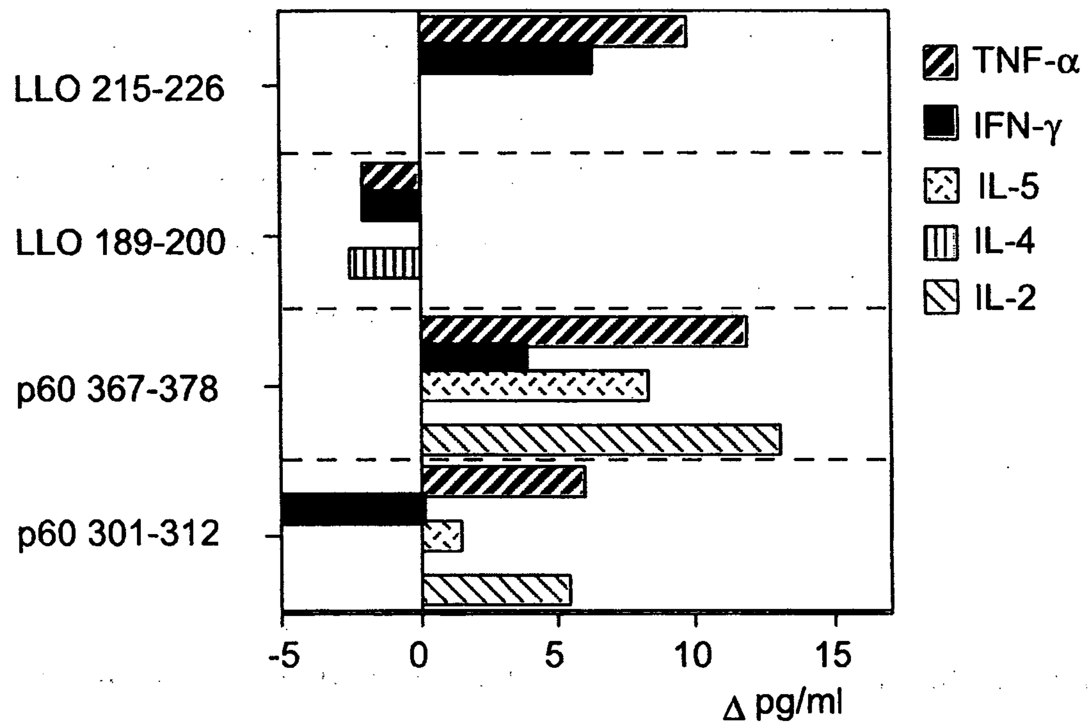


Fig. 4, Nagata et al.

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

Masato Uchijima[a], Toshi Nagata[b], and Yukio Koide[a,*]

[a]Department of Infectious Diseases, and [b]Department of Health Science,

Hamamatsu University School of Medicine,

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

*Corresponding author: Department of Infectious Diseases, Hamamatsu University School of Medicine, 1-20-1 Handa-yama, Higashi-ku, Hamamatsu, 431-3192, Japan

Tel. and Fax: +81-53-435-2335

E-mail address: koidelb@hama-med.ac.jp (Y. Koide)

Abstract

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

Keywords: DNA immunization, chemokine, tuberculosis

1. Introduction

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

Chemokines play an essential role in induction of inflammatory responses by trafficking of immune cells [3]. Chemokines bind to specific cell-surface receptors which are internalized after binding with ligands [4, 5]. Chemokine receptors are differentially expressed on a variety of immune cells. Sentinel antigen-presenting cells (APCs), such as immature dendritic cells (DCs), express chemokine receptors such as CCR5. CCR5 has been identified as the receptor for MIP-1α, RANTES, MCP-1, -2,-3,-4, and geotaxis [6]. Therefore, it should be possible to harness the receptor binding and internalization of chemokine to increase the immunogenecity of vaccines. In this study, the efficacy of MIP-1α-antigen fusion was examined by using DNA vaccine against *M. tuberculosis*. Antigen-specific T cell responses appeared to be significantly enhanced by genetic fusion of MIP-1α to MPT51, one of major protective antigens of *M. tuberculosis* [7].

