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Upregulation of Toll-like Receptor (TLR) Expression and Release of Cytokines from Mast Cells by IL-12

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Key Words
IL-12 • Toll-like receptor • Mast cell • IL-13

Abstract
Background: It has been reported that peptidoglycan (PGN) and lipopolysaccharide (LPS) can provoke mast cells to release an array of cytokines via TLR2 and TLR4, respectively. However, little is known of the regulatory mechanism of TLR2 and TLR4 mediated cytokine production in mast cells. Methods: Since IL-12 plays important roles in protection of the body from microorganism infection and mast cell is a crucial source of IL-12, we investigated effects of IL-12 on expression of TLR2 and TLR4, and cytokine production in mast cells by using quantitative real time PCR, flow cytometry analysis and cellular activation of signaling ELISA techniques. Results: The results showed that IL-12 induced significant increase in expression of TLR2 and TLR4 mRNAs and proteins, respectively. It can also synergistically enhance LPS-induced TLR4 expression in P815 cells. IL-12 not only by itself, but also synergistically enhanced LPS-induced IL-13 release from P815 cells. It appears that IL-12 induced IL-13 release and TLR4 expression is through activation of MAPK and PI3K/Akt signaling pathways, whereas IL-12 induced upregulation of TLR2 is via activation of PI3K/Akt signaling pathway, but not MAPK pathway. Conclusion: The ability of IL-12 in modulation of expression of TLR2 and TLR4 in mast cells, and in stimulation of IL-13 release from mast cells provides further evidence that this cytokine may play a role in the protective immunity against bacteria infection.

Introduction

TLRs are a group of single membrane-spanning non-catalytic receptors that recognize structurally conserved pathogen-associated molecular patterns derived from microbes, and activate immune cell responses [1-2]. Among the 11 known TLRs, TLR2 has been shown to be present in human [3] and murine mast cells [4], which responds to bacterial derived lipopeptides and PGN [5], as well as zymosan [6]. TLR4 has also been found in human [7] and murine [8] mast cells, which can be recognized by different types of LPS [9]. Ligand specificity for a number of TLR2 activators is thought to require

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heterodimerization with additional TLR molecules, TLR1 and TLR6 [10]. Distinct TLR molecules are capable of inducing differential cellular responses. However, little is known of effects of cytokines on TLR2 and TLR4 expression in mast cells.

Mast cells have long been recognized as the primary effector cells of allergy. However, recent insight into mast cells has revealed this cell type as key players in the regulation of innate [11] as well as adaptive immunity through TLRs [12-13]. It was found that PGN from Staphylococcus aureus stimulated bone marrow-derived mast cells in a TLR2-dependent manner to produce tumor necrosis factor (TNF), IL-4, IL-5, IL-6 and IL-13 [14]. Human mast cells, following PGN activation, produce substantial levels of IL-1, TNF, IL-5, IL-10, IL-13 [15], histamine [7] and granulocyte-macrophage colony-stimulating factor (GM-CSF) [6]. LPS, a classical TLR4 activator has been found to induce cytokine release from mast cells, including TNF [14, 16], IL-6, IL-13 [14], IL-5, IL-10 and Eotaxin [17-18]. However, the regulatory mechanism of TLR2 and TLR4 mediated cytokine release from mast cells remains largely unknown.

IL-12 is a cytokine that governs production of interferon (IFN)-gamma in CD4+T cells [19] and natural killer (NK) cells [20], and is involved in the induction and maintenance of T-helper 1 (Th1) cells [21-23]. IL-12 also plays important roles in protection of the body from various microbes' infection such as viruses, bacteria and parasites [24-25]. Since little is known of regulatory mechanisms of TLR2 and TLR4 expression and TLR2 and TLR4 mediated cytokine production in mast cells, and mast cells are a crucial source of functional IL-12 [26], we anticipate that IL-12 could modulate expression of these two TLRs and cytokine production in mast cells. We found that IL-12 can induce upregulation of expression of TLR2 and TLR4, and provoke IL-13 production in P815 cells through MAPK/ERK and PI3K/Akt signaling pathways in the present study.

Materials and Methods

Reagents and cells

Paraformaldehyde and 4-(4-fluorophenyl)-2-(4-methylsulfinylphenyl)-5-(4-pyridyl) -1H-imidazole (SB203580) were from Sigma Inc. (St Louis, MO, USA). Recombinant mouse IL-12, anti-mouse IL-12 monoclonal antibody (mAb), recombinant human IL-12, anti-human IL-12 mAb were from R&D Systems (Minneapolis, MN, USA). Tissue culture reagents including Dulbecco's modified Eagle's medium (DMEM), RPMI 1640 media, HEPES and fetal bovine serum (FBS) were obtained from GibcoBRL (Carlsbad, CA, USA). Cellular activation of signaling ELISA (CASE) kits for Akt, extracellular signal-regulated kinase (ERK), p38 mitogen-activated protein kinase (MAPK) and signal transducer and activators of transcription (STAT)3 were from SuperArray Bioscience Corporation (Frederick, MD, USA). Mouse IL-6, IL-10, TNF-α and IL-13 ELISA kits were from Pierce Biotechnology Inc. (Rockford, IL, USA). Human IL-13 ELISA kit was from R&D Systems (Minneapolis, MN, USA). 2-(2-Diamino)-3-methoxyphenyl-4H-1-benzopyran-4-one (PD98059), 1,4-diamino-2,3-dicyano-1,4-bis(2-aminophynyltio)butadiene (U0126), tyrphostin (AG490), 1,4-diamino-2,3-dicyano-1,4-bis(methylthio) butadiene (U0124) and 2-(4-morpholinyl)-8-phenyl-4H-1-benzopyran-4-one (LY294002) were from Cell Signaling Technology (Beverly, MA, USA). TRIzol Reagent and SYBR Green I Stain were from Invitrogen (Carlsbad, CA, USA). PGN from Staphylococcus aureus and LPS from S. minnesota were from Invivogen (San Diego, CA, USA). ExScript RT reagent kit and SYBR Premix Ex Taq (perfect real time) was from TaKaRa Biotechnology Co. Ltd (DaLian, China). FITC-conjugated rat anti-mouse TLR2 and TLR4 mAbs, FITC-conjugated rat isotype control, PE-conjugated mouse anti-human TLR2 and TLR4 mAb and PE-conjugated mouse isotype control were from eBioScience (Los Angeles, CA, USA). The mouse mastocytoma cell line (P815) was obtained from the American Type Culture Collection (ATCC, Manassas, VA, USA). HMC-1 cell line, established from a patient with mast cell leukemia, was a present from Dr. Joseph H. Butterfield (Mayo Clinic, MN, USA). Most of other reagents such as salt and buffer components were analytical grade and obtained from Sigma.

