

Common and distinct signalling cascades in the production of tumour necrosis factor- α and interleukin-13 induced by lipopolysaccharide in RBL-2H3 cells

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Summary

Background Activation of mast cells by lipopolysaccharide (LPS) results in the production of TNF- α and IL-13. TNF- α and IL-13 are key mediators in the development of neutrophilic and allergic inflammation, respectively. LPS-induced TNF- α and IL-13 production in mast cells has been reported to be mediated by Toll-like receptor 4 (TLR4) signalling, but differences in signal transduction mechanisms leading to the production of these cytokines are not clearly defined.

Objective We investigated the molecular mechanisms responsible for LPS-induced TNF- α and IL-13 production in mast cells.

Methods TNF- α and IL-13 production by LPS was assessed by transfecting RBL-2H3 cells with dominant-negative (DN) expression vectors.

Results Transfection of RBL-2H3 cells with plasmids encoding DN mutants of myeloid differentiation protein (MyD88) and TNFR-associated factor (TRAF6) inhibited both LPS-induced TNF- α and IL-13 production. I κ B α -DN inhibited LPS-induced production of TNF- α , but not IL-13. We also found that inhibition of p38 kinase suppressed both TNF- α and IL-13 induction by LPS, and inhibition of JNK reduced IL-13 production, but not TNF- α . Furthermore, we found that protein kinase R (PKR) was activated by LPS in these cells. Treatment with 2-aminopurine, a PKR inhibitor, attenuated LPS-induced nuclear factor- κ B activation and TNF- α production, whereas inhibition of PKR had little effect on IL-13 production.

Conclusion These findings indicate that the production of TNF- α and IL-13 by LPS required TLR4/MyD88/TRAF6 signalling as a common pathway of mast cell-mediated inflammation. We furthermore found that TNF- α and IL-13 production were differentially regulated by signalling cascades through PKR and mitogen-activated protein kinases downstream of TRAF6 in mast cells.

Keywords allergy, bacterial, inflammation, mast cells

Submitted 29 June 2004; revised 16 November 2004; accepted 8 February 2005

Introduction

Recent reports indicate that mammalian innate immune responses are initiated by certain pattern recognition receptors, called Toll-like receptors (TLRs) [1]. TLRs comprise a family of cell-surface proteins that stimulate pro-inflammatory cytokine gene transcription in response to various microbial ligands [2]. Lipopolysaccharide (LPS) is a critical glycolipid component of the outer wall of Gram-negative bacteria [3]. Many of the cellular signals activated by Gram-negative bacteria are attributed to TLR4-mediated recognition of LPS [4].

A number of studies demonstrate that mast cells play a crucial role in protection against Gram-negative bacteria [5–7]. We previously reported that cell-surface TLR4-mediated

activation of mast cells by Gram-negative bacteria LPS resulted in production of inflammatory cytokines including TNF- α . TNF- α secretion induces a rapid infiltration of neutrophils, followed by expansion of the inflammatory responses that leads to efficient eradication of bacteria. Mast cells are a predominant source of TNF- α which is critical for the innate immune response to bacterial infection [6, 7].

Not only do mast cells play a pivotal role in neutrophilic inflammation against bacterial infection, but they also are central effectors in allergic inflammation [8]. Mast cell activation is triggered by the aggregation of Fc ϵ RI through the multivalent binding of Ag to receptor-bound IgE in allergic inflammation. Fc ϵ RI aggregation then results in the release of Th2 cytokines, IL-4, IL-5, IL-9 and IL-13, which are important in the pathogenesis of allergic reactions [9, 10]. In particular, IL-13 was identified as a critical regulator of the allergic response. Initial studies in animal models of allergic diseases provided compelling evidence that IL-13, independent of other Th2 cytokines, was necessary and sufficient to induce all features of allergic asthma [11], in that the selective neutralization of IL-13 ameliorates airway hyper-responsiveness, eosinophil recruitment and mucus overproduction [12].

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LPS has been reported to strongly enhance the expression of TNF- α and IL-13 in bone-marrow-derived murine mast cells (BMMCs) [13, 14], and can further stimulate the production of IL-13 by BMMCs activated with cross-linked IgE [13, 14]. More recently, several groups confirmed that human mast cells response to TLR ligands, including LPS. Varadaradjalou et al. [15] reported that LPS induced significant release of TNF- α and IL-13 from human normal cord blood-derived mast cells. It has also shown the up-regulation of TLR4 expression following exposure to IL-4 [15] or IFN- γ [16] on human mast cells. These findings suggest that LPS may function as a powerful activating stimulus for mast cells in neutrophilic and allergic inflammatory reactions.

The signal transduction pathway of TLRs has been intensively studied in the macrophage. Ligand activation of TLRs, results in recruitment of the adaptor protein, myeloid differentiation protein (MyD88) [17], which in turn signals through a complex comprised of the IL-1R-associated kinases (IRAKs) and TNFR-associated factor 6 (TRAF6) [18, 19]. Through a series of kinases, TRAF6 transmits the ligand signal to activate the transcriptional regulator nuclear factor- κ B (NF- κ B) [18, 19] and mitogen-activated protein kinases (MAPKs) [20]. In mast cells, relatively little is known about the signal transduction pathway of LPS-induced pro-inflammatory cytokines and Th2 cytokines. To better understand neutrophilic and allergic inflammation associated with LPS-activated mast cells, we studied the downstream effects of activation of the TLR4 signal transduction pathway by LPS, which leads to induction of TNF- α and IL-13 production in mast cells.

