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Th2 Cytokine Production from Mast Cells Is Directly Induced by Lipopolysaccharide and Distinctly Regulated by c-Jun N-Terminal Kinase and p38 Pathways¹

Akio Masuda,² Yasunobu Yoshikai, Keiko Aiba, and Tetsuya Matsuguchi

Mast cells secrete multiple cytokines and play an important role in allergic inflammation. Although it is widely accepted that bacteria infection occasionally worsens allergic airway inflammation, the mechanism has not been defined. In this study, we show that LPS induced Th2-associated cytokine production such as IL-5, IL-10, and IL-13 from mast cells and also synergistically enhanced production of these cytokines induced by IgE cross-linking. LPS-mediated Th2-type cytokine production was abolished in mouse bone marrow-derived mast cells derived from C3H/HeJ mice, suggesting that Toll-like receptor 4 is essential for the cytokine production. Furthermore, we found that mitogen-activated protein kinases including extracellular signal-regulated kinase 1/2, c-Jun N-terminal kinase, and p38 kinase were activated by LPS stimulation in bone marrow-derived mast cells. Inhibition of extracellular signal-regulated kinase activation has little effect on LPS-mediated cytokine production. In contrast, inhibition of c-Jun N-terminal kinase activation significantly suppressed both IL-10 and IL-13 expression at both mRNA and protein levels. Interestingly, although inhibition of p38 did not down-regulate the mRNA induction, it moderately decreased all three cytokine productions by LPS. These results indicate that LPS-mediated production of IL-5, IL-10, and IL-13 was distinctly regulated by mitogen-activated protein kinases. Our findings may indicate a clue to understanding the mechanisms of how bacteria infection worsens the clinical features of asthma. *The Journal of Immunology*, 2002, 169: 3801–3810.

Mast cells are central effector and regulatory cells in Th2-dominated immune responses (1). The multivalent binding of Ag to receptor-bound IgE and the subsequent aggregation of the FcεRI provide triggers for mast cell activation. FcεRI aggregation results in the release of inflammatory mediators, including histamine, leukotriene C4, and PGD₂ (1). In addition to inflammatory mediators, mast cells can also produce a variety of cytokines including TNF-α and Th2-associated cytokines such as IL-4, IL-5, IL-6, IL-10, and IL-13, which are all important players in the pathogenesis of allergic reactions (2–4).

Besides allergic reactions, a number of studies have confirmed that mast cells play a critical role in host immune defense against Gram-negative bacteria through the release of TNF-α (5, 6). Mast cells are able to perceive a variety of infectious agents either by opsonin-dependent mechanisms or via direct binding to parasites and bacteria (7).

Pathogen-associated molecular patterns are represented mostly by molecules localized in microbial cell walls. Prominent among them is LPS, or endotoxin of Gram-negative bacteria, which induces endotoxin shock. LPS binds the soluble LPS binding protein

(LBP)³ and the complex binds CD14 (8). CD14 presents the LPS-LBP complex to the LPS receptor Toll-like receptor 4 (TLR4), a member of the Toll family of receptors originally identified in *Drosophila* (9). It has been reported that two mouse strains (C3H/HeJ and C57BL10/ScCr) that exhibit impaired ability to respond to many types of LPS have different mutations in the TLR4 gene (10, 11). Signals originating in the LPS-triggered TLR4 receptor activate several signaling pathways such as NF-κB (12, 13), AP-1 (13), and mitogen-activated protein kinase (MAPK) pathways, including extracellular signal-regulated kinases (ERKs) (14, 15), c-Jun N-terminal kinases (JNKs) (16, 17), and p38 kinases (18, 19), in target cells such as monocytes, macrophages, and B cells. In response to LPS, these cells produce a number of cytokines, including IL-1, IL-6, IL-8, IL-10, TNF-α, and Th1-associated cytokines such as IL-12 and IFN-γ, and the involvement of these three MAPK subtypes for production of these cytokines has been documented extensively (16, 17, 20–23).

Mast cells respond to LPS by producing IL-1β, IL-6, IL-13, and TNF-α without degranulation (24–26). Stimulation of mast cells by FcεRI ligation activates MAPK pathways, and important roles for these MAPK pathways are predicted on the ability of ERK1/2, JNK, and p38 to regulate the transcriptional activity of various cytokine genes (27–30). Recently, it has been reported that LPS activates NF-κB, ERK1/2, and p38 in mast cells (26). However, the molecular mechanisms for cytokine production by LPS in mast cells have remained largely unknown.

In the present study, we have demonstrated that LPS induces a significant amount of Th2-associated cytokines such as IL-5, IL-10, and IL-13, but not IL-4, from mast cells, in addition to IL-6 and

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³ Abbreviations used in this paper: LBP, LPS binding protein; TLR, Toll-like receptor; MAPK, mitogen-activated protein kinase; ERK, extracellular signal-regulated kinase; JNK, c-Jun N-terminal kinase; BMMC, bone marrow-derived mast cell; DNP, dinitrophenyl; HSA, human serum albumin; RPA, RNase protection assay; PKC, protein kinase C.

TNF- α . When combined with Ag-IgE ligation of Fc ϵ RI, LPS synergistically increased the secretion of these Th2-type cytokines. The cytokine production was abolished in bone marrow-derived mast cells (BMMCs) derived from C3H/HeJ mice, suggesting that TLR4 is essential for production of these cytokines. Furthermore, we investigated the signal transduction induced in mast cells by LPS stimulation and demonstrated that ERK, JNK, and p38 kinase were all activated by LPS. Among the activated MAPKs, JNK and p38, but not ERK, regulated cytokine production at different stages.

Recent studies suggest that LPS is considered to be a risk factor of asthma severity. Although many investigators have indicated that LPS induces airway hyperresponsiveness (31, 32) and worsens allergic airway inflammation (33, 34), the details of this mechanism have not been elucidated. Because Th2-associated cytokines have a central role in the pathogenesis of asthma, our report indicates that Th2-type cytokine production by mast cells, mediated by JNK and p38 activation, is involved in the airway hypersensitivity by LPS.

Materials and Methods

Reagents and Abs

LPS from *Escherichia coli* serotype 055:B5 and curcumin were obtained from Sigma-Aldrich (St. Louis, MO). PD98059, a specific inhibitor of ERK kinase, and SB208530, a specific inhibitor of p38 kinase, were purchased from Calbiochem (San Diego, CA). RPMI 1640 medium was from Life Technologies (Rockville, MD). FCS was purchased from Sigma-Aldrich. Synthetic *E. coli*-type lipid A, ONO4007, was kindly provided by Ono Pharmaceutical (Tokyo, Japan) and was described previously (35). Anti-phospho-ERK mAb was obtained from New England Biolabs (Beverly, MA). The polyclonal anti-JNK1 Ab, which does not cross-react with JNK2 or 3, and the anti-p38 MAPK polyclonal Ab were obtained from Santa Cruz Biotechnology (Santa Cruz, CA). The phospho-specific anti-p38 polyclonal Ab, the polyclonal anti-ERK Ab, and the anti-phospho-ERK mAb were purchased from New England Biolabs. The mouse monoclonal anti-dinitrophenyl (DNP) Ab and the DNP-human serum albumin (HSA) were purchased from Sigma-Aldrich. The biotinylated anti-mouse IgE mAb was purchased from eBioscience (San Diego, CA).