Mast cell culture and challenge

P815 cells were cultured with ATCC complete growth medium including DMEM with 4 mM L-glutamine, 1.5 mg/ml sodium bicarbonate, 4.5 mg/ml glucose, 10% FBS, 100 U/ml penicillin and 100 μg/ml streptomycin in 75-cm2 tissue culture flasks (Falcon) at 37°C. HMC-1 cells were cultured and maintained in RPMI 1640 media with 10 mM HEPES, 2 mM L-glutamine, 10% heat inactivated FBS and antibiotics in 75-cm2 tissue culture flasks at 37°C in a 5% CO2, water-saturated atmosphere. Mast cells at a density of 1.0 × 106 cells/ml were incubated with the serum-free basal medium for 6 h before being challenged by various concentrations of IL-12 (0.1-100 ng/ml) with or without its blocking antibody (10, 30 μg/ml) or isotype control antibody, PGN at 0.25 and 2.5 μg/ml and LPS at 50 and 500 ng/ml for 2, 6 or 16 h. After the supernatant (5 ml) being collected and stored at -80°C, the cell pellet containing approximately 5 ×106 cells were resuspended for immunofluorescence and real-time PCR analysis. For certain experiments, cells were preincubated with 1.0 ng/ml and 10 ng/ml of IL-12 for 1 h before adding PGN or LPS for 16 h.

For cell signalling experiments, cultured cells at a density of 1.0 × 106 cells/ml were treated with the inhibitors of cell signalling pathways including PD98059 (50 μM), U0126 (5 μM), U0124 (5 μM), SB203580 (20 μM), LY294002 (20 μM) and AG490 (40 μM) for 30 min before being challenged with IL-12 (10 and 100 ng/ml) for 15 min, 2 or 6 h [27]. Following incubation, 400 μl of cell suspension was removed for signaling ELISA analysis,

Target gene	Forward sequence (5 -3)	Reverse sequence (5 -3)	AS (bp)	AT (°C)	GA
Mouse-TLR2	TGCTTTCCTGCTGGAGATTT	CAGCTCGCTCACTACGTCTG	284	50	NM 011905
Mouse-TLR4	GCTTTCACCTCTGCCTTCAC	AGGCGATACAATTCCACCTG	259	56	NM 021297
Mouse-ß-actin	GCTACAGCTTCACCACCACAG	GGTCTTTACGGATGTCAACGTC	288	60	NM 007393
Human-TLR2	TCTCCCATTTCCGTCTTTTT	GGTCTTGGTGTTCATTATCTTC	125	60	NM 003264
Human-TLR4	CCGCTTCCTGGTCTTATCAT	TCTGCTGCAACTCATTTCAT	141	60	NM 003266
Human ß-actin	GTTGCGTTACACCCTTTCTT	ACCTTCACCGTTCCAGTTT	148	60	BC 002409

Table 1. Primers for RT-PCR and real-time RT-PCR. AS = amplicon size; AT = annealing temperature; GA = Genbank accession.

and the remaining 700 µl of cell suspension was centrifuged. The culture supernatant was collected for ELISA analysis, and cells (~ 1.0 × 106) were resuspended for immunofluorescence analysis.

Examination of expression of TLR mRNAs

The expression of TLR mRNAs in P815 and HMC-1 cells was determined with RT-PCR. Total RNA was isolated by using a TRIzol reagent kit according to the manufacturer's instruction. Briefly, cells were collected by centrifugation and lysed directly by adding TRIzol reagent (1 ml per 1 ×106 cells). After being treated with chloroform, RNA was precipitated by adding 0.5 ml of isopropyl alcohol and then resuspended with 1 ml of 75% (v/v) ethanol. Total RNA was quantified by measuring absorbance ratios at 260/280 nm. The cDNA was prepared by reverse transcriptase using a commercial RNA-PCR kit according to the manufacturer's instruction. For each reaction, 1 µg of total RNA was reversely transcribed using oligo-d (T). The cDNA was amplified using forward and reverse specific primers for amplifying mouse TLRs. β-actin was used as an internal control. Primers were designed according to the Genbank sequences for mouse and human TLRs were summarized in Table 1. The conditions for amplification were as follows: 95°C for 5 min, 30 cycles of denaturation at 95°C for 30 s, annealing temperatures as shown in Table 1 for 30 s, and extension at 72°C for 30 s. PCR products were electrophoresed on 1.5% agarose gels that were stained with SYBR Green I Nucleic Acid Gel Stain and photographed under ultraviolet (UV) light.

