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**Figure legends**

Fig. 1. The schema of murine Ii p41 molecule whose CLIP is replaced by LLO 215-226, LLO 189-200, p60 367-378, or p60 301-312 deduced from the cDNA construct (pmIi p41-LLO215m, pmIi p41-LLO189m, pmIi p41-p60 367m, or pmIi p41-p60 301m, respectively). The deduced amino acid sequences of the replaced CLIP region and the Th-epitope peptides are shown. Amino acid numbers of each domain of murine Ii p41 molecule are also shown.

Fig. 2. Individual Th-epitope-specific proliferative responses of spleen cells from mice immunized with Th-epitope expression plasmids. BALB/c mice were immunized with each plasmid by using gene gun four times at 1-week intervals. Spleen cells from the immunized mice were harvested one month after the last immunization and cultured in vitro ($5 \times 10^5$ per well) in the presence or absence of 1 μM of each Th-epitope peptide for 2 days and pulsed with 0.5 μCi of [methyl-$^3$H] thymidine for last 12 h. Results of control wild-type Ii p41 expression plasmid-immunized mice are also shown as a control. The means ± SE of stimulation index (cpm in the presence of the peptide divided by cpm in the absence of the peptide) of three mice per group are shown except for two mice for LLO 215-227 group. Asterisks indicate statistical significance ($p < 0.05$) compared with the value of control mice.

Fig. 3. Evaluation of protective immunity induced by immunization with Th-epitope expression plasmids. Mice were immunized with each Th-epitope expression plasmid four times at 1-week intervals. One month after the last immunization, the immunized mice were challenged i.v. with $2 \times 10^3$ CFU of *L. monocytogenes*. Bacterial numbers in the spleens were determined 72 h after the challenge infection by plating ten-fold dilutions of tissue homogenates on trypticase soy agar plates. Results of naive mice are also shown as a control (Naive). Results are expressed as the

mean ± SE for three to four mice for each group except for two mice for LLO 189-200 group. Asterisk indicates statistical significance ($p < 0.05$) compared with the value of mice immunized with p60 301-312 DNA vaccine.

Fig. 4. Cytokine productions by spleen cells from Th-epitope expression plasmid-immunized mice. The spleen cells of mice immunized with each Th-epitope expression plasmid were harvested one month after the last immunization and cultured in vitro in the presence or absence of 1 μM of each Th-epitope peptide for 5 days, and the culture supernatants were analyzed by CBA. The values represent the means of Δpg/ml (the value in the presence of the peptide minus the value in the absence of the peptide) of two mice per each group.