Mast cells

The MC/9 mouse mast cell line and BMMCs were cultured as previously described (36, 37). BMMCs were derived from femoral bone marrow cells of 6-wk-old BALB/c mice. BMMCs from C3H/HeJ and C3H/HeN mice were derived from femoral bone marrow cells of 6-wk-old mice. After 3 wk of culture with 10% WEHI-3-conditioned medium, the cells were harvested for the experiments and consisted of >98% mast cells assessed by toluidine blue staining and FACS analysis of cell surface expression of *c-kit* and Fc ϵ RI.

Cells were washed twice with RPMI 1640 containing 10% FCS and were incubated for 6 h before LPS stimulation. For the cross-linking of Fc ϵ RI on mast cells, cells were sensitized by incubating for 2 h with 1 μ g/ml anti-DNP IgE in RPMI 1640 containing 10% FCS and then were washed, incubated (2×10^6 cells/ml) for 6 h in RPMI 1640 containing 10% FCS, and stimulated with 50 ng/ml DNP-HSA for the indicated time.

RNase protection assay (RPA)

For RPA analysis, a RiboQuant MultiProbe RNase Protection Assay System (BD Pharmingen, San Diego, CA) was used according to the manufacturer's instructions. The DNA template set was used for T7 RNA-polymerase-directed synthesis of [α - 32 P]UTP-labeled antisense RNA probes. The probes were hybridized with 10 μ g of RNA isolated from mast cells using Trizol reagent (Life Technologies) according to the manufacturer's instructions. Samples were then digested with RNases to remove single-stranded (nonhybridized) RNA. The remaining probes were resolved on denaturing polyacrylamide gels and were exposed to Fuji RX-U films (Fuji Film, Tokyo, Japan).

RT-PCR analysis

Total cellular RNA was isolated as described above. cDNA was synthesized from 2 μ g of the total RNA by extension of random primers with 200 U (Superscript II; Life Technologies). PCR of the cDNA was performed in

a final volume of 50 μ l containing 2.5 mmol/L magnesium dichloride (MgCl $_2$), 2.5 U (AmpliTag; PerkinElmer, Norwalk, CT), and 1 μ mol/L specific primers (geneAmp 2400 PCR system; PerkinElmer). The amplification procedure performed in a temperature controller was as follows: for amplification of β -actin cDNA, after an initial denaturation step at 95°C for 5 min, 20 cycles were performed at 95°C for 1 min followed by 54°C for 1 min, and 72°C for 1 min. Five microliters of each PCR product was run on a 1.5% agarose gel (Life Technologies) for UV visualization. For amplification of IL-10 and IL-13 cDNA, PCR assays were performed for 30 cycles (95°C for 1 min followed by 54°C for 1 min and 72°C for 1 min). For amplification of IL-5 cDNA, PCR assays were performed for 35 cycles (95°C for 1 min followed by 60°C for 1 min and 72°C for 1 min). These numbers of cycles were in the linear range.

Primer sequences were as follows: IL-5 sense, 5'-GAAAGAGACCTTGACACAGCTG-3'; IL-5 anti-sense, 5'-GAACTCTGTCAGGTAATCAGG-3'; IL-10 sense, 5'-TACCTGGTAGAAGTGATGCC-3'; IL-10 anti-sense, 5'-CATCATGTATGCTTCTATGC-3'; IL-13 sense, 5'-ATGAGTCTGCAGTATCCCG-3'; IL-13 anti-sense, 5'-CCGTGGCAGACAGGAGTGTT-3'; β -actin sense, 5'-TGGAATCCTGTGGCATCCATGAAAC-3'; and β -actin anti-sense, 5'-TAAACGCAGCTCAGTAACAGTCCG-3'.

Purification of mast cells by MACS

BMMCs were sensitized by incubating for 2 h with 1 μ g/ml anti-DNP IgE in RPMI 1640 containing 10% FCS. Then cells were stained with biotinylated anti-IgE Ab and secondarily incubated with streptavidin microbeads (MACS; Miltenyi Biotec, Bergish Gladbach, Germany). BMMC cells were enriched by magnetic cell sorting according to the manufacturer's protocol (Miltenyi Biotec). More than 99.8% of the MACS-purified cells were mast cells assessed by toluidine blue staining and FACS analysis of cell surface expression of *c-kit* and Fc ϵ RI.

Northern blot analysis

Total cellular RNA was isolated as described above. Twenty-microgram aliquots of the total RNAs were fractionated on a 1% agarose gel containing 20 mM MOPS, 5 mM sodium acetate, 1 mM EDTA (pH 7), and 6% (v/v) formaldehyde and were transferred to a nylon membrane. After UV cross-linking, membranes were soaked in prehybridization solution (6 \times SSC, 5 \times Denhardt's reagent, 0.5% SDS, 100 mg/ml denatured salmon sperm DNA, and 50% formamide) for 3 h at 65°C, followed by incubation with a 32 P-labeled probe in hybridization solution (6 \times SSC, 0.5% SDS, 100 mg/ml denatured salmon sperm DNA, and 50% formamide) for 14 h at 65°C. The membranes were washed in 2 \times SSC, 0.1% SDS for 10 min twice at room temperature and in 0.1 \times SSC, 0.1% SDS for 10 min twice at 50°C and were exposed to Fuji RX-U films (Fuji Film). cDNA fragments of the coding regions of mouse TLR4 and β -actin were used as specific probes.

Western blotting analysis

Cells were lysed in ice-cold lysis buffer (50 mM HEPES (pH 7), 150 mM NaCl, 10% glycerol, 1% Triton X-100, 1.5 mM MgCl $_2$, 1 mM EGTA, 100 mM NaF, 10 mM NaPi, 1 mM Na $_3$ VO $_4$, 1 mM PMSF with aprotinin, and leupeptin at 10 μ g/ml). The lysates were separated on a 10% SDS-PAGE, and the proteins were transferred to Immobilon polyvinylidene difluoride membranes (Millipore, Bedford, MA). Membranes were blocked with 1% BSA in TBST for 1 h, and Western blot analysis was performed as described previously (38), followed by detection using an ECL system (Amersham Pharmacia Biotech, Piscataway, NJ) according to the manufacturer's instructions.

Immune complex kinase assay

Cell lysates (10^7 cells/sample) were incubated with 0.4 μ g of Ab for 2 h at 4°C, followed by incubation with protein A-Sepharose beads (Amersham Pharmacia Biotech) for additional 1 h. The beads were washed three times in lysis buffer and then once in kinase buffer (20 mM Tris-HCl (pH 7.4), 20 mM MgCl $_2$, 2 mM EGTA, 0.5 mM sodium vanadate, 10 mM β -glycerophosphate, and 1 mM DTT). The kinase reaction was initiated by the addition of 40 μ l of kinase buffer with 20 μ M ATP, 5 μ Ci of [γ - 32 P]ATP, and 0.5 μ g of GST-c-Jun(5–89) (39) for JNK or GST-activating transcription factor 2(1–109) (39) for p38 MAPK and was allowed to proceed for 20 min at 30°C. The reaction was terminated by the addition of 2 \times SDS sample buffer. Samples were boiled and resolved by SDS-PAGE, and the fixed gel was exposed to an x-ray film.