Quantitative real-time PCR

Quantitative expression of TLR mRNAs in mast cells was determined by real-time PCR following the manufacture's protocol. Briefly, after synthesizing cDNA from 1 µg of total RNA by using ExScriptTM RT reagent kit, real-time PCR was performed by using SYBR® Premix Ex Taq TM on the ABI Prism 7000 Sequence Detection System (Perkin Elmer Applied Systems, CA, USA). Each reaction contains 12.5 µl of 2 × SYBR green Master Mix, 1 µl of 10 µM of primers, 1 µl of the cDNA, to a total volume of 25 µl. The thermal cycling conditions included an initial denaturation step at 50°C for 2 min, 95°C for 10 min; 40 cycles at 95°C for 15 s, annealing temperatures as shown in Table 1 for 30 s and extension at 72°C for 30 s. Consequently, at the end of the PCR cycles, specificities of the amplification products were controlled by dissociation curve analysis. mRNA expression in each sample was finally determined after correction with β-actin expression. The gene specific threshold cycle (Ct) for each sample (ΔCt) was corrected by subtracting the Ct for the housekeeping gene β-actin. Untreated controls were chosen as the reference samples, and the ΔCt for all experimental samples were subtracted by the ΔCt for the control samples (ΔΔCt). The magnitude change of test gene mRNA was expressed as 2-ΔΔCt [28]. Each measurement of a sample was conducted in duplicate.

Flow cytometry analysis

P815 cells were pelleted by centrifugation at 450 g for 10 min, and were incubated with FITC-conjugated rat anti-mouse TLR2 and TLR4 mAb or FITC-conjugated rat isotype control (at a final concentration 4 µg/ml) at 37°C for 1 h. For HMC-1 staining, cells were incubated with PE-conjugated mouse anti-human TLR2 and TLR4 mAb or PE-conjugated mouse isotype control. Cells were finally resuspended in PBS and analyzed on a FACS Calibur flow cytometer equipped with CellQuest software (BD Biosciences).

Immunofluorescent cell staining

P815 cells were incubated with 3% normal serum blocking solution for 10 min. The same staining procedures were used as for the flow cytometry analysis. Images were obtained on a Zeiss 5 LIVE confocal laser scanning microscope (Zeiss, Germany).

Determination of levels of cytokines

IL-6, IL-10, TNF-α and IL-13 levels were measured by using ELISA kits, according to the manufacturer's instruction.

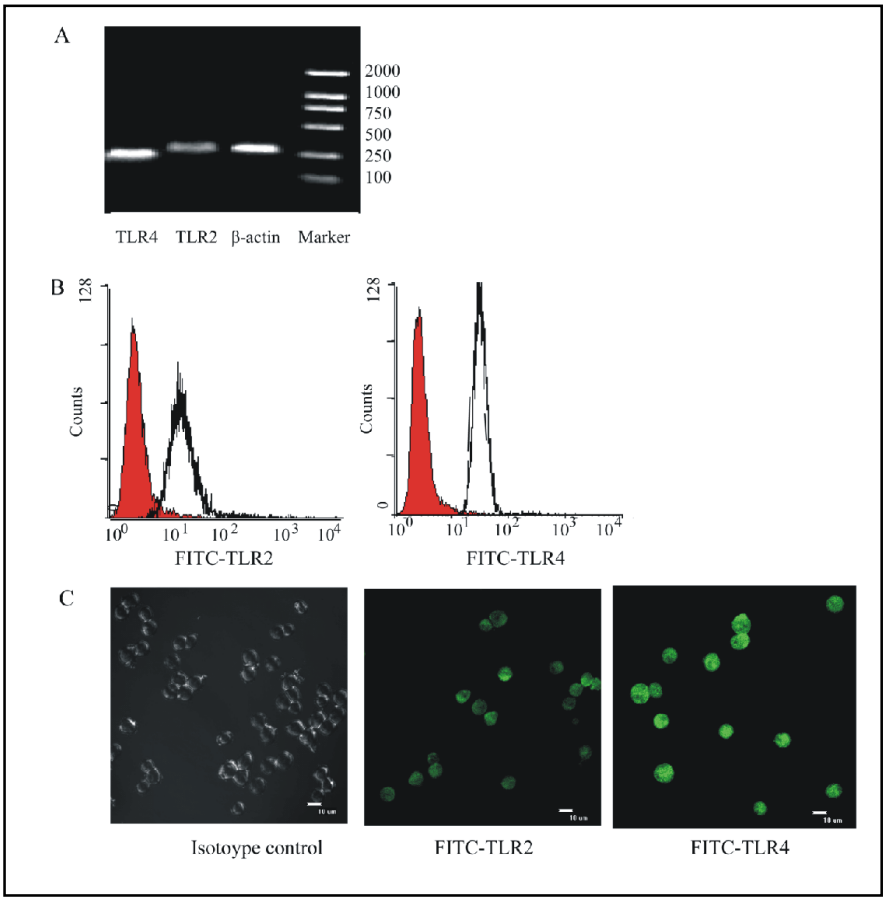

Fig. 1. Analysis of expression of TLR2 and TLR4 in P815 cells by RT-PCR (A), flow cytometry (B) and immunofluorescent microscopy (C). In (A), lane1-4 represented TLR4 (259 bp), TLR2 (284 bp), β-actin (288 bp) and DNA marker, respectively. In (B), cells were incubated with antibodies or isotype control at 37°C for 1 h. The red peak represents isotype control and white peak represents TLR2 or TLR4 positively stained cells. In (C), positively stained cells were visualized by immunofluorescent microscopy.

Analysis of signal-transduction pathways

The phosphorylation of Akt, ERK, p38 and STAT3 was analyzed by using CASE kits as described previously [27]. Briefly, before the CASE experiment was performed, P815 cells were seeded in a 96-well plate and cultured until 50-80% confluence. A total of two sets of cells were prepared with one set of cells being incubated with the phospho-protein-specific antibody to measure phosphorylated protein, and the other set of cells being incubated with the pan-protein-specific antibody to measure total protein. After being fixed with Cell Fixing Buffer, the quenching buffer and antigen retrieval buffer was added, respectively. Cells were blocked with blocking buffer for 1 h before addition of primary antibody to each appropriate well for 1 h, which was followed by adding secondary antibody for 1 h. Following addition of developing solution until the darkest staining well turns a medium- to dark-blue, the stop solution was added into each well. The plate was read at 450 nm on an ELISA Plate Reader (Max 340PC, Molecular Devices, CA, USA).

