

The diacylated lipopeptide FSL-1 induces TLR2-mediated Th2 responses

Kazuto Kiura^{1,2}, Hideo Kataoka¹, Motoaki Yasuda¹, Nobuo Inoue² & Ken-ichiro Shibata¹

¹Department of Oral Pathobiological Science, Hokkaido University Graduate School of Dental Medicine, Sapporo, Japan; and ²Department of Oral Health Science, Hokkaido University Graduate School of Dental Medicine, Sapporo, Japan

Correspondence: Ken-ichiro Shibata, Department of Oral Pathobiological Science, Hokkaido University Graduate School of Dental Medicine, Nishi 7, Kita 13, Kita-ku, Sapporo 060-8586, Japan. Tel.: +8 111 706 4240, fax: +8 111 706 4901; e-mail: shibaken@den.hokudai.ac.jp

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Abstract

The diacylated lipopeptide FSL-1 enhanced the generation of IgG antibodies in TLR2^{+/+} mice, but not in TLR2^{-/-} mice, when administered together with hen egg lysozyme as an antigen. *Escherichia coli* lipopolysaccharide enhanced the generation of antigen-specific antibodies in both TLR2^{-/-} and TLR2^{+/+} mice. In TLR2^{+/+} mice, the level of enhancement due to FSL-1 was similar to that caused by lipopolysaccharide. Analysis of the IgG antibodies subclass demonstrated that the level of Th2-type IgG1 antibodies was higher than that of Th1-type IgG2a antibodies. Both FSL-1 and lipopolysaccharide induced production of IL-10 and IL-6 by splenocytes from TLR2^{+/+} mice. Lipopolysaccharide also induced production of these cytokines by splenocytes from TLR2^{-/-} mice, but FSL-1 did not. Neither FSL-1 nor lipopolysaccharide induced IL-12p70 production by splenocytes from either type of mice. FSL-1 upregulated B7.2 expression in B220⁺ cells from TLR2^{+/+} mice but not those from TLR2^{-/-} mice, whereas lipopolysaccharide upregulated B7.2 expression in B220⁺ cells from both types of mice. FSL-1 and, to a lesser extent, lipopolysaccharide activated mitogen-activated protein kinases in splenocytes. FSL-1 and, to a lesser extent, lipopolysaccharide induced the expression of c-Fos, which is known to be involved in Th2-type responses, in splenocytes. Thus, this study demonstrated that FSL-1 possessed TLR2-mediated Th2-type responses *in vivo*.

Introduction

Toll-like receptors (TLRs) mainly expressed on phagocytes such as macrophages and dendritic cells (DCs) are involved in the activation of the innate immune response by recognizing microbial conserved structures called pathogen-associated molecular patterns (PAMPs), such as LPS, bacterial lipoprotein (BLP) and lipoteichoic acid, bacterial unmethylated CpG DNA, viral RNA, and flagellin (Medzhitov *et al.*, 1997). However, activation of the innate immune response in peripheral tissues through TLRs has a limited ability to eradicate pathogens in mammals. More effective host defense in mammals is achieved with the activation of adaptive immune response, which mainly takes place in secondary lymphoid tissues such as the lymph nodes and spleen. DCs play an essential role in the immune response by communicating between the peripheral and lymphoid tissues (Banchereau and Steinman, 1998). Recognition of PAMPs by specific receptors in the DC membrane is a crucial event in the activation of DCs and initiation of adaptive immune responses (Banchereau and Steinman, 1998). The capture

of microbial antigens in peripheral tissues and migration to the draining lymph nodes is the first step in the generation of adaptive immunity. DCs play a key role in immune surveillance and have the unique capacity among antigen-presenting cells (APCs) to stimulate naïve T cells and induce differentiation of T helper 1 (Th1)- and Th2-type CD4⁺ T cells (Banchereau and Steinman, 1998; Moser and Murphy, 2000). CD4⁺ T-lymphocytes, which can differentiate into functionally distinct subsets, are a key component of the immune defense against pathogens. Th1 cells, which generate interferon- γ and IL-2, promote the cytotoxic effector functions of natural killer (NK) cells, CD8⁺ T cells and macrophages. In contrast, Th2 cells, which produce IL-4, IL-5 and IL-10, promote humoral immunity mediated by B-cell-produced IgG4 and IgE in humans and IgG1 and IgE in mice. As each of these subsets may be involved in protection against microbial pathogens or are involved in beneficial or detrimental responses in inflammatory and autoimmune diseases, it is critical to understand the mechanisms involved in Th1/Th2 differentiation.

Release of cytokines upon recognition of microorganisms is one of the most important effects of TLR activation. There is strong evidence for an important role of TLRs in driving Th1 responses through stimulation of IL-12p70 and IFN- γ release from DCs. In this respect, the activation of TLR4 by LPS and TLR9 by CpG DNA induces strong Th1 responses through IL-12p70 release (Barton and Medzhitov, 2002), and IFN- γ production by TLR3, TLR4, TLR7, and TLR9 is thought to be an important driving force of TLR-mediated Th1 responses (Pulendran, 2004). These data suggest that TLRs control Th1 differentiation, whereas Th2 responses are generated in the absence of TLR-mediated signals (Schnare *et al.*, 2001). However, it has recently been reported that TLR2 stimulation induces the release of only small amounts of IL-12p70 (Dillon *et al.*, 2004) and that TLR2-mediated signals preferentially induce a Th2 profile in DCs (Dillon *et al.*, 2004). Consistent with a preferential role of TLR2 in the activation of Th2 responses, the triacylated lipopeptide (LPT) Pam₃Cys as a TLR2 ligand activates CD11c⁺ CD11b⁺ DCs to induce the Th2-type cytokine IL-10 (Dillon *et al.*, 2004). Thus, activation of DCs via TLRs do not always result in Th1 responses (Medzhitov and Janeway, 2000) but can also induce a skew toward Th2 responses. However, we have recently demonstrated that mycoplasmal diacylated LPT FSL-1, which is known to activate macrophages, monocytes and fibroblasts (Shibata *et al.*, 1997; Shibata *et al.*, 2000), activates bone marrow-derived dendritic cells (BMDCs) to induce production of the Th1-type cytokine IL-12p70 in a TLR2-dependent manner (Kiura *et al.*, 2006). We were therefore interested in knowing which type of immune response, Th1 or Th2, is induced by FSL-1 *in vivo*. To address this question, FSL-1 and *Escherichia coli* LPS for a comparative study were injected into mice together with hen egg lysozyme (HEL) as an antigen, and then subclasses of IgG antibodies produced and cytokine profiles produced by spleen cells in response to FSL-1 were investigated in this study. This study demonstrated that the diacylated LPT FSL-1 possesses TLR2-mediated adjuvant activity to induce Th2-type responses *in vivo* and that IL-10, mainly produced by CD11b⁺, CD11b⁻ or B220⁺ cells existing in the spleen, plays an important role in the induction.

Materials and methods

Reagents and antibodies (Abs)

HEL was purchased from Wako Pure Chemicals (Tokyo, Japan). Kits of Cytotfix/Cytoperm and the mouse inflammation cytometric bead array (CBA), GolgiStop, FITC-conjugated monoclonal antibodies (mAbs) against CD11c, CD11b, B220 and CD8 α , PE-conjugated mAbs against B7.1, B7.2, I-A^b, and IL-10 and appropriate isotype controls were purchased from BD Pharmingen (San Diego, CA).