Plasmids

The hemagglutinin-tagged mouse JNK3 expression vector (40) was used for in vitro mutagenesis using two-step PCR procedures (41) to generate

T183A and Y185F mutants. The mutant hemagglutinin-mJNK3 cDNA was inserted into the pcDNA3 vector (dominant-negative JNK). MC/9 cells were transfected with dominant-negative JNK by DMRIE-C Reagent (Life Technologies) according to the manufacturer's instructions. Two days posttransfection, cells were selected in medium containing 1 mg/ml G418. After 3 wk of selection, resistant cell populations were pooled and cultured continuously in the presence of 0.1 mg/ml G418.

Cytokine ELISA

The cell-free culture supernatants were measured for the concentration of IL-5 (BD PharMingen), IL-10, and IL-13 (R&D Systems, Minneapolis, MN) by ELISA according to the manufacturer's instructions.

Measurement of cell viability

Cell viability was determined by counting viable cells. The viable cells were determined by trypan blue exclusion. At least 200 total (live and dead) cells were counted from each individual culture.

Results

LPS up-regulated Th2 cytokine mRNA expression in mast cells

It has been well documented that mast cells produce a variety of cytokines such as TNF- α , IL-4, 5, 6, 10, and 13 after Fc ϵ RI ligation (2–4). When stimulated with LPS, mast cells have been reported to produce IL-1 β , TNF- α , and IL-6 (24, 25). Recently, Supajatura et al. (26) have reported that mast cells also secrete IL-13, a Th2-type cytokine, in response to LPS. To examine LPS-induced cytokine production from mast cells more extensively, MC/9 cells, a well-established mouse mast cell line, were stimulated with LPS, IgE cross-linking, or a combination of both, or BMMCs were stimulated with LPS. Total RNA was isolated and RPA analysis was performed. A panel of cytokines including IL-2, IL-4, IL-5, IL-6, IL-9, IL-10, IL-15, and IFN- γ was screened in these cells. As shown in Fig. 1A, in MC/9 cells, LPS up-regulated IL-5, 6, 10, and IL-13 mRNA as seen in IgE cross-linking, and a combination of LPS and IgE cross-linking stimulation further up-regulated mRNA expression of these cytokines. BMMCs simulated with LPS also showed increases of IL-5, 6, 10, and 13 mRNA expressions similar to MC/9 cells. Although IgE cross-linking slightly up-regulated IL-4 mRNA in MC/9 cells, LPS could not significantly affect IL-4 mRNA expression in either MC/9 cells or BMMCs. As shown in Fig. 1B, LPS up-regulated IL-5, 10, and 13 mRNA expression as early as 1 h after stimulation, and the up-regulation lasted for at least for 8 h in MC/9 cells. These results indicate that LPS induces mRNA up-regulation of Th2-associated cytokines such as IL-5, 10, and 13, but not IL-4, in mast cells. LPS could also further increase mRNA levels of IL-5, 6, 10, 13, and TNF- α , when combined with IgE cross-linking.

Mast cells secreted IL-5, 10, and 13 in culture supernatants in response to LPS

To determine whether LPS stimulates Th2 cytokine protein secretion from mast cells, we measured Th2 cytokine concentration in the culture supernatants of mast cells incubated with LPS. MC/9 cells or BMMCs were stimulated with LPS, IgE cross-linking, or the combination of both for 16 h. After the 16-h incubation, ~90% of the cells are viable and the viabilities were similar among differently stimulated cells (Fig. 2A). As shown in Fig. 2B, MC/9 cells produced IL-13 in response to LPS in a concentration-dependent manner. A combination of LPS and IgE cross-linking stimulation induced a large amount of IL-13 production from MC/9 cells in a synergistic manner. MC/9 cells that we examined did not produce detectable amounts of IL-5 and 10, even when stimulated with not only LPS but also IgE cross-linking and the combination of these stimulations (data not shown). BMMCs also produced a

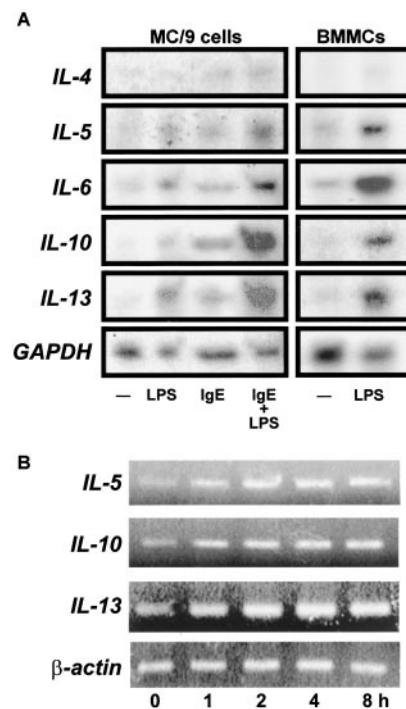


FIGURE 1. Cytokine mRNA expression by LPS stimulation in mast cells. *A*, MC/9 cells (2×10^6 cells/ml) were stimulated with LPS ($1 \mu\text{g/ml}$), IgE cross-linking (IgE), or costimulation of LPS ($1 \mu\text{g/ml}$) and IgE cross-linking (IgE plus LPS) for 4 h. BMMCs (2×10^6 cells/ml) were stimulated with LPS ($1 \mu\text{g/ml}$) or not for 4 h. Total RNA ($10 \mu\text{g}$ each) was isolated and RPA analysis was performed. *B*, MC/9 cells (2×10^6 cells/ml) were stimulated with LPS ($1 \mu\text{g/ml}$) for the indicated time. Total RNA ($2 \mu\text{g}$ each) was reverse transcribed and PCR amplified as described in *Materials and Methods*.

significant amount of IL-13 and also IL-5 and IL-10 in response to LPS in a concentration-dependent manner (Fig. 2B). The amounts of cytokines induced by LPS are at similar levels to those induced by IgE cross-linking, which is a strong inducer of Th2-associated cytokines in mast cells (2, 3). The combination of LPS and IgE cross-linking worked synergistically, as in mRNA levels. Although we also examined IL-4 concentration in the same supernatants, we could not detect any IL-4 protein except for the positive control, which is the supernatant of BMMCs stimulated with ionomycin (data not shown). Although purity of BMMCs cultured with IL-3 is >98%, a small population of other cell types were also included. To rule out the possibility that cells other than mast cells were the source of Th2 cytokines, we further purified mast cells using a MACS cell separator. After this procedure, >99.8% of the MACS-purified cells were mast cells assessed by toluidine blue staining and FACS analysis of cell surface expression of *c-kit* and Fc ϵ RI (data not shown). Fig. 2C shows that these purified mast cells responded to LPS within 2 h and produced a significant amount of Th2 cytokines. These data provide evidence that LPS is a potent inducer of IL-5, IL-10, and IL-13, but not IL-4, from mast cells.