In order to determine relative cell number, the assay solution was removed from each well. The cell staining buffer was then placed into each well for 30 min, followed by adding sodium dodecyl sulfate for 1 h. The plate was read at 595 nm. To normalize the antibody reading to the relative cell number, the OD450 reading for each well was divided by its OD595 reading. To determine the relative extent of target protein phosphorylation, the OD450:OD595 ratio for phosphor-protein-specific antibody is normalized by the ratio for pan-protein-specific antibody under the same experimental condition.

Statistics

Data are expressed as mean ± SEM for the indicated number of independently performed duplicated experiments. Statistical significance between means was analyzed by one-way analysis of variance or the Student's t test utilizing the SPSS 13.0 version. $P < 0.05$ was taken as statistically significant.

Results

Expression of TLRs in P815 cells

In order to ensure if P815 cells are the appropriate cells for the investigation of regulatory effect of IL-12 on TLR expression, we first examined the expression of TLRs in these cells. With RT-PCR analysis, we found that P815 cells express mRNAs of TLR2 and TLR4 (Fig. 1A). Using flow cytometry analysis (Fig. 1B) and immunofluorescent cell staining (Fig. 1C) techniques, we confirmed that P815 cells also express TLR2 and TLR4 proteins.

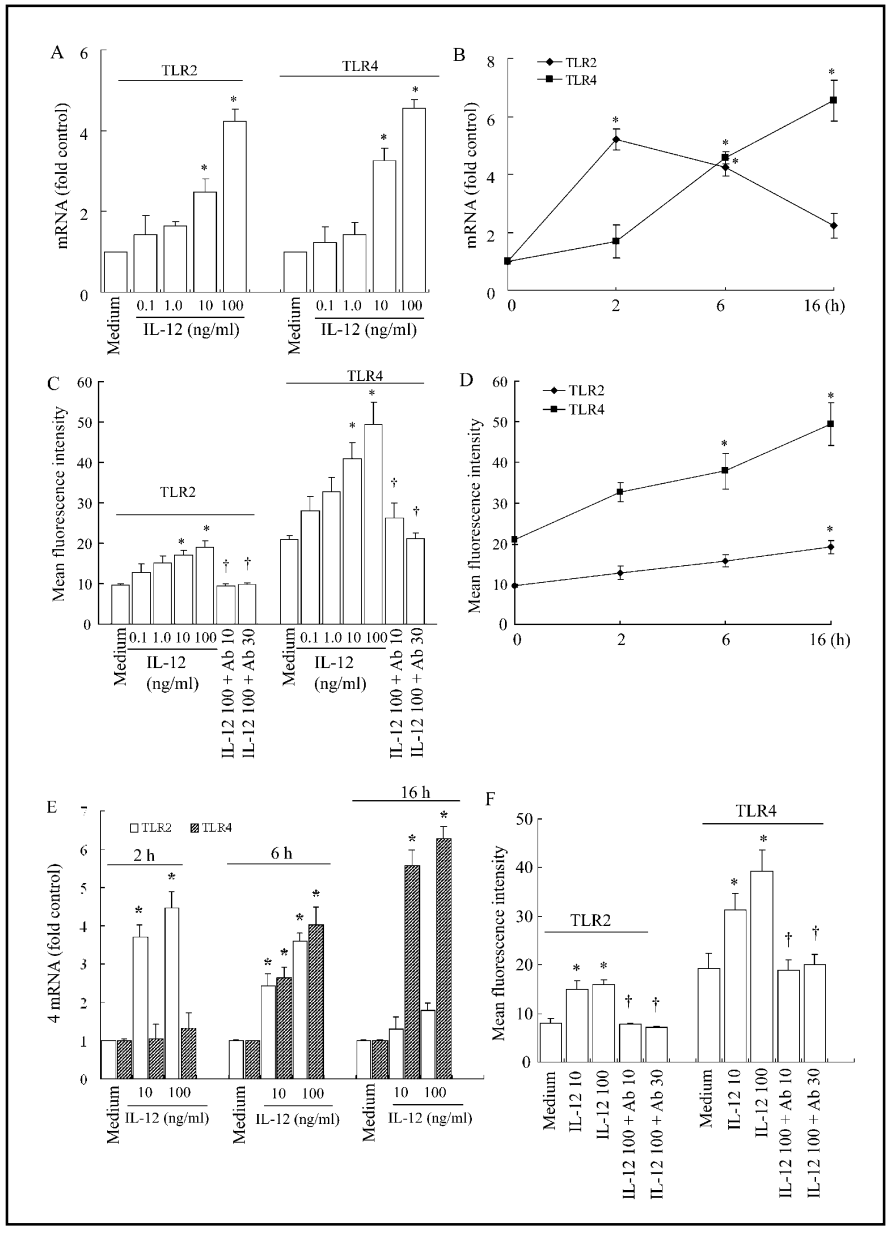

Fig. 2. Induction of upregulated expression of TLR2 and TLR4 in mast cells by IL-12. In (A), P815 cells were incubated with IL-12 for 6 h and expression of TLR2 and TLR4 was examined with real-time PCR analysis. In (B), time course study for IL-12 induced expression of TLR2 and TLR4 mRNAs in P815 cells. Cells were incubated with 100 ng/ml of IL-12 for 2, 6 and 16 h before being collected for real-time PCR analysis. In (C), P815 cells were incubated with IL-12 for 16 h and expression of TLR2 and TLR4 was examined with flow cytometry analysis. In (D), time course study for IL-12 induced expression of TLR2 and TLR4 in P815 cells. Cells were incubated with 100 ng/ml of IL-12 for 2, 6 and 16 h before being collected for flow cytometry analysis. (E) Real-time PCR analysis of expression of TLR2 and TLR4 mRNAs in HMC-1 cells. (F) HMC-1 cells were incubated with IL-12 for 16 h before expression of TLR2 and TLR4 being examined with flow cytometry analysis. Values shown are Mean ± SE for four separate experiments performed in duplicate. * $P < 0.05$ compared with the response to the corresponding medium alone control. † $P < 0.05$ compared with the response to the corresponding uninhibited control. Ab = anti-IL-12 antibody (μg/ml).