RPMI1640 medium, penicillin/streptomycin and *Escherichia coli* LPS O55:B5 were purchased from Sigma (St Louis, MO). FSL-1 [S-(2,3-bispalmitoyloxypropyl) Cys-Gly-Asp-Pro-Lys-His-Pro-Lys-Ser-Phe, Pam₂CGDHPKPKSF] was synthesized as described previously (Shibata *et al.*, 2000) and originally dissolved in PBS containing 10 mM n-octyl- β -glucopyranoside (OG) in PBS and diluted with PBS to reduce the OG concentration to less than 0.5 mM when used for stimulation as described previously (Okusawa *et al.*, 2004). All of the mAbs to mouse MAPKs (phospho-specific p38, p38 α , phospho-specific JNK and JNK1/2, phospho-specific ERK1/2 and ERK1) were obtained from BD Pharmingen. Anti-c-Fos Ab was purchased from Santa Cruz Biotechnology (Santa Cruz, CA).

All of the other chemicals were obtained from commercial sources and were of analytical or reagent grade.

Mice

Sex-matched 8-week-old C57BL/6 mice (TLR2^{+/+} mice) were purchased from Japan Clea (Tokyo, Japan). TLR2^{-/-} mice of the same genetic background were kindly provided by Dr S. Akira, Department of Host Defense, Research Institute for Microbial Diseases, Osaka University (Suita, Osaka, Japan). All mice were maintained in specific pathogen-free conditions at the animal facility of Hokkaido University, and all experiments were approved by the regulations of Hokkaido University Animal Care and Use Committee.

Immunization

Groups of six mice each of TLR2^{+/+} or TLR2^{-/-} were intraperitoneally (i.p.) or intranasally (i.n.) immunized on days 1, 7, 14 and 21 with 1 mg of HEL in the absence or presence of 1 μ g of *E. coli* LPS or 1 μ g of FSL-1, which was dissolved in PBS. Two hundred and fifty or 30 μ L of the solution was injected i.p. or i.n. into the mice, respectively. Blood was collected at different time points (days 6, 13, 20 and 27) after immunization from each mouse by the tail artery and was allowed to clot at 37 °C for 1 h, and then clots were removed by centrifugation at 500 g for 10 min. Sera were stored at -70 °C prior to determination of HEL-specific Abs.

Enzyme-linked immunosorbent assay (ELISA)

ELISA was performed for measurement of total immunoglobulin G (IgG), IgG1 and IgG2a in sera. Each well of a 96-well flat-bottomed plate (MaxSorp, Nunc-Immuno) was coated overnight at 4 °C with 100 μ L of HEL dissolved in carbonate-bicarbonate buffer (pH 10.0) at a concentration of 1 μ g mL⁻¹. The plate was washed twice with PBS containing 0.05% Tween 20, and then blocked at room temperature

(RT) for 2 h with PBS containing 5% (w/v) skimmed milk. The plate was then washed twice with PBST, and then 100 μ L of 10-fold serial dilutions of sera samples was added to each well and the plate was incubated at RT for another 1 h. Following that, the plate was washed four times with PBST and incubated at RT for 1 h with a 1:3000 dilution of peroxidase-conjugated rabbit antimouse IgG. Then the plate was washed four times and color development was achieved by the addition of 100 μ L of a 1:1 mixture of chromogen (TMB) and substrate (H_2O_2) to each well. After incubation at 37 °C for 15 min, the reaction was stopped by adding 100 μ L of 1.8 N sulfuric acid per well. The optical densities at 450 nm were measured using a microplate reader (Bio-Rad, Heidelberg, Germany). Ab titers are expressed as the reciprocal of the geometric mean end point titer. Endpoint titers were expressed as the reciprocal of the last dilution, which gave an optical density at 450 nm of 0.2 U above the values of the negative controls.

Measurement of TNF- α in sera

Groups of six mice each were i.p. immunized with 1 mg of HEL in the absence or presence of 1 μ g of LPS or 1 μ g of FSL-1. Sera were prepared from blood collected at different time points after immunization from each mouse by the tail artery. Sera were stored at -70 °C until used. TNF- α was detected using an ELISA kit (BD PharMingen). The range of detection was 15.6–1000 pg mL⁻¹.

Cytokine determination. Spleen cell suspensions were prepared by depletion of tissue fragments by passage through fine nylon mesh. The suspensions were washed twice with RPMI 1640 medium without serum and then resuspended in RPMI 1640 medium supplemented with 10% FBS, penicillin (100 U mL⁻¹) and streptomycin (100 μ g mL⁻¹). The cells (1×10^6 cell per well) were cultured at 37 °C for 15, 48, 72 and 96 h with or without various concentrations of LPS (10, 100 or 1000 ng mL⁻¹) or FSL-1 (1, 10 or 100 ng mL⁻¹) in a 96-well flat-bottomed plate in a humidified 5% CO₂ atmosphere. Culture supernatants were collected by centrifugation at 500 g for 10 min and stored at -70 °C until used. IL-6, IL-10, TNF- α , MCP-1, IL-12p70 and IFN- γ were detected using a mouse inflammation CBA kit (BD PharMingen). Briefly, 50 μ L of each sample was mixed with 50 μ L of mixed capture beads and 50 μ L of PE-conjugated antimouse Abs to IL-6, IL-10, TNF- α , MCP-1, IL-12p70 and IFN- γ . The samples were incubated at RT for 3 h in the dark. After washing once, the samples were resuspended in 300 μ L of wash buffer before acquisition on the a FACS Calibur (BD Biosciences, Sunnyvale, CA). Data were analyzed using CBA software (BD PharMingen). Standard curves were generated for each cytokine using the mixed cytokine standard provided with the kit. The concentration for each cytokine in cell supernatants was deter-

mined by interpolation from the corresponding standard curve. The range of detection was 20–5000 pg mL⁻¹ for each cytokine measured by CBA.

B cell isolation. B cells were isolated from spleen cells using a B cell isolation kit (Miltenyi Biotec, San Diego, CA) according to the manufacturers' instructions. Briefly, non-B cells were labeled with a cocktail of biotin-conjugated mAbs against CD43 (Ly-48), CD4 (L3T4) and Ter-119 as a primary labeling reagent and antibiotin mAbs conjugated to MicroBeads as a secondary labeling reagent. The magnetically labeled non-B cells were depleted by retaining them on a MACS (Magnetic Cell Sorting) column in the magnetic field of a MACS separator, while the unlabeled B cells passed through the column.

FACS analysis of intracellular cytokines

Spleen cells were plated in a 24-well plate in RPMI 1640 containing 10% FBS. Spleen cells were stimulated at 37 °C for 43 h with FSL-1 (100 ng mL⁻¹) or *E. coli* LPS (100 ng mL⁻¹). The cells were incubated for 5 h with a GolgiStop solution (BD Pharmingen). The cells (10^6 cells in 200 μ L of PBS with 1% BSA) were incubated on ice with antimouse CD32/CD16 (Fc γ II/III receptor). After 30 min incubation, the cells were washed and resuspended in 200 μ L of PBS with 1% BSA and incubated with FITC-conjugated mAb against CD11c, CD11b, B220 or CD8 α and appropriate isotype controls (BD Pharmingen). The cells were fixed and permeabilized using a Cytofix/Cytoperm kit (BD Pharmingen). After washing, cells were incubated with a PE-conjugated mAb against IL-10 and appropriate isotype controls (BD Pharmingen). Data for 30 000 cells falling within appropriate forward and side light scatter gates were collected from each sample with a FACS Calibur (BD Biosciences, Sunnyvale, CA). Data were analyzed using CellQuest software (BD Biosciences).