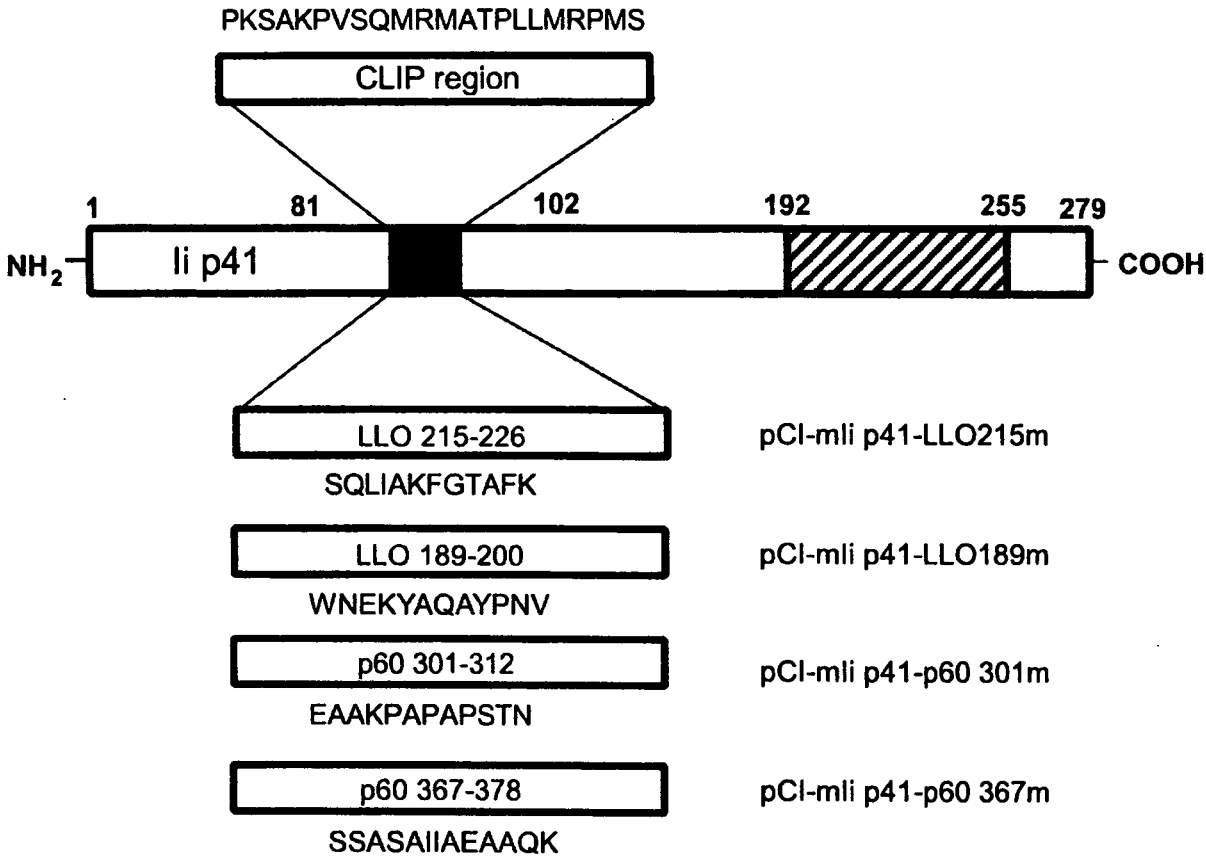


Fig. 1, Nagata et al.

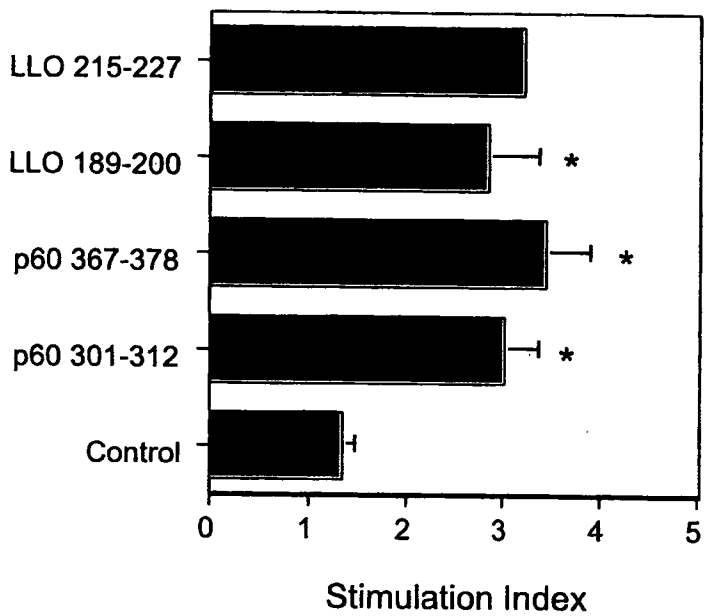


Fig. 2, Nagata et al.

