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The synthetic analogue of mycoplasmal lipoprotein FSL-1 induces dendritic cell maturation through TLR2

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Key words: Dendritic cells, Toll-like receptor 2, lipopeptide, lipopolysaccharide

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## Abstract

GM-CSF-differentiated bone marrow-derived DCs (BMDCs) were stimulated with FSL-1 or *E. coli* LPS. FSL-1 induced the production of TNF- $\alpha$  and IL-12 by C57BL/6-derived BMDCs but not by BMDCs from Toll-like receptor 2-deficient (TLR2<sup>-/-</sup>) mice, whereas LPS induced the production of TNF- $\alpha$  and IL-12 by BMDCs derived from either type of mice. FSL-1 did not induce production of IL-10 by BMDCs from either type of mice, whereas LPS induced small amounts of IL-10 by BMDCs from both types of mice. FSL-1 upregulated the expression of CD80, CD86 and the MHC class II molecule IA<sup>b</sup> in both dose- and time-dependent manners on the surfaces of C57BL/6-derived BMDCs but not on the surface of TLR2<sup>-/-</sup>-derived BMDCs, whereas LPS upregulated the expression of them on the surfaces of BMDCs from both types of mice. The expression of CD11c on the surfaces of C57BL/6-derived BMDCs was upregulated by stimulation with both FSL-1 and LPS up to 12 h and then the expression was downregulated.

The results suggest that FSL-1 has activity to accelerate maturation of BMDCs and that the activity of FSL-1 is mediated by TLR2.

## Introduction

Mycoplasmas are cell wall-less and the smallest self-replicating microorganisms. Mycoplasmal membrane-bound lipoproteins (LP) have been suggested to be one of pathogenic factors because of their capability to activate macrophages, monocytes and fibroblasts [1-6]. The active site of mycoplasmal LP has been shown to be the N-terminal lipopeptide moieties [4, 5]. We have synthesized a diacylated lipopeptide S-(2,3- bispalmitoyloxypropyl)-CGDPKHSPKSF, called FSL-1, on the basis of the structure of a 44-kDa lipoprotein of *Mycoplasma salivarium* that is capable of activating human gingival fibroblasts [4]. The synthesized lipopeptide FSL-1 has activity for inducing the production of inflammatory cytokines such as IL-6, IL-8 and MCP-1 by normal human gingival fibroblasts and the production of tumor necrosis factor (TNF)- $\alpha$  by monocytes/macrophages [7].

The recognition of microbial products by the host system is mediated by members of the Toll-like receptors (TLRs) family. They are involved in the innate immune response by recognizing microbial conserved structures called pathogen-associated molecular patterns (PAMPs), [8, 9] such as LPS, bacterial lipoprotein, peptidoglycan, lipoteichoic acid, bacterial unmethylated CpG DNA, mycobacterial lipoarabinomannan, and yeast mannans. The recognition of PAMPs by the pattern recognition receptor leads to the activation of various intracellular signaling cascades which modulate nuclear translocation of the transcription factor NF- $\kappa$ B[8, 9], induction of cytokines, and expression of effector molecules, such as the costimulatory molecules B7-1 (CD80) and B7-2 (CD86) . Therefore, activated innate immunity subsequently leads to effective adaptive immunity.

Dendritic cells (DCs) are one of key players that bridge innate immunity to adaptive immunity [10, 11]. DCs, which are the most effective antigen-presenting cells capable of inducing robust CD4<sup>+</sup> and CD8<sup>+</sup> T cell immunity, are one of key regulators in determination of Th1/Th2 balance [10,

11]. It is well known that signaling via TLRs, especially TLR3, 4, 7 and 9, induces IL-12 (p70) and IFN- $\alpha$  from DCs, which subsequently stimulate Th1 responses [10-13]. However, it is controversial whether TLRs can also induce Th2 responses. It has recently been reported that TLR2 ligands such as LP and lipopeptides are able to activate DCs to induce production of the Th2-restricted cytokine IL-10 and elicit Th2 responses *in vivo* [14-17]. There are several types of DCs that can differentially induce Th1 and Th2 responses [10, 11]. We have therefore been interested in the interaction of the diacylated lipopeptide FSL-1 with DCs and the type of immune response induced *in vivo* by FSL-1, because it has recently been demonstrated that diacylated lipopeptides, including FSL-1, are recognized by TLR2 in combination with TLR6 [8, 7, 18].

