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Immunization with a gene encoding granulocyte-macrophage colony-stimulating factor inserted with a single helper T-cell epitope of an intracellular bacterium induces a specific T-cell subset and protective immunity

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Abstract

We evaluated here the effect of immunization with a gene encoding granulocyte-macrophage colony-stimulating factor (GM-CSF) inserted with a helper T cell (Th) epitope, listeriolysin O (LLO) 215–226 derived from *Listeria monocytogenes* on induction of a specific Th by gene gun bombardment. Immunization of C3H/He mice with pGM215m plasmid encoding murine GM-CSF inserted with LLO 215–226 Th epitope gave the epitope-specific proliferative responses of $CD4^+$ T lymphocytes. In addition, specific interferon-γ production from the splenocytes was observed. Concomitantly, pGM215m-immunized mice showed significant protective immunity against lethal listerial challenge. These results suggest that immunization of a gene for GM-CSF inserted with a Th epitope is useful for eliciting a specific Th subset in vivo.

Keywords: DNA immunization; GM-CSF; Th epitope

1. Introduction

Helper T cells (Th) play pivotal roles in many aspects of infection immunity, especially for modulating immune responses by producing special sets of cytokines. For protection against intracellular bacteria, activation of macrophages is indispensable and type 1-helper T cells (Th1) are important for the activation. The DNA vaccination method which induces only a particular Th population without production of antibodies may be advantageous as antibodies could, in some cases, give undesirable consequences [1]. Here, we evaluated the effect of immunization with a gene encoding murine granulocyte-macrophage colony-stimulating factor (GM-CSF) inserted with a single H2-E^k-restricted Th epitope [residues 215–226 of listeriolysin O (LLO)] derived from *Listeria monocytogenes* [2] by gene gun bombardment.

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2. Materials and methods

2.1. Animals

C3H/He mice (between 6 and 18 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 Guidelines for Animal Experimentation, Hamamatsu University School of Medicine.

2.2. Plasmid construction

The eukaryotic expression vector, pCI (Promega, Madison, WI) was used as a backbone plasmid for construction of plasmids for DNA immunization. The oligonucleotides used for p215m plasmid were, 5′-CCCGGG ATG AGC CAG CTG ATC GCC AAG TTT GGC ACC GCC TTT AAG TAG CCCGGG-3′ and the opposite-strand oligonucleotide,

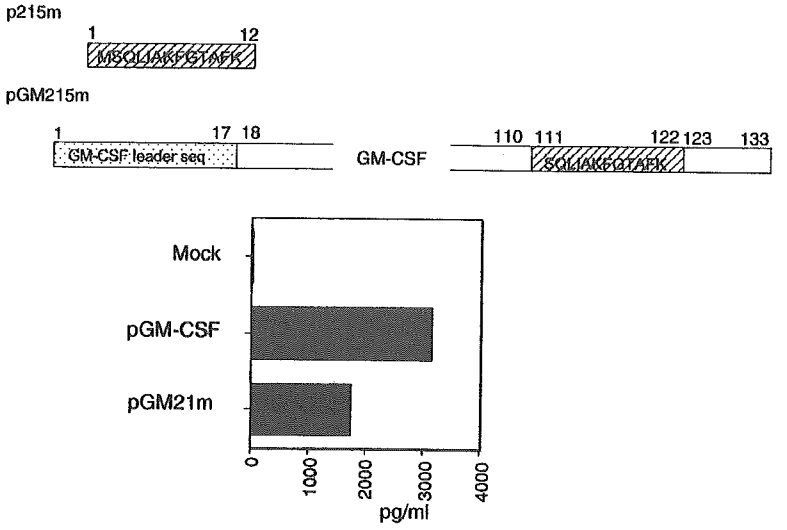

Fig. 1. (A) The schema of gene products deduced from the cDNA constructs prepared in this study (p215m and pGM215m). The hatched boxes indicate LLO 215–226 peptide and a dotted box indicate murine GM-CSF leader sequence. Amino acid numbers of each domain were shown above each schema. (B) Expression of GM-CSF inserted with LLO 215–226 peptide (GM215) in transfected cells. Supernatants of pGM-CSF- or pGM215m-transfected 293T cells were examined with ELISA specific to murine GM-CSF.