Upregulation of expression of TLRs in mast cells by IL-12

Quantitative real time RT-PCR showed that IL-12 at the concentrations of 1.0, 10 and 100 ng/ml induced a dose-dependent up-regulation of TLR2 and TLR4 mRNA expression in P815 cells following 6 h incubation period. The maximum enhanced expression of TLR2 and TLR4 mRNAs was 4.2 and 4.6 fold over baseline control, respectively (Fig. 2A). The time course study showed that up-regulated TLR2 mRNA expression was markedly increased at 2 h, began to decline at 6 h and dropped almost to baseline level at 16 h following incubation (Fig. 2B). On the other hand, IL-12 provoked enhancement of TLR4 mRNA expression appeared a relatively slow process, which initiated at 6 h and reached the peak at 16 h following incubation (Fig. 2B). In order to confirm the above findings, IL-12 induced expression of TLR2 and TLR4 mRNAs was also examined with HMC-1 cells. The result showed that IL-12 induced expression of TLR2 initiated at 2 h and declined at 6 h, whereas IL-12 induced expression of TLR4 started at 6 h and continuously increased till 16 h (Fig. 2E), which was similar to those observed with P815 cells.

Flow cytometry analysis showed that IL-12 at 10 and 100 ng/ml provoked upregulation of expression of TLR2 and TLR4 in P815 cells following 16 h incubation period (Fig. 2C). Approximately up to 2.0 and 2.4 fold upregulated expression of TLR2 and TLR4 was observed

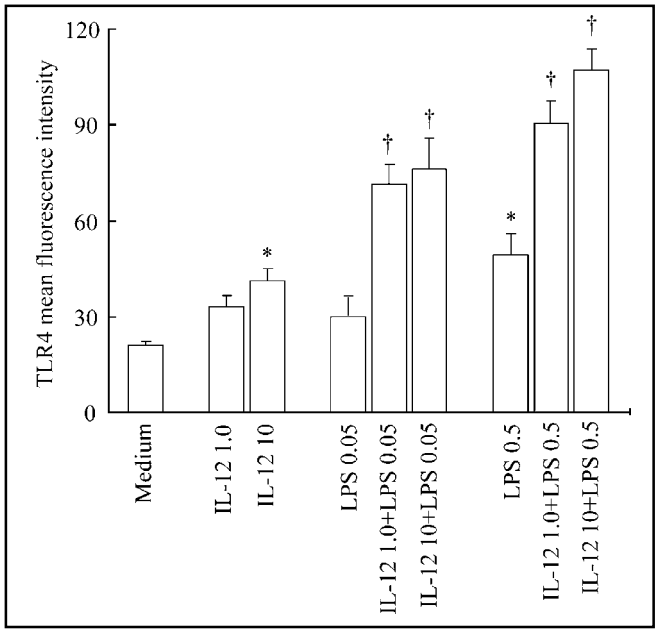

Fig. 3. Influence of IL-12 on LPS induced TLR4 expression in P815 cells. Cells were preincubated with IL-12 (ng/ml) for 1 h before LPS (μg/ml) being added for 16 h at 37°C. Values shown are Mean ± SE for four separate experiments performed in duplicate. * $P < 0.05$ compared with the response to medium alone control. † $P < 0.05$ compared with the response to the corresponding LPS alone.