In the present study, we first focused on biological activities of FSL-1 toward bone marrow-derived DCs (BMDCs), because the method for preparing BMDCs is well established. We found that FSL-1 is capable of activating BMDCs to induce production of the Th1-restricted cytokine IL-12 and expression of costimulatory molecules and MHC class II in a TLR2-dependent manner.

## **2. Materials and methods**

### *2.1. Mice*

Sex-matched 8-week-old C57BL/6 mice (TLR2<sup>+/+</sup>) were purchased from Japan Clea (Tokyo, Japan). TLR2-deficient mice (TLR2<sup>-/-</sup>) on the same genetic background were generated by gene targeting as described previously [19]. All mice were maintained in specific pathogen-free conditions at our animal facility at Hokkaido University, and all experiments were carried out in accordance with the regulations of the Hokkaido University Animal Care and Use Committee.

### *2.3. Synthesis of the diacylated lipopeptide FSL-1*

FSL-1 was synthesized according to the method described previously [4]. Briefly, side chain-protected Cys-Gly-Asp-Pro-Lys-His-Pro-Lys-Ser-Phe was built up with an automated peptide synthesizer (model 433; PE Applied Biosystems, Foster City, CA, USA).

F-moc-*S*-(2,3-bispalmitoyloxypropyl)-cysteine (Novabiochem, Laeufelfingen, Switzerland ) was manually coupled to the peptide-resin using a solvent system of 1-hydroxy-7-azabenzotriazole-1-ethyl-3-(3-dimethylaminopropyl)-carbodiimide/CH<sub>2</sub>Cl<sub>2</sub>-DMF. The F-moc and resin were removed from the lipopeptide by trifluoroacetic acid. The lipopeptides were purified by preparative HPLC with a reverse-phase C<sub>18</sub> column (30 x 250 mm). The purity of FSL-1 was confirmed by analytical HPLC with a reverse-phase C<sub>18</sub> column (4.6 x 150 mm) to be 98%. The lipopeptide FSL-1 was dissolved in PBS.

### *2. 4. Preparation of bone marrow-derived DCs (BMDCs) and cultures*

BMDCs were prepared according to the method described previously [20] with minor modifications. Briefly, bone marrow cells (BM cells) were obtained by flushing femurs and tibias with RPMI 1640 using a 26-gauge needle. The BM cells (1 x 10<sup>6</sup> cells/ml) were cultured overnight in each well of 24-well plates in RPMI 1640 supplemented with 10% FBS. Nonadherent cells were harvested and cultured in the same medium containing 10 ng/ml recombinant mouse GM-CSF (Pepro Tec., Rocky Hill ,NJ, USA). On day 3, the medium was changed to a fresh medium containing 10 ng/ml GM-CSF. On day 6, nonadherent cells and loosely adherent cells were harvested and used for experiments as immature DCs. Immature DCs suspended in fresh RPMI

1640 were used for stimulation by FSL-1 or *Escherichia coli* LPS O55:B5 (Sigma., St. Louis, MO, USA).

### *2.5 Stimulatioin of BMDCs by FSL-1 or LPS*

BMDCs ( $5 \times 10^5$ ) were plated in a 24-well plate in RPMI 1640 containing 10% FBS. BMDCs were stimulated at 37 °C for 12 h with various concentrations of FSL-1 or *E. coli* LPS and centrifuged at 400 g for 10 min to separate the cells and culture supernatants. The cells were recultured for an additional 24 h with the same concentration of FSL-1 or LPS and centrifuged again to separate the cells and culture supernatants. This method was used to avoid secondary effects of cytokines produced and LPS- or FSL-1-induced tolerance. Pooled culture supernatants were used for measuring cytokine concentration by ELISA, and the cells were used for analysis of the expression of cell surface antigens, costimulatory molecules and MHC class II molecules by flow cytometry as described below.