TLR4 is essential for lipid A-induced Th2-associated cytokine production from mast cells

To rule out the possibility that other substances contaminated in the commercial grade of LPS were responsible for the Th2-associated cytokine production from mast cells, we stimulated mast

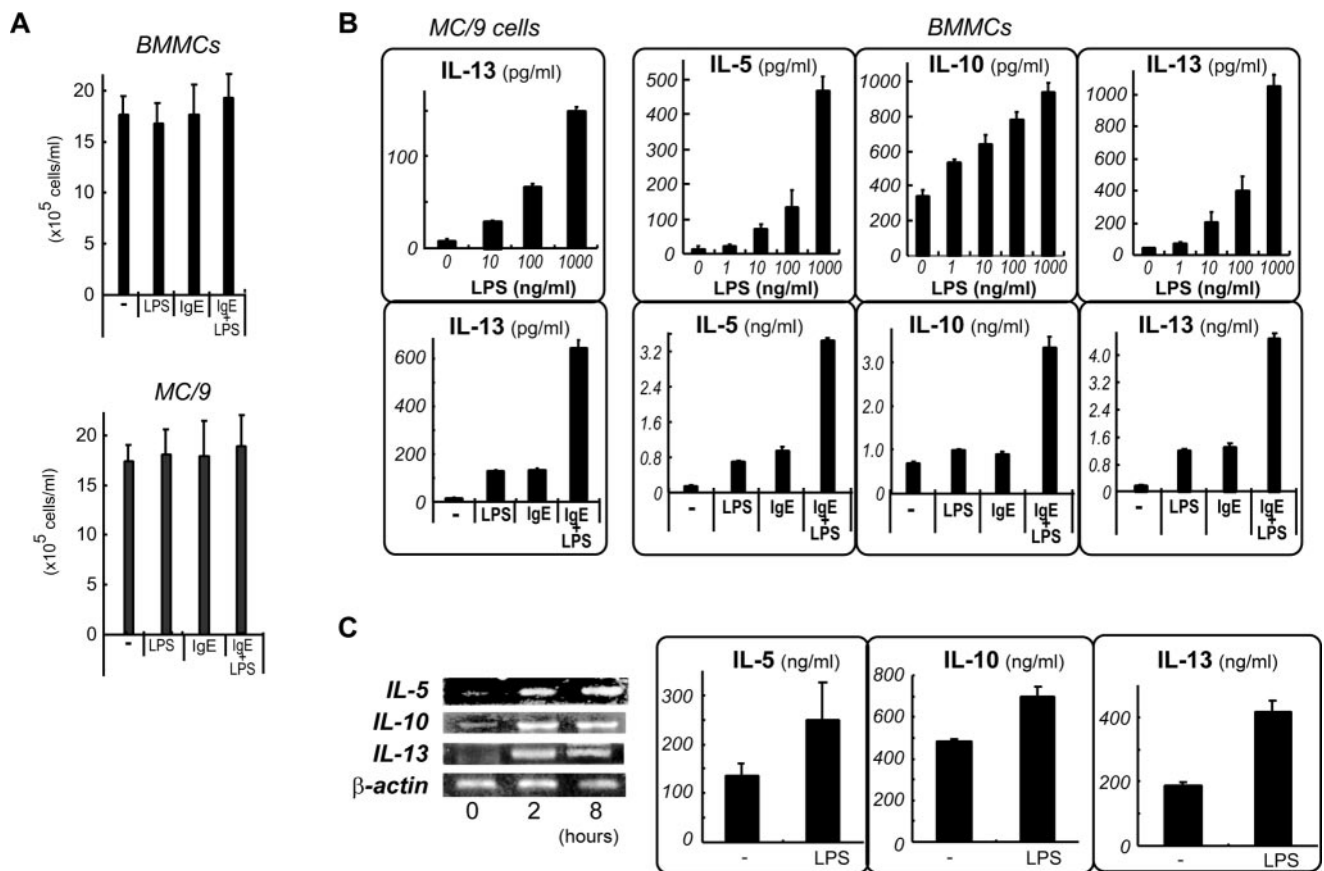


FIGURE 2. A, Cell viability of mast cells after stimulation. Cells (2×10^6 cells/ml) were stimulated with LPS ($1 \mu\text{g/ml}$) (LPS). For IgE cross-linking study, the cells sensitized with anti-DNP IgE were stimulated with 50 ng/ml DNP-HSA (IgE). Costimulation of IgE cross-linking and LPS (IgE+LPS) was done by the simultaneous addition of DNP-HSA and LPS ($1 \mu\text{g/ml}$). After 16 h, total cell counts were determined by trypan blue exclusion. The error bars represent SD values. B, LPS induced Th2-associated cytokine production from mast cells. Cells (2×10^6 cells/ml) were stimulated with various concentrations of LPS (upper panels) or with $1 \mu\text{g/ml}$ LPS as indicated (lower panels). For IgE cross-linking study, the cells sensitized with anti-DNP IgE were stimulated with 50 ng/ml DNP-HSA (IgE). Costimulation of IgE cross-linking and LPS (IgE+LPS) was done by addition of DNP-HSA and LPS ($1 \mu\text{g/ml}$) at the same time. After 16 h, the cell-free culture supernatants were collected and cytokine ELISAs were performed. The experiments were done in triplicate using three separate BMMC batches. The error bars represent SD values. C, Mast cells purified using MACS cell separator responded to LPS. Mast cells further purified by MACS (2×10^6 cells/ml) were stimulated with LPS ($1 \mu\text{g/ml}$) for the indicated time. Total RNA ($2 \mu\text{g}$ each) was reverse transcribed and PCR amplified as described in *Materials and Methods*. For ELISA, cells were stimulated for 16 h and cell-free supernatants were collected. The error bars represent SD values.

cells with synthetic lipid A for 16 h and cytokine ELISAs were performed. As shown in Fig. 3A, BMMCs produced IL-5, 10, and 13, and MC/9 cells also produced IL-13 in response to lipid A in a concentration-dependent manner. These results showed that Th2-associated cytokine production from mast cells by LPS is mediated at least partly by lipid A, the bioactive center of LPS.

It has been well known that TLR4 recognizes the lipid A portion of LPS and has a critical role in LPS-mediated bioactivity (10, 11). We confirmed that the *TLR4* gene was expressed in both BMMCs and MC/9 cells (Fig. 3B). To determine whether the LPS-induced Th2 cytokine production is mediated by TLR4, we examined BMMCs from C3H/HeJ mice that contain a nonfunctional mutation in the *TLR4* gene. When BMMCs isolated from this mouse strain were stimulated with synthetic lipid A, IL-13 production was severely impaired compared with that of BMMCs isolated from the closely related C3H/HeN mice (Fig. 3C). Because we could not detect sufficient amounts of IL-5 and IL-10 in the culture supernatants from these mice for ELISA, the change after lipid A stimulation was evaluated at the mRNA level using RT-PCR assay. As shown in Fig. 3D, IL-5, 10, and 13 mRNA increases after lipid A stimulation were also severely impaired in BMMCs isolated from

C3H/HeJ mice. These findings suggest that TLR4 is essential for the lipid A-induced production of IL-5, IL-10, and IL-13.

LPS stimulation activates MAPK pathways, including ERK, JNK, and p38 kinase in mast cells

LPS stimulation induces the activation of MAPK pathways, including ERK, JNK, and p38 kinase in various cell types (14–19). Because MAPKs play an important role in the regulation of cytokine production in mast cells (27–30), we sought to confirm the activation of MAPKs in LPS-stimulated mast cells. Factor-deprived MC/9 cells were stimulated with various concentrations of LPS for 30 min. We examined the ERK and p38 kinase phosphorylation, which is closely related to kinase activity, by phospho-specific Abs for each, and the JNK kinase activity was examined by *in vitro* kinase assay. As shown in Fig. 4A, LPS induced ERK, JNK, and p38 kinase phosphorylation or activation in a concentration-dependent manner. In the time course analysis, the maximum activation of these MAPKs occurred at 30 min of LPS stimulation (Fig. 4A). LPS similarly activated these MAPK pathways in BMMCs (Fig. 4B). To confirm the degrees of MAPK activation,

