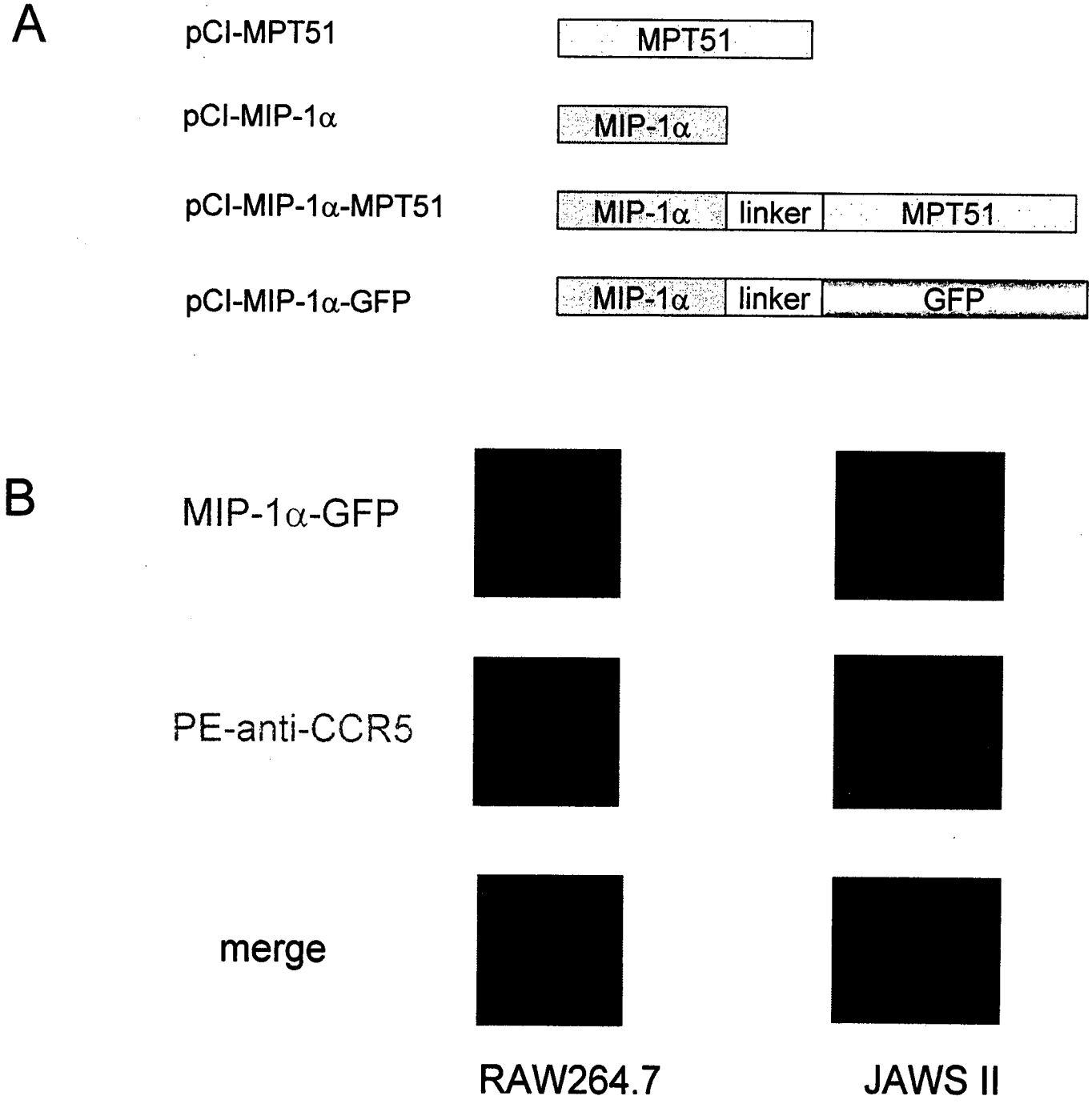
B. Chemokine receptor binding assay. MIP-1α-fused GFP proteins was prepared from the pCI-MIP-1α-GFP transfected HEK293 cells. RAW264.7 cells or JAWS II cells were incubated with the GFP fusion protein and PE-labeled anti-CCR5 antibody for 30 min on ice and 15 min at room temperature. Binding analysis was performed by using laser confocal microscopy.

Fig. 2. Detection of MPT-51-specific $CD8^+$ T cells with MPT51 24-32/H2-D^d tetramer. Naïve and immune splenocytes were stained with PE-conjugated H2-D^d-MPT51 peptide tetramer complexes and FITC-conjugated anti-CD8 antibodies for 30 min at 4℃. Stained cells were analyzed by a digital flow cytometer.

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

obtained in three independent experiments.

Fig. 4 IFN-γ production from immune splenocytes in response to MPT51 peptide stimulation. Immune spleen cells were cultured for 3 days at 1 x 10^7/ml in the presence of MPT51 peptide. Concentration of IFN-γ in the culture supernatants was determined by ELISA.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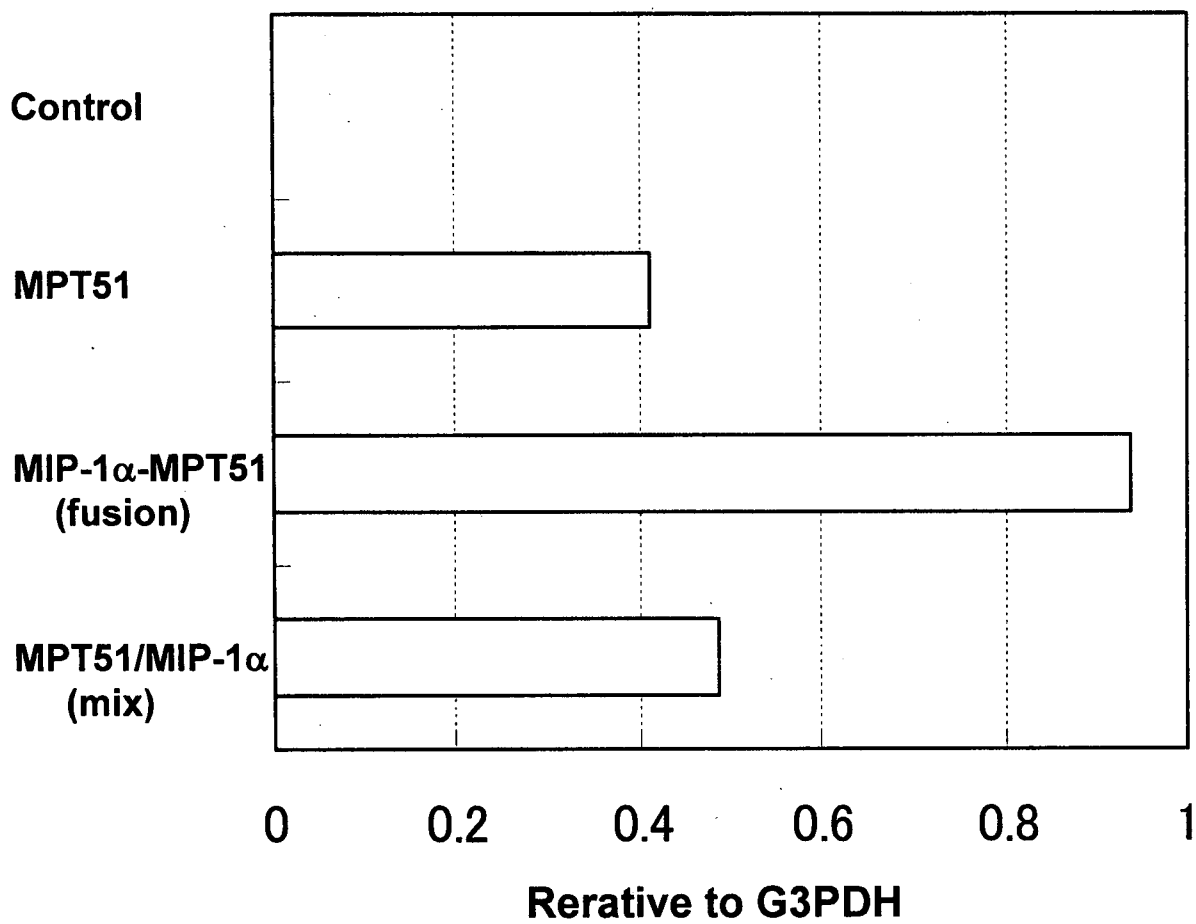
MPT51
pCI-MIP-1α
MIP-1α
pCI-MIP-1α-MPT51
MIP-1α
linker
MPT51
pCI-MIP-1α-GFP
MIP-1α
linker
GFP
B
MIP-1α-GFP
PE-anti-CCR5
merge
RAW264.7
JAWS II