### *2.5. Determination of cytokines*

Cell culture supernatants were assayed for various cytokines, including TNF- $\alpha$ , IL-12(p70) and IL-10, by using ELISA kits purchased from PharMingen (San Diego, CA, USA), Pepro Tech (Rocky Hill, NJ, USA) and R&D Systems (Minneapolis, MN, USA), respectively. Briefly, flat-bottomed 96-well Nunc-Immuno MaxSorp assay plates were coated overnight with the appropriate anti-cytokine antibodies. After blocking the plates with bovine serum albumin, the plates were incubated for 2 h with the culture supernatants and then incubated for 1 h with each of

biotin-conjugated anti-cytokine antibodies. Then horseradish peroxidase-conjugated streptavidin was added and developed with TMB peroxidase substrate. The optical densities were measured at 405 nm using a microplate reader.

## 2.6. Flow cytometry

For flow cytometric analysis, BMDCs were incubated on ice ( $10^6$  cells in 200  $\mu$ l of PBS with 1% BSA) with anti-mouse CD32/CD16 (Fc $\gamma$ II/III receptor). After 30 min, aliquots were washed and incubated in 200  $\mu$ l of of PBS with 1% BSA with FITC-conjugated monoclonal antibodies (mAb) against CD11c, CD11b or CD8 $\alpha$  and PE-conjugated mAbs against CD80 (B7.1), CD86 (B7.2) or I-A<sup>b</sup> (MHC class II ) and appropriate isotype controls (BD Pharmingen, San Diego, CA, USA). The cells were fixed in 0.1 M phosphate buffer containing 0.5% formaldehyde and then analyzed by a FACScan flow cytometer (BD Bioscience, Mountain View, CA, USA). Data for 10000 cells falling within appropriate forward and side light scatter gates were collected from each sample. Data were analyzed using CellQuest software (BD Bioscience).

## 3. Results and discussion

In order to determine the interaction of FSL-1 with DCs, we prepared BMDCs from TLR2<sup>+/+</sup> and TLR2<sup>-/-</sup> mice according to the method of Inaba et al. [20]. GM-CSF-differentiated BM cells derived from TLR2<sup>+/+</sup> (Fig. 1) and TLR2<sup>-/-</sup> (data not shown) were examined for surface expression of CD8 $\alpha$  as well as CD11b and CD11c (Fig.1 *left panels*), because these antigens are used for characterization of DCs [11]. The BMDCs strongly expressed CD11c and CD11b, but not CD8 $\alpha$ , on their surfaces (Fig. 1 *right panels*). This finding suggests that BM cells are differentiated into immature BMDCs (imBMDCs), because CD11c and CD11b are important marker antigens of

mouse BMDCs [21].

The next experiment was carried out to determine whether FSL-1 or *E. coli* LPS activates imBMDCs. The imBMDCs were stimulated for 12 h with FSL-1 or *E. coli* LPS and then washed and recultured for another 24 h in the presence of FSL-1 or LPS. The amounts of TNF- $\alpha$ , the Th1-restricted cytokine IL-12 and the Th2-restricted cytokine IL-10 released in the culture supernatant were measured by ELISA. FSL-1 induced the production of TNF- $\alpha$  by B6-derived BMDCs but not by TLR2KO-derived BMDCs, whereas LPS induced production of TNF- $\alpha$  by BMDCs from both types of mice (Fig. 2). Interestingly, it was found that the BMDCs were no longer capable of producing TNF- $\alpha$  after 12-h stimulation (Fig. 2). It was also found that LPS induced production of IL-12 by both B6- and TLR2KO-derived BMDCs, whereas FSL-1 induced production of IL-12 by only B6-derived BMDCs (Fig. 2). In contrast to TNF- $\alpha$ , both FSL-1 and LPS induced IL-12 production even after 12-h stimulation (Fig. 2). LPS also induced production of IL-10 by BMDCs derived from both types of mice, but the level of production was much lower than those of IL-12 and TNF- $\alpha$  (Fig. 2). FSL-1 did not induce production of IL-10 by BMDCs derived from either type of mice under the condition used in this experiment (Fig. 2).