2. Materials and Methods

2.1. Fusion gene cloning and plasmid constructions

The eukaryotic expression vector, pCI (Promega, Madison, WI) was used for construction for DNA vaccines. Murine MIP-1α gene was cloned by reverse transcription (RT)-PCR from total RNA of DCs. MIP-1α gene was fused with MPT51

gene via 14-amino acids spacer sequence and cloned into pCI vector (pCI-MIP-1α-MPT51). A plasmid expressing MIP-1α alone was constructed for control experiments. MIP-1α-fused GFP expression plasmid, pCI-MIP-1α-GFP, was constructed by the same strategy.

2.2. 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 phycoerythrin (PE)-labeled anti-CCR5 antibody (BD PharMingen, San Jose, CA) for 30 min on ice and 15 min at room temperature. The samples were washed three times with phosphate-buffered saline (PBS) containing 1 % fetal calf serum (FCS). Binding analysis was performed by using laser confocal microscopy (Olympus Fluoview , Tokyo, Japan).

2.3. Animals and immunization

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

For DNA immunization with Helios gene gun system (Bio-Rad Laboratories, Hercules, CA), 0.5 mg of gold particles was coated with 1 μg of plasmid. BALB/c mice were injected with 2 μg of plasmid DNA three times at one-week intervals.

2.4. Analysis of $CD8^+$ T cells H2-D^d-peptide tetramer complexes

An H2-D^d-peptide tetramer complex was kindly supplied by the NIH Tetramer Facility. MHC/peptide tetramer assay was performed as described previously [8]. In brief, three days after the last immunization, spleen cells were prepared and stained with phycoerythrin (PE)-conjugated H2-D^d-peptide tetramer complexes and fluorescein isothiocyanate (FITC)- conjugated anti-CD8 (BD PharMingen) monoclonal antibody for

30 min at 4°C. After washing, cells were resuspended in PBS containing 1% bovin serum albumin, and then analyzed on an EPICS XL digital flow cytometer (Beckman Coulter, Miami, FL).

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

Two weeks after last immunization, spleen cells were prepared and plated at 1 x 10^7 cells/well in the presence of 1 μM of MPT51 24-32 peptide for 16 hr. Total RNA was prepared by using ISOGEN (Nippon gene, Tokyo, Japan), and then quantitative RT-PCR was performed as described previously[9]. The sequence of primers used in this study are as follows: IFN-γ forward, 5'-TCTGAGACAATAAACGCTAC-3'; IFN-γ reverse, 5'-GAATCAGCAGCGACTCCTTT-3'; G3PDH forward, 5'-ACCACAGTCCAT CCATCAC-3'; G3PDH reverse, 5'- TCCACCACCCTGTTGCTGTA-3'.

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

Spleen cells were prepared from the immunized mice and plated in 96-well plates at 1 x 10^6 cells/well. Cells were stimulated with 1 μM of MPT51 24-32 peptide for 3 days. Concentration of IFN-γ in the culture supernatants was determined by a sandwich ELISA as described previously[10].

3. Results

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

To investigate receptor binding and internalization of chemokine fusion protein, we constructed a MIP-1α-GFP expression plasmid (Fig. 1A). HEK293T cells were transiently transfected with pCI- MIP-1α-GFP plasmid and the cell lysates were used for receptor binding assay by using confocal microscopy. Most of the MIP-1α-GFP fusion proteins localized on the surface of murine macrophage-like RAW264.7 cells (Fig. 1B, left). Co-staining of the cells with PE-labeled ant-CCR5 antibody showed co-localization of the MIP-1α-GFP protein and CCR5. In constant, the MIP-1α-GFP

proteins readily localized in the cytosol of JAWS II, a murine dendritic cell line, in the same experimental condition (Fig. 1B right). CCR5 co-localized with the GFP fused MIP-1α in the cytoplasm, which indicated in yellow. Taken together, these data suggested that MIP-1α fusion protein is capable of binding to CCR5 and is efficiently internalized especially into DCs.