Control
MPT51
MIP-1α-MPT51
(fusion)
MPT51/MIP-1α
(mix)
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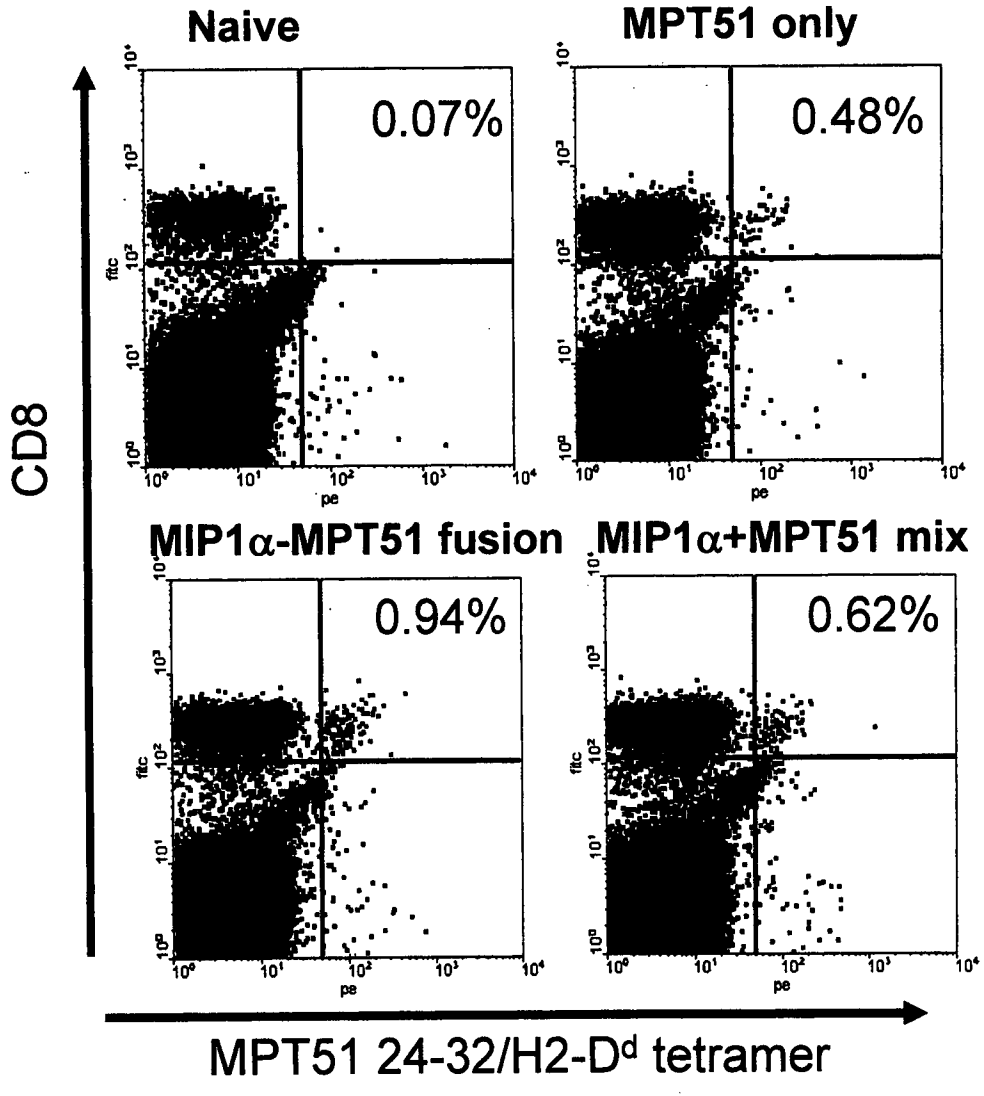
0.4
0.6
0.8
1
Rerative to G3PDH


Naive
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MIP1α-MPT51 fusion
MIP1α+MPT51 mix
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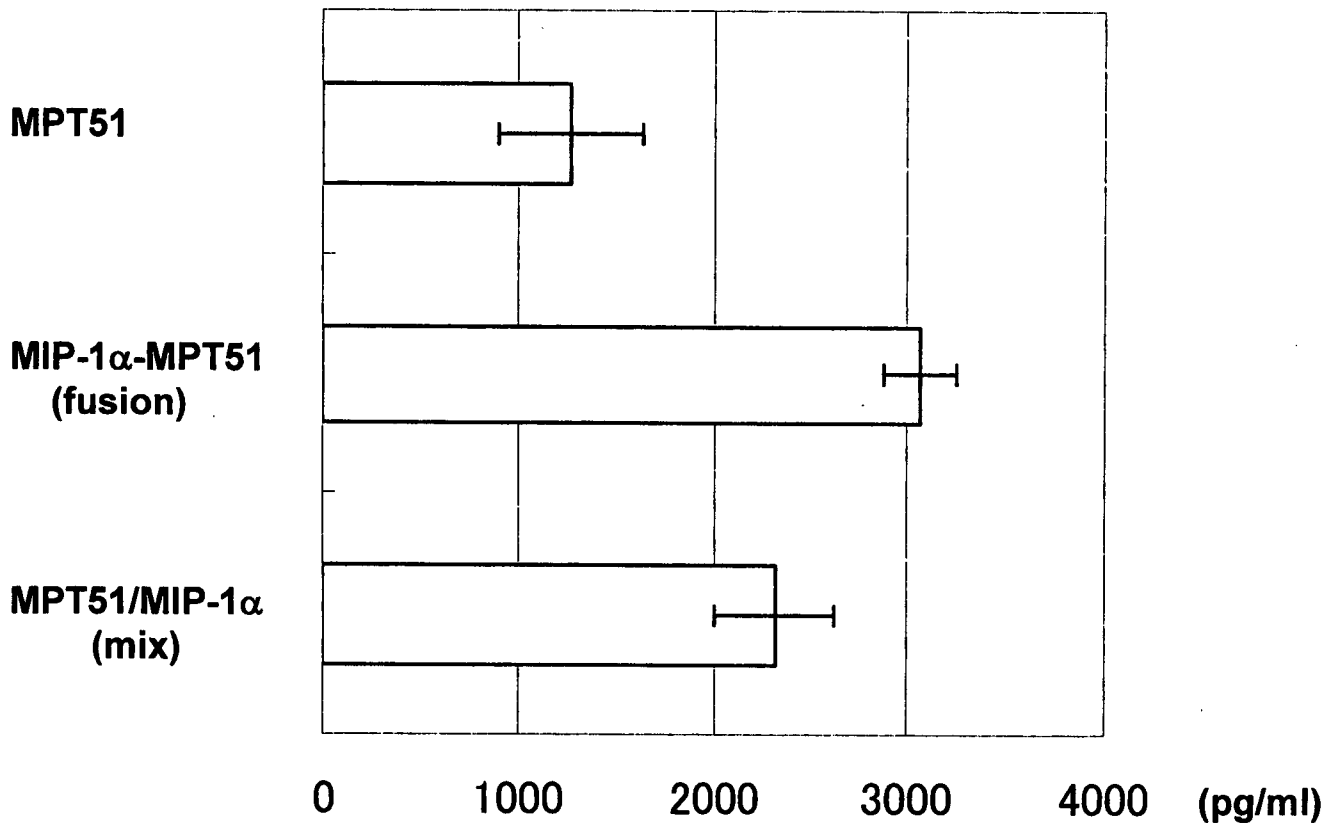
0.48%
0.94%
0.62%
CD8
MPT51 24-32/H2-Dd tetramer
fitc
pe


MPT51
MIP-1α-MPT51
(fusion)
MPT51/MIP-1α
(mix)
0
1000
2000
3000
4000
(pg/ml)

RESEARCH ARTICLE

Immunization with dendritic cells loaded with α-galactosylceramide at priming phase, but not at boosting phase, enhances cytotoxic T lymphocyte activity against infection by intracellular bacteria

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Keywords
α-galactosylceramide; dendritic cell; cytotoxic T lymphocyte; memory cell; *Listeria monocytogenes*.

Abstract

We evaluated the effect of immunization with dendritic cells (DCs) pulsed with α-galactosylceramide (αGalCer) and listeriolysin O (LLO) 91-99 peptide, a dominant cytotoxic T lymphocyte (CTL) epitope of *Listeria monocytogenes* by observing the responses of specific $CD8^+$ T cells and *in vivo* CTL activity. DCs were pulsed with various combinations of αGalCer and LLO91-99 peptide and administered to BALB/c mice. Immunization with DCs pulsed with αGalCer and LLO91-99 at priming phase and with DCs pulsed with LLO91-99 alone at boosting phase induced stronger *in vivo* CTL activity, reduced the bacterial load in spleens of *Listeria*-challenged mice and augmented $CD62L^+$ $CD8^+$ central memory T cells compared with other immunization protocols. The blockade of interferon-γ (IFN-γ) at boosting phase reversed the induction of $CD8^+$ central memory T cells and reduced the bacterial load in spleens of *Listeria*-challenged mice immunized with DCs pulsed with αGalCer and LLO91-99 at both phases, suggesting that αGalCer at boosting phase has deleterious effects through IFN-γ production. These results indicate that immunization with DCs pulsed with CTL epitope peptide together with αGalCer at priming phase, but not at boosting phase, is feasible for eliciting a specific CTL activity and protective immunity against infection of intracellular bacteria.