After 12-h stimulation, the culture supernatants were removed and then stimulated for another 24 h as described in Materials and Methods. This method was used to avoid secondary effects of cytokines produced and LPS- or FSL-1-induced tolerance. At present, it remains unknown why BMDCs were no longer capable of producing TNF- $\alpha$  after being stimulated for 12 h with FSL-1 or LPS. There is a possibility that this phenomenon is attributed to tolerance of BMDCs induced by LPS or FSL-1. If this is the case, it is not explainable that IL-12 was produced even after 12-h stimulation. It has been known that both IL-12 and TNF- $\alpha$  are regulated by the transcription factor NF- $\kappa$ B [22]. There are other possibilities that the signaling pathway leading to the transcription of IL-12 upstream activation of NF- $\kappa$ B differs in that leading to TNF- $\alpha$  and BMDCs responsible for IL-12 production are different from those for TNF- $\alpha$  production because of heterogeneity of

BMDCs prepared in this study. Further studies are required to explain this phenomenon.

The mechanism of the antigen presentation correlates with the ability to upregulate the expression of costimulatory molecules such as CD80 (B7.1) and CD86 (B7.2) on the surfaces of the APCs. T cell activation generally requires a signal delivered via interaction of the T cell receptor (TCR) with a specific antigen on MHC molecules and a costimulatory signal. Therefore, we examined the expression of costimulatory molecules such as CD80 (B7.1) and CD86 (B7.2), IA<sup>b</sup> (MHC class II) and CD11c, a DC marker, on the surfaces of BMDCs after stimulation with FSL-1 and LPS. FSL-1 upregulated the expression of these molecules in both dose- and time-dependent manners on the surfaces of TLR2<sup>+/+</sup>-derived BMDCs, but not on the surfaces of TLR2<sup>-/-</sup>-derived BMDCs (Fig. 3A and 3B), whereas LPS upregulated the expression of CD80 (B7.1), CD86 (B7.2) and IA<sup>b</sup> (MHC class II) on the surfaces of BMDCs derived from both types of mice (Fig. 3B). In addition, it was found that the expression of CD86 (B7.2) was upregulated more strongly than that of CD80 (B7.1) (Fig. 3A and 3B). The expression of CD11c on the surfaces of TLR2<sup>+/+</sup>-derived BMDCs was upregulated by stimulation with both FSL-1 and LPS up to 12 h and then the expression was downregulated. This is in good agreement with the previous finding that CD11c is downregulated as DCs mature [10].

Judging from the previous findings that imDCs express low levels of MHC class II proteins and almost no costimulatory molecules [10], our results suggest that FSL-1 has the activity to accelerate maturation of BMDCs and that the activity of FSL-1 is mediated by TLR2.

LPS upregulated the expression of CD86 and IA<sup>b</sup> more strongly in DC derived from TLR2<sup>+/+</sup> than in TLR2<sup>-/-</sup> (Fig. 3B). This appeared to be unreasonable judging from the fact that LPS are recognized by TLR4 in combination with MD2 and CD14 [8]. However, it has previously been reported that LPS preparation is contaminated with proteinous substances that are extremely biologically active [23-28]. More recently, it was demonstrated that LPS prepared from *Porphyromonas gingivalis* LPS are contaminated with lipoproteins [29]. Therefore, the unusual

upregulation of the expression of CD86 and IA<sup>b</sup> by LPS might be explained by the possibility that BMDCs were stimulated by both LPS and the contaminated lipoproteins through both TLR2 and TLR4.

It has been reported that signaling via TLRs, especially TLR3, 4, 7 and 9, stimulates Th1 responses [11-14]. However, it is not clear whether TLRs can also induce Th2 responses. It has recently been reported that TLR2 ligands such as LP and lipopeptides are able to activate DCs to induce production of the Th2-restricted cytokine IL-10 and elicit Th2 responses *in vivo* [15-18]. Although the present finding that FSL-1 is able to activate BMDCs to produce the Th1-restricted cytokine IL-12 strongly suggests that FSL-1 induces Th1 responses, the results were obtained by *in vitro* experiments. Therefore, *in vivo* experiments should be carried out to conclusively determine whether FSL-1 is able to induce Th1 responses.

