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Original Article

Bacterial chemoattractant synergizes with LPS to induce inflammation via upregulating TLR4

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Abstract: The principal challenge for the host is to efficiently detect the invading pathogen and mount a rapid defensive response. Leukocytes recognize invading pathogens by directly interacting with pathogen-associated molecular patterns *via* Toll-like receptors (TLRs) expressed on the leukocyte surfaces. In the present study we show for the first time that fMLP synergized with LPS to induce an inflammatory response *via* up-regulation of TLR4 *in vitro* and *in vivo*. Moreover, we show that fMLP-induced TLR4 up-regulation was mediated by FPR and Gi/Go class of heterotrimeric G proteins. Interestingly, low molecular weight GTPase Rho A acted as a regulator for FPR-mediated TLR4 induction, thereby implying a critical role for Rho A GTPase in inflammatory response during bacterial infection. Our data indicate a possible important role of fMLP that bacterial chemoattractant fMLP not only attracts leukocytes but also contributes directly to inflammation by synergizing with bacterial LPS.

Keywords: Bacterial chemoattractant, receptor, signal transduction, inflammation, gene expression

Introduction

Leukocytes constitute the first line of host defense against invading microorganisms and are a major cellular component of the inflammatory reaction. When exposed to chemoattractants, such as formyl peptide, leukocytes rapidly become activated. The bacterial tripeptide fMet-Leu-Phe (fMLP) is able to activate all major functions of leukocytes and is a prototypical ligand for the N-formyl peptide receptor (FPR), which contains 7 putative transmembrane domains characteristic of the G protein-coupled receptor (GPCR) of the rhodopsin family [1, 2]. More recently, fMLP-stimulated PBMC have been shown to express a defined set of gene products, including IL-1, IL-1ß, and IL-6 [3].

Previous works have shown that synergistic activation is achieved through the signaling pathways by different ligands [4-6], however, most research on the production of pro-inflammatory cytokines has been focused on understanding how cytokines are produced by a single bacterial product. Work performed in our laboratory involved the use of single bacterial product such as lipopolysaccharide (LPS), or formylated peptides (fMLP), a bacterial chemoattractant, to induce pro-inflammatory cytokine gene expression [7-11]. Recently, we found that mixtures of bacterial products synergistically induced inflammatory response. However, little is known about the molecular mechanisms underlying regulation of synergy by bacterial products/components.

Toll-like receptors (TLRs) are a class of pathogen recognition receptors that mediate recognition of pathogen-associated molecular patterns. Among the 11 mammalian TLRs, TLR4 plays an especially critical role due to its capability to detect pathogen-associated molecular patterns from gram-negative bacteria, lipopolysaccharide (LPS) [12-15]. We previously reported that TLR4 is a key receptor by recognizing LPS and is required for LPS-induced pro-inflammatory cytokine gene expression [9]. Given the fact that fMLP co-exists with LPS in vivo and synergistically enhances LPS-induced inflammation, it is of particular interest to investigate

the molecular mechanism by which TLR4 is induced by fMLP. In the present study, we show that fMLP induces TLR4 up-regulation through a FPR, Gi/Go class of heterotrimeric G proteins dependent signaling pathway. Moreover, fMLP induces activation of small GTPase Rho A, which in turn regulates fMLP-induced TLR4 expression. These studies thus bring new insights into synergistic regulation of host TLRs during bacterial infections.

Methods

Reagents

FMLP was obtained from Sigma (St. Louis, MO). Ultra pure LPS (E. coli, 0111:B4) was obtained from InvivoGen (San Diego, CA). Clostridium botulinum C3 exotoxin was from List Biological Laboratories, Inc. (Campbell, CA). Pertussis and cholera toxins were purchased from Calbiochem (San Diego, CA). Recombinant IFNγ was obtained from CP Biotech (Santa Cruz, CA). A monoclonal antibody against Rho A was purchased from Santa Cruz Biotechnology (Santa Cruz, CA).

Preparation of monocytes from human peripheral blood and monocytic cell lines

Heparinized human peripheral blood from health donors was fractionated on Percoll (Pharmacia) density gradients. Monocytes were prepared from the mononuclear cell population as described [16]. The monocytic cell line, THP1 cells were cultured in RPMI 1640 (Irvine Scienfic, Santa Ana, CA) with 10% (V/V) heat-inactivated fetal bovine serum, penicillin (100 units/ml), streptomycin (100 μg/ml), and L-glutamine (2 mM; Irvine Scienfic, Santa Ana, CA) and 2-mercaptoethanol (complete media).

Quantitative real-time PCR (QRT-PCR) analysis of TLR4 and IL-1β

Human peripheral blood monocytes were stimulated with fMLP and LPS or fMLP/LPS for 2 hours. Total RNA was isolated by using TRIzol reagent (Invitrogen) by following the manufacturer's instructions. For the reverse transcription reaction, TaqMan reverse transcription reagents (Applied Biosystems) were used. The reverse transcription reaction was performed for 60 min at 37°C, followed by 60 min at 42°C by using oligo (dT) and random hexamers. PCR amplification was performed by using TaqMan Universal Master Mix. Predeveloped TaqMan assay reagents (probe and primer mixture of TLR4 and IL-1β) were used to detect expression of the gene. In brief, reactions were performed in duplicate containing 2 x Universal Master Mix, 2 μl of template cDNA, 200 nM primers, and 100 nM probe in a final volume of 25 μl, and they were analyzed in a 96-well optical-reaction plate (Applied Biosystems). Probes include a fluorescent reporter dye, 6-carboxy-fluorescein (FAM), on the 5' end and labeled with a fluorescent quencher dye, 6-carboxytetramethyl-rhodamine (TAMRA), on the 3' end to allow direct detection of the PCR product. Reactions were amplified and quantified by using an ABI 7500 sequence detector and the manufacturer's corresponding software (Applied Biosystems). Relative quantity of TLR4 and IL-1β mRNA was obtained by using the comparative Ct Method (for details, see User Bulletin 2 for the ABI PRISM 7500 sequence-detection system) and was normalized by using predeveloped TaqMan assay reagent human cyclophilin as an endogenous control (Applied Biosystems).