5′-CCCGGG CTA CTT AAA GGC GGT GCC AAA CTT GGC GAT CAG CTG GCT CAT CCCGGG-3′, which encode amino acid residues 215–226 of LLO, MSQLIAKFGTAFK and a termination codon. These oligonucleotides were annealed and inserted into the SmaI site of pCI (Fig. 1A). The codon usage of the oligonucleotide for LLO 215–226 peptide was optimized to that of *Mus musculus* [3]. pGM-CSF was constructed by inserting murine GM-CSF gene into the EcoRI/NotI sites of pCI. For pGM215m plasmid, a double-stranded oligonucleotide encoding LLO 215–226 was inserted in the unique EcoRV site of murine GM-CSF gene in pGM-CSF (Fig. 1A). The region is located in the region which should not affect the function of GM-CSF [4,5]. The nucleotide sequences of the resultant plasmids were confirmed by dideoxy sequencing by using ABI PRISM 310 Genetic Analyzer (Applied Biosystems; Foster City, CA).

2.3. Mice immunization

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

2.4. Enzyme-linked immunosorbent assay (ELISA) for GM-CSF

293T cells (human embryonal kidney cells) (approximately 5×10^6) were transfected with 2 μg of pGM-CSF or pGM215m using SuperFect Transfection Reagent (QIAGEN GmbH, Hilden, Germany). The supernatants were prepared 48 h after transfection and were assayed for GM-CSF using AN'ALYZA mouse GM-CSF Immunoassay Kit (G-T, Mineapolis, MN) according to the instruction manual.

2.5. Lymphocyte proliferation assay

Spleen cells (5×10^5 cells per well) from the immunized mice were incubated in RPMI1640 medium supplemented with 10% fetal calf serum (FCS) at 37 °C in a humidified 5% CO_2 atmosphere 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/well of [methyl-^{3}H] thymidine (10 Ci/mmol; ICN Biochemicals, Irvine, CA) for the last 12 h of culture. Triplicate cultures were harvested onto glass fiber filters, and the [methyl-^{3}H] thymidine incorporation was determined by counting the radioactivity (cpm) using a liquid scintillation counter.

2.6. ELISA for IFN-γ

Spleen cells were harvested from the immunized mice. Recovered cells were plated in 24-well plates at 2×10^6 cells/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 a sandwich ELISA as described in our previous report [6].

2.7. Intracellular IFN-γ staining

The number of LLO 215–226-specific $CD4^+$ T-cell subset was examined by intracellular IFN-γ staining. Spleen cells

from the immunized mice were treated with Tris-buffered ammonium chloride solution to remove red blood cells as described in our previous report [7].

2.8. Bacterial infection

A seed of *L. monocytogenes* EGD strain was cultured overnight in trypticase soy broth (Beckton Dickinson and Company, Cockeysville, MD) at 37 °C in a bacterial shaker and suitably diluted with PBS. The exact infection dose was assessed retrospectively by plating. Mice were immunized four times with DNA vaccine plasmids as described above, or immunized by a single intraperitoneal injection with a sub-lethal dose (1×10^4 CFU) of *L. monocytogenes*. One month later, the mice were challenged intraperitoneally 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 (Beckton Dickinson and Company).

2.9. Statistics

Data from multiple experiments were expressed as the mean ± S.D. Statistical analyses were performed by using StatView-J 5.0 statistics program (SAS Institute, Inc., Cary, NC). Data were analysed with one factor-analysis of variance followed by the Fisher's protected least significant difference (PLSD) test.

3. Results

3.1. Construction of plasmids for DNA immunization

In order to evaluate vaccination with plasmid DNA encoding murine GM-CSF inserted with a dominant Th epitope, we constructed pGM215m plasmid (Fig. 1A). As a control, we prepared another plasmid, p215m, a minigene plasmid for expression of LLO 215–226 peptide alone (Fig. 1A). In order to confirm expression of the GM-CSF-LLO 215–226 protein (GM215) by transfection of pGM215m, we transiently transfected 293T cells with pGM215m or pGM-CSF control plasmid and prepared the culture supernatants. As shown in Fig. 1B, we were able to detect expression of GM215 protein in the culture supernatant of pGM215m-transfected 293T cells by using GM-CSF-specific ELISA.

3.2. Proliferative responses of spleen cells of mice immunized with pGM215m plasmid and the epitope-specific IFN-γ production by the spleen cells

In order to examine the effect of immunization with pGM215m, we performed lymphocyte proliferation assay after immunization of C3H/He mice with the plasmid by using gene gun bombardment. We chose the immunization method as it is an appropriate vaccination route to evaluate the effect of GM-CSF expression plasmid on Langerhans cells and also it is a reliable and reproducible method from our previous experience [8]. As shown in Fig. 2, immunization with pGM215m plasmid induced LLO 215–226-specific proliferative responses of spleen cells from the immunized mice. Immunization with p215m plasmid showed less proliferative responses to LLO 215–226 peptide stimulation, which were not significantly different from those observed in naïve mice (data not shown).