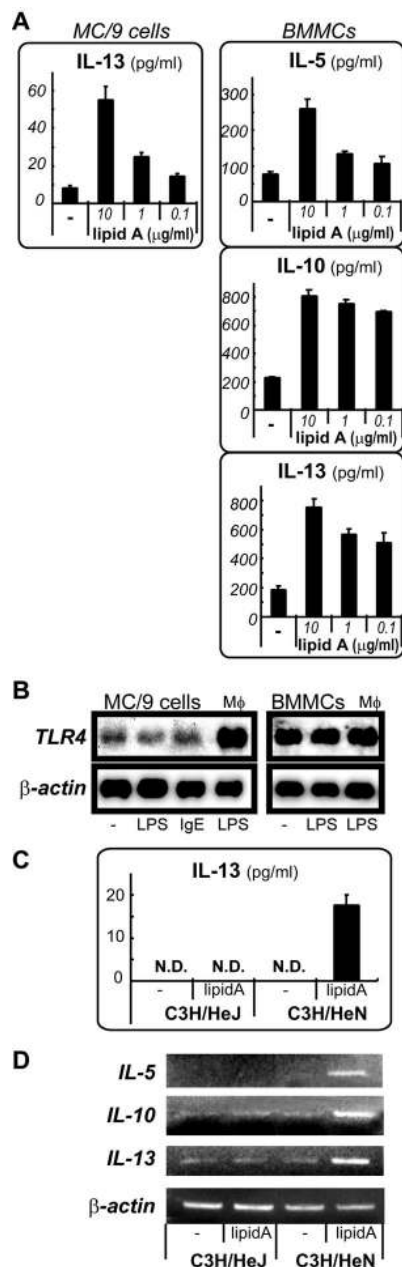


FIGURE 3. A, Lipid A induced Th2-associated cytokine production from mast cells. Cells (2×10^6 cells/ml) were stimulated with various concentrations of lipid A. After 16 h, the cell-free culture supernatants were collected and cytokine ELISAs were performed. The experiments were done in triplicate. The error bars represent SD values. B, TLR4 gene was expressed in mast cells. Cells (2×10^6 cells/ml) were stimulated with LPS (1 μ g/ml) or not for 4 h. For IgE cross-linking study, the cells sensitized with anti-DNP IgE were stimulated with 100 ng/ml DNP-HSA for 4 h (IgE). As a positive control, the mouse macrophage cell line RAW264.7 was stimulated with LPS for 4 h (M ϕ). Total RNA (20 μ g) from mast cells or RAW264.7 cells was used in Northern blot analysis. C, IL-13 production induced by lipid A was severely impaired in BMMCs derived from C3H/HeJ mice. BMMCs (2×10^6 cells/ml) derived from C3H/HeJ or N mice were stimulated with synthetic lipid A (10 μ g/ml) or not. After 16 h, the cell-free culture supernatants were collected and cytokine ELISAs were performed. The experiments were done in triplicate. The error bars represent SD values. D, Th2-associated cytokine mRNA up-regulation induced by lipid A was severely impaired in BMMCs derived from C3H/HeJ mice. BMMCs (2×10^6 cells/ml) derived from C3H/HeJ or N mice were stimulated with synthetic lipid A (10 μ g/ml) or not for 4 h. Total RNA (2 μ g/each) was reverse transcribed and PCR amplified as described.

MC/9 cells were stimulated with LPS, IgE cross-linking, or a combination of these stimulations. As shown in Fig. 4C, ERK activation by LPS was weak in comparison with that by IgE cross-linking. In contrast, JNK and p38 were significantly activated by LPS, and the combination of LPS and IgE cross-linking activated these MAPKs synergistically. The synergistic activation by the two stimulations was not observed for ERK.

JNK activation is essential for LPS-mediated IL-10 and IL-13 mRNA induction and protein production

To investigate possible involvement of MAPK pathways in LPS-induced Th2-type cytokine production from mast cells, cells were pretreated with inhibitors of each MAPK pathway or were transfected with a dominant-negative (kinase-inactive) mutant form of JNK. The viability of these cells post-treatment with inhibitors was similar, as shown in Fig. 5A. When MC/9 or BMMCs were pretreated with a specific inhibitor of ERK (PD98059), LPS-mediated ERK phosphorylation was abolished (Fig. 5B). However, PD98059 pretreatment did not affect Th2-associated cytokine production from mast cells (Fig. 5C), indicating that ERK activation is not essential for Th2-type cytokine production by LPS-treated mast cells.

We then pretreated the cells with curcumin, which has recently been reported to inhibit JNK activation in various cell types at a concentration of 5 or 10 μ M (42). As shown in Fig. 6A, 10 μ M curcumin inhibited activation of JNK induced by LPS in BMMCs. Treatment of BMMCs with 10 μ M curcumin inhibited production of IL-10 and IL-13 induced by LPS (Fig. 6A). We then established three independent MC/9 cell lines stably expressing the dominant-negative mutant of JNK. As shown in Fig. 6B, introduction of the dominant-negative JNK significantly inhibited LPS-stimulated JNK activation in both MC/9 cell lines. In these transfectants, LPS-mediated IL-10 and IL-13 production was significantly decreased compared with empty vector-transfected cells (Fig. 6B). To confirm whether the inhibition of cytokine production was due to the transcriptional regulation, we performed RT-PCR analyses. As shown in Fig. 6C, inhibition of JNK activation decreased IL-10 and IL-13 mRNA up-regulation by LPS. These observations suggest that JNK is critically involved in IL-10 and IL-13 production by LPS through mRNA up-regulations in mast cells. Inhibition of JNK activation did not affect IL-5 production of BMMCs by LPS, although it slightly up-regulated IL-5 mRNA after LPS stimulation in both BMMCs and MC/9, suggesting that JNK is not essential for IL-5 production by LPS-treated mast cells.

Activation of p38 kinase is not necessary for LPS-mediated Th2-associated cytokine mRNA induction but is essential for their protein production

To investigate whether the p38 kinase pathway is involved in LPS-induced Th2-type cytokine production from mast cells, cells were pretreated with a specific inhibitor of p38 kinase (SB208530). As shown in Fig. 7A, p38 kinase activation by LPS was clearly inhibited by incubation with a specific inhibitor (SB208530) in both BMMCs and MC/9 cells. The inhibition of p38 kinase activation in BMMCs clearly inhibited the production of IL-10 and moderately inhibited IL-5 and IL-13 induced by LPS. Also in MC/9 cells, SB208530 pretreatment inhibited IL-13 production (Fig. 7B). Surprisingly, this pretreatment did not decrease mRNA of Th2-associated cytokine in both BMMCs and MC/9 cells (Fig. 7, C and D). In MC/9 cells, it seemed to moderately increase the mRNA levels. These findings suggest that p38 kinase activation is involved in the post-transcriptional processing of Th2-type cytokine production. This is true even in IL-5, whose production was not affected by