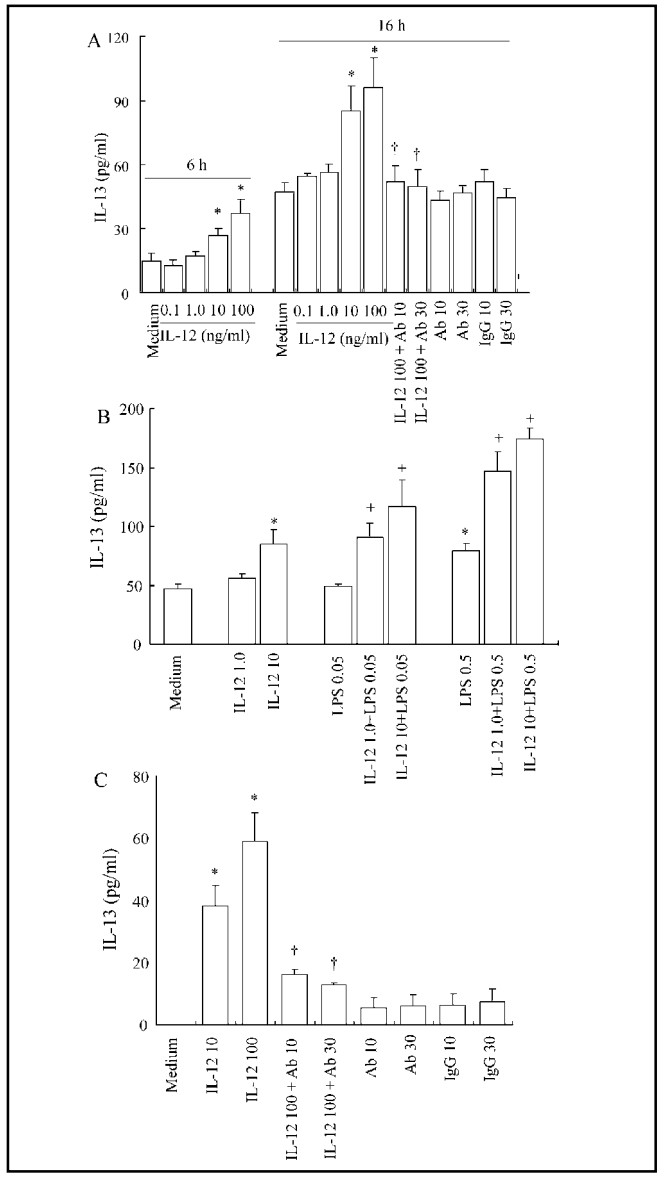

Fig. 4. Induction of IL-13 release from mast cells by IL-12. (A) P815 cells were incubated with various concentrations of IL-12 (ng/ml) or IL-12 with its blocking antibody (Ab, μg/ml) or isotype control antibody (IgG, μg/ml) at 37°C for 6 and 16 h. (B) Cells were preincubated with IL-12 for 1 h before LPS (μg/ml) being added for 16 h at 37°C. (C) HMC-1 cells were incubated with various concentrations of IL-12 (ng/ml) or IL-12 with its blocking antibody (Ab, μg/ml) or isotype control antibody (IgG, μg/ml) at 37°C for 16 h. Values shown are Mean ± SE for four separate experiments performed in duplicate. * $P < 0.05$ compared with the response to medium alone control. † $P < 0.05$ compared with the response to the corresponding uninhibited control. + $P < 0.05$ compared with the response to the corresponding LPS alone.

when cells were incubated with 100 ng/ml of IL-12 for 16 h (Fig. 2C). The time course study showed that IL-12 induced up-regulation of expression of TLR2 was only observed at 16 h, whereas the expression of TLR4 initiated at 6 h following incubation (Fig. 2D). In the parallel experiments, immunofluorescent analysis showed similar pattern of increased expression of TLR2 and TLR4 in P815 cells following 2, 6 and 16 h incubation periods (data not shown). IL-12 induced expression of TLR2 and TLR4 proteins was also examined with HMC-1 cells following 16 h incubation period, which showed that IL-12 could induce up-regulation of expression of TLR2 and TLR4 in HMC-1 cells (Fig. 2F). In order to determine if IL-12 induced up-regulation of expression of TLR2 lasts longer than 16 h, P815 cells were incubated with 100 ng/ml of IL-12 for 24 and 48 h. The results showed that approximately up to 1.8 and 1.6 fold increase in expression of TLR2 were observed when cells were incubated for 24 and 48 h, respectively.

Enhancement of LPS induced expression of TLR4 in P815 cells by IL-12

In order to determine if IL-12 affects PGN or LPS induced TLR expression, P815 cells were preincubated with IL-12 for 1 h before adding PGN or LPS for 16 h. The results showed that IL-12 synergistically enhanced

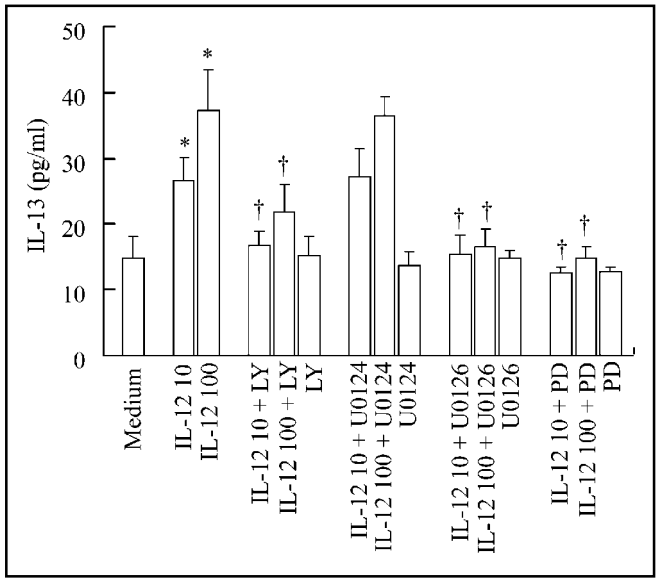

Fig. 5. Effect of PD98059 (PD), U0126, U0124 and LY294002 (LY) on IL-12 induced IL-13 secretion from P815 cells. Cells were preincubated with PD (50 μM), U0126 (5 μM), U0124 (5 μM) or LY (20 μM), respectively for 30 min before IL-12 (ng/ml) being added for 6 h at 37°C. Values shown are Mean ± SE for four separate experiments performed in duplicate. * $P < 0.05$ compared with the response to medium alone control. † $P < 0.05$ compared with the response to corresponding uninhibited control.

LPS-induced TLR4 expression in P815 cells. Approximately up to 2.5 fold upregulated expression of TLR4 was observed when cells were preincubated with 1.0 ng/ml of IL-12 for 1 h before adding LPS for 16 h (Fig. 3). In contrast, preincubation of IL-12 had little influence on PGN induced TLR2 expression (data not shown).

Induction of cytokine secretion from mast cells by IL-12

IL-12 at 1.0, 10 and 100 ng/ml induced a concentration dependent release of IL-13 from P815 cells following 6 and 16 h incubation periods. Approximately up to 2.5 fold increases in IL-13 release was achieved when cells were incubated with 100 ng/ml of IL-12 (Fig. 4A). Once being added at the same time with IL-12, anti-IL-12 antibody was able to block IL-12 induced IL-13 secretion (Fig. 4A). IL-12 at the concentrations examined had little effect on IL-6, IL-10 and TNF-α secretion from P815 cells (data not shown). HMC-1 cells showed little baseline IL-13 release. However, once they were incubated with IL-12 at 10 and 100 ng/ml for 16 h, HMC-1 released substantial quantity of IL-13 (Fig. 4C).