3.2. Construction of plasmid for DNA immunization

To construct a DNA vaccine against TB, we used MPT51, a major secreted protein of *M. tuberculosis*, since we demonstrated that the MPT51 could induce T-cell-mediated immune responses and protective immunity upon challenge with *M. tuberculosis*[7]. MIP-1α and MPT51 genes were ligated via short 14-amino acid spacer sequence and cloned into mammalian expression vector pCI (Fig. 1A). A plasmid expressing MIP-1α alone (pCI- MIP-1α) was also constructed for control experiments

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

In order to evaluate the effect of immunization with MIP-1α-fused DNA vaccine, epitope specific CTL responses were monitored by quantitating MHC/peptide tetramer binding to $CD8^+$ T cells following DNA immunization. A representative experiment is shown Fig. 2. The antigen specific $CD8^+$ T cells were higher in number in spleen cells of mice immunized with the fusion DNA vaccine as compared to those of mice immunized with DNA vaccine encoding MPT51 alone or combination with the MIP-1α expression plasmid. These experiments demonstrate that MIP-1α fusion DNA vaccine efficiently induces antigen-specific $CD8^+$ T cells.

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

We next examined the ability of antigen-specific IFN-γ mRNA expression in the DNA vaccine immunized spleen cells. Two weeks after last immunization, spleen cells from immunized mice were stimulated with MPT51 24-32 peptide, $CD8^+$T cell epitope

derived from MPT51, for 16 hr. and the IFN-γ mRNA expression level was determined by real time quantitative RT-PCR. Amounts of antigen-specific IFN-γ mRNA considerably increased in spleen cells from the fusion DNA vaccine-immunized mice as compared with those of pCI-MPT51- and pCI-MPT51 +pCI-MIP1α - immunized mice. (Fig.3). Furthermore, we evaluated the production of IFN-γ of immunized mice after 3-days in vitro stimulation with MPT51 24-32 peptide employing ELISA. As shown in Fig. 4, mice immunized with the fusion DNA vaccine produced the highest level of MPT51-specific IFN-γ protein among these three DNA vaccination patterns consisting with the mRNA induction data (Fig. 3). A plasmid encoding MIP-1α showed adjuvant effect to some extent.

4. Discussion

The potency of vaccine presumably relies on the ability to recruit APCs and deliver antigens to them, leading to efficient antigen presentation to specific T cells. DCs are crucial in the activation of naïve T cells and induction of T cell-dependent immune responses. For this reason, experimental modification of vaccines, in particular genetic antigen delivery, has attracted much interest. Immature DCs, which are known to sentinel APCs, preferentially express CCR1, CCR2, CCR5, and CCR6 [11, 12]. Chemokines upon receptor ligation induce intracellular signals and endocytosis of chemokine receptors [4, 5]. In this study, we evaluated the genetic fusion of MIP-1α to MPT51 to enhance DNA vaccine efficacy.

We here demonstrated that MIP-1α-GFP protein was quickly internalized and found in the cytosol, co-localized with CCR5 when a murine DC line, JAWS II cells were incubated at room temperature (Fig. 1B). Similar results were obtained when bone marrow –derived DCs were incubated with the MIP-1α-GFP protein (data not shown). These data suggested that the fusion proteins not only retained its chemokine receptor binding properties of their nonfused chemokine counterparts, but also were efficiently

internalized to cytosol in immature DCs despite being linked to a relatively large antigen. The fate of the internalized MIP-1α fusion protein during receptor internalization remains unknown. Biragyn and his colleagues reported that MIP-3α–fused melanoma-associated antigen are internalized via CCR6 to early/late endosomal and lysosomal compartement through a clathrin-dependent process and subsequently delivered to the cytosol for proteasomal processing, facilitating efficient cross-presentation to TAP-dependent MHC class I presentation pathway[13]. It is, therefore, possible that such cross-presentation is involved in the antigen-specific $CD8^+$T cells induced with our MIP-1α-fused antigen.