Introduction

Dendritic cells (DCs) are the most potent antigen-presenting cells (APCs) that patrol all tissues of the body, with the possible exceptions of the brain and testis (Banchereau & Steinman, 1998). DCs possess the distinct ability to prime naïve helper T lymphocytes (Th) and cytotoxic T lymphocytes (CTL). Thus, there has been much interest on their use for the immune modulation of diseases. A number of groups have demonstrated that DC-based vaccines, such as those pulsed with tumor-associated antigens (Ags), can generate specific antitumor immunity *in vivo* in murine tumor models (Mayordomo *et al.*, 1995; Celluzzi *et al.*, 1996). In the field of infectious diseases, several studies exploring the efficacy of DC-based vaccines have been reported (Manickan *et al.*, 1997; Ahuja *et al.*, 1999; Ranieri *et al.*, 1999; Kikuchi *et al.*, 2000; Shaw *et al.*, 2001).

Infection with intracellular pathogens, such as *Mycobacterium tuberculosis*, poses serious health problems worldwide. Efficient protection against such intracellular bacteria critically depends on the induction of cellular immune responses. Thus far, only live attenuated vaccines are considered to be effective. However, because of the low safety of live vaccines in immunocompromised individuals and their variable effectiveness, the development of new, improved vaccines against intracellular pathogens has become a current research priority (Seder & Hill, 2000). DC vaccination would be one of the potent vaccine strategies against infection by intracellular pathogens.

Listeria monocytogenes is a Gram-positive facultative intracellular bacterium that causes life-threatening infections during pregnancy and in immunocompromised individuals (Gelin & Broome, 1989). A well-characterized *in vivo* mouse model of *L. monocytogenes* infection has yielded significant

insight into the nature of innate and adaptive cell-mediated immunity, the latter of which primarily associated with specific $CD8^+$ CTL (Pamer, 2004). *Listeria monocytogenes* enters eukaryotic cells in membrane-bound vesicles, then escapes from the vesicles by virtue of the function of listeriolysin O (LLO), multiplies within the cell cytoplasm and spreads directly to adjacent cells. Previously, Harty & Bevan (1992) showed that the adoptive transfer of $CD8^+$ CTL specific for LLO91-99 confers protection against *L. monocytogenes* infection. Consistent with this observation, our previous studies demonstrated that immunization with a minigene plasmid DNA encoding a single dominant CTL epitope, LLO91-99, or with DCs retrovirally transduced with LLO91-99, induced strong CTL activity and conferred partial protection against murine *L. monocytogenes* infection (Uchijima *et al.*, 1998; Nakamura *et al.*, 2003).

Natural killer T (NKT) cells represent a subset of T lymphocytes expressing both T-cell receptor and NK-cell receptor, and play a role in bridging innate immunity to adaptive immunity (Kronenberg & Gapin, 2002; Kronenberg, 2005), and have been reported to be involved in early immune responses against various pathogens (Gumperz & Brenner, 2001), including *L. monocytogenes*, *Mycobacterium bovis* bacillus Calmette-Guérin and *Leishmania major* (Emoto *et al.*, 1999; Ishikawa *et al.*, 2000; Ranson *et al.*, 2005). Among several NKT cell subsets, NKT cells that have T-cell receptors with invariant Vα14-Jα18 rearrangements (murine *i*NKT cells) possess reactivity to a glycosphingolipid, α-galactosylceramide (αGalCer), when presented by the class Ib molecule, CD1d (Kronenberg & Gapin, 2002; Kronenberg, 2005). αGalCer administration has been extensively examined for the enhancement of tumor immunotherapy (Fujii *et al.*, 2002; Silk *et al.*, 2004; Ishikawa *et al.*, 2005). There have been several reports on αGalCer employment with vaccination against pathogens (Gonzalez-Aseguinolaza *et al.*, 2000; Kakimi *et al.*, 2000). These investigators administered free αGalCer itself in their experiments. In the present study, we examined the effect of immunization with DCs pulsed with αGalCer together with LLO91-99 immunodominant CTL epitope peptide on the induction of $CD8^+$ T cells against *L. monocytogenes*.

Materials and methods

Mice

BALB/c mice were purchased from SLC Japan (Hamamatsu, Japan). These mice were maintained in specific pathogen-free conditions at the Laboratory Animal Center, Hamamatsu University School of Medicine. All mice used in this study were between 6 and 14 weeks of age. All animal experiments were performed according to the animal care guidelines of Hamamatsu University School of Medicine.

Culture of bone marrow-derived DCs

Bone marrow-derived DCs (BM-DCs) were cultured in RPMI-1640 supplemented with 10% fetal calf serum (FCS), 50 μM 2-mercaptoethanol, 1000 U mL^{-1} mouse rGM-CSF (kindly provided by Kirin Brewery, Gumma, Japan), and 1000 U mL^{-1} mouse rIL-4 (R&D Systems, Minneapolis) (complete RPMI medium) using methods described by Inaba *et al.* (1992) with some modifications, as in our previous study (Nakamura *et al.*, 2003). Approximately 75% of the resultant cells were CD11c-positive DCs (data not shown).

Preparation of αGalCer and/or LLO91-99 peptide-pulsed DCs

αGalCer was kindly provided by Kirin Brewery. αGalCer was suspended in phosphate-buffered saline (PBS) supplemented with 0.5% polysorbate-20 (w/v). The LLO91-99 peptide, GYKDGNEYI, representing an H2-K^d-restricted immunodominant CTL epitope spanning amino acid residues 91–99 of LLO, was synthesized by BEX (Tokyo, Japan). BM-DCs from BALB/c mice after 6 days of culture were resuspended in RPMI 1640 medium supplemented with 10% FCS and 50 μM 2-mercaptoethanol (RPMI/10FCS) at a concentration of 1×10^7 cells mL^{-1} and pulsed with 100 ng mL^{-1} αGalCer or the control vehicle for 1 h at 37 °C in 5% CO_2 atmosphere. After washing twice in PBS, BM-DCs were resuspended in RPMI/10FCS at a concentration of 1×10^7 cells mL^{-1}, and pulsed with or without 5 μM LLO91-99 peptide in the presence of human β2-microglobulin (Sigma Chemical, St Louis, MO) for 1 h at 37 °C in 5% CO_2 atmosphere.

Immunization with DCs

After washing twice in PBS, 5×10^5 αGalCer and/or LLO91-99 peptide-pulsed DCs in 0.2 mL PBS were injected intravenously into BALB/c mice twice, at 2 week intervals (Fig. 1a). As controls, naïve mice were immunized with 1×10^3 CFU (*c.* 0.1 LD_{50}) of *L. monocytogenes* i.v. once or with 2 mg of LLO91-99 expression plasmid (p91mam) three times at 1 week intervals using Helios gene gun system (Bio-Rad Laboratories, Hercules, CA) as described by Uchijima *et al.* (1998).

Monitoring CTL responses with MHC tetramer

Phycoerythrin-conjugated tetrameric H2-K^d/LLO91-99 peptide complex was synthesized by Proimmune Ltd (Oxford, UK). Three days after the boost immunization, splenocytes from immune mice were stained directly with the tetrameric complex and fluorescein isothiocyanate (FITC)-conjugated antimouse CD8α mAb (BD Biosciences PharMingen, San Diego, CA, USA). Subsequently, the cells were analyzed with an EPICS XL digital flow cytometer (Beckman Coulter, Fullerton, CA).