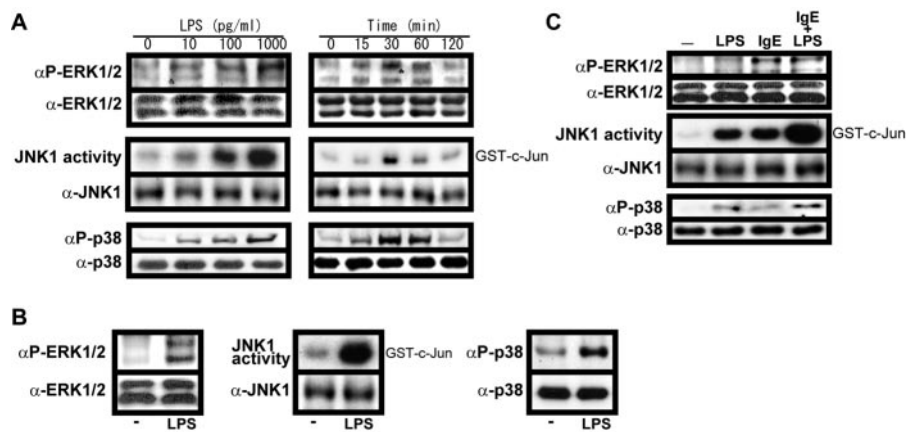


FIGURE 4. A, LPS induced ERK, JNK, and p38 activation in MC/9 cells. MC/9 cells were incubated in RPMI + 10% FCS for 6 h and were stimulated with various concentrations of LPS for 30 min (*left*) or with LPS (1 μg/ml) for the indicated times. LPS-mediated ERK phosphorylation was measured by Western blot using an anti-phospho-ERK Ab. LPS-mediated JNK activation was measured by the *in vitro* kinase assay using GST-c-Jun as substrate. LPS-mediated p38 phosphorylation was measured by Western blot using an anti-phospho-p38 Ab. B, LPS induced ERK, JNK, and p38 activation in BMMCs. BMMCs were incubated in RPMI + 10% FCS for 6 h and were stimulated with LPS (1 μg/ml) for 10 min. Phosphorylation of ERK and p38 kinase activity were measured as in A. C, MAPK activation induced by LPS or IgE cross-linking. MC/9 cells were incubated in RPMI + 10% FCS for 6 h and were stimulated with LPS (1 μg/ml), IgE cross-linking (IgE), or costimulation of LPS (1 μg/ml) and IgE cross-linking (IgE+LPS) for 30 min. Phosphorylation of ERK and p38 kinase and JNK1 kinase activity were measured as in A.

blocking the JNK pathway, in that SB208530 pretreatment moderately decreased IL-5 protein but not mRNA level after LPS stimulation.

Discussion

In this study, we have demonstrated that LPS induces Th2-associated cytokine production such as IL-5, IL-10, and IL-13, but not IL-4, from mast cells. Also, when mast cells were treated with LPS in combination with the Ag-IgE ligation of FcεRI, Th2-cytokine induction by FcεRI ligation was synergistically promoted. The Th2-type cytokine induction by LPS is mediated by TLR4, a recently identified component of the LPS receptor complex, in that it was abolished in BMMCs derived from C3H/HeJ mice, which have a nonfunctional mutation in the cytoplasmic domain of TLR4. Significantly, both the MC/9 mast cell line and BMMCs expressed TLR4 mRNA. Furthermore, we have investigated the signal transduction in LPS-treated mast cells and have demonstrated that MAPKs, including ERK1/2, JNK, and p38 kinase, are activated by LPS stimulation. Although inhibition of ERK activation showed little effect, inhibition of JNK activation by either curcumin or the dominant-negative JNK expression significantly suppressed IL-10 and IL-13 at both mRNA and protein levels. Inhibition of p38 activation suppressed IL-5, IL-10, and IL-13 protein secretion, although the amounts of these cytokine mRNAs were not decreased. These results indicate that these cytokine productions were differently regulated by JNK and p38.

Recent studies suggest that LPS is considered to be a risk factor of asthma severity. LPS up-regulated airway responsiveness in humans (31, 32). There was a correlation between clinical asthma scores and household LPS levels, but not dust mite allergen levels (43–45). Acute inhalation of LPS by asthmatics induced bronchial obstruction and hyperresponsiveness (46). In a mouse model of allergic airway inflammation, inhalation of Ag with LPS promoted airway inflammation, up-regulated airway responsiveness, and gathered more eosinophils to airways (33). Furthermore, mice deficient in LBP, which is essential for proper response to LPS, have weak airway responsiveness against allergens in comparison with wild-type mice (34). Taken together, these reports indicate that allergens induce stronger airway inflammation in the presence of

LPS. However, little is known about the molecular and cellular mechanisms.

Because mast cells are essential players in the development of airway responsiveness (47), we would like to propose that LPS-mediated production of IL-5 and IL-13 by mast cells is an important mechanism. IL-5 has been well known to be a critical factor for the activation of eosinophils that have a central role in allergic airway inflammation (48). IL-13 also has been well known to induce airway hyperresponsiveness and allergic inflammations (49) and to have an important role in asthma (50–52).

The cytoplasmic domain of the Toll family proteins is homologous to the cytoplasmic domain of the IL-1R family (53). This family also includes IL-18R. Yoshimoto et al. (54) reported that IL-18 stimulates IL-4 release from basophils. In this manuscript, they reported that basophils stimulated with IL-18 produced a significant amount of IL-4 and IL-13 and that mast cells stimulated with IL-18 produced a moderate amount of IL-13, but not IL-4. Quite recently, it has been reported that peptidoglycan stimulated mast cells in a TLR2-dependent manner to produce TNF-α, IL-4, IL-5, IL-6, and IL-13 (55). It has been reported that mast cells stimulated with LPS produced IL-13 in a TLR4-dependent manner (26), and we have reported in the present manuscript that TLR4 is essential for IL-5, IL-10, and IL-13 production induced by LPS. It seems that stimulation through the IL-1R family generally promotes Th2 cytokine production in mast cells. Although we shown some clues in this manuscript, further examinations are necessary to elucidate the exact molecular mechanisms for the Th2-type cytokine expression in mast cells.

The dose of LPS required for effective mast cell activation is a matter of some controversy. Several authors suggest that a relatively high dose of LPS is necessary (24, 56, 57), whereas a recent report suggests a much lower dose is sufficient (26). We have shown in our present manuscript that Th2 cytokine production by LPS from mast cells is dose dependent and that LPS concentrations as low as 1–10 ng/ml still induced a significant amount of Th2 cytokine production. Thus, our results have indicated that mast cells respond to physiological concentrations of LPS in Gram-negative bacterial infection *in vivo*. The reasons for the discrepancy of LPS concentration among reports are unknown. We