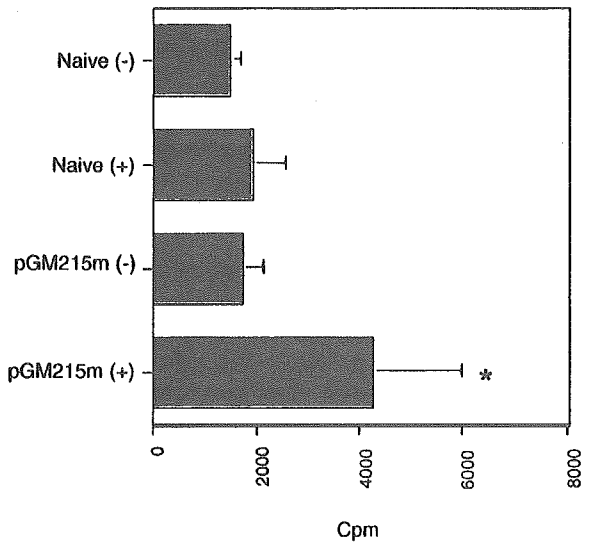

Fig. 2. Specific proliferative responses of spleen cells from pGM215m-immunized mice. C3H/He mice were immunized with pGM215m plasmid by using gene gun four times at 1-week intervals. Spleen cells from the immunized mice were harvested three weeks after the last immunization and cultured in vitro (5×10^5 cells/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 last 12 h. Results of naïve C3H/He mice are also shown as a control. The values indicate cpm per well. The mean ± S.D. of cpm of four mice per group are shown. Asterisks indicate statistical significance ($p \leq 0.001$) compared with the value of spleen cells of naïve mice without LLO 215–226 stimulation.

Furthermore, we analyzed IFN-γ amounts in the supernatants of spleen cell culture after 5-day in vitro stimulation with LLO 215–226 peptide. Again, immunization with pGM215m induced higher amounts of IFN-γ than those of mice immunized with p215m after the in vitro stimulation (Table 1). We did not detect IL-4 pro-

Table 1
IFN-γ production by splenocytes from C3H/He mice immunized with pGM215m plasmid

Immunization	Stimulation[a]	IFN-γ (pg/ml)[b]
Naïve	–	85.1
	LLO 215	74.1
pGM215m	–	95.9
	LLO 215	1318.7

[a] Spleen cells of immunized mice (2×10^6 cells/well) were cultured in the absence (–) or presence of 1 μM of LLO 215–226 peptide (LLO 215).

[b] After 4 days, cytokine concentrations in culture supernatants were quantified with sandwich ELISA. The mean of duplicate wells of representative data was shown.

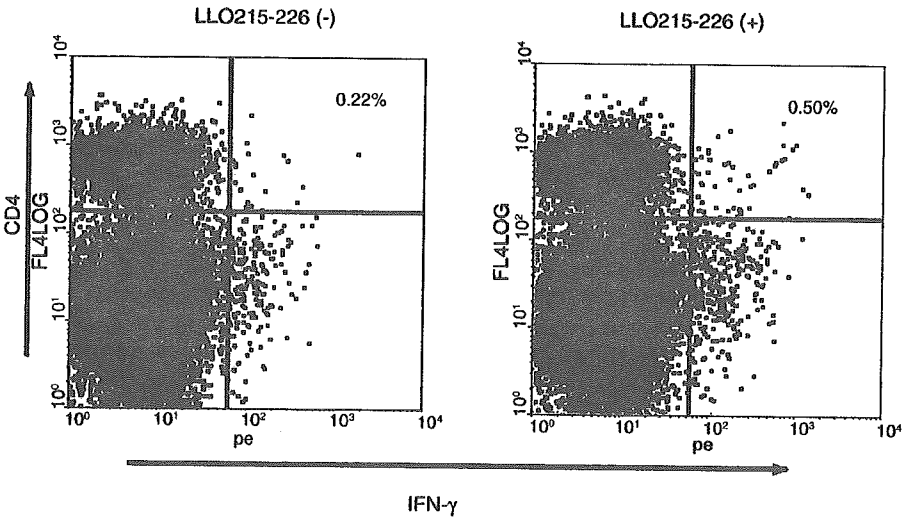

Fig. 3. Induction of LLO 215-specific $CD4^+$ T cells after pGM215m immunization. Intracellular IFN-γ staining on $CD4^+$ T-cell subset was performed using spleen cells after pGM215m immunization in the presence or absence of LLO 215–226 peptide. The percentages of IFN-γ-positive cells in $CD4^+$ T cells are shown.

duction from spleen cells of the immunized mice with ELISA (the detection limit of IL-4 in our ELISA system was approximately 100 pg/ml; data not shown). In addition, we performed intracellular IFN-γ staining with spleen cells of mice immunized with pGM215m. After in vitro stimulation with LLO 215–226 peptide, $CD4^+$ IFN-γ-producing cells was induced in the immune spleen cells (Fig. 3).