Flow cytometry

Spleen cells were plated in a 24-well plate in RPMI 1640 containing 10% FBS. The cells were stimulated at 37 °C for 15 h with FSL-1 (100 ng mL⁻¹) or *E. coli* LPS (100 ng mL⁻¹). The cells (10^6 cells in 200 μ L of PBS with 1% BSA) were incubated on ice with antimouse CD32/CD16 (Fc γ II/III receptor). After 30 min incubation, the cells were washed and resuspended in 200 μ L of PBS with 1% BSA and incubated with FITC-conjugated mAb against CD11c, CD11b, B220 or CD8 α and PE-conjugated mAbs against B7.1, B7.2 or I-A^b (MHC class II) and appropriate isotype controls (BD Pharmingen). The cells were fixed in 0.1 M phosphate buffer containing 0.5% formaldehyde and then analyzed by a FACS Calibur (BD Bioscience). Data for 30 000 cells falling within appropriate forward and side light

scatter gates were collected from each sample and analyzed using CellQuest software (BD Bioscience).

Western blotting

Spleen cells from TLR2^{+/+} mice were stimulated for 20, 40, 60 and 120 min with either LPS (1 µg mL⁻¹) or FSL-1 (1 µg mL⁻¹) and lysed by a SDS sample buffer. Proteins in the cell lysates were separated by electrophoresis on 12% SDS-polyacrylamide gels and transferred to a nitrocellulose membrane. The membrane was incubated at 4 °C overnight with mAbs to phosphorylated and nonphosphorylated ERK1/2, SPAK/JNK, p38 (BD Pharmingen) or c-Fos (Santa Cruz Biotechnology). Bands were visualized with a secondary horseradish peroxidase (HRP)-conjugated Ab. Immunoreactive proteins were detected by the use of ECL detection reagents (Amersham Pharmacia Biotech., Place, UK).

Results

Upregulation of the production of Ab against HEL by co-administration of FSL-1

Evidence indicating that microbial LPTs act as effective adjuvants in parenteral and mucosal immunization has been accumulating (Rharbaoui *et al.*, 2002; Redecke *et al.*, 2004). In this study, we first evaluated the adjuvant activity of FSL-1 to stimulate humoral immune responses by determining the serum titers of Ab against HEL in immunized mice, since we have recently found that FSL-1 activates macrophages, fibroblasts and BMDCs to produce inflammatory cytokines (Shibata *et al.*, 1997; Shibata *et al.*, 2000; Kiura *et al.*, 2006) and is recognized by TLR2. Therefore, experiments using both TLR2^{+/+} and TLR2^{-/-} mice were also carried out to determine whether TLR2 is involved in expression of the adjuvant activity of FSL-1 to enhance anti-HEL Ab. It was found that FSL-1 enhanced the generation of anti-HEL Ab in TLR2^{+/+} mice, but not in TLR2^{-/-} mice, when administered i.p. (Fig. 1a) and i.n. (Fig. 1b) together with HEL. In TLR2^{+/+} mice, the level of enhancement was similar to that obtained by coadministration of LPS (Figs 1a and b) which is a well-known microbial adjuvant (Wiedemann *et al.*, 1991). The level of anti-HEL IgG Ab in TLR2^{+/+} mice obtained by coadministration of FSL-1 increased immediately after the second immunization and reached maximum after the third immunization, whereas such an increase was not observed in TLR2^{-/-} mice (Figs 1a and b). However, LPS accelerated the generation of anti-HEL IgG Ab in both TLR2^{-/-} and TLR2^{+/+} mice. These results demonstrated that FSL-1 possessed adjuvant activity to enhance humoral immune responses mediated by TLR2.

Most immune responses are regulated by the activity of two functionally polarized T helper cell types, Th1 and Th2 cells. In order to determine which type of immune response,

Th1 or Th2, is induced by FSL-1 *in vivo*, we analyzed the subclass distribution of the HEL-specific IgG in the sera of TLR2^{+/+} and TLR2^{-/-} mice immunized with HEL in the presence of FSL-1 or LPS. In mice, the production of IgG2a and IgG1 reflects Th1-type and Th2-type responses, respectively. As shown in Fig. 1, the level of IgG1 Ab was found to be higher than that of IgG2a regardless of the immunization route, i.p. or i.n. This finding suggests that both FSL-1 and LPS are capable of inducing a Th2 response *in vivo*.

Toxicity of FSL-1

As stated above, FSL-1 possessed adjuvant activity, the level of which was similar to that of LPS. It is well known that LPS plays a role in the induction and pathology of septic shock. LPS does not injure host tissues directly but, rather, through the actions of various inflammatory mediators induced by exposure to LPS (Beutler *et al.*, 1985). Of these mediators, TNF-α has been the most extensively studied and is thought to be considered a central mediator of septic shock (Beutler *et al.*, 1985). Therefore, we carried out an experiment to determine whether FSL-1 induces TNF-α in sera after immunization i.p. together with HEL. It was found that LPS induced production of serum TNF-α in both TLR2^{+/+} and TLR2^{-/-} mice immunized with LPS, and the production peaked at 1 h after immunization (Fig. 2), whereas FSL-1 failed to induce TNF-α, even in TLR2^{+/+} mice. These findings suggest that the *in vivo* toxicity of FSL-1 to mice is significantly lower than that of LPS despite the same levels of adjuvant activity.

Activation of spleen cells by FSL-1

The finding that FSL-1 possessed the TLR2-mediated adjuvant activity suggests that FSL-1 is capable of activating antigen-presenting cells (APCs) such as macrophages, DCs and B cells. Therefore, experiments were carried out to determine whether FSL-1 is capable of activating spleen cells derived from TLR2^{+/+} and TLR2^{-/-} mice, since spleen cells contain various types of APCs. It was found that FSL-1 was capable of upregulating TNF-α production by spleen cells derived from TLR2^{+/+} mice, but not those from TLR2^{-/-} mice (Fig. 3), whereas LPS upregulated TNF-α production by spleen cells from both types of mice (Fig. 3). These results demonstrated that FSL-1 activated spleen cells in a TLR2-dependent manner.