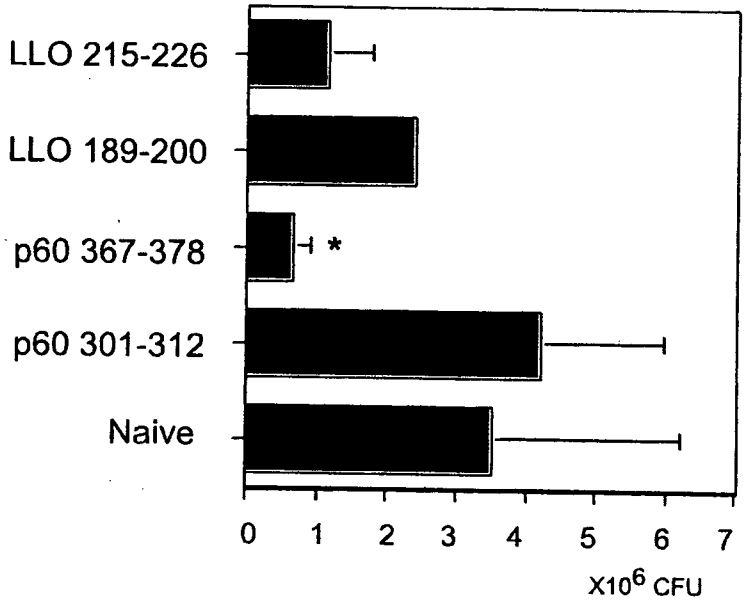


Fig. 3, Nagata et al.

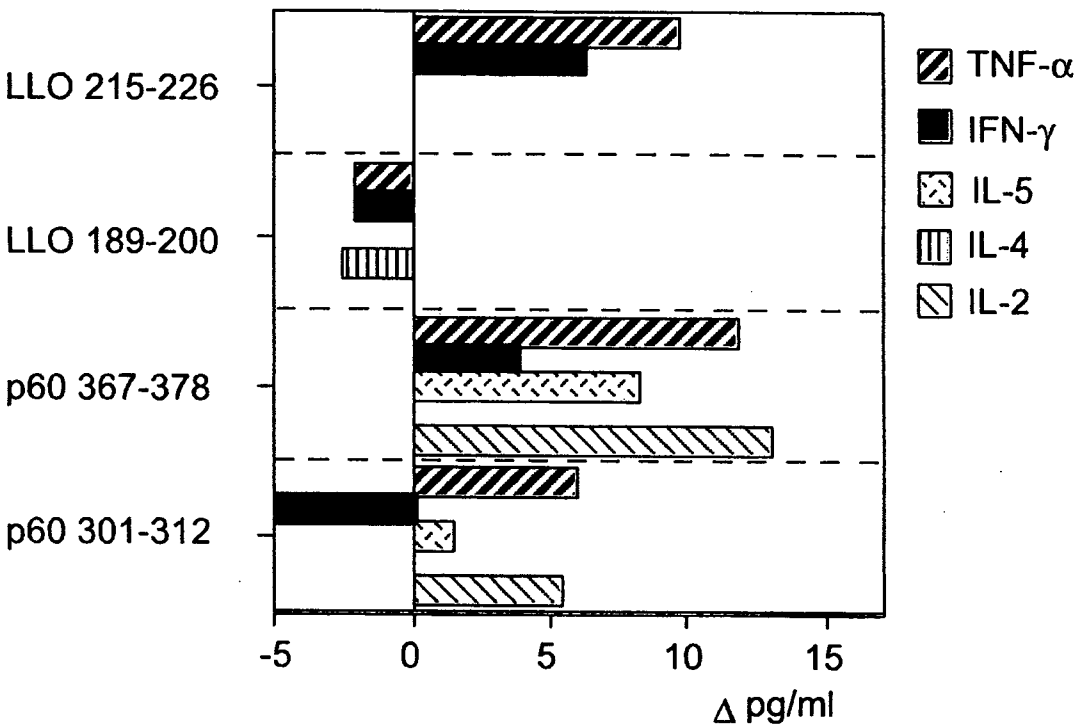


Fig. 4, Nagata et al.

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

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**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.

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