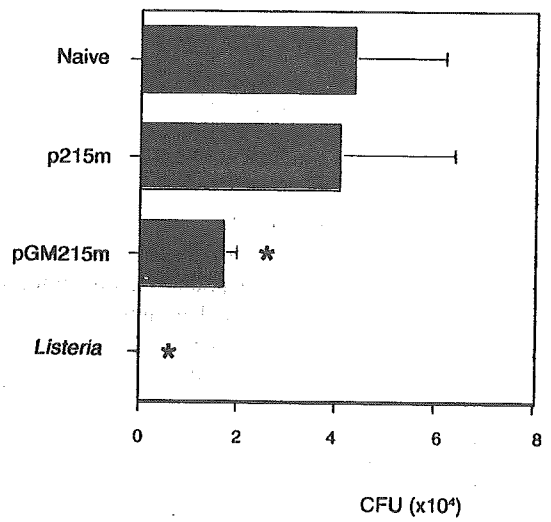

Fig. 4. Protective immunity induced by immunization with pGM215m. Mice were immunized with pGM215m four times at one-week intervals. One month after the last immunization, the immunized mice were challenged intraperitoneally with 1×10^5 CFU of *L. monocytogenes*. Bacterial numbers in the spleens were determined 72 h after the challenge infection by plating 10-fold dilutions of tissue homogenates on trypticase soy agar plates. Results of naïve mice and mice immunized with a sublethal dose of *L. monocytogenes* 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 naïve mice.

3.3. Induction of protective immunity against listerial infection after immunization with pGM215m plasmid

In order to examine whether the immunity evoked by immunization with pGM215m plasmid 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 immunized with pGM215m plasmid were sacrificed and CFU from the spleens were counted. As shown in Fig. 4, immunization with p215m did not show significant protective effects. On the contrary, mice immunized with a sublethal dose of *L. monocytogenes* were able to eliminate challenged *L. monocytogenes* from the spleens. Immunization with pGM215m conferred moderate, but significant protective immunity against lethal listerial challenge.

4. Discussion

DNA vaccination may work through direct transfection of antigen presenting cells (APCs), or by secretion of the encoded protein by muscle or skin cells for the uptake by APCs. Therefore, two different strategies have been considered to induce a particular epitope-specific Th by DNA vaccination. One is an intracellular targeting of antigens [9–11]. Another strategy is taking an advantage of secreted proteins. In general, secreted proteins are phagocytosed by APCs and presented on MHC class II molecules. In this study, we used a plasmid expressing murine GM-CSF, a cytokine which is indispensable for development of APCs such as dendritic

cells and macrophages. GM-CSF is one of most studied cytokines for vaccine adjuvants [12,13]. GM-CSF expression plasmid injected into mouse muscle has been reported to lead to a local infiltration of potential APCs [14]. We therefore reasoned that immunization of a gene for GM-CSF-Th epitope fusion molecule may work well for induction of the epitope-specific Th subset.

Several reports showed that immunization with a DNA vaccine co-expressing both antigen and GM-CSF [15] or a bicistronic plasmid DNA for antigen and GM-CSF [16,17] is superior to co-immunization of DNA vaccines for antigen alone and for GM-CSF in terms of strength of the specific immunity induced by the vaccines. Linking antigen and GM-CSF expression closely in vivo may provide a microenvironment suitable for the uptake and presentation of antigen by dendritic cells or macrophages. Immunization of pGM215m plasmid fits this condition.

It has been reported that $CD4^+$ T cells, especially LLO 215–226-specific T cells, are involved in protective immunity against listerial challenge. Verma et al. [18] demonstrated that induction of $CD4^+$ T cell population responsive to LLO 215–226 in vivo elicits partial protective immunity by using *Salmonella* carrier system. They showed one-log order reduction in numbers of the bacterium in spleens and livers of the immunized mice. In another approach, we showed that significant induction of protective immunity to *L. monocytogenes* by immunization with a plasmid DNA expressing LLO 215–226 Th epitope that replaces the class II-associated invariant chain (Ii) peptide (CLIP) of the Ii [10] or immunization with a plasmid encoding LLO 215–216 Th epitope fused with the endosomal/lysosomal targeting signal of lysosome-associated membrane protein (LAMP)-1 [11]. pGM215m immunization shown here was more effective than LLO215–LAMP fusion DNA immunization [11] in terms of induction of the protective immunity (data not shown).