Depending on the profiles of cytokines secreted during the interaction between DCs and T cells, the immune response can polarize toward either a cellular or a humoral response mediated by Th1 or Th2 cells, respectively. IL-12 promotes the induction of a Th1-type response and inhibits the induction of a Th2 type response. Conversely, IL-10 is critical for the induction of a Th2-type response and inhibits the induction of a Th1-type response. In addition, IL-10 can

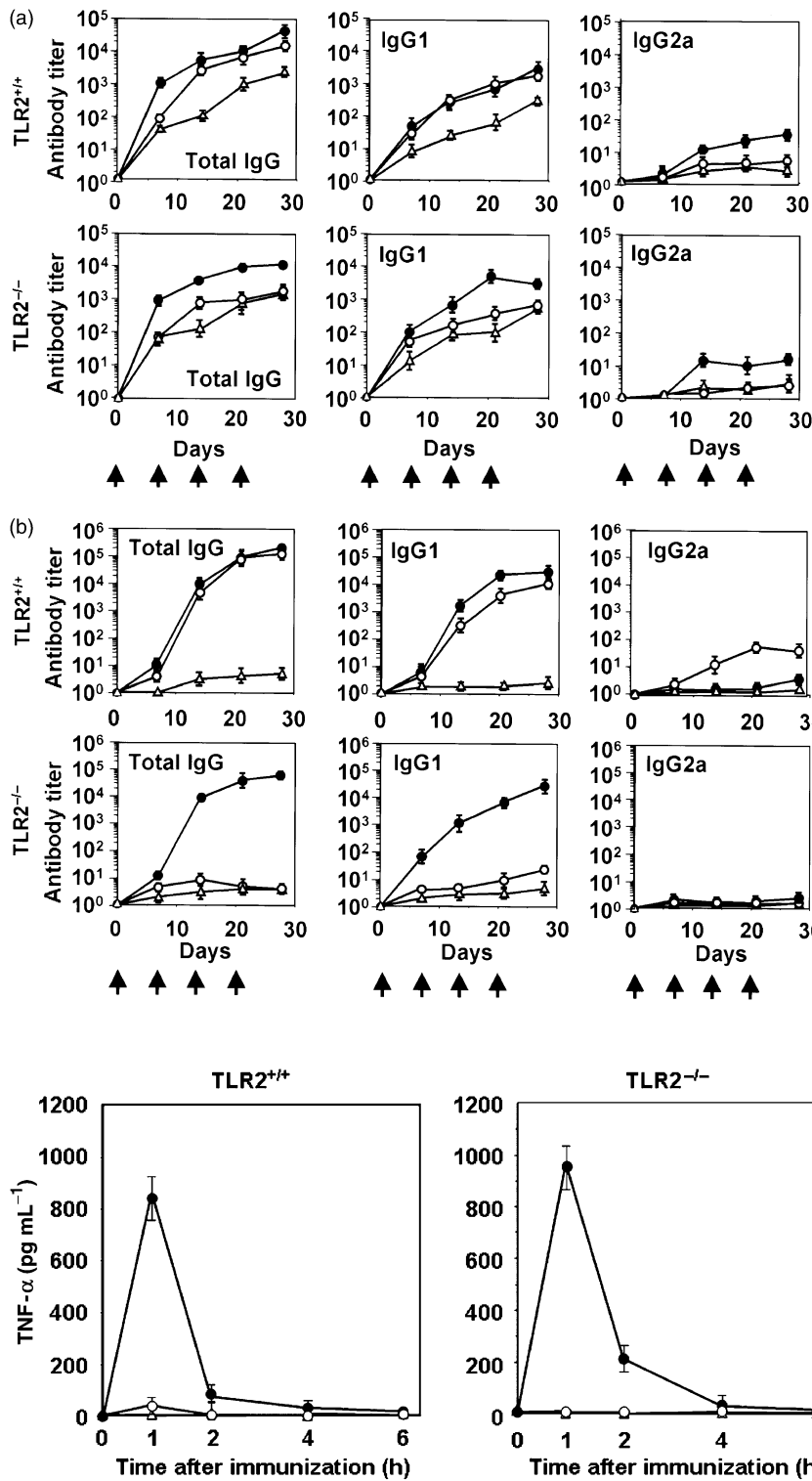


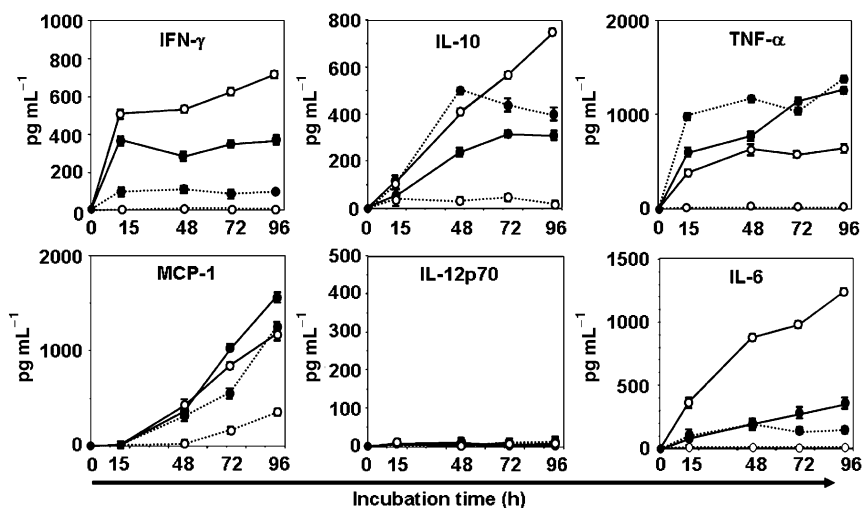
Fig. 1. Kinetics of HEL-specific total IgG, IgG1 or IgG2a antibodies in sera of mice immunized with FSL-1 or LPS. Groups of six mice, each of TLR2^{+/+} or TLR2^{-/-}, were immunized i.p. (a) or i.n. (b) on days 0, 6, 13 and 20, which are shown by arrows, with 1 mg HEL alone (Δ), HEL plus 100 ng or 1 μ g LPS (\bullet) or HEL plus 100 ng or 1 μ g FSL-1 (\circ). Ab titers were measured by ELISA as described in Materials and methods. See text for details. Ab titers are expressed as the reciprocal of the geometric mean end point titer. Endpoint titers were expressed as the reciprocal of the last dilution, which gave an optical density at 450 nm of 0.2 U above the values of the negative controls. This is a representative data obtained by 1 μ g mL⁻¹ of LPS and FSL-1. Results are expressed as the means and standard deviations of three determinations.

Fig. 2. Kinetics of TNF- α production in sera of mice immunized with FSL-1 or LPS together with HEL. Groups of six mice each of TLR2^{+/+} or TLR2^{-/-} were immunized with HEL alone (Δ), HEL plus 100 ng or 1 μ g LPS (\bullet) or HEL plus 100 ng or 1 μ g FSL-1 (\circ). Sera were collected at different time points (1, 2, 4, and 6 h) and assayed for TNF- α secretion by ELISA as described in Materials and methods. See text for details. This is a representative data obtained by 1 μ g mL⁻¹ of LPS and FSL-1. Results are expressed as the means and standard deviations of three determinations.

also drive the development of regulatory T cells (Treg) (Sakaguchi, 2000), the main function of which is thought to be the maintenance of tolerance to self-antigens. In contrast, Treg should not interfere with the induction of

pathogen-specific protective immune responses. One mechanism that allows the activation of pathogen-specific T cells in the presence of Treg is a TLR-mediated block of suppression. This mechanism is mediated by IL-6 produced