Gene gun immunization is an efficient method for the administration of DNA vaccines [14]. Direct transfection of APCs or cross-presentation of exogenous antigen acquired from transfected nonimmune cells enables MHC class I-restricted activation of $CD8^+$ T cells [15, 16]. Previously, we have reported that MPT51 possesses one $CD8^+$ T cell epitope, p24-32, in MALB/c mice [17]. Therefore, we are able to examine the efficacy of MIP-1α-MPT51 DNA vaccine in inducing $CD8^+$ T cells using the epitope peptide. Using MPT51 p24-32 peptide/H2-Dd tetramer, we demonstrated that gene gun immunizations into the skin of mice with plasmid DNA encoding MIP-1α-MPT51 protein induced high level of epitope-specific $CD8^+$ T cells (Fig. 2). The efficient uptake of antigens by APCs may be particularly important for DNA vaccine, which typically expresses low amounts of antigen that are largely restricted to the local site of inoculation [19, 20]. We also demonstrated that splenocytes from BALB/c mice immunized with DNA vaccine encoding MIP-1α-MPT51 secreted more IFN-γ in response to the peptide p24-32 than those immunized with a DNA vaccine encoding MPT51 or with a mixture of two DNA vaccine encoding either MPT51 or MIP-1α. Several reports showed that co-immunization with DNA vaccines encoding antigens and chemokines enhanced the efficacy of vaccine by recruiting DCs to the inoculation

sites [18]. In our hands, immunization with a mixture of pCI-MPT51 and pCI-MIP-1α also enhanced the effects although this activity was less than that induced by pCI-MIP-1α -MPT51 DNA vaccination.

In summary, our data suggest that MIP-1α-antigen fusion proteins encoded by DNA vaccine vector are efficiently internalized into APCs and induce higher level of antigen specific T cell responses.

Acknowledgements

We are grateful to Kiyoshi Shibata (Hamamatsu Univ.) for excellent technical assistance. This work was supported by a grant-in-aid for scientific research and a grant-aid for centers of excellence (COE) research program from the Ministry of Education, Culture, Sports, Science and Technology of Japan.

References

[1] Dyc C, Scheele S, Dolin P, Psthania V, Raviglione MC. Global burden of tuberculosis: estimated incidence, prevalence, and mortality by country. JAMA 1999;282:677-86

[2] Colditz GA, Brewer TF, Berkey CS, Wilson ME, Burdick E, Fineberg HV, *et al.* Efficacy of BCG vaccine in the prevention of tuberculosis. JAMA 1994;271:698–702

[3] Rollins BJ. Chemokines Blood 1997;90: 909-28

[4] Mueller A, Kelly E, Strange PG. Pathways for internalization and recycling of the chemokine receptor CCR5. Blood. 2002;99:785-91

[5] Richardson RM, Pridgen BC, Haribabu B, Snyderman R. Regulation of the human chemokine receptor CCR1: cross-regulation by CXCR1 and CXCR2. J Biol Chem. 2000;275:9201-08

[6] Blanpain C, Migeotte I, Lee B, Vakili J, Dranz BJ, Govaerts C, Vassart G, *et al.* CCR5 binds multiple CC-chemokines: MCP-3 acts as a natural antagonist. Blood 1999;94:1899-905

[7] Miki K, Nagata T, Tanaka T, et al. Induction of protective cellular immunity against *Mycobacterium tuberculosis* by recombinant attenuated self-destructing *Listeria monocytogenes* strains harboring eukaryotic expression plasmids for antigen 85 complex and MPB/MPT51. Infect Immun 2004;72(4):2014-21.