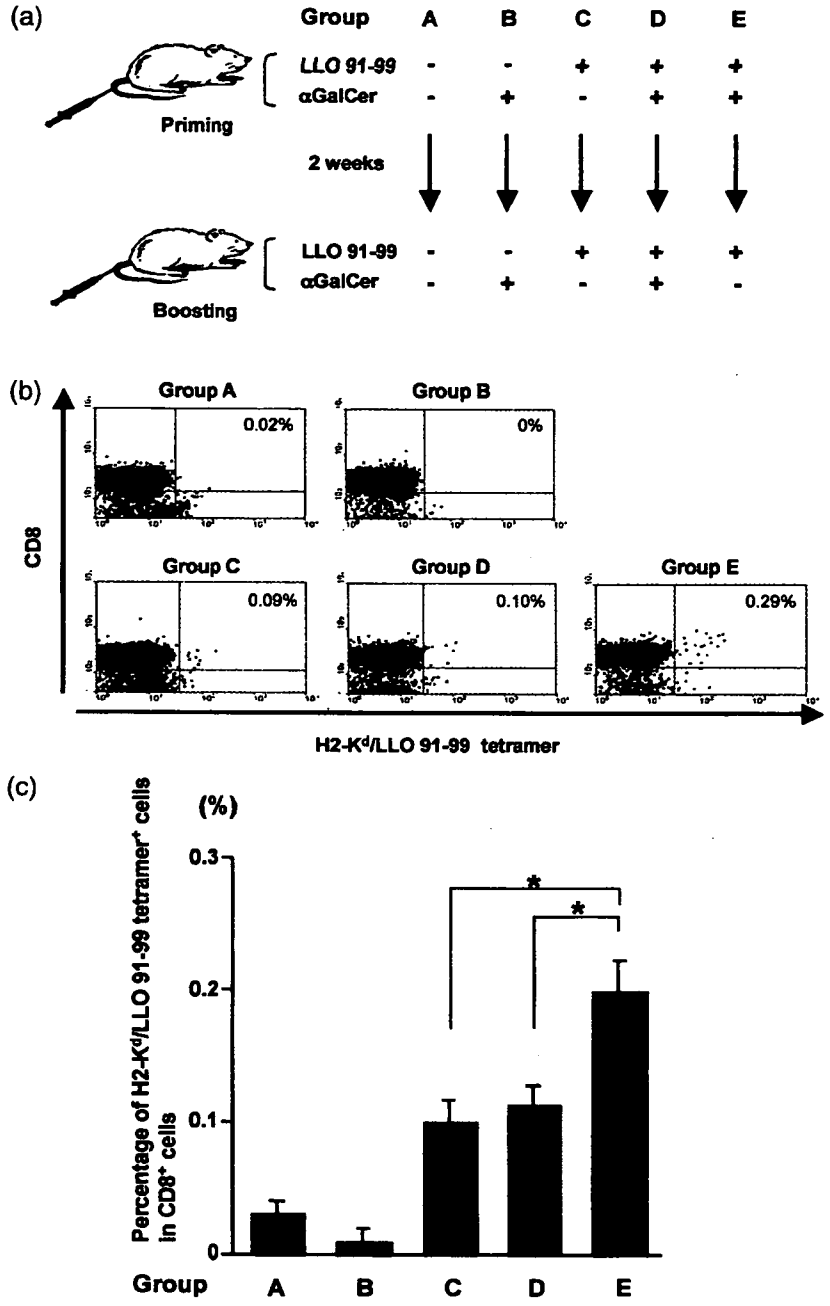

Fig. 1. Induction of LLO91-99-specific $CD8^+$ T cells in the spleens of LLO91-99 peptide and/or αGalCer-pulsed DC-immune mice. (a) Immunization schedule of mice with DCs pulsed with LLO91-99 peptide and/or αGalCer. Mice were immunized with DCs pulsed with LLO91-99 peptide and/or αGalCer twice with a 2 week interval. Group A, mice given untreated DCs twice; group B, mice given DCs pulsed with αGalCer twice; group C, mice given DCs pulsed with LLO91-99 peptide alone twice; group D, mice given DCs pulsed with both LLO91-99 peptide and αGalCer twice; group E, mice given DCs pulsed with both LLO91-99 peptide and αGalCer at priming phase and DCs pulsed with LLO91-99 peptide alone at boosting phase. (b) Flow cytometric analysis for LLO91-99-specific $CD8^+$ T cells after boost DC immunization. H2-K^d/LLO91-99 tetramer staining was performed 3 days after boost immunization with LLO91-99 peptide and/or αGalCer-pulsed DCs. The percentages of the $tetramer^+$ cells in $CD8^+$ T cells of each experimental group are shown. (c) Summary of the percentages of H2-K^d/LLO91-99 $tetramer^+$ cells in $CD8^+$ T cells of each experimental group. The means ± SE of five mice per group are shown. Asterisks indicate significant difference ($P < 0.01$).

Quantitation of IFN-γ in splenocyte culture supernatants by sandwich ELISA

Pools of spleen cell suspensions (2×10^6 cells mL^{-1}) from groups of mice immunized with DCs were cultured in RPMI/10FCS in 24-well plates in the presence or absence of 1 μM LLO91-99 peptide at 37 °C in 5% CO_2 atmosphere. Supernatants were harvested after 3 days and stored at − 20 °C until assayed. The concentration of interferon-γ (IFN-γ) in the culture supernatants was determined using sandwich enzyme-linked immunosorbent assay (ELISA) (Quantikine®; R&D Systems).

Quantitation of serum cytokines using a cytometric beads array

Blood was obtained from the lateral tail veins of mice, before and 6, 12, 24 and 36 h after the prime and boost immunizations. The concentrations of cytokines [IFN-γ, interleukin (IL)-12p70, IL-4, IL-10, tumor necrosis factor-α (TNF-α) and IL-6] in each serum were evaluated with a BD mouse inflammation cytometric bead array kit and a BD Th1/Th2 cytokine cytometric bead array kit (BD Biosciences PharMingen) according to the manufacturer's instructions.

Monitoring CTL memory cells

Four weeks after the boost immunization, splenocytes from immunized mice were stained with the phycoerythrin-conjugated tetrameric H2-K^d/LLO91-99 peptide complex and phycoerythrin-Cy5-conjugated antimouse CD8α monoclonal antibody (mAb) (BD Biosciences PharMingen). Simultaneously, these cells were stained with FITC-conjugated antimouse CD44 mAb (eBioscience, San Diego, CA) or FITC-conjugated antimouse CD62L mAb (eBioscience), and analyzed using an EPICS XL digital flow cytometer (Beckman Coulter).

In vivo CTL assay

The *in vivo* CTL assay was as originally reported by Aichele *et al.* (1997). Spleens from naïve BALB/c mice were removed aseptically and homogenized. Erythrocytes were depleted from the spleen cell suspension by lysis with 0.83 M NH_4Cl solution, washed in RPMI 1640, resuspended at 2×10^7 cells mL^{-1} in RPMI/10FCS, and incubated for 1 h at 37 °C in 5% CO_2 atmosphere in the presence or absence of 1 μM LLO91-99 peptide. After the incubation, the cells were washed, resuspended in PBS at 2×10^7 cells mL^{-1}, and labeled for 5 min at 37 °C in the dark with 5 (6)-carboxyfluorescein diacetate succinimidyl ester (CFSE; Dojindo, Kumamoto, Japan) either at 0.5 μM (unpulsed cells; CFSE^{lo}) or 5 μM (peptide-pulsed cells; CFSE^{hi}). Cells were washed with PBS supplemented with 5% FCS and resuspended in PBS at 5×10^7 cells mL^{-1}. Ten million cells of a 1 : 1 mixture of CFSE^{lo} : CFSE^{hi} were adoptively transferred into immune BALB/c mice. Eighteen hours later, the adoptively transferred mice were sacrificed, the spleens were removed and homogenized, the erythrocytes were depleted, and the cells were washed and resuspended in PBS. The cells were then analyzed with an EPICS XL digital flow cytometer (Beckman Coulter). To evaluate the percentage of specific lysis, the ratio of CFSE^{lo} : CFSE^{hi} in immune mice was compared with that in the transferred naïve control mice. For each experimental group, at least three animals were tested.

Bacterial infection and evaluation of antilisterial immunity

Four weeks after boosting immunization, the immunized mice were challenged with 5×10^4 CFU (*c.* 5 LD_{50}) of *L. monocytogenes* i.v. Seventy-two hours after the challenge infection, bacterial numbers in the spleens were determined by plating 10-fold dilutions of the tissue homogenates on trypticase soy agar plates.

Blocking boost immunization with IFN-γ neutralizing mAb

Two days before boost immunization, 3 mg per mouse of IFN-γ neutralizing mAb, which was obtained from R4-6A2 cells (ATCC) culture supernatant, were injected i.p. Subsequently, we monitored the CD8$^+$ T-cell proliferation with MHC tetramer 3 days after the boost immunization, and the CD8$^+$ memory T cells and antilisterial immunity 4 weeks after boost immunization.

Statistical analysis

Data from multiple experiments were expressed as the mean ± SE. Data were analyzed by Fisher's protected least significant difference (PLSD). Statistical analysis was performed using STATVIEW-J 4.5 (SAS Institute Inc., Cary, NC). All tests were two-sided and performed at the 0.05 significance level.

Results

Immunization with LLO91-99 peptide-loaded DC pulsed with αGalCer at priming phase, but not at boosting phase, augmented specific CD8$^+$ T-cell responses

In order to see the effects of αGalCer-pulsed DCs on specific CD8$^+$ T-cell induction *in vivo*, we first evaluated LLO91-99 peptide-specific CD8$^+$ T-cell induction in the spleen cells of mice immunized with DCs pulsed with LLO91-99 peptide alone, or with LLO91-99 peptide and αGalCer by H2-K^d/LLO91-99 tetramer analysis. We immunized BALB/c mice twice (at priming and boosting phases) with a 2 week

interval with syngeneic BM-DCs pulsed with LLO91-99 peptide and/or αGalCer, as shown in Fig. 1(a).