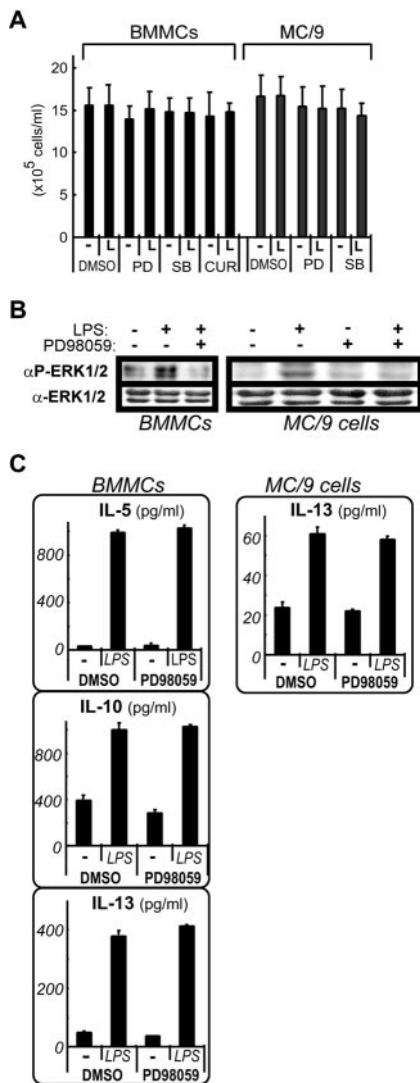


FIGURE 5. A, Cell viability of mast cells after stimulation. Cells (2×10^6 cells/ml) were preincubated with 0.1% DMSO, 10 μ M PD98059 (PD), 10 μ M SB208530 (SB), 10 μ M curcumin (CUR), or not for 30 min followed by 1 μ g/ml LPS stimulation (L) or they were left untreated (-). After 16 h, total cell counts were determined by trypan blue exclusion. The error bars represent SD values. B, Effective inhibition of the ERK phosphorylation by a specific inhibitor of ERK pathway (PD98059). Cells were preincubated with DMSO (0.1%) or PD98059 (10 μ M) for 30 min and then were stimulated with LPS (1 μ g/ml) for 10 min (BMMCs) or 30 min (MC/9 cells). LPS-mediated ERK phosphorylation was measured by Western blot using the anti-phospho-ERK Ab. C, ERK activation is not essential for the Th2-associated cytokine production from mast cells. Cells (2×10^6 cells/ml) were preincubated with DMSO (0.1%) or PD98059 (10 μ M) for 30 min followed by 1 μ g/ml LPS stimulation. After 16 h, the cell-free culture supernatants were collected and cytokine ELISAs were performed. The experiments were done in triplicate. The error bars represent SD values.

found that LPS from some serotypes of *E. coli* needs a significantly higher concentration than other serotypes to stimulate Th2 cytokine production from mast cells. Thus, one possible explanation is the different bacterial serotypes used in the previous reports. Furthermore, as shown in Figs. 2B and 3C, BMMCs from C3H/HeN mice reacted to LPS much less in comparison with BMMCs from BALB/c mice. Thus, it is also possible that the differences in cytokine responses were caused by the different strains of mice.

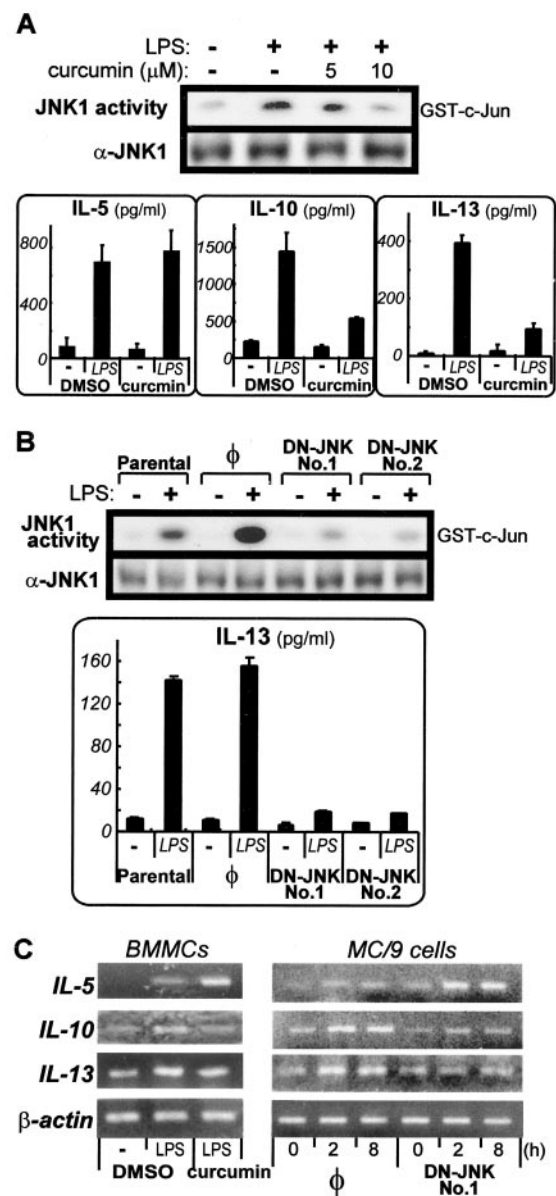


FIGURE 6. A, Inhibition of the JNK activation decreased IL-10 and IL-13 production from BMMCs. For measurement of JNK activity, BMMCs were preincubated with various concentrations of curcumin for 30 min followed by a 10-min stimulation with 1 μ g/ml LPS. LPS-mediated JNK activation was measured by the in vitro kinase assay using GST-c-Jun as the substrate. For cytokine ELISAs, BMMCs (2×10^6 cells/ml) were preincubated with DMSO (0.1%) or curcumin (10 μ M) for 30 min followed by 1 μ g/ml LPS stimulation. After 16 h, the cell-free culture supernatants were collected and cytokine ELISAs were performed. The experiments were done in triplicate. The error bars represent SD values. B, Expression of the dominant-negative JNK inhibited the production of IL-13 from MC/9 cells. MC/9 cells were transfected with the plasmids encoding a kinase-inactive JNK3 cDNA. As controls, MC/9 cells were also transfected with vector alone (ϕ). Two G418-resistant clones were obtained (DN-JNK No. 1 and No. 2). For measurement of JNK activity, in vitro kinase assay was performed using GST-c-Jun as the substrate with whole cell extracts from the parental, ϕ , or DN-JNK No.1 or No.2. Cells were stimulated with 1 μ g/ml LPS for 30 min. For cytokine ELISAs, BMMCs (2×10^6 cells/ml) were incubated with 1 μ g/ml LPS. After 16 h, the cell-free culture supernatants were collected and cytokine ELISAs were performed. The experiments were done in triplicate. The error bars represent SD values. C, Inhibition of the JNK activation impaired the mRNA up-regulation of IL-10 and IL-13. Cells (2×10^6 cells/ml) were stimulated with LPS (1 μ g/ml) or not for 4 h (BMMCs) or for the indicated time (MC/9 cells). Total RNA (2 μ g each) was reverse transcribed and PCR amplified as described.