Fig. 3. Profiles of production of various cytokines by spleen cells in response to FSL-1 and LPS. Spleen cells from TLR2^{+/+} (—) or TLR2^{-/-} (---) mice were stimulated with for 15, 48, 72 and 96 h with LPS (100 ng mL⁻¹) (●) or FSL-1 (100 ng mL⁻¹) (○) and supernatants were collected by centrifugation at 400 g for 10 min. The amounts of these cytokines produced in supernatant were measured by CBA as described in Materials and methods. This is a representative data obtained by 100 ng mL⁻¹ of FSL-1, although spleen cells were stimulated with 1, 10 or 100 ng mL⁻¹ of FSL-1. See text for details. Results are expressed as the means and standard deviations of three determinations.



by DCs in response to TLR ligands (Pasare and Medzhitov, 2003). A series of experiments was therefore carried out to characterize the type of Th responses induced by stimulation with FSL-1 or LPS. First, profiles of cytokines produced by spleen cells containing several DC subsets in response to FSL-1 or LPS were determined. As shown in Fig. 3, both FSL-1 and LPS induced production of IL-10 and IL-6 by spleen cells from TLR2^{+/+} mice. LPS also induced production of IL-10 and IL-6 by spleen cells from TLR2^{-/-} mice, but FSL-1 did not (Fig. 3). Moreover, the production levels of both cytokines induced by FSL-1 were higher than those induced by LPS (Fig. 3). However, neither FSL-1 nor LPS induced IL-12p70 in the cells. Monocyte chemoattractant protein-1 (MCP-1) has been shown to suppress IL-12 secretion by monocytes/macrophages and to be associated with Th2 development (Matsukawa *et al.*, 2000).

Taken together, these results suggest that both FSL-1 and LPS are able to induce Th2-type responses under the experimental conditions used in this study.

Subpopulations of spleen cells responsible for IL-10 production

As described above, FSL-1 induced production of Th2 cytokines, IL-10 and IL-6, by spleen cells. Therefore, experiments were performed to determine what cell populations in spleen cells produce IL-10 in response to FSL-1 or LPS. The mice spleen contains several DC subsets, such as CD8 α ⁺ DCs (CD11c⁺ CD11b⁻ B220⁻), CD8 α ⁻ DCs (CD11c⁺ CD11b⁻ B220⁻) and plasmacytoid DCs (CD11c^{dull} CD11b⁻ B220⁺). Proportions of IL-10-producing cells were determined by FCM analysis after intracellular IL-10 staining in conjunction with cell surface CD11c, CD11b, B220 or CD8 α staining. Stimulation with FSL-1 or LPS generated a significant increase in proportions of IL-10-producing

CD11b⁺ cells, CD11b⁻ cells and B220⁺ cells compared with proportions of unstimulated cells (Fig. 4).

B cells characterized by cell surface expression of B220 are important lymph node APCs normally responsible for activating secondary immune responses and producing Abs. Therefore, B cells were purified from spleen cells of TLR2^{+/+} mice by MACS and stimulated with FSL-1 or LPS. It was found that B cells produced a large amount of IL-10 in response to these stimuli (Fig. 5). This finding suggests that IL-10 produced by splenic B cells in response to FSL-1 or LPS may play a crucial role in the shift toward Th2-type responses.

Upregulation of cell surface expression of costimulatory molecules on spleen cells by FSL-1

The adjuvant activity correlates with the ability to upregulate the expression of costimulatory molecules such as B7.1 and B7.2 on the surface of APCs. T cell activation generally requires at least two signals delivered via interaction of the T cell receptor (TCR) with a specific antigen on an MHC molecule and via interaction of CD28 with the costimulatory molecules B7.1 and B7.2. Therefore, we carried out an experiment to determine whether FSL-1 induced upregulation of MHC class II molecules as well as B7.1 and B7.2 on CD11c⁺, CD11b⁺, B220⁺ or CD8 α ⁺ spleen cells. It was found that FSL-1 selectively induced upregulation of B7.2 expression on the surface of B220⁺ cells from TLR2^{+/+} mice, but not those from TLR2^{-/-} mice, whereas LPS induced B7.2 on the surface of B220⁺ cells from both types of mice (Fig. 6a). Moreover, FSL-1 induced upregulation of both B7.1 and B7.2 expression on the surface of CD11b⁺ cells from TLR2^{+/+} mice, but not those from TLR2^{-/-} mice, whereas LPS induced upregulation of both B7.1 and B7.2 on the surface of CD11b⁺ cells from both types of mice