First, we examined the specific $CD8^+$ T-cell induction 3 days after boosting immunization (Fig. 1b and c). As expected, LLO91-99 peptide-pulsed (groups C to E), but not unpulsed (groups A and B), DC immunization induced H2-K^d/LLO91-99 $tetramer^+$ $CD8^+$ T cells. The spleen cells of group E mice showed a significantly higher percentage of $tetramer^+$ cells in the whole $CD8^+$ T-cell population when compared with those of group C mice ($P = 0.0019$) or group D mice ($P = 0.0042$). These percentages of $tetramer^+$ cells in the $CD8^+$ T-cell population correlated well with the absolute numbers of $tetramer^+$ cells in the $CD8^+$ T-cell population (data not shown).

Immunization with DCs pulsed with αGalCer induced large amounts of IFN-γ production from immune spleen cells

Free αGalCer administration has been reported to induce large amounts of IFN-γ *in vivo* (Fujii *et al.*, 2002). In order to see the effects of immunization of DCs pulsed with αGalCer on IFN-γ production, we examined IFN-γ production by immune spleen cells after the immunization. Three days after the boost injection of DCs, spleen cells from the immunized mice were obtained and cultured with or without LLO91-99 peptide for 3 days. Supernatant of each sample was harvested after 3 days of culture, and the amounts of IFN-γ in it were determined by sandwich ELISA. Immunization with unpulsed DCs alone induced high amounts of IFN-γ from the immune spleen cells (Fig. 2).

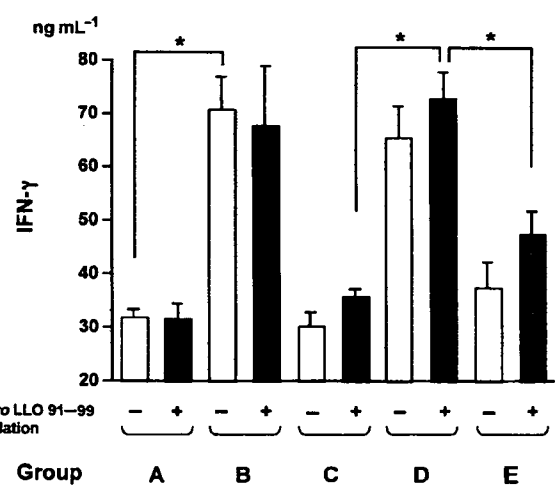

Fig. 2. Immunization with αGalCer-pulsed DCs induced large amounts of IFN-γ production by the immune spleen cells. The immune spleen cells were removed 3 days after boost immunization and the cells were cultured with or without LLO91-99 peptide. The supernatants were harvested after 3 days of culture and the concentration of IFN-γ of the supernatants was determined by sandwich ELISA. The means ± SE of five mice per group are shown. The asterisks indicate statistical significance ($P < 0.01$).

Immunization with αGalCer-pulsed DCs induced more than two times higher amounts of IFN-γ compared with the amounts induced by immunization with untreated DCs (Fig. 2: A(–) and B(–), $P = 0.0001$). When LLO91-99 peptide was also pulsed on DCs, LLO91-99 peptide stimulation *in vitro* increased the amounts of IFN-γ especially in group E, but the level of increase was minimal compared with the baseline level, especially in group D. Therefore, large amounts of IFN-γ produced by immune spleen cells were mainly attributable to αGalCer pulsed on DCs. We tried to observe induction of NKT cells by αGalCer-pulsed DC injection, but observed a rapid decrease of NKT cell number after the injection (data not shown). This would appear to be due to down-modulation of T-cell receptor and NK1.1 molecule on NKT cells rather than activation-induced cell death (Crowe *et al.*, 2003; Wilson *et al.*, 2003; Harada *et al.*, 2004).

Immunization with DCs pulsed with αGalCer at priming phase significantly reduced the bacterial load in the spleens of LLO91-99 peptide-pulsed DC immunized mice after *Listeria* challenge

We next examined whether αGalCer treatment of LLO91-99 peptide-pulsed DC affects the antilisterial protective immunity by the DC immunization. We immunized BALB/c mice with DCs loaded with LLO91-99 peptide and/or αGalCer at priming and boosting phases. Four weeks after the boost immunization, the immune mice were challenged with a lethal dose of *L. monocytogenes*. Three days later, bacterial CFUs in the spleens were evaluated. As shown in Fig. 3, group A and B mice did not show a significant decrease in CFU compared with naïve mice. The group C mice showed a significant decrease in CFU ($P = 0.0158$) to a level equivalent to the CFU in mice given LLO91-99 minigene-DNA vaccine (p91mam plasmid) three times. Group D mice tended to show a lower number of CFU compared with group C mice, but not significantly so ($P = 0.309$). Surprisingly, the group E mice showed the lowest number of CFU among the DC-immunized mice groups, and the difference in the number of CFU between groups C and E was significant ($P = 0.0379$).

Immunization with DCs pulsed with αGalCer at priming phase significantly enhanced the specific CTL activity by LLO91-99 peptide-pulsed DC immunization *in vivo*

We then analysed the specific CTL activity *in vivo* in these immune mice 4 weeks after the last immunization. As shown in Fig. 4, the group E mice showed the highest LLO91-99 peptide-specific CTL activity *in vivo* among the DC-immunized mice groups (groups C and E, $P = 0.029$; groups D and E, $P = 0.0005$; LLO91-99 DNA and group E,

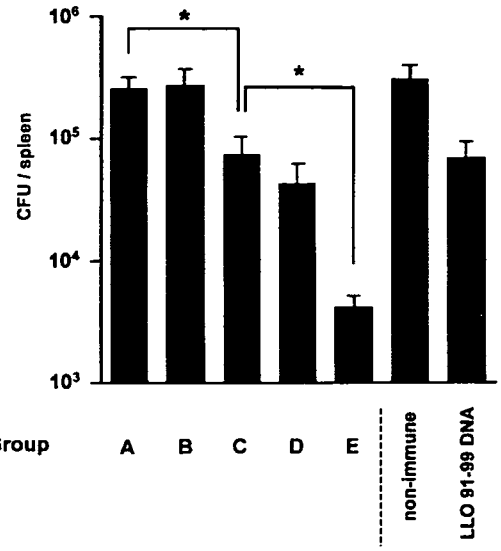

Fig. 3. *In vivo* protection of DC-immune mice against lethal *L. monocytogenes* challenge infection. Four weeks after the last immunization, the mice were challenged with 5×10^4 CFU of *L. monocytogenes* i.v. The antilisterial protective immunity induced by the DC immunization was evaluated by counting the bacterial numbers in the spleens 72 h after challenge infections by plating 10-fold dilutions of tissue homogenates. The means ± SE of eight mice per group are shown. The asterisks indicate statistical significance ($P < 0.05$).

$P < 0.0001$), although the *in vivo* CTL activity in the mice immunized with a sublethal dose of *L. monocytogenes* was much higher. This result suggests that the specific CTL activity *in vivo* reflects the protective immunity against lethal listerial challenge.

Immunization with DCs pulsed with αGalCer at the priming phase alone augmented the specific central memory CD8$^+$ T-cell number produced by LLO91-99 peptide-pulsed DC immunization

The induction of memory T cells is the most critical issue in vaccination against intracellular pathogens. Memory T cells have been divided into two major subtypes, effector and central memory T cells, which are characterized with effector memory (CD44$^+$ CD62L$^-$) and central memory (CD44$^+$ CD62L$^+$) phenotypes, respectively (Lefrançois & Masopust, 2002), and the latter has been reported to be critical for effective responses to secondary pathogen challenge (Wherry *et al.*, 2003; Zaph *et al.*, 2004; Roberts *et al.*, 2005; Vaccari *et al.*, 2005). Therefore, we examined the effect of αGalCer-pulsed DC immunization on the induction of specific memory CD8$^+$ T cells. Four weeks after boost immunization, immune spleen cells were prepared, and CD44 and CD62L (memory T-cell markers) expression on the H2-K^d/LLO91-99 tetramer$^+$ CD8$^+$ T-cell subset was examined. The percentage of CD44$^+$ cells in H2-K^d/LLO91-99 tetramer$^+$ CD8$^+$ T cells was significantly higher in the group E mice than the group C mice ($P = 0.0381$, Fig. 5a). No difference between group D mice and group E mice was observed in the percentage of CD44$^+$ cells in H2-K^d/LLO91-99 tetramer$^+$ CD8$^+$ T cells (Fig. 5a). However, the percentage of CD62L$^+$ cells in H2-K^d/LLO91-99 tetramer$^+$ CD8$^+$ T cells was significantly higher in the group E mice compared with those in the group D ($P = 0.0092$) mice and LLO91-99 DNA (p91mam)-immunized mice ($P = 0.0098$) (Fig. 5b). This result suggests that the percentage of central memory cells in total memory CD8$^+$ T cells in group E was higher than that in group D, although the percentage of total memory cells in LLO91-99-specific CD8$^+$ T cells was similar in groups D and E.