Materials and methods

Cells and reagents

RBL-2H3 cells were kindly provided by H. Suzuki (Nihon University School of Medicine, Tokyo, Japan). RBL-2H3 cells were maintained in MEM containing 10% FCS.

LPS (*Escherichia coli* serotype 055:B5) was obtained from Sigma (St Louis, MO, USA). Anti-phospho-specific p38 MAPK Ab, anti-p38 MAPK, anti-phospho-specific JNK Ab, anti-JNK Ab (Cell Signaling Technology, Beverly, CA, USA) [21]. The specific inhibitor of JNK, SP600125 [22], was obtained from BIOMOL Research Laboratories (Plymouth Meeting, PA, USA). The specific inhibitor of p38 MAPK, SB203580 [23], were obtained from Calbiochem (San Diego, CA, USA). Anti-protein kinase R (PKR) rabbit polyclonal ab (Biosource International Inc., Camarillo, CA, USA), anti-FLAG M2 mouse mab (Upstate Biotechnology, Lake Placid, NY, USA), anti-Myc-Tag Rabbit polyclonal ab (Cell Signaling Technology), anti-haemagglutinin (HA) rat mab, clone3F10 (Roche Molecular Biochemicals, Mannheim, Germany), anti-MyD88 polyclonal ab (BD PharMingen, San Diego, CA, USA), anti-TRAF6 polyclonal ab and anti-I κ B α mab (Santa Cruz Biotechnology, Santa Cruz, CA, USA).

Western blot analysis

In brief, whole-cell lysates containing 10 μ g of protein were separated by 15% SDS-PAGE and then electrophoretically

transferred to a nylon membrane. The membrane was first incubated with indicated specific antibodies, and then incubated with a secondary HRP-conjugated anti-rabbit IgG and with an HRP-conjugated ab specific for biotin to detect biotinylated protein markers. Blots were incubated in enhanced chemiluminescence solution for 1 min and exposed on Kodak XAR film (Tokyo, Japan).

Retroviral vectors, constructs and transfections

MyD88-dominant negative (DN) was kindly provided by M. Muzio (Mario Negri Institute, Milan, Italy). MyD88-DN encodes only the COOH-terminal Toll-IL-1R-like domain that abrogates LPS-induced NF- κ B activation [24]. MyD88-DN was tagged with the HA epitope. TRAF6-DN, kindly provided by H. Nakano (Juntendo University, Tokyo, Japan), encompasses amino acids 1–274 of TRAF6, in which the N-terminal RING finger sequence required for TRAF6 signalling is deleted. TRAF6-DN was tagged with the FLAG epitope. I κ B α -DN was a kind gift from P. Baeuerle (Tularik Inc., San Francisco, CA, USA) [25]. The mutated gene has two serines in the N-terminal domain of I κ B α that are critical for stability, Ser-32 and Ser-36 replaced with alanine to render I κ B α resistant to degradation by cellular activators.

DN mutant proteins were subcloned into pcDNA3.0 (Invitrogen, Carlsbad, CA, USA) and the Moloney murine leukaemia virus-based retroviral vector, pMX-puro [26]. To generate retroviruses, retroviral vectors were transiently transfected using Eugene 6 (Roche Molecular Biochemicals) into an ecotropic retrovirus packaging cell line, PLAT-E

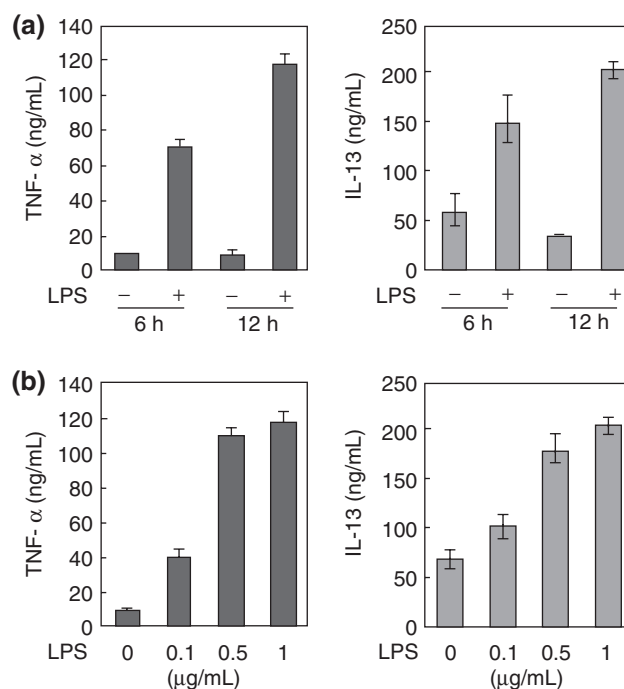


Fig. 1. Lipopolysaccharide (LPS) induces TNF- α and IL-13 production. (a) RBL-2H3 cells (1×10^6 cells/mL) were cultured in medium without or with LPS (1 μ g/mL), and the concentrations of TNF- α and IL-13 in the culture supernatants were determined after 6 and 12 h. (b) RBL-2H3 (1×10^6 cells/mL) cells were grown in medium alone or with LPS (0.1, 0.5 or 1.0 μ g/mL) and the concentrations of TNF- α and IL-13 in the culture supernatants were determined after 12 h. The values are the mean $\pm$ SD of five replicates.