The immunization strategy shown here, i.e., immunization with GM-CSF gene inserted with a double-stranded oligonucleotide encoding a Th epitope, would be applicable to DNA vaccination for induction of CTL or antibodies as a molecular adjuvant for supplying Th. Investigators add a universal Th epitope such as 13 aminio-acid Pan HLA-DR Epitope (PADRE) [19] in multi-CTL epitope plasmid DNA construction for efficient CTL induction [20]. GM-CSF-Th epitope DNA vaccination would be the alternative strategy for induction of Th.

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Brief Communication

IFN-γ overcomes low responsiveness of myeloid dendritic cells to CpG DNA

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Summary Dendritic cells (DC) are professional APC that have an extraordinary capacity to prime naive T cells. It has been reported that human DC subsets express distinct toll-like receptor (TLR), which influences their function. In mice, we observed that plasmocytoid DC (pDC) express a higher level of TLR9 compared with myeloid DC (mDC) cultured with GM-CSF. However, we demonstrated that stimulation with IFN-γ is capable of upregulating TLR9 expression in mDC to a level comparable with expression in pDC. Consistent with this observation, IL-12 p40 and IL-6 mRNA expression and IL-12 p70 secretion in response to CpG-oligodeoxynucleotides are enhanced in mDC pretreated with IFN-γ compared with untreated cells. Therefore, TLR-mediated responses of DC subsets may be influenced not only by signals delivered by pathogens but also by regulatory signals from cytokines such as IFN-γ.

Key words: CpG-oligodeoxynucleotide, IFN-γ, myeloid dendritic cell, plasmacytoid dendritic cell, toll-like receptor 9.

Introduction

Dendritic cells (DC) have a pivotal role in the interplay between the innate and adaptive immune responses against pathogens and tumours. They are a unique group of bone marrow-derived leucocytes that are specialized for uptake, transport, processing and presentation of antigen to T cells.[1] Although triggering of T cells into cell cycle progression is a central function of DC, it has also been suggested that DC subsets can influence the subsequent development of these dividing T cells. In humans, CD40 ligand-activated monocyte-derived DC, but not plasmacytoid DC (pDC) produced a large amount of IL-12 and induced Th1 rather than Th2 responses.[2] In mice, pDC ($CD11c^+$ $CD11b^-$ $B220^+$) induced Th1 cells and myeloid DC (mDC) ($CD11c^+$ $CD11b^+$ $B220^-$) induced Th2 cells when activated with CpG-DNA; however, both DC subsets have been shown to induce flexibly Th1 and Th2 cell development depending on antigen dose and differential toll-like receptor (TLR) ligation.[3] It has been reported that a given DC subset can induce either a Th1 or a Th2 response depending on the type of stimulation and pathogen.[4] Therefore, it seems likely that these DC subsets themselves may not have intrinsic capacity to direct either Th1 or Th2 cell development.

Recognition of the pathogen-associated molecular patterns involves members of the TLR family.[5] In humans, 11 TLR have been identified, and distinct DC subsets express different TLR.[6,7] For example, mDC express TLR2 and TLR4 whereas pDC express TLR7 and TLR9, indicating that distinct DC subsets respond to distinct microbial products.[8] In mice, pDC express low levels of TLR2, TLR3 and TLR4, and high levels of TLR7 and TLR9.[9] In contrast, mDC express high levels of TLR4 and low levels of TLR9.[3] Bacterial CpG DNA and CpG-oligodeoxynucleotides (ODN) have been shown to stimulate mammalian immune cells through TLR9.[10] Therefore, pDC produce type I IFN, IL-6 and IL-12 p70 when activated with TLR9 ligand CpG.[3] In contrast, LPS stimulates mDC to produce IL-12 p70.[3]

In this study, we showed that IFN-γ significantly increases TLR9 expression in mDC and thereby overcomes low responsiveness to CpG DNA.

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Materials and methods

Mice

Female BALB/c mice (Japan SLC, Hamamatsu, Japan) were kept under specific pathogen-free conditions and were fed autoclaved food and water ad libitum at the Institute for Experimental Animals of the Hamamatsu University School of Medicine. Two-month-old female mice were used in all experiments. All animal experiments were performed according to the Guidelines for Animal Experimentation, Hamamatsu University School of Medicine.