Rapid induction of various cytokines in sera of mice immunized with DCs pulsed with αGalCer

We were curious about why group E mice induced significant levels of specific CTL activity *in vivo* and the protective immunity. Cytokine milieu in the induction of *in vivo* CTL may affect the nature of CTL. We therefore examined several cytokine levels in sera of immune mice immediately after the first and second DC injections (Fig. 6). At priming phase, a rapid increase of the serum IFN-γ level was observed in group D or E mice compared with group C mice at 12 h after DC injection ($P = 0.0316$). At boosting phase, much greater amounts of Th1-type cytokines (IFN-γ, IL-12p70) were detected in the sera of group D mice compared with group C and E mice at 6 h after injection ($P < 0.0001$ respectively). Serum IFN-γ level in group D mice was almost 100-fold greater than that in group C and E mice at boosting phase. In addition, the serum levels of Th2-type cytokine (IL-4) as well as inflammatory cytokines (TNF-α, IL-6) were also significantly enhanced in group D mice compared with group C and E mice at boosting phase ($P < 0.0001$ respectively). Intriguingly, group E mice showed low levels of these cytokines, as well as IFN-γ and IL-12p70, at boosting phase.

Blockade of IFN-γ at boosting phase retrieved the induction of LLO91-99-specific CD8$^+$ central memory T cells and the reduction of the bacterial load in the spleens of *Listeria*-challenged mice

Among the variety of cytokines produced in sera after boost DC injection, we speculated that IFN-γ was the key cytokine influencing the subsequent T-cell responses because IFN-γ amounts were saliently produced in sera at boosting phase in group D, which showed the attenuated protective immunity. We were therefore interested in the effects of IFN-γ burst at boosting phase in the subsequent immune responses. To clarify the effects, we injected IFN-γ-neutralizing mAb 2 days before boost DC immunization to block the

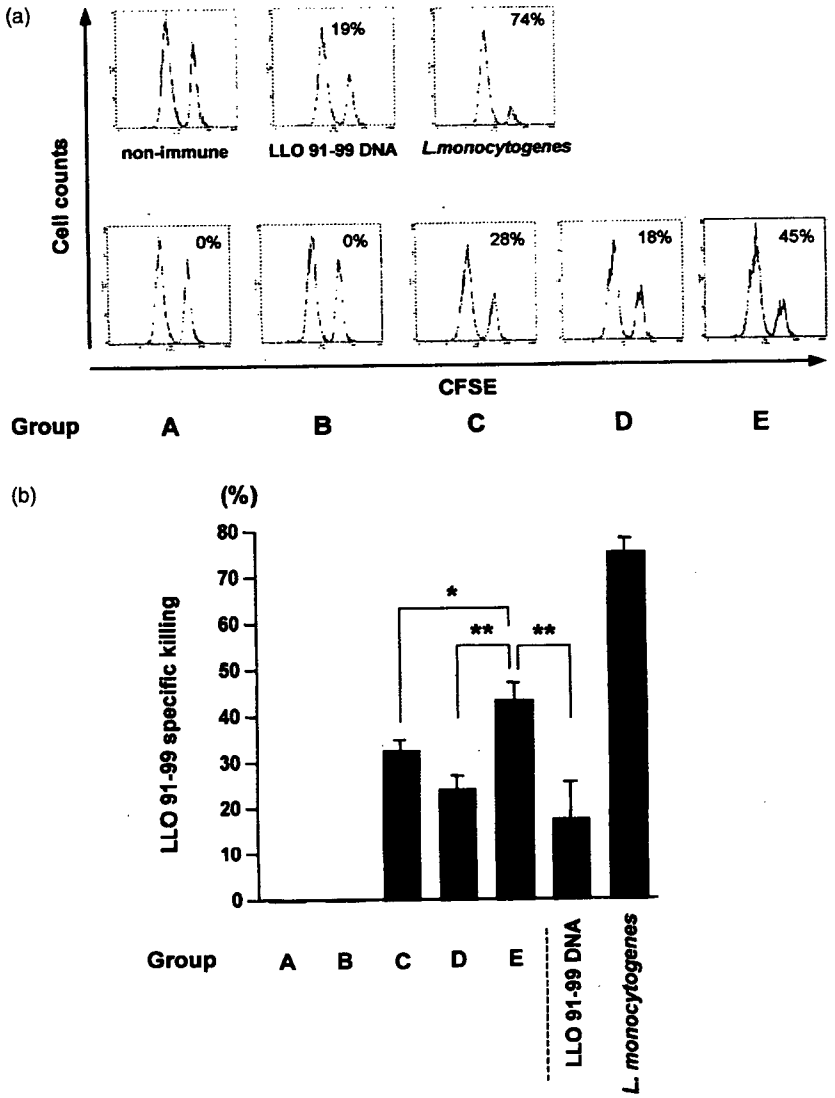

Fig. 4. *In vivo* CTL activity specific for LLO91-99 peptide-pulsed spleen cells. (a) Flow cytometric analysis of *in vivo* CTL activity specific for LLO91-99-pulsed spleen cells. Four weeks after boost immunization, LLO91-99 peptide and/or αGalCer-pulsed DC-immune mice were adoptively transferred with a 1 : 1 mixture of unpulsed $CFSE^{lo}$-labeled and the peptide-pulsed $CFSE^{hi}$-labeled naïve spleen cells 18 h before the analysis. The percentage of lysis for each experimental group is shown. The data of LLO91-99-expressing plasmid (p91mam) DNA-immune, or a sublethal dose (10^3 CFU) of *Listeria*-immune mice are also shown as controls. (b) Summary of the percentage of lysis of each experimental group obtained with *in vivo* CTL assay. The means ± SE of six mice per group are shown. Single asterisk and double asterisks indicate significant differences of $P < 0.05$ and $P < 0.01$, respectively.

IFN-γ burst at boosting phase, and examined the effects on the subsequent immune responses. We observed an increased number of LLO91-99-specific $CD8^+$ T cells in the mAb-treated group D mice compared with the mAb-untreated mice group (Fig. 7a). Furthermore, the number of $CD62L^+$ central memory $CD8^+$ T cells specific for LLO91-99 peptide in the mAb-treated group D mice was significantly increased ($P = 0.0158$), and was comparable with the number of these cells in group E mice (Fig. 7b). In parallel, the bacterial load in the spleens of *Listeria*-challenged mice 4 weeks after the boost immunization was improved in the mAb-treated group D mice ($P = 0.0380$, Fig. 7c). Administration of neutralizing mAb against IFN-γ in boosting phase did not affect the protective immunity in group E mice. Taken together, these results suggest that IFN-γ burst at boosting phase is involved in the exacerbating effect on the establishment of the effective specific $CD8^+$ memory T-cell pool.

Discussion

As DCs are the most powerful APCs that can initiate primary immune responses, DC vaccination has been an

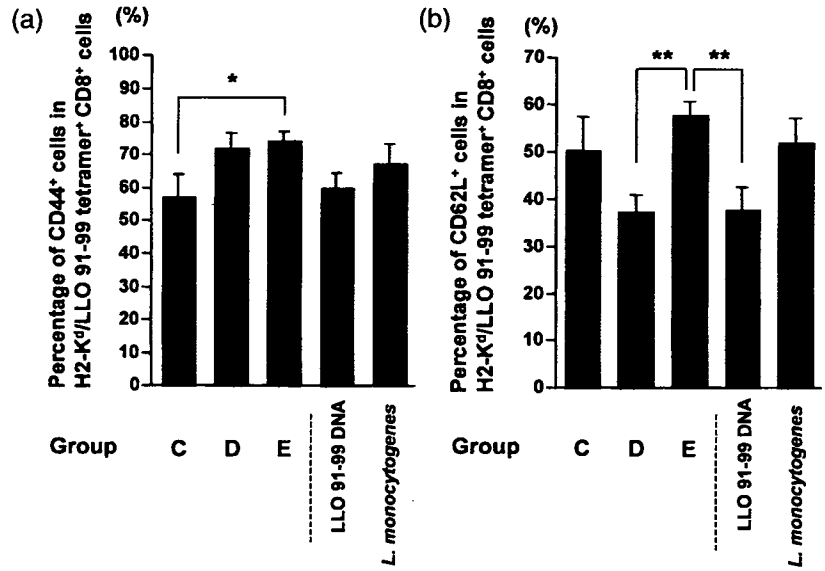

Fig. 5. Expression of CD44 and CD62L on LLO91-99-specific $CD8^+$ T cells in spleens of LLO91-99 peptide and/or αGalCer-pulsed DC-immune mice. (a) Summary of the percentage of $CD44^+$ cells in the H2-K^d/LLO91-99 $tetramer^+$ $CD8^+$ T cells. Flow cytometric analysis for H2-K^d/LLO91-99 tetramer and CD44 staining was performed 4 weeks after boost immunization. The means ± SE of five mice per group are shown. A single asterisk indicates a significant difference ($P < 0.05$). (b) Summary of the percentage of $CD62L^+$ cells in the H2-K^d/LLO91-99 tetramer-positive $CD8^+$ T cells. The immune spleen cells were analyzed as in (a) using FITC-conjugated antimouse CD62L mAb. The means ± SE of five mice per group are shown. Double asterisks indicate a significant difference ($P < 0.01$).

attractive strategy for combating tumors and infectious diseases (Mayordomo *et al.*, 1995; Celluzzi *et al.*, 1996). Several reports have tried to take advantage of αGalCer administration in order to strengthen the immunization effects of DC vaccination. αGalCer strongly activates *i*NKT cells. *i*NKT cells interact with DCs through CD40–CD40 ligand interaction (Fujii *et al.*, 2004) and IL-12 signaling (Kitamura *et al.*, 1999), resulting in DC maturation. We first examined the effects of treatments of DCs using lipopolysaccharides or CpG oligonucleotides to induce DC maturation *in vitro* before injection to mice. We did not observe significant enhancement of immune responses by these treatments (data not shown). We consider that immunization with αGalCer-pulsed DCs is sufficient for activation of DC itself *in vivo* through feedback effects by activated *i*NKT cells.