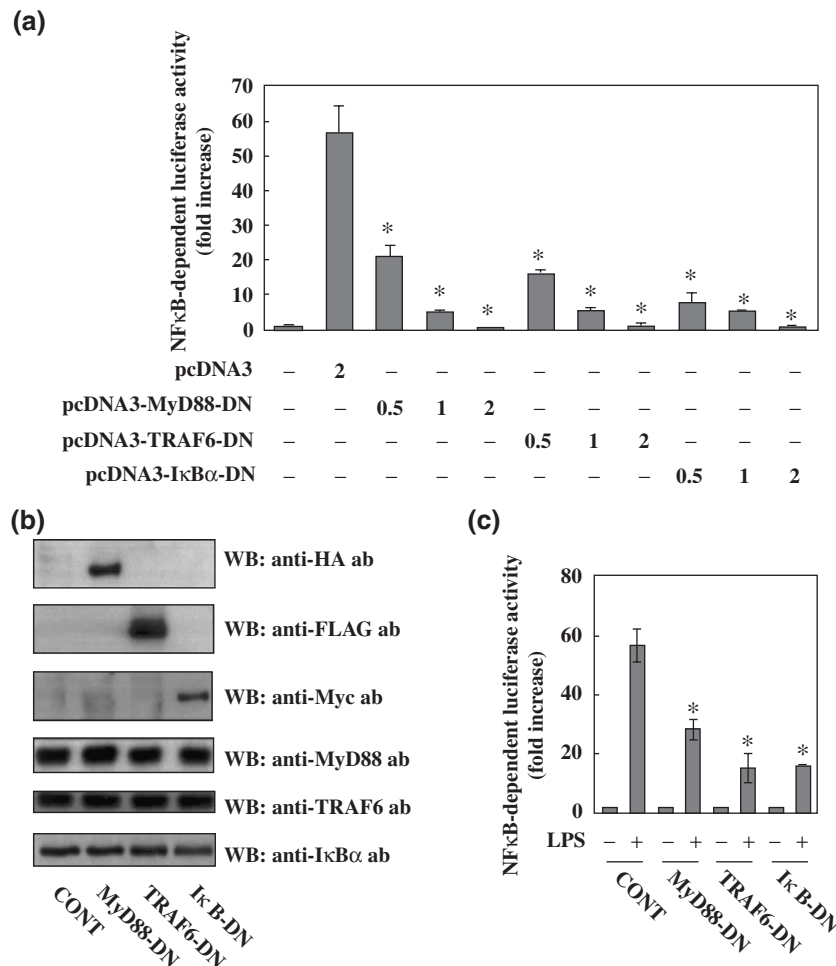


Fig. 2. MyD88-DN, TRAF6-DN, IκBα-DN inhibits LPS-induced NF-κB-dependent gene expression. (a) Effect of dominant-negative MyD88, TRAF6 and IκBα on LPS-induced activation of an NF-κB promoter. RBL-2H3 cells (5×10^5 cells) were transiently transfected with 0.5, 1 or 2 μg of each plasmids encoding DN MyD88, TRAF6 or IκBα as indicated, along with NF-κB-luciferase plasmid. Following stimulation with LPS (1 μg/mL), extracts were prepared and measured for luciferase activity. Results are represented as fold increase over non-stimulated empty vector control. (b) Expression of MyD88, TRAF6, IκBα, MyD88-DN, TRAF6-DN and IκBα-DN proteins. Whole-cell lysates were prepared from the transfected cells. Equal amounts of whole-cell lysates (10 μg/lane) were subjected to SDS-PAGE, and then western blots were performed using a specific antibody against HA, FLAG, Myc, MyD88, TRAF6 and IκBα. MyD88, TRAF6 and IκBα were used as loading controls. (c) Dominant-negative MyD88, TRAF6 and IκBα inhibit LPS-induced activation of an NF-κB promoter in RBL-2H3 cells. RBL-2H3 cells expressing individual dominant mutant proteins were grown on 12-well plates, transfected with NF-κB-luciferase reporter plasmids, and incubated for 12 h prior to stimulation with LPS (1 μg/mL) for 6 h. Luciferase activity in the cytoplasmic extracts is given as the fold increase of mean luciferase light units with reference to unstimulated cells. The values are the mean $\pm$ SD of three independent experiments. * $P < 0.01$ compared with the empty vector transfected cells. MyD88, myeloid differentiation protein; DN, dominant negative; TRAF6; TNFR-associated factor 6; LPS, lipopolysaccharide; NF-κB, nuclear factor-κB.

(SD3444, American Type Culture Collection, Manassas, VA, USA). Cells expressing the marker gene were selected with 1.5 μg/mL puromycin (Sigma). The pMX-puro vector alone was transfected for preparation of control protein extracts. Twenty-four hours post-transfection, viral supernatants were harvested and concentrated by centrifugation. The pellets were resuspended in 0.1 volume of culture medium for subsequent infection of RBL-2H3 cells.

Measurement of TNF- α and IL-13

RBL-2H3 cells were stimulated with LPS in the presence of 10% FCS. Six hours after treatment, the culture supernatants were cleared by centrifugation for the determination of TNF- α and IL-13 concentrations using commercially available ELISA kits (Biosource International Inc.). All samples were assayed in duplicate.