#### **4. Conclusion**

FSL-1 is capable of activating imBMDCs to produce the Th1-restricted IL-12 and to upregulate the expression of costimulatory molecules, CD80 (B7.1) and CD86 (B7.2) and IA<sup>b</sup> (MHC class II), in a TLR2-dependent manner.

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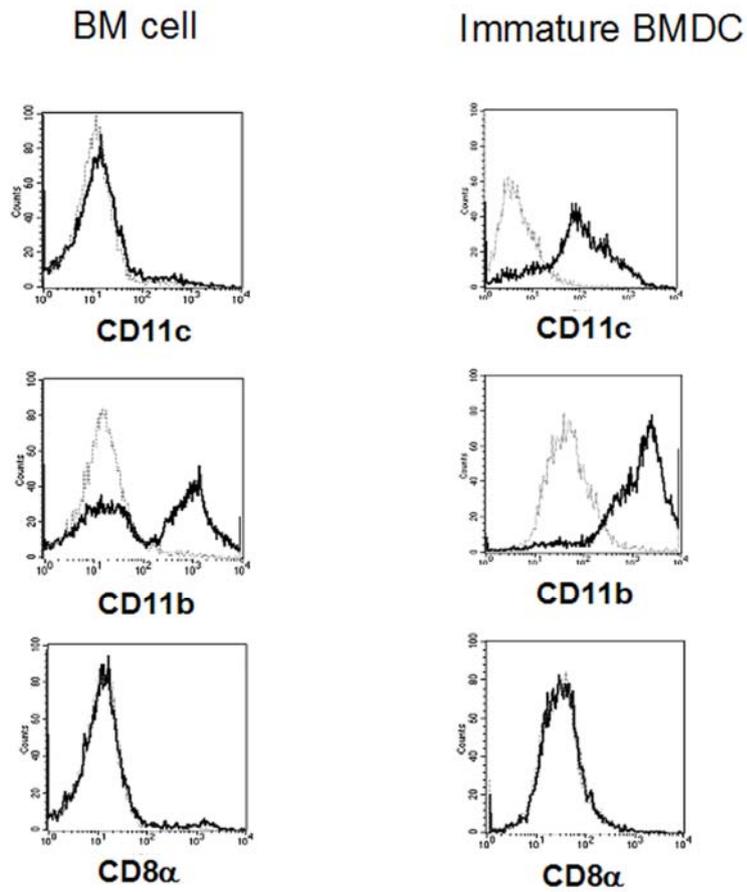
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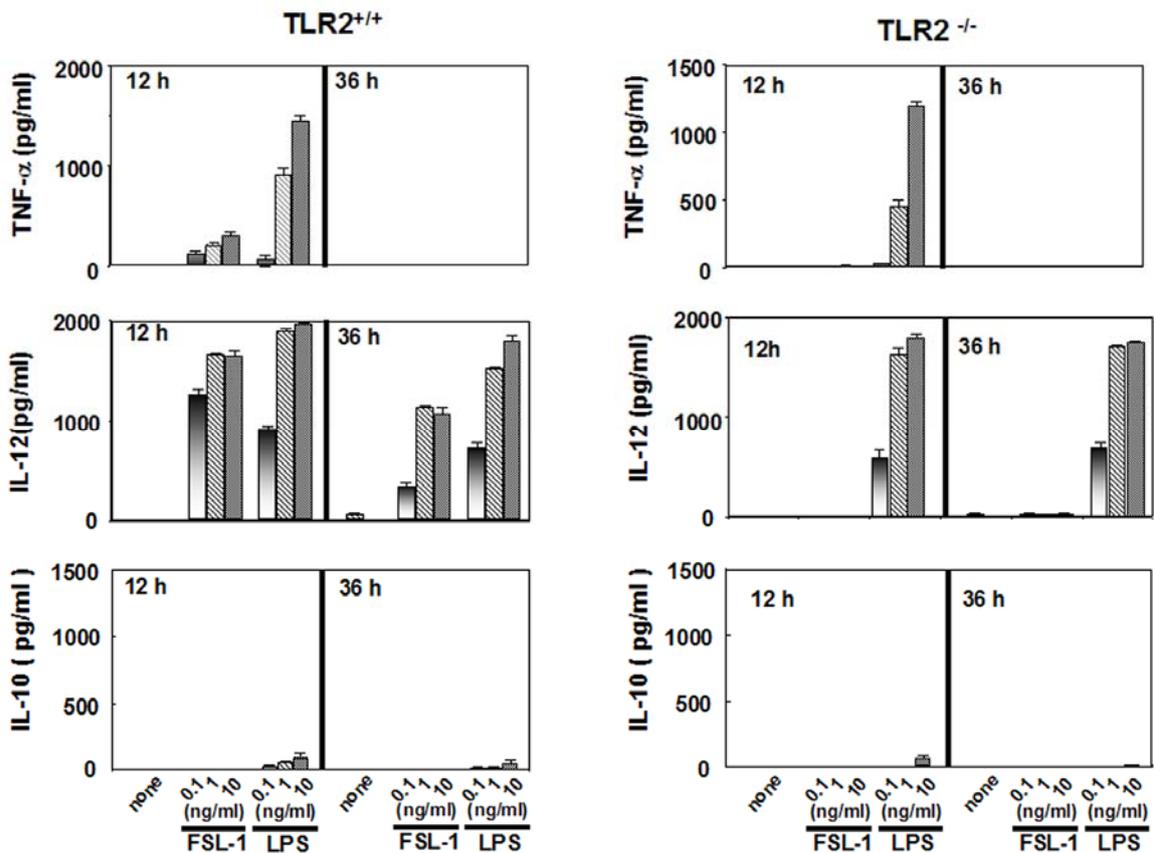
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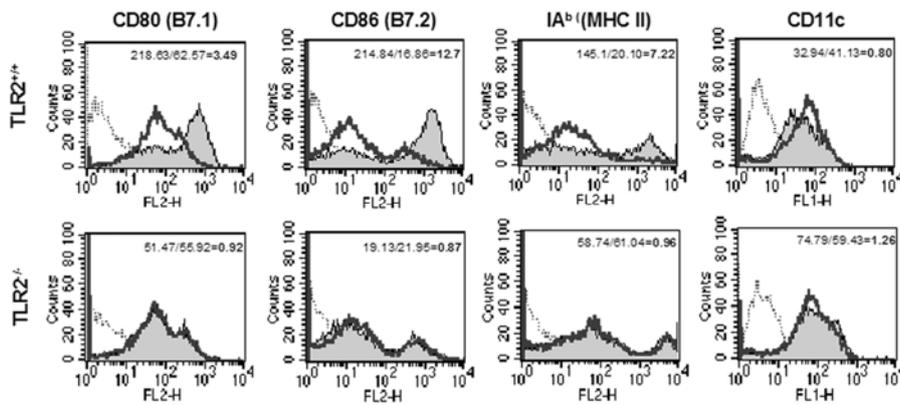