In order to determine if IL-12 affects PGN or LPS induced IL-13 release, P815 cells were preincubated with

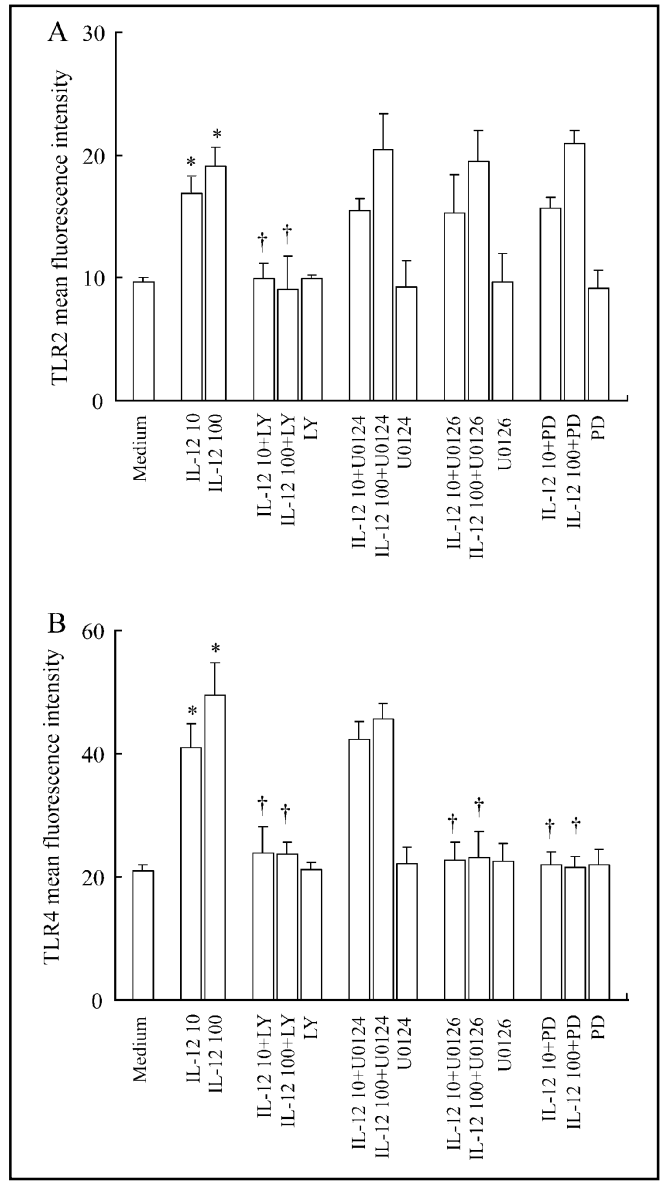

Fig. 6. Effect of PD98059 (PD), U0126, U0124 and LY294002 (LY) on IL-12 induced upregulation of TLR2 (A) and TLR4 (B) expression in P815 cells. Cells were preincubated with PD (50 μM), U0126 (5 μM), U0124 (5 μM) or LY (20 μM), respectively for 30 min before IL-12 (ng/ml) being added for 6 h at 37°C. Values shown are Mean ± SE for four separate experiments performed in duplicate. * $P < 0.05$ compared with the response to medium alone control. † $P < 0.05$ compared with the response to corresponding uninhibited control.

IL-12 for 1 h before adding PGN or LPS for 16 h. The results showed that IL-12 at 1.0, but not 10 ng/ml synergistically enhanced LPS-induced IL-13 release from

P815 cells (Fig. 4B). IL-12 at the concentrations examined had little effect on PGN induced IL-13 secretion from P815 cells (data not shown).

Effect of cell signaling inhibitors on IL-12-induced release of cytokines and upregulated expression of TLR2 and TLR4

Our former findings that IL-12 induced IL-4 release through activation of ERK and Akt signaling pathways in P815 mast cell line suggest us to explore the possible signaling pathways of IL-12 induced IL-13 release and TLR2 and TLR4 expression in P815 cells [27]. We employed PD98059 a MAPK pathway inhibitor, U0126 an inhibitor of MEK and thus a MAPK pathway inhibitor, SB203580 a selective inhibitor of p38 MAPK, LY294002 a PI3K inhibitor and AG490 a Janus kinase (JAK)/STAT3 pathway inhibitor to investigate the potential GM-CSF signaling pathways in P815 cells. The results showed that PD98059, U0126 and LY294002 almost completely abolished IL-12-induced IL-13 release from P815 cells when they were preincubated with the cells for 30 min (Fig. 5), indicating that IL-12 induced IL-13 release is through activation of MAPK and PI3K/Akt signaling pathways. In contrast, SB203580, U0124 a structural analogue negative control of U0126 and AG490 had little influence on IL-12 induced IL-13 release (data not shown). All inhibitors tested did not significantly affect basal IL-13 release ability of P815 cells (Fig. 5).

PD98059 and U0126 completely abolished also IL-12 induced upregulation of TLR4 expression (Fig. 6B), indicating that the actions of IL-12 are via activation of MAPK signaling pathway. Similarly, LY294002 completely abolished IL-12 induced upregulation of TLR2 (Fig. 6A) and TLR4 (Fig. 6B) expression, indicating IL-12 induced upregulation of TLR2 and TLR4 expression is through activation of PI3K/Akt signaling pathway. As expected, SB203580, U0124 and AG490 had little influence on IL-12 induced upregulation of TLR2 and TLR4 expression (data not shown). All inhibitors tested did not significantly affect basal TLR2 and TLR4 expression in P815 cells.

Inhibition of IL-12-induced phosphorylation of ERK and Akt by signaling inhibitors

PD98059 and U0126 inhibited approximately up to 50 and 46.7% of IL-12 induced phosphorylation of ERK in P815 cells, respectively following 30 min preincubation period (Table 2). LY294002 diminished IL-12-induced phosphorylation of Akt in P815 cells by approximately 76.2%, following 30 min preincubation period (Table 3).