Transfection and luciferase assays

RBL-2H3 cells were cultured for 16 h prior to transfection with Fugene6 (Roche Molecular Biochemicals). The plasmid NF-κB-luciferase (Stratagene, La Jolla, CA, USA), containing five copies of consensus NF-κB site linked to a minimal promoter-luciferase gene, functioned as a reporter for signal activation by LPS. Duplicate or triplicate wells were set up for each transfection, with DNA amounts reported in the figure legends. Cells were allowed to recover for 24 h post-transfection and then were left unstimulated or were stimulated with LPS as described in the figure legends. Cell lysates were prepared, and luciferase levels were measured using Promega's Luciferase Assay System according to manufacturer's instructions. In some experiments, pRL-Tk (Promega) plasmid DNA was cotransfected as an internal control for normalization of differences in transfection efficiency.

Statistics

Statistical significance was assessed by analysis of variance (ANOVA). A *P*-value of less than 0.05 was considered to be significant.

Results

LPS induces TNF- α and IL-13 production from RBL-2H3 mast cells

RBL-2H3 mast cells were cultured with 1 $\mu\text{g/mL}$ of LPS. The concentrations of TNF- α and IL-13 in the cultured supernatants from RBL-2H3 mast cells were determined by ELISA after 6 or 12 h of treatment. As shown in Fig. 1a, the concentrations of TNF- α and IL-13 protein increased with LPS in a time-dependent manner. We also examined the LPS dose dependency of TNF- α and IL-13 production using the supernatants from RBL-2H3 mast cells stimulated for 12 h with various concentrations of LPS. The concentrations of TNF- α and IL-13 in the culture supernatants from LPS-stimulated cultures increased in a dose-dependent manner (Fig. 1b).

MyD88, TRAF6 and I κ B α mediate the activation of NF- κ B by LPS

MyD88 and TRAF6 have been reported to be transducers for the TLR superfamily, a recently defined and expanding group of receptors that participate in innate immune and inflammatory responses [17–19]. We examined the role of MyD88, TRAF6 and I κ B α in LPS-induced transactivation of NF- κ B. DN mutants of MyD88, TRAF6 and I κ B α were used in cotransfection experiments with the NF- κ B-luciferase plasmid, and their effects on LPS-induced NF- κ B activation were assessed. Transient transfection of RBL-2H3 cells with MyD88-DN, TRAF6-DN, I κ B α -DN completely inhibited the LPS-induced NF- κ B-dependent luciferase gene expression in a dose-dependent manner, and 2 μg (per 5×10^5 cells) of the DN plasmids was completely abolished LPS-mediated NF- κ B activation (Fig. 2a). Overall, these results imply that MyD88, TRAF6 and I κ B α are required for the effect of LPS on the transactivation of gene expression by NF- κ B.

To study the role of components of the TLR4 signalling complex in LPS-induced TNF- α and IL-13 production in mast cells, we next introduced antigen-tagged, MyD88-DN, TRAF6-DN and I κ B α -DN proteins into RBL-2H3 cells using a retrovirus expression system. Constructs encoding HA-tagged MyD88-DN, FLAG-tagged TRAF6-DN and Myc-tagged I κ B α -DN were transfected into RBL-2H3 cells. Western immunoblots with HA, FLAG or Myc tag-specific antibodies demonstrated the expression of each of these molecules in transfected cell extracts (Fig. 2b). LPS induction of NF- κ B-dependent luciferase gene expression is inhibited 50–90% in cells expressing any of the mutant proteins (Fig. 2c).

MyD88 and TRAF6 are required for LPS-induced TNF- α and IL-13 production, but activation of NF- κ B is not essential for LPS-induced IL-13 production

Figure 3 shows the effect of MyD88-DN, TRAF6-DN or I κ B α -DN overexpression on LPS-induced TNF- α and IL-13