[8] Aoshi T, Suzuki M, Uchijima M, Nagata T, Koide Y. Expression mapping using a retroviral vector for $CD8^+$ T cell epitopes: definition of a *Mycobacterium tuberculosis* peptide presented by $H2\text{-}D^d$. J Immunol Methods. 2005;298:21-34

[9] Uchijima M, Nagata T, Aoshi T, Koide Y. IFN-γ overcomes low responsiveness of myeloid dendritic cells to CpG DNA. Immunol Cell Biol. 2005;83:92-5

[10] Yoshida A, Koide Y, Uchijima M, Yoshida TO. Dissection of strain difference in

acquired protective immunity against Mycobacterium bovis Calmette-Guerin bacillus (BCG). J Immunol. 1995;155:2057-66

[11] Sallusto F, Palermo B, Lenig D, Matikainen S, Julkunen I, *et al.* Distinct patterns and kinetics of chemokine production regulate dendritic cell function. Eur J Immunol. 1999;29:1617-25

[12] Dieu MC, Vanbervliet B, Vicari A, Oldham E, Ait-Yahia S, *et al.* Selective recruitment of immature and mature dendritic cells by distinct chemokines expressed in different anatomic sites. J Exp Med. 1998;188:373-86.

[13] Sohiavo R, Baatar D, Okuhanud P, Indig FE, Restifo N, Taub D, *et al.* Chemokine receptor targeting efficiently directs antigens to MHC class I pathways and elicits antigen-specific CD8+ T-cell responses. Blood. 2006 ;107:4597-605

[14] Yoshida A, Nagata T, Uchijima M, Koide Y. Advantage of gene gun-mediated over intramuscular inoculation of plasmid DNA vaccine in reproducible induction of specific immune responses. Vaccine. 2000;18:1725-9

[15] Heath WR, Carbone FR. Cross-presentation in viral immunity and self-tolerance. Nat Rev Immunol. 2001;1:126–34

[16] Ulmer JB, Otten GR. Priming of CTL responses by DNA vaccines: direct transfection of antigen presenting cells versus cross-priming. Dev. Biol. 2000;104:9–14

[17] Suzuki M, Aoshi T, Nagata T, Koide Y. Identification of murine H2-D^{d}- and H2-A^{b}-restricted T-cell epitopes on a novel protective antigen, MPT51, of Mycobacterium tuberculosis. Infect Immun. 2004;72:3829-37

[18] Kutzler MA, Weiner DB. Developing DNA vaccines that call to dendritic cells. J Clin Invest. 2004;114:1241-4

[19] Casares S, Inaba K, Brumeanu TD, Steinman RM, Bona CA. Antigen presentation by dendritic cells after immunization with DNA encoding a major

histocompatibility complex class II—restricted viral epitope. J Exp Med. 1997;186:1481–86

[20] Barouch DH, Truitt DM, Letvin NL. Expression kinetics of the interleukin-2/immunoglobulin (IL-2/Ig) plasmid cytokine adjuvant. Vaccine. 2004;22:3092–97

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.