αGalCer administration has been carried out as free αGalCer administration or as αGalCer-pulsed DC administration. Free αGalCer administration induces the rapid increase of serum IFN-γ, IL-4 and IL-12 concentrations (Fujii *et al.*, 2002), but repeated αGalCer administration induces splenic T cells to secrete IL-4 and IL-10, and dramatically reduced levels of IFN-γ (Burdin *et al.*, 1999). In addition, αGalCer presentation by B cells suppresses DC–*i*NKT interaction when αGalCer is given as a free drug (Bezbradica *et al.*, 2005). Conversely, immunization with αGalCer-loaded DCs induces sustained IFN-γ production (Fujii *et al.*, 2002). Our experiments described here show that after the injection of DCs pulsed with αGalCer at priming phase, the serum IFN-γ level was significantly elevated. *i*NKT cells produce large amounts of IFN-γ and the production requires direct contact between *i*NKT cells and DCs through CD40–CD40 ligand interactions (Nishimura *et al.*, 2000). IFN-γ produced by *i*NKT cells has been shown to have a critical role in the antimetastatic effect of αGalCer in murine tumor models (Hayakawa *et al.*, 2001; Smyth *et al.*, 2002). The rapid IFN-γ production has been reported to stimulate the development of $CD8^+$ T-cell responses strongly, and also regulates $CD8^+$ T-cell contraction, and subsequently affects the formation of $CD8^+$ T-cell memory (Whitmire *et al.*, 2005). This finding is supported by our results that strong stimulation with DCs pulsed with αGalCer at priming phase but not at boosting phase leads to rapid IFN-γ production and induced stronger *in vivo* CTL activity. It is also conceivable that in addition to IFN-γ, direct DC activation by *i*NKT cells through CD40 signaling may affect the subsequent $CD8^+$ T-cell responses (Hermans *et al.*, 2003). Here, we showed that immunization with DCs pulsed with αGalCer together with an antigenic peptide at priming phase induced more $CD44^+$ memory-phenotype $CD8^+$ T cells, indicating that effective and strong priming of naïve T cells was elicited by αGalCer on DCs.

Another important finding here is that the consecutive use of αGalCer on DC immunization at both priming and boosting phases had deleterious effects on the effective memory T-cell response. *In vivo* CTL activity and the

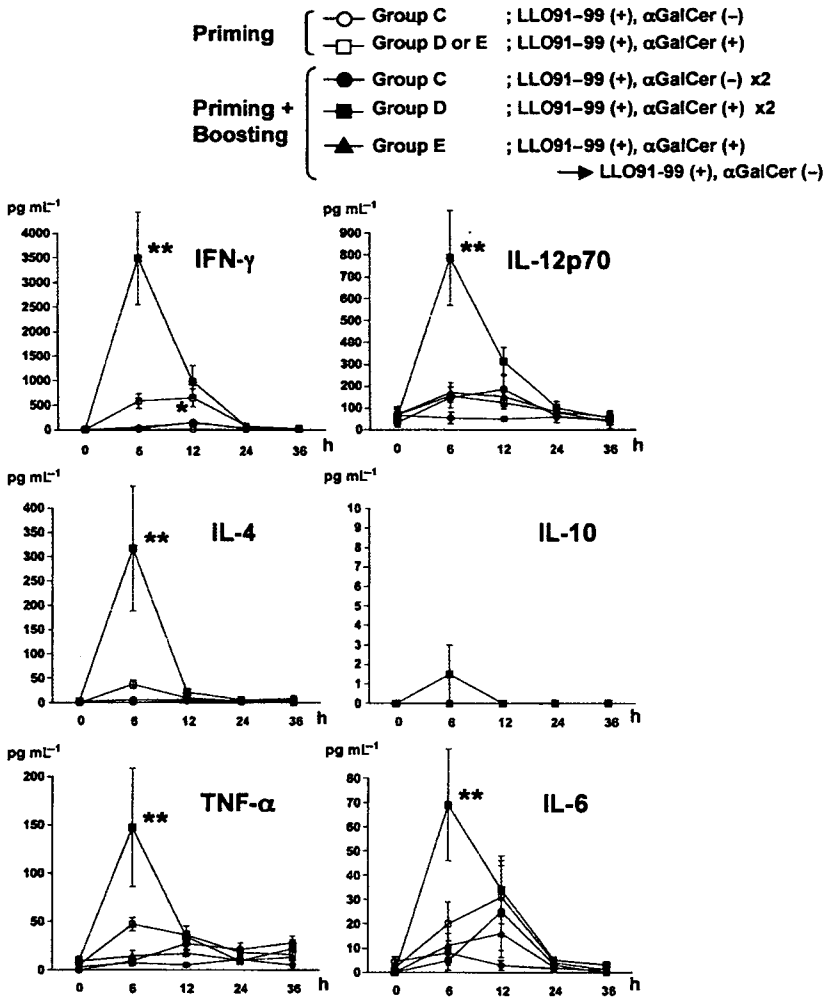

Fig. 6. Induction of cytokines in sera of LLO91-99 peptide and/or αGalCer-pulsed DC-immune mice. Sera were obtained from the immune mice at indicated time points after priming and boosting DC injections. Amounts of IFN-γ, IL-12p70, IL-4, IL-10, TNF-α and IL-6 in sera of the immune mice were evaluated with a cytometric bead array. Open symbols indicate cytokine levels after priming DC injection, and closed symbols indicate those after boosting DC injection. The means ± SE of five mice per group at each time point are shown. Single asterisk and double asterisks indicate significant differences of $P < 0.05$ and $P < 0.01$ compared with other groups at the same time point, respectively.

bacterial load in the spleens of *Listeria*-challenged mice, and also central memory $CD8^+$ T-cell pool formation were diminished by the consecutive use of αGalCer on DC immunization. Recent studies indicated that $CD8^+$ central memory T cells, but not $CD8^+$ effector memory T cells, play a pivotal role in protective immunity against the pathogen (Wherry *et al.*, 2003; Zaph *et al.*, 2004; Roberts *et al.*, 2005; Vaccari *et al.*, 2005). Therefore, diminished central memory $CD8^+$ T-cell formation in group D may reduce antilisterial immunity. Serum levels of Th1-type cytokines (IFN-γ and IL-12p70) were mostly elevated after the boost immunization in mice given DCs pulsed with LLO91-99 peptide and αGalCer at both phases (group D) (Fig. 6). In addition, serum levels of inflammatory cytokines (TNF-α and IL-6) and Th2-type cytokine (IL-4) were also elevated in the same group of mice, although the Th1-dominant immune response was maintained even at boosting phase (Fig. 6). Recently, Badovinac *et al.* (2005) reported that LLO91-99 peptide-pulsed DC vaccination generated $CD8^+$ T cells with the phenotype and function of memory cells within 4–6 days, leading to elevated numbers of effector and memory T cells and enhanced protective immunity compared with immunization with a sublethal dose of *L. monocytogenes*. In their system, they showed that inflammation events, such as infection with a sublethal dose of *L. monocytogenes* or CpG treatment, which lead to IFN-γ production, prevented the accelerated generation of memory $CD8^+$ T cells. Although early inflammatory signals, including IFN-γ production, are required to mediate activation of immature DCs and promote Ag presentation to naïve T cells, inflammatory