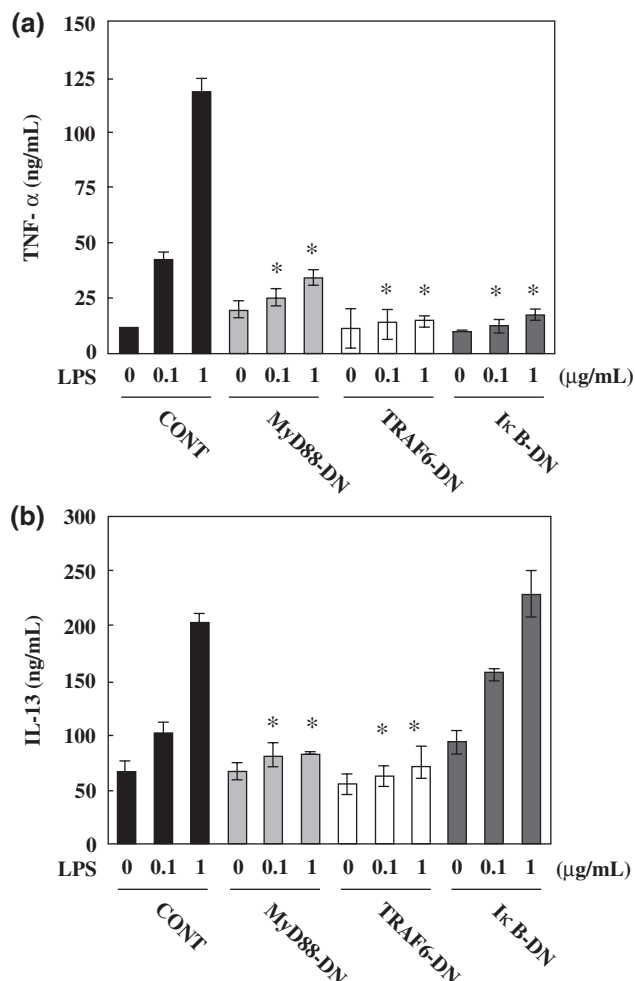


Fig. 3. MyD88 and TRAF6 are necessary for LPS-induced TNF- α and IL-13 production, but activation of NF- κ B is not essential for LPS-induced IL-13 production. RBL-2H3 cells expressing MyD88-DN, TRAF6-DN or I κ B α -DN mutant proteins grown on 12-well plates (1×10^6 cells/mL) were stimulated with 0.1 or 1 $\mu\text{g/mL}$ LPS. After 12 h, the culture supernatants were collected for TNF- α and IL-13 cytokine ELISAs. Experiments were performed done in triplicate, and the error bars represent standard deviation from the mean. **P* < 0.01 compared with the empty vector transfected cells. MyD88, myeloid differentiation protein; TRAF6, TNFR-associated factor 6; LPS, lipopolysaccharide; NF- κ B, nuclear factor- κ B; DN, dominant negative.

production in RBL-2H3 cells. Overexpression of either MyD88-DN, TRAF6-DN or I κ B α -DN reduced LPS-induced TNF- α production in RBL-2H3 cells compared with vector-only transfected control cells (Fig. 3a). MyD88-DN and TRAF6-DN also reduced induction of IL-13 by LPS in RBL-2H3 cells, whereas overexpression of I κ B α -DN had no effects (Fig. 3b).

p38 MAPK activation is required for both of LPS-induced TNF- α and IL-13 synthesis, but JNK activation is essential only for LPS-mediated IL-13 synthesis

LPS has been reported to induce the activation of MAPK pathways, including ERK, JNK and p38 MAPK in various cell types [13, 20, 27]. MAPKs play an important role in the regulation of inflammatory and Th2 cytokine production from mast cells [14, 28–30]. To investigate possible involve-

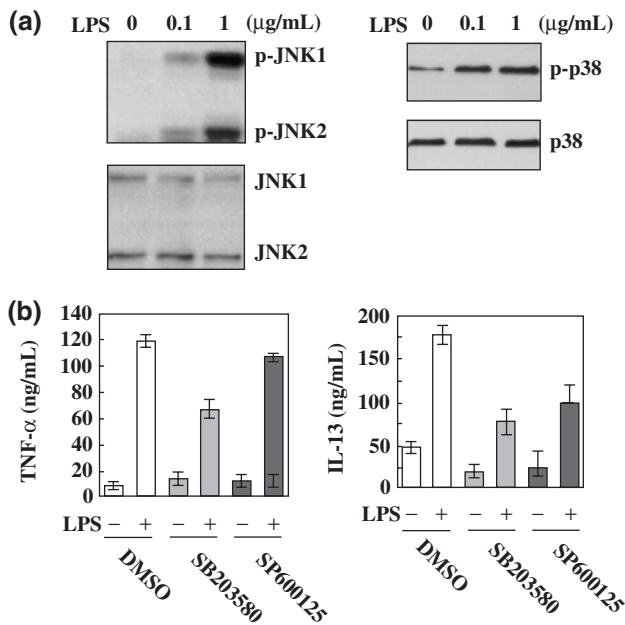


Fig. 4. JNK and p38 MAPK is involved in LPS-induced TNF- α and IL-13 production. (a) RBL-2H3 cells were stimulated with 0.1 or 1 μ g/mL of LPS for 15 min. LPS-mediated JNK activation was evaluated by western blots using an antibody to phosphorylated JNK (left upper panel), and LPS-mediated p38 MAPK phosphorylation was evaluated using antibodies specific to phosphorylated p38 MAPK (right upper panel). Each blot was then stripped and reprobed with a phosphorylation state-independent JNK-specific antibody (left lower panel), or a phosphorylation state-independent p38 MAPK-specific antibody (right lower panel), to determine the amount of total protein loaded. Blots shown represent one example of three identical experiments performed independently. (b) RBL-2H3 (1×10^6 cells/mL) cells were incubated with 0.1% DMSO only, or 10 μ M JNK-specific inhibitor SP600125 and 10 μ M p38 MAPK-specific inhibitor SB203580 in 0.1% DMSO for 1 h, and then stimulated with 1 μ g/mL LPS. After 12 h, the culture supernatants were collected and cytokine ELISAs were performed. The experiments were performed in triplicate, and the error bars represent standard deviation from the mean. MAPK, mitogen-activated protein kinases; LPS, lipopolysaccharide.

ment of MAPK pathways in LPS-induced TNF- α and IL-13 production from mast cells, we tested for activation of MAPKs in LPS-stimulated mast cells. Extracts from RBL-2H3 cells stimulated with 0.1 or 1 μ g/mL of LPS for 15 min were analysed by western immunoblot with ERK-, p38 MAPK- or JNK-specific antibodies. Levels of phosphorylated JNK and p38 MAPK increased in a dose-dependent manner (Fig. 4a, upper panel). Increases in JNK and p38 MAPK phosphorylation occurred in the absence of changes in protein levels, regardless of incubation time (Fig. 4a, lower panel).