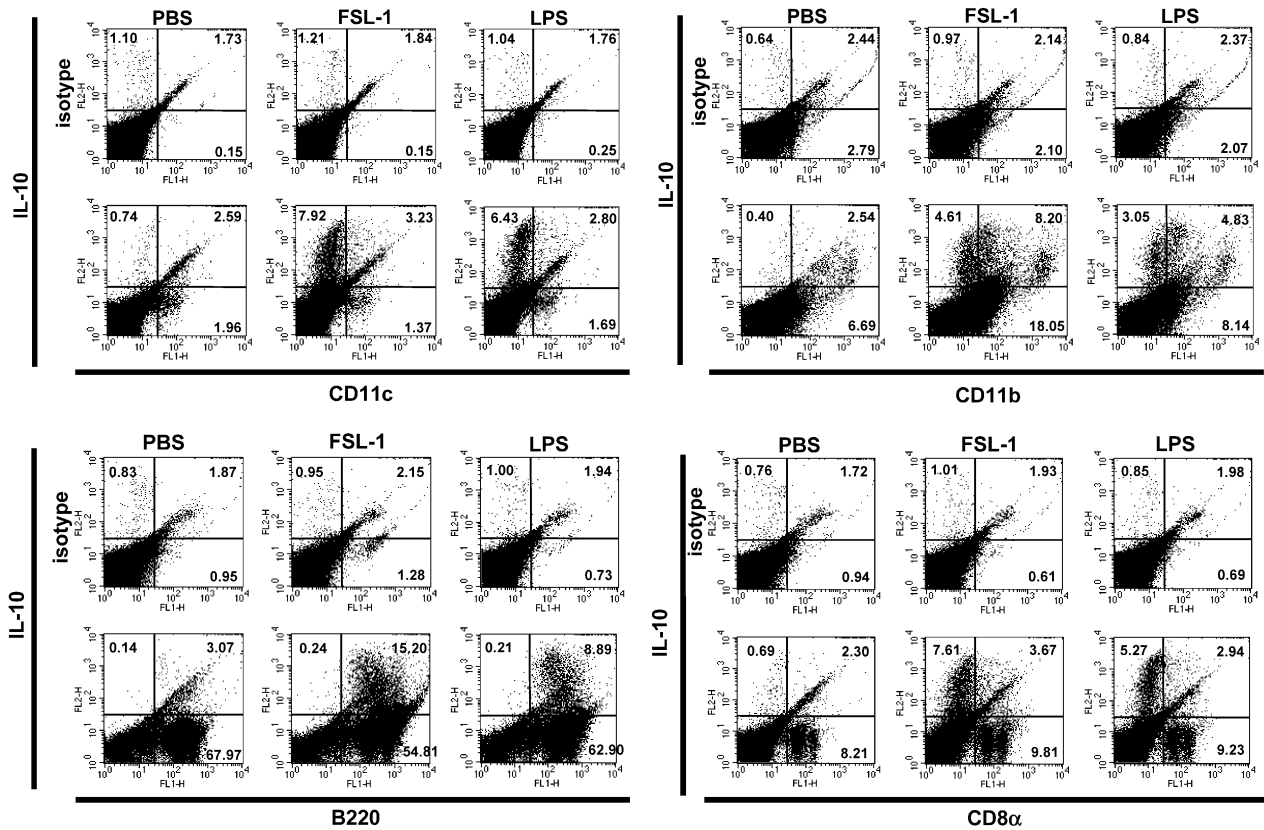


Fig. 4. IL-10 production by various subpopulations in spleen cells in response to FSL-1 and LPS. Spleen cells from TLR2^{+/+} mice were stimulated for 43 h with FSL-1 (100 ng mL⁻¹) or LPS (100 ng mL⁻¹), and then were incubated for 5 h with GolgiStop reagent, followed by being stained with anti-IL-10 Ab and Abs to CD11c, CD11b, CD8 α or B220. See text for details. FACS data expressed as dot plots show intracellular expression of IL-10 in CD11c⁺, CD11b⁺, B220⁺ or CD8 α ⁺ cells. The percentages of cells that were positive for both the phenotype marker and intracellular IL-10 are indicated in the top right quadrant. The result is a representative of three experiments.

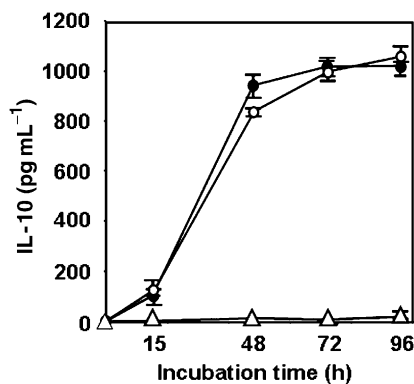


Fig. 5. IL-10 production by splenic B cells isolated from TLR2^{+/+} mice in response to FSL-1 and LPS. B cells isolated from spleen cells of TLR2^{+/+} mice by MACS were incubated for 15, 48, 72 and 96 h with LPS (100 ng mL⁻¹) (●), FSL-1 (100 ng mL⁻¹) (○) or PBS (Δ). IL-10 production in supernatants was measured by ELISA as described in Materials and methods. See text for details. The result is a representative of three experiments.

(Fig. 6b). Neither LPS nor FSL-1 upregulated B7.1, B7.2 and MHC class II on the surface of CD11c⁺ cells or CD8 α ⁺ cells from TLR2^{+/+} and TLR2^{-/-} mice (data not shown).

Activation of ERK, JNK, and p38 MAP kinase in spleen cells by FSL-1

We focused on the MAP kinase (MAPK) signaling pathway, one of the most ancient signal transduction pathways in mammalian cells. MAPK consists of three major groups, p38, ERK1/2, and JNK1/2. Previous studies indicated a critical role of MAPK in regulating Th1/Th2 balance in T cells, and emerging evidence suggests a role of MAPK in regulating cytokine production from APCs (Yi *et al.*, 2002). We therefore examined whether p38, ERK1/2, and JNK1/2 are activated in spleen cells from TLR2^{+/+} mice in response to FSL-1 or LPS. FSL-1 induced the activation of ERK1/2 and JNK1/2 20 min after stimulation. LPS also induced the activation, but the level of activation by LPS was significantly lower than that of by FSL-1. FSL-1 and LPS appeared

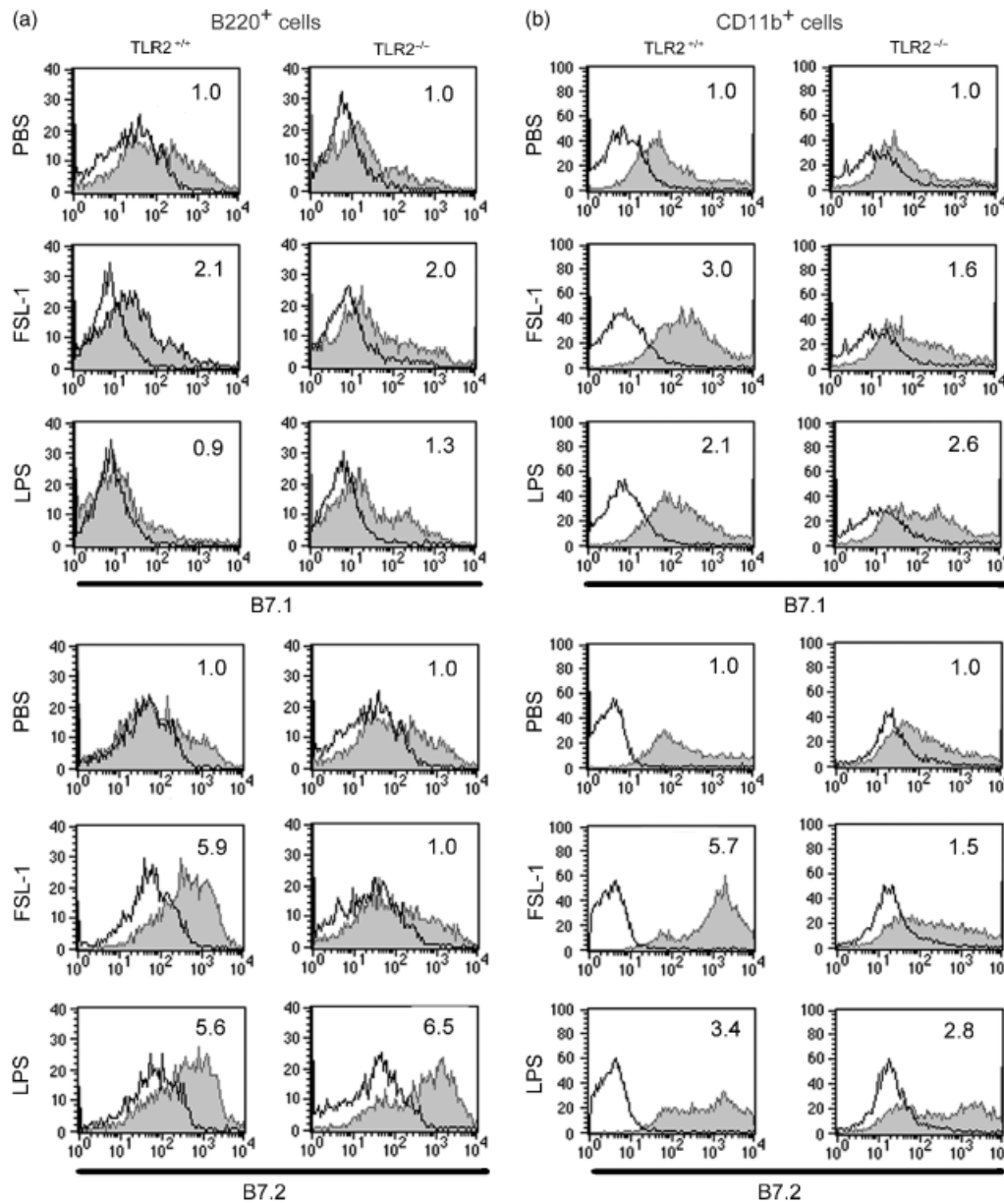


Fig. 6. Expressions of B7.1 and B7.2 in B220⁺ or CD11b⁺ spleen cells in response to FSL-1 or LPS. Spleen cells derived from TLR2^{+/+} and TLR2^{-/-} mice were stimulated for 15 h in the absence (PBS) or the presence of FSL-1 (100 ng mL⁻¹) or LPS (100 ng mL⁻¹). The cells were stained with Abs to B220 (a) or CD11b (b) and then with Abs to B7.1 and B7.2 (filled) or isotype Abs (solid line). The values in the histograms are the ratios of mean fluorescence intensities of stimulated cells stained with anti-B7.1 or anti-B7.2 mAb to that of non-stimulated cells. The result is a representative of three experiments.

to enhance the activation of p38 MAPK 20 min after stimuli, although p38 was activated in the absence of these stimuli (Fig. 7).