A

pCI-MPT51

pCI-MIP-1α

pCI-MIP-1α-MPT51

pCI-MIP-1α-GFP

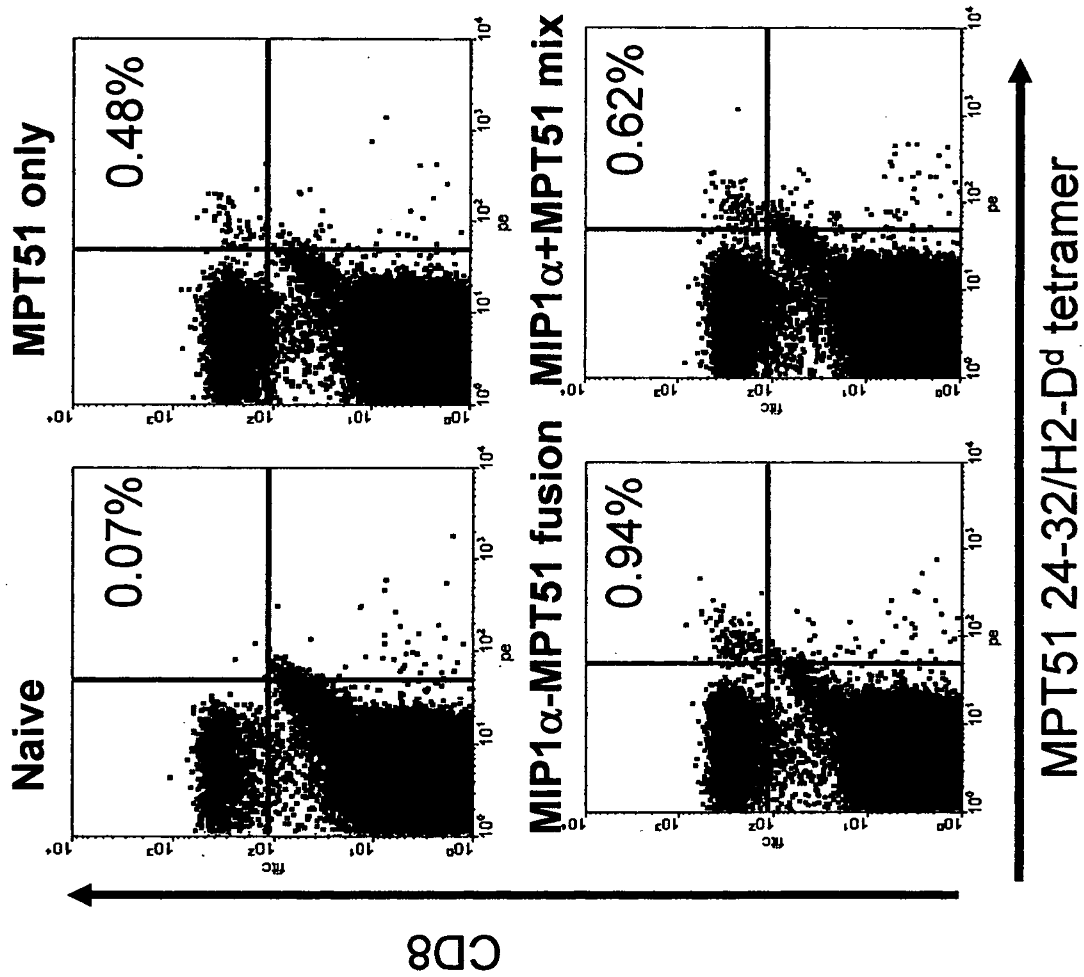
B

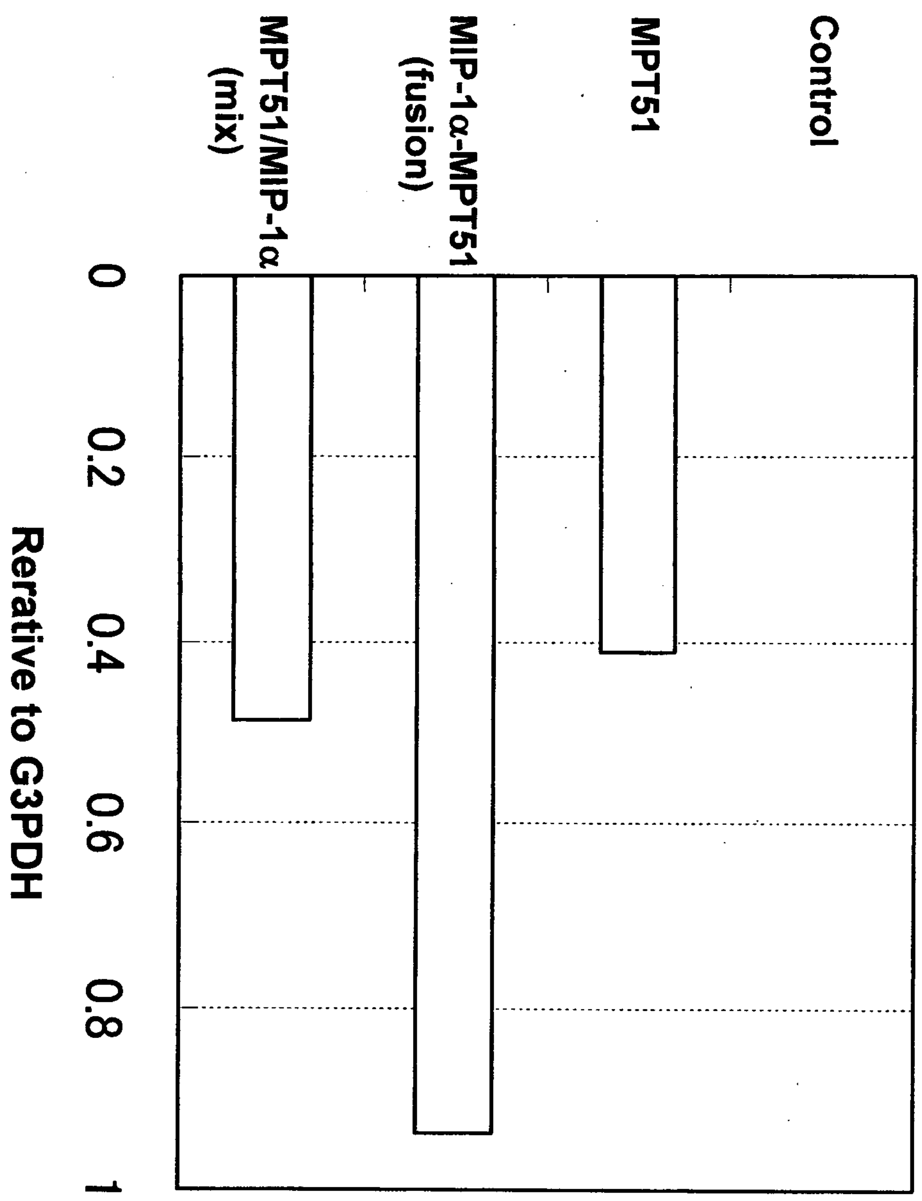
MIP-1α-GFP

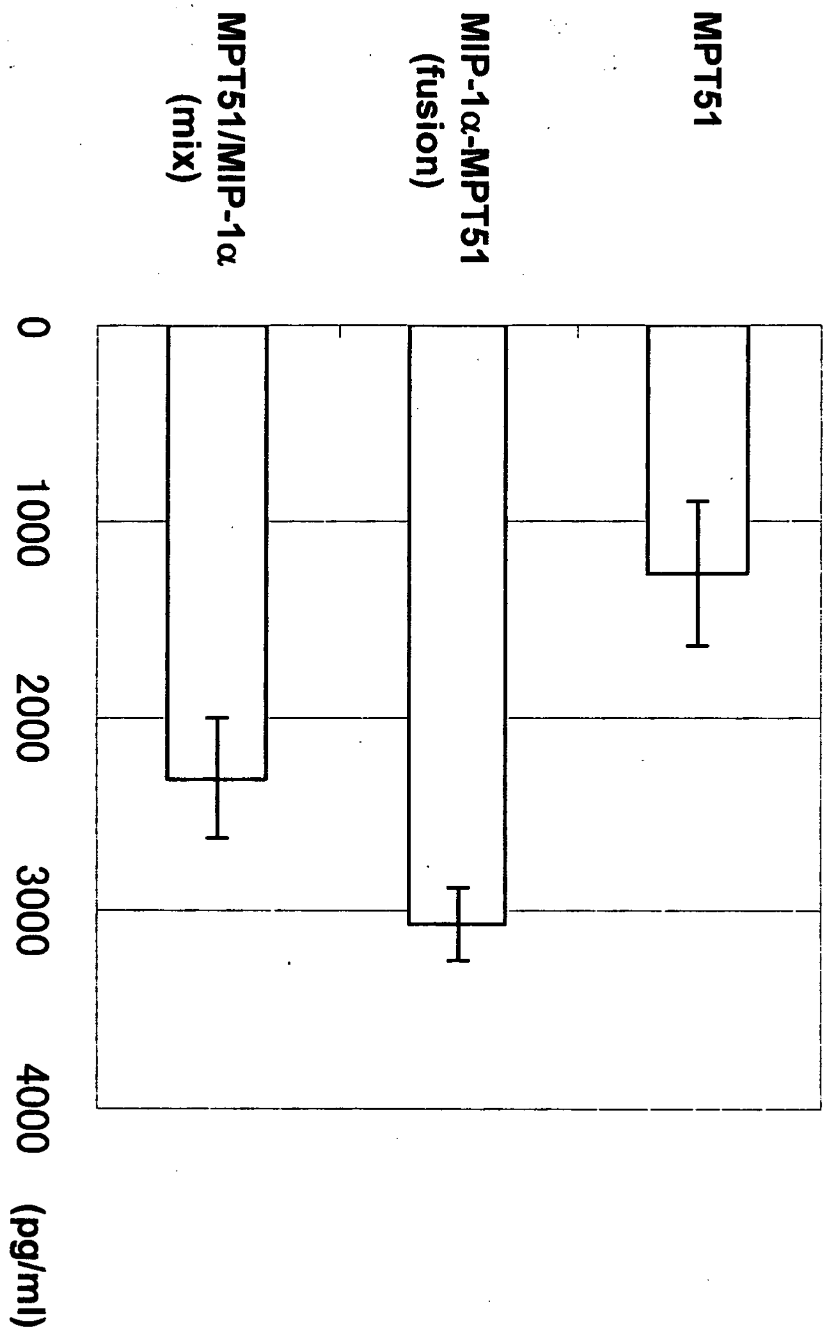
PE-anti-CCR5

merge

RAW264.7 JAWS II


Naive
0.07%
MPT51 only
0.48%
MIP1α-MPT51 fusion
0.94%
MIP1α+MPT51 mix
0.62%
CD8
MPT51 24-32/H2-D^d tetramer


Control
MPT51
MIP-1α-MPT51
(fusion)
MPT51/MIP-1α
(mix)
0
0.2
0.4
0.6
0.8
1
Rerative to G3PDH


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

Noriyuki Enomoto[1], Toshi Nagata[2], Takafumi Suda[1], Masato Uchijima[3], Yutaro Nakamura[1], Kingo Chida[1], Hirotoshi Nakamura[1] & Yukio Koide[3]

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

Correspondence: Toshi Nagata, Department of Health Science, Hamamatsu University School of Medicine, 1-20-1 Handa-yama, Higashi-Ku, Hamamatsu 431-3192, Japan. Tel.: +81 53 435 2332; fax: +81 53 435 2332; e-mail: tnagata@hama-med.ac.jp

Received 10 February 2007; revised 19 May 2007; accepted 9 July 2007.
First published online October 2007.

DOI:10.1111/j.1574-695X.2007.00316.x

Editor: Patrick Brennan

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 cyotoxic 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

© 2007 Federation of European Microbiological Societies
Published by Blackwell Publishing Ltd. All rights reserved

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).

© 2007 Federation of European Microbiological Societies
Published by Blackwell Publishing Ltd. All rights reserved