To investigate which kinases may regulate LPS-induced TNF- α and IL-13 expression in RBL-2H3 cells, we examined the effects of specific inhibitors of p38 MAPK (SB203580) and/or JNK (SP600125). RBL-2H3 cells pre-incubated with SP600125 and SB203580 were stimulated with LPS for 12 h, and the concentrations of TNF- α and IL-13 in the culture supernatants were determined by ELISA. Pre-incubation of the cells with both SP600125 (10 μ M) and SB203580 (10 μ M) for 1 h resulted in the inhibition of IL-13 production in response to stimulation with LPS. SB203580 alone inhibited LPS-induced TNF- α production, whereas SP600125 had no effects (Fig. 4b).

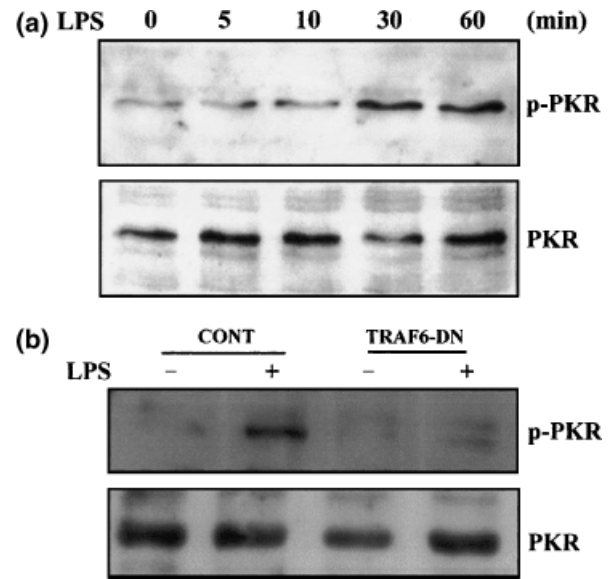


Fig. 5. LPS induces PKR activation in RBL-2H3 cells. (a) RBL-2H3 cells were stimulated with 0.1 or 1 μ g/mL LPS for indicated time periods. LPS-mediated PKR activation was evaluated by western blot using antibodies to phosphorylated PKR (upper panel). The blot was then stripped and reprobed with a phosphorylation state-independent PKR-specific antibodies (lower panel) to determine the amount of phosphoprotein relative to total protein. Blots are representatives of three identical experiments performed independently. (b) TRAF6-DN-overexpressing RBL-2H3 cells were stimulated with 1 μ g/mL of LPS for 30 min. PKR activation was evaluated by comparison of western blots using antibodies to phosphorylated PKR (upper panel) and a phosphorylation state-independent PKR-specific ab (lower panel). LPS, lipopolysaccharide; PKR, protein kinase R; TRAF6, TNFR-associated factor 6; DN, dominant negative.

PKR activation is involved in LPS-induced TNF- α production, but not in IL-13 production

Because LPS has been reported to activate PKR in human macrophages [31], and PKR, in addition to MAPKs, is considered as a component of the TLR4 signalling pathway [32], the effect of LPS on the activation of PKR in RBL-2H3 cells was examined. RBL-2H3 cells were stimulated with LPS and harvested at 0, 5, 10, 15, 30 and 60 min to evaluate PKR phosphorylation using antibodies specific to phosphorylated PKR by western immunoblots. PKR activity increased at 30 min and peaks at 60 min (Fig. 5a). Stimulation of LPS did not induce PKR autophosphorylation in TRAF6-DN-expressing RBL-2H3 cells, suggesting that PKR is downstream of TRAF6 in TLR4 signalling (Fig. 5b).

2-aminopurine (2-AP), a serine/threonine protein kinase inhibitor and competitive inhibitor of ATP during the autophosphorylation of activated PKR [33, 34], was used to test the effect of PKR activation on LPS-induced TNF- α and IL-13 expression. 2-AP pre-treatment of RBL-2H3 cells reduced LPS-induced PKR autophosphorylation (Fig. 6a), as well as NF- κ B-driven luciferase gene expression in these cells (Fig. 6b). To elucidate whether the production of TNF- α or IL-13 depends on the activation of PKR, 2-AP-treated RBL-2H3 cells were stimulated with LPS, and the levels of TNF- α and IL-13 protein were determined by ELISA. Pre-treatment of cells with 2-AP inhibited the LPS-induced expression of TNF- α in mast cells, whereas almost no inhibition of IL-13 expression was observed (Fig. 6c).