Generation of bone marrow-derived mDC and pDC

Bone marrow-derived $CD11c^+$ $CD11b^+$ $B220^-$ mDC were generated as described previously.[11] In brief, bone marrow cells were isolated by flushing femurs with culture medium and red blood cells were lysed using 0.83% ammonium chloride. The cells (1×10^6) were placed in 12-well plates in 2 mL medium with 10 ng/mL GM-CSF and 10 ng/mL IL-4 (Pepro Tech EC, London, UK). The cultures were fed every 2 days by gently swirling the plates, aspirating the medium and adding fresh medium back. At day six, non-adherent cells were collected and resuspended in fresh medium and cultured for an additional 1 day in 12-well plates. $CD11c^+$ $CD11b^-$ $B220^+$ pDC were generated by culturing bone marrow cells in culture medium containing 100 ng/mL Flt3 ligand (R & D Systems, Minneapolis, MN, USA)

for 10 days at 2 × 10^6 cells in 12-well plates in a volume of 2 mL. At day five, 1 mL medium was replaced by 1 mL fresh medium containing Flt3 ligand.[12] The purity after culture was always >85%.

CpG-ODN

Lipopolysaccharide-free phosphorothioate oligodeoxynucleotides (BEX, Tokyo, Japan) were used in all experiments. The nucleotide sequence of CpG-ODN used in this study is 5′-TGACTGTGAACGTTCGAGATGA-3′ (underlining indicates the immunostimulatory DNA sequence).

Preparation of total RNA and semiquantitative RT–PCR

Reverse transcriptase (RT)–PCR was performed as described previously.[13] The sequences of the primers used in this study are as follows: TLR9 forward, 5′-TGTTGCATAAGGCACAGAGC-3′; TLR9 reverse, 5′-CCTGAGCTATTCTGCTGTAGG-3′; IL-12 p40 forward, 5′-GGGACATCATCAAACCAGACC-3′; IL-12 p40 reverse, 5′-CCCAACCAAGCAGAATGCAGC-3′; IL-6 forward, 5′-TATGAAGTTCCTGTGTGCAA-3′; IL-6 reverse, 5′-CTTTGTATCTCTGGAAGTTT-3′; G3PDH forward, 5′-ACCACAGTCCATGCCATCAC-3′; and G3PDH reverse, 5′-TCCACCACCCTGTTGCTGTA-3′.

Real-time quantitative RT–PCR

One μL of total RNA from each sample was used for cDNA synthesis. Nineteen μL of LightCycler mastermix (Fast Start DNA master SYBR Green I; Roche Diagnostics, Basel, Switzerland) was filled in the glass capillaries and 1 μL cDNA was added as a template. Real-time quantitative PCR was performed using a LightCycler rapid thermal cycler (Roche Diagnostics). The manufacturer's LightCycler experimental run protocol was used.

Western blotting

Bone marrow-derived mDC and pDC were stimulated with 5 ng/mL IFN-γ for 12 h. After stimulation, cells were harvested and resuspended in lysis buffer. Protein concentrations were measured by Bio-Rad Protein Assay Kit (Bio-Rad, Hercules, CA, USA). Equal amounts of protein were loaded in each lane and separated by SDS–PAGE. Gels were transferred onto Hybond-P membranes (Amersham, Piscataway, NJ, USA) and blotted with antimouse TLR9 antibody (IMGENEX, San Diego, CA, USA).

ELISA

ELISA was performed as described previously.[14] To measure IL-12 concentration, antimurine IL-12 p70 mAb (PharMingen, San Jose, CA, USA) and antimurine IL-12 p40/p70 (PharMingen) were used as capture and detection mAb, respectively.

Results and discussion

First, we investigated the level of TLR9 expression in murine bone marrow-derived mDC in comparison with that in pDC. As shown in Figure 1a,b, both semiquantitative RT–PCR and real-time quantitative RT–PCR analyses revealed that TLR9 mRNA was faintly expressed in mDC. In marked contrast to mDC, TLR9 mRNA was highly expressed in pDC. This observation agrees well with a published report of murine DC subsets with respect to TLR expression.[3] To investigate the effect of IFN-γ on expression of TLR9 mRNA in subsets