Expression of c-Fos in spleen cells by FSL-1

AP-1 is one of the transcription factors activated by the different MAPK pathways. AP-1 consists of heterodimers between the nuclear protooncogenes of the Jun and Fos

families of transcription factors and homodimers between different Jun proteins. It has recently been suggested that enhanced ERK signaling results in the phosphorylation and stabilization of the immediate early gene product c-Fos, in a fibroblast cell line (Murphy *et al.*, 2002). In fact, ERK phosphorylation appears to be essential for enhanced c-Fos expression (Murphy *et al.*, 2002; Dillon *et al.*, 2004). Therefore, we carried out an experiment to determine whether the expression of c-Fos was induced in spleen cells

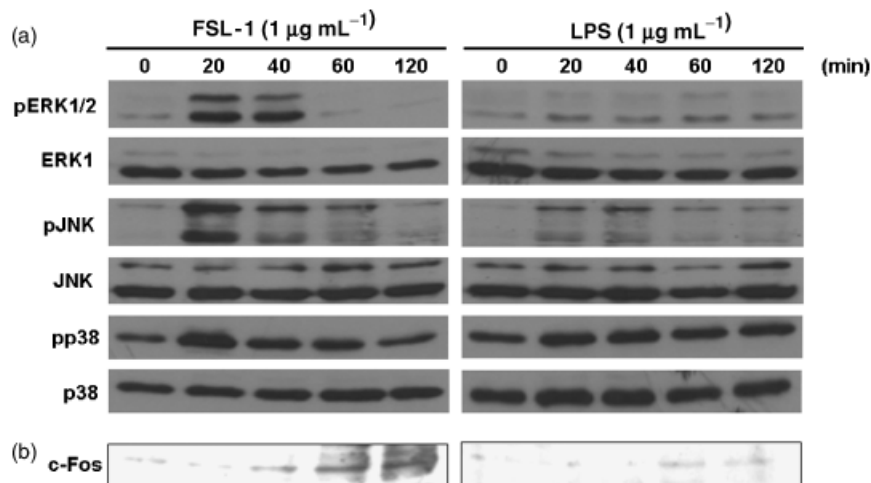


Fig. 7. Activation of MAPK and c-Fos in spleen cells in response to FSL-1 and LPS. Spleen cells from TLR2^{+/+} were stimulated for 20, 40, 60 and 120 min with or without FSL-1 (1 µg mL⁻¹) or LPS (1 µg mL⁻¹). The cells were lysed in an SDS sample buffer and the proteins were separated by SDS-PAGE (12%), followed by staining with Abs to either phosphorylated or nonphosphorylated MAPK (a) and c-Fos (b). The result is a representative of three experiments.

from TLR2^{+/+} mice in response to FSL-1 or LPS. The expression of c-Fos in spleen cells was significantly upregulated by FSL-1, but not by LPS (Fig. 7b).

Discussion

Microbial components that activate the host immune system have been designated as adjuvants. Adjuvants have often been used for immunization with a pure antigen for potential induction of antibody production, cytolytic T lymphocytes, and activation of NK cells (Wiedemann *et al.*, 1991; Azuma and Seya, 2001). The role of adjuvants in effective immune potentiation had not been identified at the molecular level until TLRs were discovered in mammals (Medzhitov *et al.*, 1997). The majority of adjuvants are microbial components such as LPS and bacterial CpG-DNA as ligands for TLRs. The recognition of microbes by innate immune cells initiates activation of the whole immune system. TLRs expressed on many types of cells, including macrophages and DCs, are known to recognize various components of invading pathogens. The recognition of invading microbes by TLRs on DCs induces proinflammatory cytokine production, enhances antigen presentation to naive T cells and finally activates antigen-specific adaptive immune responses. Thus, numerous lines of evidence have been indicated that TLRs are adjuvant receptors that bridge between innate immunity and adaptive immunity (Kaisho and Akira, 2002; Seya *et al.*, 2003; Iwasaki and Medzhitov, 2004).

LPTs of bacterial origin are potent activators of monocytes/macrophages and fibroblasts *in vitro* (Shibata *et al.*, 2000). They are efficient immunoadjuvants in parenteral, nasal and oral immunization when given in combination or covalently linked to antigens as demonstrated in mice, rats, rabbits, chickens, sheep and other species (Baier *et al.*, 2000; Redecke *et al.*, 2004). In all cases, coapplication of LPTs with

antigen enhanced the humoral immune response to a level comparable or superior to that in the case of Freund's adjuvant (Wiedemann *et al.*, 1991). Recently, we have also characterized the diacylated LPT FSL-1 responsible for activation of human gingival fibroblasts and monocytes/macrophages (Shibata *et al.*, 2000; Okusawa *et al.*, 2004). Bacterial LPTs have been shown to stimulate innate immunity through the recognition by TLR2 on APCs (Akira and Takeda, 2004). We have demonstrated that TLR2 plays a key role in the recognition of FSL-1 (Fujita *et al.*, 2003; Into *et al.*, 2004; Okusawa *et al.*, 2004). Therefore, this study was carried out to determine whether FSL-1 possessed adjuvant activity and whether the activity was mediated by TLR2 if this was the case. TLR2^{+/+} and TLR2^{-/-} mice were immunized with HEL in the presence or the absence of FSL-1 or LPS. FSL-1 enhanced the generation of anti-HEL Ab in TLR2^{+/+} mice, but not in TLR2^{-/-} mice, when immunized i.p. or i.n. together with HEL (Figs 1a and b). On the other hand, LPS, which is recognized by TLR4, accelerated the generation of the Ab in both TLR2^{+/+} and TLR2^{-/-} mice (Figs 1a and b). We also analyzed the subclass distribution of the HEL-specific IgG in the sera of TLR2^{+/+} and TLR2^{-/-} mice. Both FSL-1 and LPS induced a greater increase in anti-HEL IgG1 response (Th2 type) than in the anti-HEL IgG2a response (Th1 type) regardless of the immunization routes, i.p. or i.n. (Figs 1a and b). These findings suggest that both FSL-1 and LPS induce Th2-mediated adjuvant activity *in vivo*.