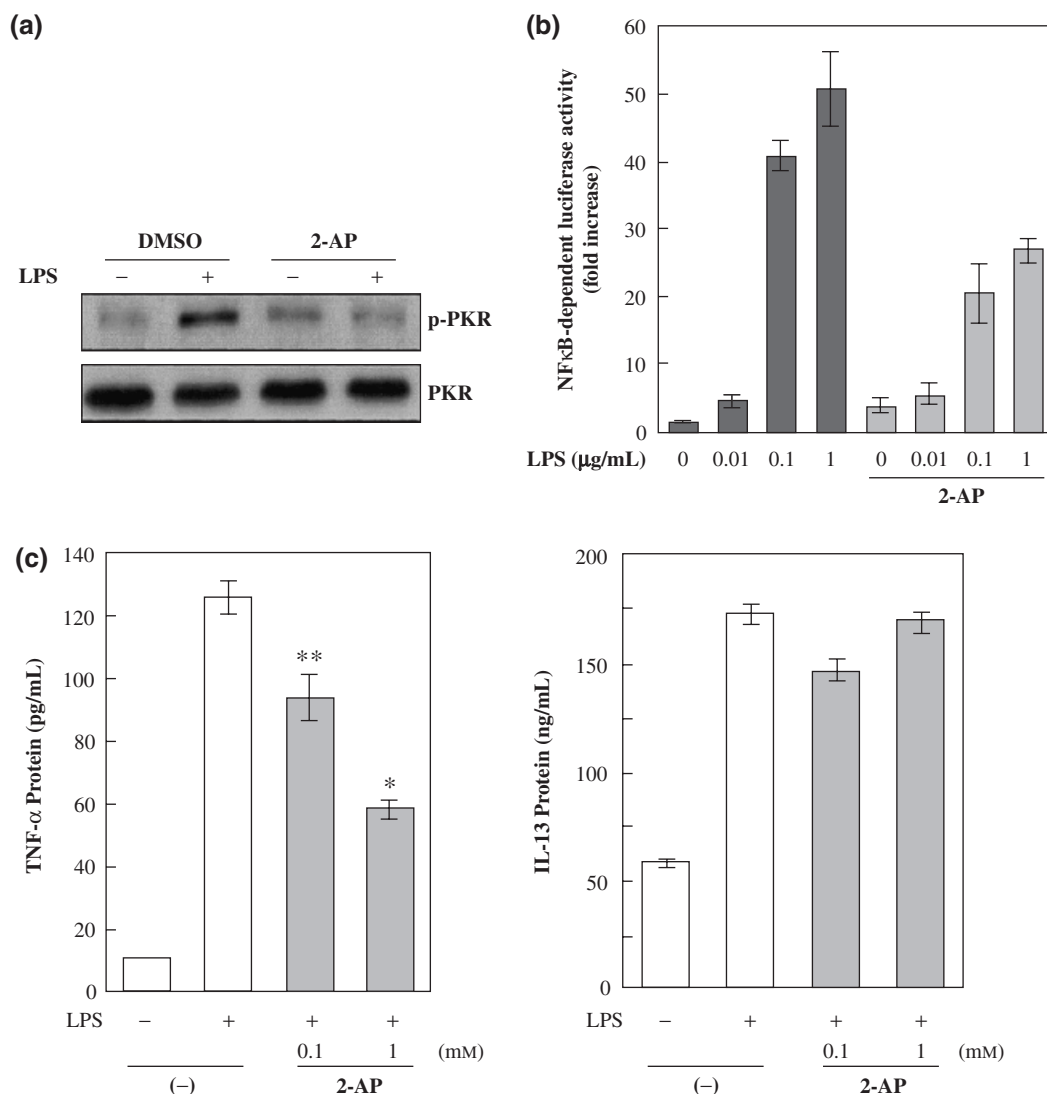


Fig. 6. PKR is involved in LPS-induced TNF- α production. (a) Effects of PKR inhibitor on LPS-induced PKR phosphorylation in RBL-2H3 cells. RBL-2H3 cells were incubated with 2-AP (1 mM) for 2 h and then stimulated with LPS (1 μ g/mL). PKR activation was evaluated by comparison of protein signals in western blots using antibodies specific to phosphorylated PKR (upper panel) relative to signals with phosphorylation state-independent PKR-specific ab (lower panel). (b) Effects of PKR inhibitor on NF- κ B-dependent transcription in LPS-stimulated RBL-2H3 cells. RBL-2H3 cells grown on 12-well plates were transfected with NF- κ B-luciferase reporter plasmids. After 12 h, the cells were incubated for 2 h with 1 mM of 2-AP prior to stimulation with the indicated dose of LPS for 6 h. Luciferase activity measured in the cytoplasmic extracts is represented as the fold increase of mean luciferase light units with reference to cells not treated with LPS. The results are the mean $\pm$ SD of three independent experiments. (c) Effects of PKR inhibitor on LPS-induced TNF- α and IL-13 production from RBL-2H3 cells. RBL-2H3 cells (1×10^6 cells/mL) were incubated for 2 h in PBS with 0.1 or 1 mM of 2-AP, and then transferred into medium alone or medium plus 1 μ g/mL of LPS. After 12 h, the culture supernatants were collected, and cytokine ELISAs were performed. The experiments were done in triplicate, and error bars represent standard deviation from the mean. ** $P < 0.05$ compared with the control cell culture (medium only). * $P < 0.01$ compared with the control cell culture (medium only). PKR, protein kinase R; LPS, lipopolysaccharide; NF- κ B, nuclear factor- κ B.

Discussion

The signalling pathway activated by LPS which results in production of inflammatory and Th2 cytokines in murine mast cells has been characterized [14, 35]. It seems that LPS-dependent up-regulation of mast cell-derived TNF- α production gives a role to this cytokine in neutrophilic inflammation, whereas LPS-induced IL-13 production enhances allergic inflammation.

MyD88 and TRAF6 act as key regulators in the LPS/TLR4 signal transduction pathway. Using retrovirus vectors encoding inactive and dominant inhibiting mutants of MyD88 and TRAF6, we clearly demonstrated a role for these proteins in enhancing the production of TNF- α and IL-13 from LPS-

stimulated mast cells. Previous studies for MyD88, IRAK-1 and TRAF6 in TLR4 signal transduction demonstrated that the TIR domain of MyD88 interacts via a homotypic interaction in the TLR4 receptor complex, and that IRAK-1 and TRAF6 are subsequently recruited to the activated complex [18, 19]. In this study, DN MyD88 and TRAF6 mutant proteins inhibited LPS-induced TNF- α and IL-13 expression in RBL-2H3 mast cells. These results demonstrate that TLR4, MyD88 and TRAF6 participate in a common pathway that activates both TNF- α and IL-13 expression in mast cells in response to LPS.