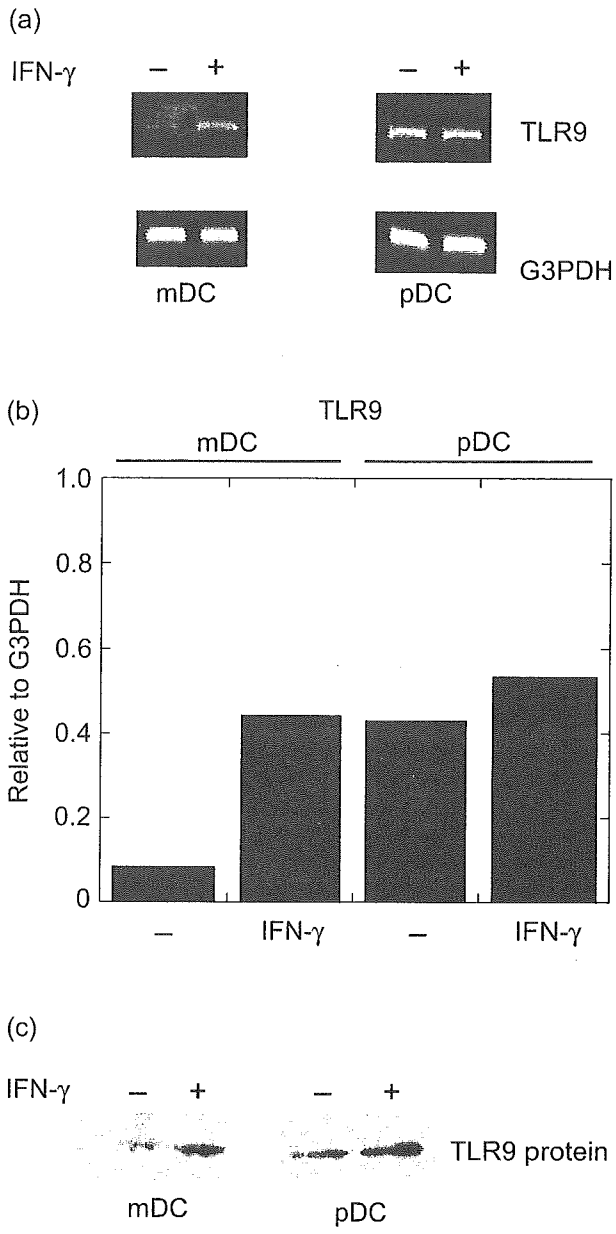

Figure 1 Upregulation of toll-like receptor (TLR)9 expression in myeloid and plasmacytoid dendritic cells (mDC, pDC) by IFN-γ. (a) DC were stimulated with IFN-γ (5 ng/mL) for 6 h and total RNA was isolated for first strand cDNA synthesis. Then, semiquantitative reverse transcriptase (RT)–PCR analysis were performed. (b) IFN-γ mRNA expression was further analysed by quantitative real-time PCR using the same cDNA used in (a). Expression is relative to G3PDH. (c) mDC and pDC were treated with IFN-γ for 12 h. Equal amounts of cell lysates were subjected to SDS–PAGE and immunoblotting for TLR9. Similar results were observed in three independent experiments.

of DC, murine bone marrow-derived mDC and pDC were stimulated with 5 ng/mL IFN-γ for 6 h. After stimulation with IFN-γ, mDC appeared to upregulate TLR9 mRNA expression to a level that was comparable with pDC (Fig. 1a,b). Expression peaked at 12 h and remained constant for 24 h after IFN-γ