**Fig. 1.** Surface expression of CD11c, CD11b and CD8 $\alpha$  on bone marrow-derived dendritic cells differentiated by GM-CSF. Bone marrow cells (BM cells) were cultured overnight in RPMI 1640 containing 10% FBS. Nonadherent cells were harvested and cultured in the same medium supplemented with 10 ng/ml recombinant mouse GM-CSF. On day 3, the medium was changed to a medium containing 10 ng/ml GM-CSF. On day 6, nonadherent cells and loosely adherent cells were harvested and used for experiments as immature DCs.

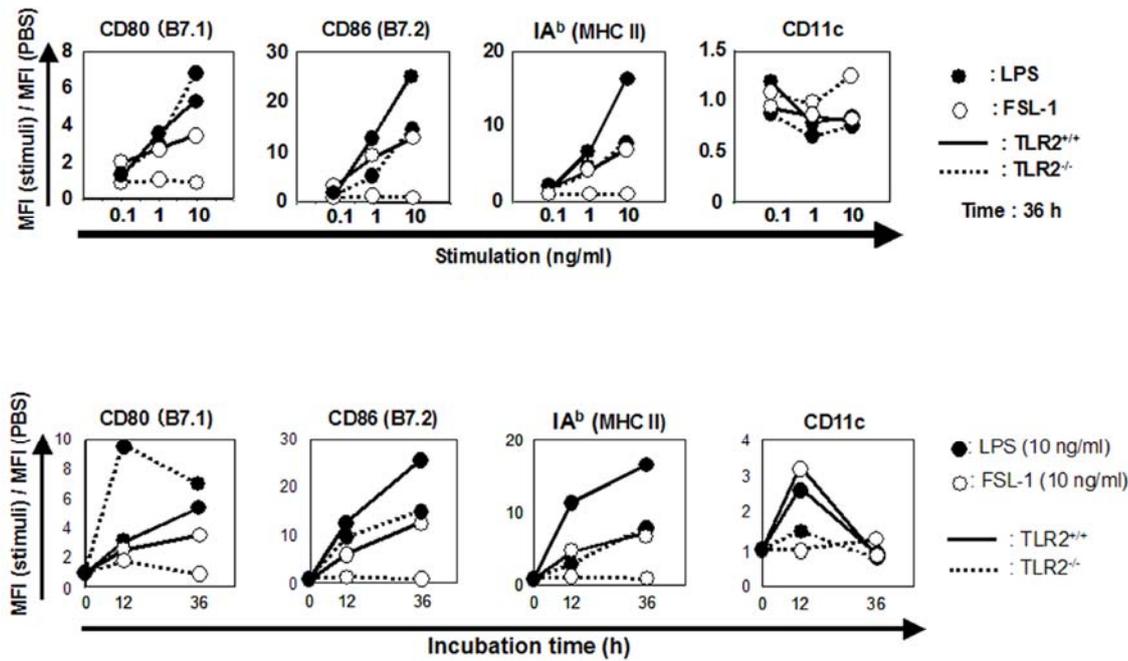


**Fig. 2.** Differential cytokine production by TLR2<sup>+/+</sup> and TLR2<sup>-/-</sup> BMDCs stimulated with FSL-1 and *E. coli* LPS. BMDCs were stimulated at 37 °C for 12 h with various concentrations of FSL-1 or *E. coli* LPS and centrifuged at 400 g for 10 min to separate the cells and culture supernatants. The cells were recultured for an additional 24 h with the same concentration of FSL-1 or LPS and centrifuged again to separate the cells and culture supernatants. Pooled culture supernatants were used for measuring TNF-α, IL-12p70 and IL-10 by ELISA. Results are representative of five experiments.

(A)



(B)



**Fig. 3.** Surface expression of CD11c, CD80 (B7.1), CD86 (B7.2) and IA<sup>b</sup> (MHC class II) on BMDCs stimulated with FSL-1 or *E. coli* LPS. BMDCs were cultured for 0, 12 and 36 h in the absence or the presence of FSL-1 or *E. coli* LPS. Cells were washed and stained with anti-mouse CD11c, CD80 (B7.1), CD86 (B7.2), IA<sup>b</sup> (MHC class II) or appropriate isotype-specific antibodies, and analyzed by flow cytometry. Data profiles were obtained after analysis of 10,000 events. Results are representative of five experiments.

(A) Representative histograms of CD11c, CD80 (B7.1), CD86 (B7.2) and IA<sup>b</sup> (MHC class II) expression on BMDCs for 36 h with medium alone (bold line), 10 ng/ml FSL-1 (filled) and isotype control (dotted line). The values in the histograms are the ratios of mean fluorescence intensities of FSL-1-stimulated BMDCs to unstimulated BMDCs.

(B) BMDCs were incubated in the presence of indicated concentration of FSL-1 or LPS for 12 h or 36 h. Shown is the ratios of mean fluorescence intensities of stimulated BMDCs to unstimulated BMDCs.