Since the predominant outcome of cellular stimulation by LPS is the activation of NF- κ B [36], we next studied the involvement of NF- κ B on the regulation of TNF- α and

IL-13 production in LPS-stimulated mast cells. We showed that overexpression of I κ B α -DN, a mutant that associates irreversibly with NF- κ B, inhibited the production of TNF- α by LPS stimulation in RBL-2H3 cells. These results demonstrate the essential role of NF- κ B in LPS-stimulated expression of TNF- α in mast cells. In contrast to its effects on TNF- α production, I κ B α -DN did little in reduction of IL-13 production in RBL-2H3 cells, confirming the recent finding that the production of mast cell IL-13 is NF- κ B independent [37].

In RBL-2H3 cells, LPS-induced phosphorylation of JNK and p38 MAPK, and inhibition of JNK activation decreased IL-13, but not TNF- α production. In this study, a p38 MAPK inhibitor attenuated the production of TNF- α and IL-13. These results indicate different roles for JNK and p38 MAPK in the regulation of neutrophilic inflammation and allergic inflammation. Masuda et al. [14] reported that JNK regulates IL-13 production by LPS at the transcriptional level. Masuda et al. [14] reported that JNK regulates IL-13 production by LPS at the transcriptional level. They showed that LPS-induced IL-13 secretion from BMMC and the murine mast cell line, MC/9 seemed to be dependent on both JNK and p38 MAPK activity [14]. On the other hand, they reported that the production of IL-5 from BMMC and MC/9 cells seemed to be independent of JNK activation and partially dependent on p38 MAPK activity. In the present study, we observed that LPS-induced TNF- α production seemed to be also regulated by a JNK-independent and p38 MAPK-dependent manner. This result suggests the possible common features between signalling pathways in LPS-mediated TNF- α and IL-5 production from mast cells. We demonstrated that the LPS-induced TNF- α production is dependent on the NF- κ B activation. Though there are a few previous studies focused on the role of NF- κ B on IL-5 regulation in mast cells, but most reports have demonstrated that NF- κ B seemed to be less important for the transcription of IL-5 [38, 39]. These findings indicate that JNK- and NF- κ B-independent other pathways seem to be involved in the regulation of IL-5.

PKR was originally identified as an antiviral serine-threonine kinase that is activated by viral double-stranded RNA (dsRNA) [40] and more recently implicated in other aspects of TLR-mediated signalling [32, 41]. In the present study, we found that PKR was phosphorylated in LPS-treated RBL-2H3 cells. Expression of a DN PKR mutant protein attenuated LPS-induced NF- κ B activation in RBL-2H3 cells.

Activation of PKR is dependent on phosphorylation of the kinase on serine residues [40]. We demonstrated that 2-AP caused a strong inhibition of LPS-induced PKR phosphorylation, most probably by competitive inhibition of ATP at the PKR-ATP binding site [33, 34]. In this study, it was shown that pre-treatment with 2-AP attenuated LPS-induced NF- κ B activation in RBL-2H3 cells, and overexpression of TRAF6-DN inhibited LPS-induced PKR phosphorylation. The main regulatory mechanism of NF- κ B activation is mediated through its interaction with the I κ B family that retains NF- κ B in the cytoplasm [42]. I κ B proteins are phosphorylated by the I κ B kinase (IKK) complex at two serine residues in response to a variety of stimuli, thus tagging them for ubiquitin-proteasome-mediated degradation [43]. It

has been shown that NF- κ B activation by PKR involves the IKK pathway [44–46], but the exact mechanism is unknown. Recently, it has been shown that the members of the TRAF family proteins including TRAF6 interact with PKR and that this association is needed for NF- κ B activation by PKR [47]. We demonstrated that LPS-induced TNF- α , but not IL-13 production from mast cells requires NF- κ B activation. Consistent with these results, pre-treatment of the cells with 2-AP attenuated LPS-induced TNF- α production, which suggests PKR plays an important role in LPS-induced TNF- α production. In contrast, LPS-induced IL-13 production, which is regulated in an NF- κ B-independent manner, was not inhibited by 2-AP. The activation of PKR by dsRNA has reported to be involved in the several pathways, including those activating p38 MAPK and JNK [48]. TLR3 has been shown to recognize dsRNA and activate NF- κ B, p38 MAPK and JNK [49]. These findings suggested that the possibility that PKR might be involved in the LPS-mediated JNK activation, but, in the present study, we found that the inhibition of PKR did not affect LPS-induced IL-13 production regulated by JNK. This result indicated that TLR4-mediated PKR activation may not be an essential step in JNK activation.

In conclusion, our data provide the evidence that LPS-induced mast cell activation is dependent on TLR4 signalling, and the expression of TNF- α and IL-13 is regulated by MyD88/TRAF6 dependent common pathway in mast cell-induced inflammation. LPS-induced TNF- α production is regulated by PKR through NF- κ B activation at downstream of MyD88/TRAF6. p38 MAPK regulates both TNF- α and IL-13 synthesis in LPS-activated mast cells, suggesting a various roles for p38 MAPK in the inflammatory process. JNK was involved in LPS-induced IL-13, but not TNF- α synthesis, suggesting that JNK has a critical role in mast-cell-induced allergic inflammation.

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