Compound	Phospho-ERK (OD450:OD595)/ Pan(OD450:OD595)	
	15 min	120 min
IL-12 10	0.19 ± 0.02	0.30 ± 0.05
IL-12 100	0.18 ± 0.03	0.32 ± 0.03
IL-12 10 + PD98059	0.13 ± 0.02*	0.15 ± 0.02*
IL-12 100 + PD98059	0.12 ± 0.01*	0.19 ± 0.02*
IL-12 10 + U0126	0.11 ± 0.02*	0.16 ± 0.02*
IL-12 100 + U0126	0.13 ± 0.01*	0.20 ± 0.03*
IL-12 10 + U0124	0.17 ± 0.03	0.27 ± 0.02
IL-12 100 + U0124	0.18 ± 0.02	0.29 ± 0.02

Table 2. Effect of the inhibitors of MAPK/ERK signal-transduction pathways on IL-12-induced phosphorylation of ERK. Values shown are Mean ± SE for four separate experiments. P815 cells were pretreated with PD98059 (PD, 50 μM), U0126 (5 μM) or U0124 (5 μM) for 30 min before being challenged with IL-12 (ng/ml) for 15 min or 2 h. Phosphorylation of ERK was determined by using signaling ELISA analysis. * $P < 0.05$ in comparison with the uninhibited control.

Compound	Phospho-Akt (OD450:OD595)/ Pan (OD450:OD595)	
	15 min	120 min
IL-12 10	0.21 ± 0.09	0.44 ± 0.04
IL-12 100	0.14 ± 0.03	0.42 ± 0.04
IL-12 10+Ly294002	0.05 ± 0.01*	0.11 ± 0.01*
IL-12 100+LY294002	0.06 ± 0.03*	0.10 ± 0.02*

Table 3. Effect of LY294002 on IL-12-induced phosphorylation of Akt. P815 cells were pretreated with 20 μM of LY294002 for 30 min before being challenged with IL-12 (ng/ml) for 15 min or 2 h. Phosphorylation of Akt was determined by using signaling ELISA analysis. * $P < 0.05$ in comparison with the uninhibited control.

Discussion

It was found for the first time that IL-12 is able to up-regulate expression TLR2 and TLR4 on mast cells, and provoke IL-13 release from mast cells. Since TLR2 and TLR4 play key roles in recognizing bacterial agents and IL-12 has the ability to regulate innate resistance against microbes [24], our finding may implicate a novel regulatory mechanism in innate immunity against bacterial pathogens. Moreover, since mast cells and IL-13 a Th2 cytokine are actively involved in the pathogenesis of

allergy, our finding may suggest a new link between bacterial infection and allergic inflammation.

IL-12 induced upregulation of TLR4 expression occurred at 2 h following incubation and consistently increased at least to 16 h. However, significantly upregulated expression of TLR2 was only observed at 16 h following incubation. IL-12 induced augmentation of TLR4 expression seems a relatively fast process as LPS provoked upregulation of TLR4 protein expression occurred at 8 h and maintained at least to 48 h following incubation with LAD2 mast cells [16].

It is fascinating to know that IL-12 and LPS can synergistically induce TLR4 expression in P815 cells. The action appears a selective one as IL-12 showed little effect on PGN induced TLR2 expression in the parallel experiments. The reason for IL-12 not affecting the actions of PGN on TLR2 expression is hard to explain, but it may be due to 1 h treatment by IL-12 is too short as IL-12 induced significant enhancement of TLR2 expression was only observed at 16 h following incubation. Obviously, more work is required to address this issue further. To our knowledge, this is the first work which demonstrates cytokine and LPS has synergistic effect on TLR4 expression. IL-12 also showed additive effect on LPS, but not PGN induced IL-13 release from P815 cells, suggesting that IL-12 and LPS induced IL-13 is most likely via different mechanisms, most likely through different receptors. Several previous reports may support our above observations. Thus, peptidoglycan (PGN) from Staphylococcus aureus and LPS from Escherichia coli was found to stimulate bone marrow-derived mast cells (BMMCs) in a TLR2- and TLR4-dependent manner to produce IL-13, respectively [14]; LPS and PGN was demonstrated to induce significant release of IL-13 from human normal cord blood-derived mast cells [7]. Moreover, LPS has been showed to synergistically enhanced production of IL-13 from mast cells by IgE cross-linking [17]. Following costimulation with LPS and IgE/Ag, IL-13 expression in BMMCs can be dramatically increased [18].

Since IL-13 is emerging as an important mediator in the development of Th2 cell responses, which can induce IgE secretion from activated human B cells [29] and acts more prominently as a molecular bridge linking allergic inflammatory cells to the non-immune cells [30]. Our current finding that IL-12 can modulate IL-13 production in P815 cells may implicate a novel cross talk mechanism between Th1 and Th2 cytokines. Since mast cells are a crucial source of functional IL-12 in response to LPS but not by FcepsilonRI cross-linking [26], our observations may suggest a novel mechanism in bacteria-induced asthmatic attack: LPS induces IL-12 release from mast cells, secreted IL-12 stimulates IL-13 release from adjacent mast cells, secreted IL-13 acts on B cells and other cells to trigger allergic inflammation. Obviously, further work is required to prove our speculation.

IL-12 induced IL-13 secretion appeared to be through activation of ERK and Akt signaling pathways, similar to the mechanism of IL-4 release induced by IL-12 through activating Akt and ERK signaling pathways. While little information on IL-12 signal pathways in mast cells is available, the studies which demonstrated that C3a stimulated substantial MCP-1 and RANTES/CCL5 production, and ERK and Akt phosphorylation in human LAD 2 mast cells [31], ERK 1/2, c-Jun N-terminal kinase, and p38 kinase were activated by LPS stimulation in bone marrow-derived mast cells [17] may help to understand our current findings.

It was previously reported that LPS and PGN induced significant release of IL-10 from human mast cells through TLR4 and TLR2, respectively [7] and that LPS induced Th2-associated IL-10 release from mouse bone marrow-derived mast cells [17]. However, we did not observe increased IL-10 release from P815 cells in the present study. The discrimination between these studies may be due to different types of mast cells were used.

In conclusion, the ability of IL-12 in modulation of expression of TLR2 and TLR4 in mast cells and in stimulation of IL-13 release from mast cells provides further evidence that this cytokine not only contributes to the pathogenesis of allergic inflammation, but also plays a role in the protective immunity against bacteria infection through alteration of mast cell behavior.

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