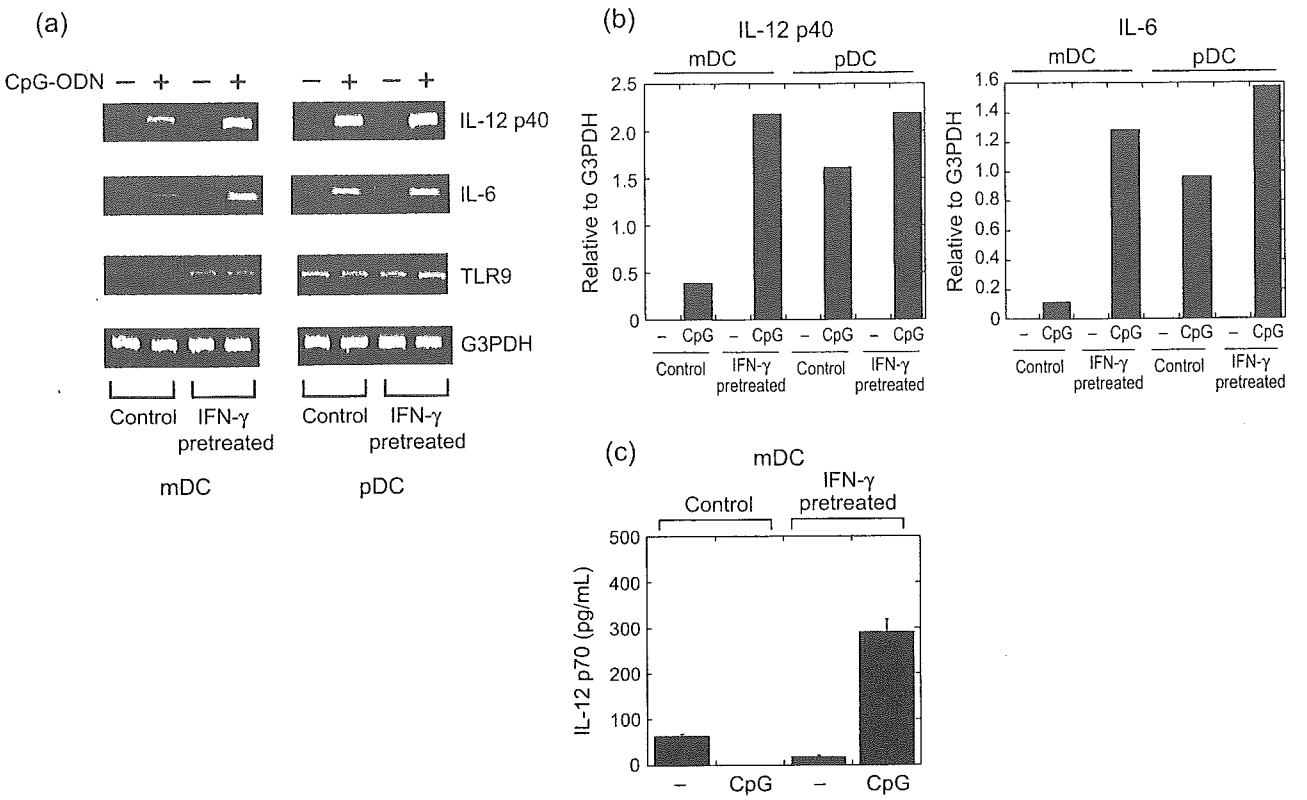

Figure 2 Effect of IFN-γ pretreatment on CpG-oligodeoxynucletide (ODN)-inducible gene expression. (a) Myeloid and plasmacytoid dendritic cells (mDC, pDC) were treated with IFN-γ (5 ng/mL) for 12 h and washed. The cells were incubated for 2 h before stimulation with CpG-ODN for 6 h. Total RNA was isolated and semiquantitative reverse transcriptase (RT)–PCR was performed. (b) IL-12 p40 and IL-6 expressions were further analysed by quantitative real-time PCR using the same cDNA used in (a). Expression is relative to G3PDH. (c) IL-12 p70 protein concentrations were measured by ELISA. Control or IFN-γ pretreated mDC were stimulated with CpG for 4 days and the supernatants were subjected to analysis.

stimulation (data not shown). The effect of IFN-γ was confirmed by the observation that no IFN-γ-induced increase of mRNA levels for TLR9 was observed in mDC and pDC from IFN-γ receptor-deficient mice (data not shown).

Given that treatment of mDC with IFN-γ significantly augmented TLR9 mRNA, we next investigated TLR9 protein expression in response to IFN-γ (Fig. 1c). Control mDC showed only a detectable level of TLR9 protein. Consistent with mRNA expression, TLR9 protein expression in mDC was upregulated after 12 h treatment with IFN-γ.

Because expression levels of TLR were expected to be reflected by their ability to produce cytokines in response to binding of their ligands, we next investigated cytokine mRNA induction of IFN-γ pretreated mDC by CpG-ODN stimulation. As shown in Figure 2a, we observed that IL-12 p40 and IL-6 mRNA expression in response to CpG-ODN was enhanced in IFN-γ pretreated mDC compared with expression in untreated cells. To obtain highly accurate quantitative data, we applied real-time RT–PCR analysis. Real-time quantitative RT–PCR confirmed the results obtained by semiquantitative RT–PCR (Fig. 2b). We also confirmed enhanced IL-12 p70 secretion from IFN-γ pretreated mDC by CpG-ODN stimulation (Fig. 2c). It seems most likely that the enhanced cytokine mRNA and protein expression depends on enhanced TLR9 expression but not on pretreated IFN-γ, because CpG DNA induced IL-12 p40 gene expression has been reported to be independent of STAT1 or IFN consensus sequence binding protein.[15] In addition, DC pretreated with IFN-γ did not induce expression of IL-12 p40 and IL-6 mRNA (Fig. 2a,b).

In humans, TLR9 expression is restricted to plasmacytoid DC.[16] In mice, however, TLR9 is highly expressed on bone marrow-derived and spleen plasmacytoid pDC, and to a lesser extent on GM-CSF cultured mDC.[3] Consistent with this report, we observed high levels of TLR9 mRNA and protein expression in pDC and significantly lower levels of expression in mDC.

Our data indicate that a high level of TLR9 expression is induced in mDC after stimulation with IFN-γ, resulting in acquisition of enhanced responsiveness to CpG. In fact, many studies support a high degree of plasticity in the capacity of DC to prime T cells and drive their functional differentiation.[17] The mechanism of IFN-γ inducible TLR9 in mDC remains unknown and awaits elucidation of the promoter sequence of the TLR9 gene.

Thus, TLR-mediated responses of DC may be influenced not only by signals delivered by pathogens such as LPS and CpG DNA, but also by regulatory signals from cytokines such as IFN-γ.

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