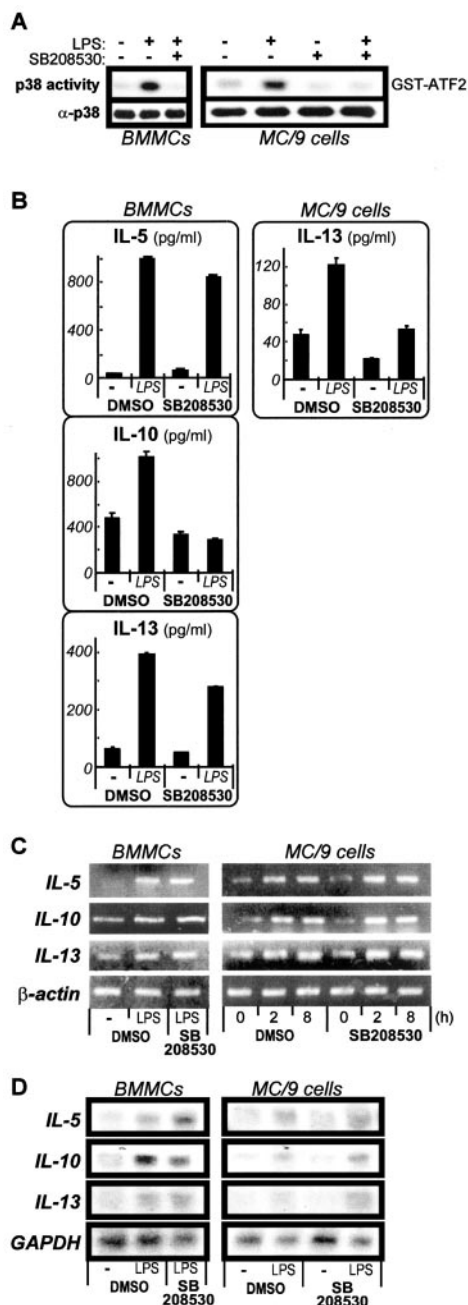


FIGURE 7. A, Effective inhibition of the p38 activation by a specific inhibitor of p38 (SB208530). Cells were preincubated with DMSO (0.1%) or SB208530 (10 μ M) for 30 min and then were stimulated with LPS (1 μ g/ml) for 10 min (BMMCs) or 30 min (MC/9 cells). LPS-mediated p38 activation was measured by the in vitro kinase assay using GST-activating transcription factor 2 as the substrate. B, Inhibition of the p38 pathway inhibited Th2-associated cytokine production. Cells (2×10^6 cells/ml) were preincubated with DMSO (0.1%) or SB208530 (10 μ M) for 30 min followed by 1 μ g/ml LPS stimulation. After 16 h, the cell-free culture supernatants were collected and cytokine ELISAs were performed. The experiments were done in triplicate. The error bars represent SD values. C, Inhibition of the p38 activation did not impair the mRNA up-regulation of Th2-associated cytokines. Cells (2×10^6 cells/ml) were preincubated with DMSO (0.1%) or SB208530 (10 μ M) for 30 min and were stimulated with LPS (1 μ g/ml) or not for 4 h (BMMCs) or for the indicated time (MC/9 cells). Total RNA (2 μ g each) was reverse transcribed and PCR amplified as described. D, Inhibition of p38 activation did not impair the mRNA up-regulation of Th2-associated cytokines. Cells (2×10^6 cells/ml) were preincubated with DMSO (0.1%) or SB208530 (10 μ M) for 30 min and were stimulated with LPS (1 μ g/ml) or not for 4 h. Total RNA (10 μ g each) was isolated and RPA analysis was performed.

Although both IL-4 and IL-13 are major Th2-type cytokines and are considered to have similar activity (58), we could hardly detect IL-4 production after LPS stimulation in contrast with IL-13 production. It has previously been reported that as in T cells, sequences between bp -87 and -70 of IL-4 gene upstream regions are critical for protein association and activation-dependent gene transcription in mast cells. In the protein complex binding this region, AP-1 family members are unique to the T cell stimulation-dependent complex, whereas mast cell complexes contain factors NF-ATp and anti-NF-ATc (59), indicating that AP-1, which is strongly activated by LPS, may not be involved in the IL-4 gene expression in mast cells. Very little is known about the transcriptional regulation of the IL-13 gene. Recently, it has been reported that IL-13 is not always coexpressed with other Th2 cytokines, such as IL-4 and IL-5, in normal Th2 cells on a single-cell basis (60). Only IL-13, but not IL-4, was produced at a significant level by the GATA-3-expressing Th1 cells, and both GATA-3 and c-maf were needed for the full induction of IL-4. These observations indicate that the expression of IL-13 is regulated by a mechanism distinct from that regulating the expression of IL-4. Because mast cells do not express GATA-3 (61), regulation of IL-13 production is different from that in T cells.

It should be noted that LPS induces other cytokines such as IL-1 β , IL-6, IL-10, and TNF- α from mast cells. It has been shown that mast cell-derived TNF- α -dependent recruitment of circulating leukocytes is crucial for a full response against acute infection (6, 62). IL-10, which is classified as a Th2-associated cytokine, has a regulatory role to inhibit excess reaction against LPS (63). Interestingly, IL-13 has also been reported to regulate excessive reactions against LPS and to prevent endotoxin lethality in mice (64). Down-regulation of TNF- α , IFN- γ , and IL-12 production by various cell types including macrophages seemed to be important for this regulatory effect of IL-13 (65). Thus, it is likely that mast cells orchestrate various types of immune reaction in the presence of LPS.

We have shown that MAPKs including ERK1/2, JNK, and p38 were activated by LPS in both the MC/9 cell line and BMMCs. Among these MAPKs, ERK activation induced by LPS was weak in comparison with that induced by Ag-IgE ligation of Fc ϵ R1. Inhibition of ERK activation did not affect IL-5, IL-10, or IL-13 production after LPS stimulation, indicating that ERK is not essential for the cytokine induction. In contrast, JNK and p38 kinase were activated by LPS to similar degrees as by IgE ligation (Fig. 4C). Inhibition of JNK activation decreased IL-10 and IL-13 mRNA and protein release from mast cells induced by LPS. These results indicate that JNK regulates cytokine production at the transcriptional level. Both IL-10 and IL-13 gene regulatory regions contain AP-1 binding sites. Because AP-1 transcriptional activity is directly regulated by JNK, JNK may regulate translation of IL-10 and IL-13 mRNA through AP-1. In a recent report (26), it was shown that LPS failed to induce JNK phosphorylation in BMMCs, in contrast with our present findings. The discrepancy is probably due to the difference in assay sensitivity (phospho-specific Western blot vs in vitro kinase assay). In fact, we could hardly detect JNK phosphorylation by the phospho-specific Ab in LPS-treated BMMCs (data not shown).

Although the inhibition of p38 kinase activity down-regulated the production of IL-5, 10, and 13 induced by LPS, the mechanism of the inhibition seems different from that of JNK, in that the inhibition of p38 kinase did not decrease the mRNA levels of the cytokines. Thus, p38 activity seems essential for the post-transcriptional regulation of these cytokines. It has been reported that p38 kinase regulates translation of mRNA in a 3'-UTR-dependent manner (66–68). The p38 kinase may regulate translation of

mRNA, whereas JNK may regulate transcription and perhaps stability of the mRNA. In contrast with IL-10, IL-5 production by LPS was only slightly decreased by inhibition of the p38 pathway and was not decreased by the inhibition of the JNK pathway. Thus, the mechanisms of IL-5 mRNA translation and production remain largely unknown. There are several reports that show that IL-5 mRNA induction and protein production are dependent on the protein kinase C (PKC) pathway (69, 70). Because LPS activates PKC in various cell types including mast cells (71–74) and some PKC isoforms activate p38 kinases (75), IL-5 production may be regulated by sequential activation of PKC-p38 kinase.

Our data also demonstrate that costimulation of LPS and IgE cross-linking synergistically promotes Th2-associated cytokine production. It has been reported that LPS strongly enhances the expression of IL-9 in addition to IL-13 in mast cells activated with IgE cross-linking and that NF- κ B has a critical role for this synergistic IL-9 production (76). Because our data suggest that not only IL-5, 10, and 13 but also IL-6 and TNF- α mRNA are synergistically up-regulated by costimulation of LPS and IgE cross-linking, most cytokines that are able to be produced by mast cells, except for IL-4, are synergistically produced by this costimulation. Although p38 kinase and JNK are synergistically activated by costimulation of LPS and IgE cross-linking and these two MAPKs have a critical role for IL-10 and IL-13 production, it is reasonable to speculate that these two MAPK pathways are involved in this synergistic cytokine production.

Taken together, we have demonstrated that LPS alone could induce IL-5, 10, and 13 production and that the combination of LPS and Ag-IgE ligation of Fc ϵ RI promoted these cytokine productions synergistically. Our report may indicate an important clue to connect missing link between asthma and LPS.

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