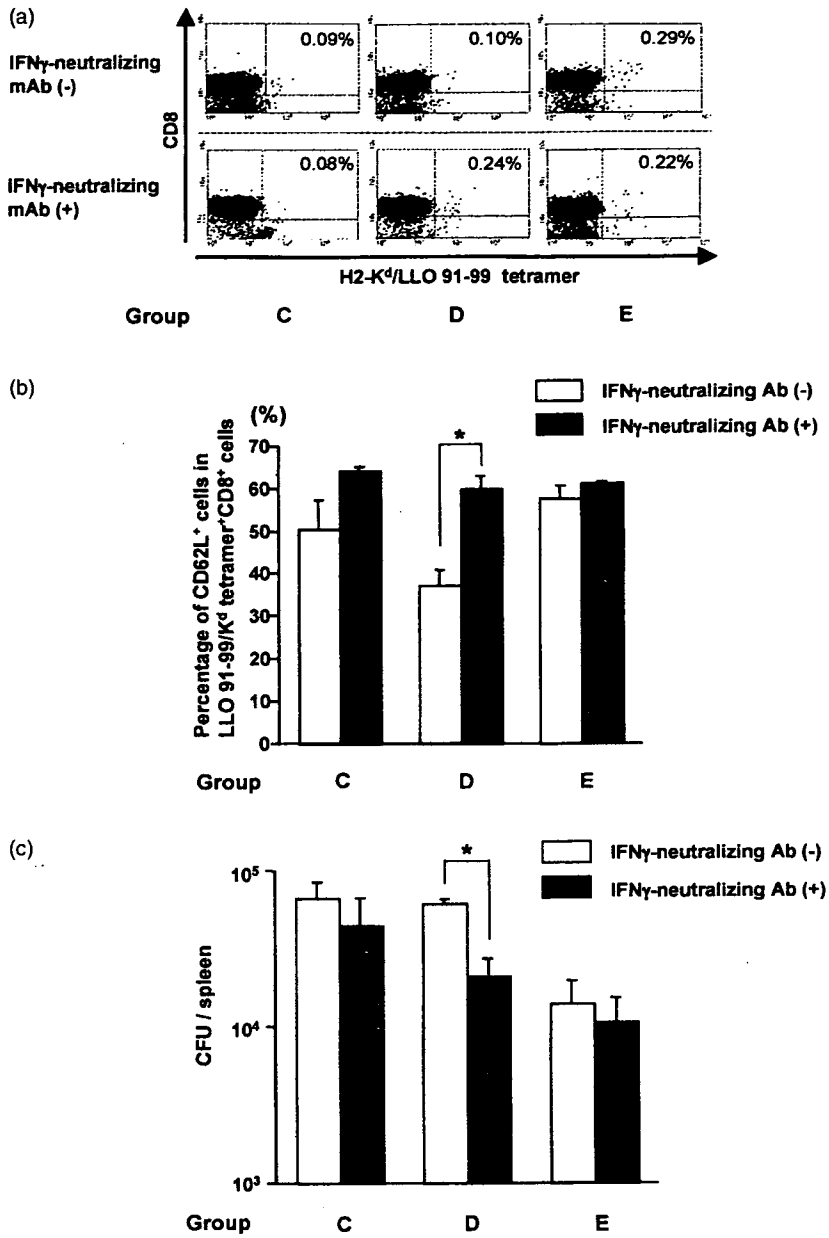

Fig. 7. Blockade of IFN-γ at boosting phase in mice given αGalCer-pulsed DC at both priming and boosting phases ameliorated specific CD8$^+$ T-cell induction, specific central memory T-cell formation, and induction of antilisterial immunity. Anti-IFN-γ neutralizing mAb was administered 2 days before boosting immunization. (a) Three days after boosting immunization, the spleen cells from the immune mice were prepared and analyzed for the appearance of H2-K^d/LLO91-99 tetramer$^+$ CD8$^+$ T cells with flow cytometry. The percentages of the tetramer$^+$ cells in CD8$^+$ T cells of each experimental group are shown. Representative data are shown. (b) The percentage of CD62L$^+$ cells in H2-K^d/LLO91-99 tetramer$^+$ CD8$^+$ T cells was examined 4 weeks after boost immunization. The asterisk indicates a significant difference ($P < 0.05$). (c) *In vivo* protection assay of antimouse IFN-γ mAb-treated mice against lethal *L. monocytogenes* challenge infection. The means ± SE of three mice per group are shown. Asterisks indicate significant differences ($P < 0.05$).

signals in the late phase of the immune response may act to enhance the proliferation and also the contraction of the T-cell response through apoptosis, leading to a delay in the generation of stable T-cell memory (Badovinac *et al.*, 2005; Woodland & Blackman, 2005). Blocking IFN-γ with the neutralizing mAb at boosting phase in mice given DCs pulsed with αGalCer at priming and boosting phases improved CTL responses, the formation of specific central memory T-cell pool and the reduction of the bacterial load in the spleens of *Listeria*-challenged mice. This suggests that

excessive amounts of IFN-γ at boosting phase deteriorate CTL responses and generation of the central memory T-cell pool. Too much inflammation and the subsequent induction of specific CTL would eliminate Ag-loaded APCs, including DCs at boosting phase, and the elimination would limit the sustenance of effective T-cell responses (Hermans *et al.*, 2000; Ronchese & Hermans, 2001). The prevention of the elimination of APCs at the boosting phase might be important for the induction of an effective stable T-cell memory. Too much inflammation would also induce activation-induced cell death of CTL (Woodland & Blackman, 2005). The results described here suggest that the removal of inflammatory signals at boosting phase may be important for an effective vaccine strategy. Although administration of αGalCer was often repeated for the enhancement of tumor immunotherapy (Ishikawa *et al.*, 2005), the deleterious effect of αGalCer at boosting phase should be taken into account. It would be preferable to add an additional combination group-LLO91-99 peptide (+)/αGalCer (−) at priming phase and LLO91-99 peptide (+)/αGalCer (+) at boosting phase to confirm the deleterious effect of αGalCer at boosting phase on peptide-pulsed DC immunization, although we were not able to add the group in this study.

The result described here that the immunization of Ag peptide-pulsed DCs with αGalCer at priming phase, but not at boosting phase, is feasible for an effective specific $CD8^+$ T-cell memory pool and the induction of protective immunity will shed light on effective vaccination strategies for specific $CD8^+$ T-cell induction. This effective immunization method using αGalCer-treated DCs would be also applicable to tumor immunotherapy.

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リファンピシンを含む結核治療におけるシクロスポリン投与量の検討

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要旨：〔目的〕すでにプレドニゾロン（PSL）やシクロスポリン（CyA）が投与されている結核患者にRFPを投与する場合，これら先行薬剤の増量を要する。PSLは2倍量程度が推奨されているが，CyAについては未だ定説がない。そこでRFP投与時のCyAの必要投与量を知るために，CyAがすでに投与されていた結核患者における投与量について検討した。〔方法と結果〕2001年から2003年の間に当院でRFPを含む肺結核の化学療法を行った患者のうち，治療前からCyAを使用していた4例の患者において結核治療前と同等のトラフ値が得られたCyAの投与量を検討した。全例PSLが投与されており，最初から2倍に増量されていた。CyAはトラフ値を測定して投与量を調節されており，2.5～3.5（平均3）倍量で結核治療開始前のトラフ値に到達し，臨床所見も安定した。CyA投与量の決定までに，トラフ値は5～12（平均8.3）回測定され，6～27（平均12）週を要していた。〔結論〕CyAの投与量決定には長期間，頻回のトラフ値測定を要したが，3倍量前後で目標値に達していた。したがって結核治療の最初からCyAは3倍に増量すると効率がよいと思われた。

キーワーズ：リファンピシン，シクロスポリン，プレドニゾロン，薬物相互作用，結核

はじめに

リファンピシン(RFP)は，主にチトクローム P450ⅢA（CYP3A）系酵素を誘導し，プレドニゾロン（PSL）やシクロスポリン（CyA）と併用した場合，これらの代謝を促進し半減期を短縮させる。したがってPSLやCyA投与患者に抗結核療法を行う場合は薬剤の増量を必要とする。

PSLは2倍量程度の増量が推奨されているが[1)2)]，CyAについては約3～5倍に増量[3)]や約2.5～3倍に増量[4)]したとの報告があるが，いずれも海外での報告であり，本邦の報告は未だない。

そこで今回われわれは，CyA使用中に発症した肺結核患者において，RFP併用後のCyAの投与量変更がどのようになされていたのかを検討したので報告する。

対象と方法

2001年から2003年の間に当院でRFPを含む肺結核の化学療法を行った患者のうち，すでにCyAを使用していた4例について後ろ向きに検討した。内訳は，男性2例，女性2例，体重46～63 kg，年齢51～78歳，間質性肺炎2例，全身性エリテマトーデス（SLE）1例，強皮症1例である。治療レジメは，イソニアジド（INH）＋RFP＋エタンブトール（EB）3剤（HRE）が3例，HRE＋ピラジナミド（PZA）4剤（HREZ）が1例である。全例RFPは450 mg/日で投与されていた。

これら4例についてCyAの投与量変更の方法と臨床経過について検討した。

結　果

全例，PSLが併用されており，1日1回投与であった。

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