FSL-1 and LPS had almost the same levels of adjuvant activity. However, FSL-1 did not induce TNF-α in sera after immunization i.p. together with HEL (Fig. 2), but LPS did. Judging from the fact that TNF-α is one of principal mediators of toxic effects of endotoxin (Beutler *et al.*, 1985), FSL-1 may be less toxic *in vivo* than LPS. This may be explained by the recent findings that TLR2 as a receptor for FSL-1 recruits the adaptor molecule MyD88, leading to

production of inflammatory cytokines, whereas TLR4 as a receptor for LPS recruits TRIF/TICAM-1 as well as MyD88, leading to production of type I IFN as well as inflammatory cytokines. That is, LPS activates the transcription factors NF- κ B and IRF3 through MyD88 and TRIF/TICAM-1, respectively, whereas FSL-1 activates only NF- κ B. On the basis of these findings, FSL-1 is considered to be superior to LPS when used as an adjuvant.

FSL-1 did not induce TNF- α in sera after immunization *i.p.* together with HEL (Fig. 2), whereas FSL-1 induced TNF- α production by spleen cells *in vitro* (Fig. 3). Several reasons are considered to explain this discrepancy. There is a big difference in experimental conditions between them. That is, FSL-1 was *i.p.* injected into mice in the former, but FSL-1 was directly added to spleen cells isolated from mouse in the latter. Therefore, the discrepancy may be explained by the differences in the experimental conditions, because it is highly possible that TNF- α produced as well as FSL-1 in the former are much more diluted by various body fluids such as peritoneal exudate fluid and blood when compared with those in the latter.

DCs play a key role in immune surveillance and have the unique capacity among APCs to stimulate naive T cells and induced differentiation of Th1- and Th2-type CD4⁺ T lymphocytes (Banchereau and Steinman, 1998). Activation of immature DCs through binding of conserved microbial molecules to pathogen recognition receptors, such as TLRs, is accompanied by maturation and homing to the lymph nodes, where the mature DCs present antigen to naive T cells (Banchereau and Steinman, 1998; Iwasaki and Medzhitov, 2004). DCs exposed to certain microenvironmental stimuli are known to promote the differentiation of CD4⁺ T cells into Th1, Th2, or Treg cell subsets. IL-12 production is required for Th1 cell development, whereas IL-10 is known as a regulator of IL-12 production and plays a key role in Th2 polarization. IL-10 production by DCs is also known to induce Treg cell development (Pasare and Medzhitov, 2003). However, it has been demonstrated that IL-6 plays a major role in T cell activation because of its ability to overcome suppression mediated by Treg (Pasare and Medzhitov, 2003). Our present investigations demonstrated that both FSL-1 and LPS induced production of IL-10 and IL-6 by spleen cells containing several DC subsets from TLR2^{+/+} mice, whereas neither FSL-1 nor LPS induced IL-12p70 in the cells (Fig. 3). Thus, these results suggest that both FSL-1 and LPS are able to induce Th2 responses *in vivo*. These results are in accordance with the finding that IgG1-type Ab is preferentially produced *in vivo* in response to both FSL-1 and LPS (Figs 1a and b). When used as adjuvants, FSL-1 and LPS are speculated to induce Th2 responses *in vivo* as evidenced by the cytokine profile and the production of specific IgG subclasses. Recent studies using MyD88-deficient mice have demonstrated that TLRs play a key role

in the generation of Th1 responses and that Th2 responses are MyD88-independent, suggesting that TLR signaling is not required for the induction of Th2 responses (Schnare *et al.*, 2001). Moreover, it has been reported that the absence of MyD88 results in Th2-biased responses (Kaisho *et al.*, 2002). However, recent investigations have shown that a number of factors influence the Th1/Th2 balance, including the amount of antigen to which DC have been exposed [High and low doses of antigen are usually associated with the generation of Th1 and Th2 responses, respectively (Boonstra *et al.*, 2003).], the ratio of T cells to DCs [Low ratios favor Th1 development (Tanaka *et al.*, 2000).], and the costimulatory molecules preferentially expressed by DCs (Expression of OX40L or a high B7.2-to-B7.1 ratio generally promotes Th2 responses (Akiba *et al.*, 2000).). In addition, high and low doses of LPS used as an adjuvant induce Th2 and Th1 responses, respectively (Eisenbarth *et al.*, 2002). However, recent evidence has indicated that *P. gingivalis* LPS, which is at present known to be contaminated with LP (Hashimoto *et al.*, 2004), flagellin (Didierlaurent *et al.*, 2004) and triacylated LPTs (Pulendran *et al.*, 2001; Dillon *et al.*, 2004) are able to induce Th2 responses. This study also demonstrated that LPS as well as FSL-1 *in vitro* induced the production of Th2-polarizing cytokine IL-10, but not Th1-polarizing cytokine IL-12p70 (Fig. 3), by spleen cells from TLR2^{+/+} mice. These results are in contrast to the results of a recent study by Dillon *et al.* (2004) showing that LPS *in vitro* activates mouse splenic DCs to secrete IL-12 and only a little IL-10 in favor of Th1 responses. However, the present study clearly demonstrated that FSL-1 and LPS activated mouse spleen cells to induce production of IL-10, but not IL-12p70, in favor of Th2 responses (Fig. 3). These disparate findings might be explained as follows. Dillon *et al.* used splenic CD11c⁺CD11b⁻ and CD11c⁺CD11b⁺ DC subsets derived from the spleen by cell sorting (Dillon *et al.*, 2004), whereas the whole spleen cells that we used contain various types of APCs, such as CD8 α ⁺ DCs (CD11c⁺ CD11b⁻ B220⁻), CD8 α ⁻ DCs (CD11c⁺ CD11b⁻ B220⁻), plasmacytoid DCs (CD11c^{dull} CD11b⁻ B220⁺) and B cells. Since different types of APCs, including DCs, express distinct sets of TLRs, it is thought that whole spleen cells produce a variety of cytokines in response to FSL-1 or LPS. In addition, there is a possibility that cytokines produced by APCs may lead in an autocrine or paracrine manner to production of secondary cytokines that possess various biological activities.

MAPKs consisting of p38, JNK and ERK play an important role in regulation of survival and maturation of DCs and their cytokine secretion. ERK responds to mitogens and growth factors that regulate cell proliferation and differentiation, whereas JNK and p38 MAPK are predominantly activated by inflammatory cytokines in response to cellular stress. Activation of ERK has been shown to promote IL-10

production and mediate negative feedback regulation of IL-12 production. Moreover, the inhibition of ERK activation in DCs results in suppression of IL-10 and enhancement of IL-12 production (Puig-Kroger *et al.*, 2001). These findings suggest that ERK phosphorylation may play an important role in the regulation of two key cytokines involved in the induction of Th cell responses. A recent study has shown that signaling through TLR2 induced DCs to secrete IL-10, by a mechanism dependent on the MEK kinase ERK, which leads to the expression of c-Fos, without secretion of IL-12p70 and thus favored Th2 responses, whereas a TLR4 ligand induced potent p38 MAPK activation, which is critical for the induction of IL-12p70, and less ERK activation, and thus favored Th1 responses (Dillon *et al.*, 2004). The present study also demonstrated that stimulation of spleen cells with the diacylated LPT FSL-1 resulted in activation of ERK and in increased c-Fos expression and, to a lesser extent, LPS stimulation also induced them. Thus, the results of the present study are consistent with the findings that TLR2 is preferentially involved in the activation of Th2 responses (Pulendran *et al.*, 2001; Dillon *et al.*, 2004).

Studies are in progress to further clarify the mechanisms by which TLR2 ligands such as FSL-1 induce Th2 responses.

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