Detection of cellular GTP-Rho

When activated, Rho undergoes GDP-GTP exchange, and activated Rho can thus be detected by analyzing GTP-bound Rho. Rho A activity was detected by the method recently described by Ren et al [17]. This assay utilizes the Rho-binding domain (RBD) from the effector protein Rhotekin as a probe to specifically isolate the active forms of Rho A. Human peripheral blood monocytes (1 × 106) were stimulated with LPS, or control media and then lysed (lysis buffer: 50 mM Tris-HCl, pH 7.5, 10 mM $MgCl_2$, 500 mM NaCl, 1% Triton X-100, 0.5% sodium deoxycholate, 0.1% SDS, 1 mM phenylmethylsulfonyl fluoride, 10 mg/ml each of leupeptin and aprotinin). Equal volumes of lysates were incubated with GST-RBD (20 mg) beads at 4°C for 45 min. The beads were washed three times with a Tris buffer containing 1% Triton X-100, 10 mM $MgCl_2$, 150 mM NaCl, 1 mM phenylmethylsulfonyl fluoride, 10 mg/ml each of leupeptin and aprotinin. Bound Rho proteins were detected by Western blotting using a monoclonal antibody against Rho A (Santa Cruz, Inc). The amount of RBD-bound Rho was normalized to the total amount of Rho

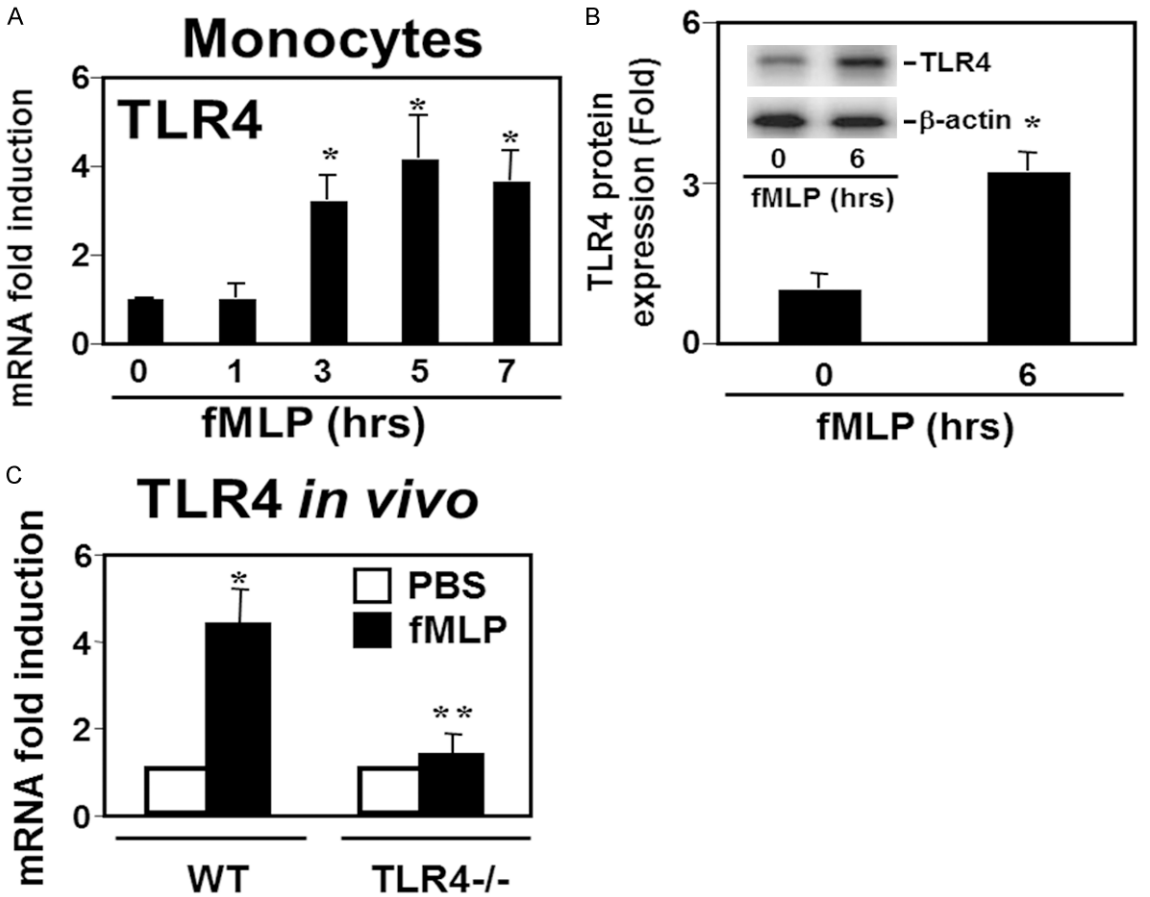

Figure 1. fMLP induces TLR4 expression *in vitro* and *in vivo*. A. Time dependence of TLR4 up-regulation in response to fMLP. Human blood monocytes were treated with 100 nM fMLP for 1, 3, 5 and 7 hours, and relative quantity of TLR4 mRNA expression was measured by Q-PCR analysis. B. Protein expression of TLR4 is up-regulated following fMLP treatment. TLR4 protein expression was measured from the monocytes stimulated with 100 nM fMLP by western blotting analysis 6 hours after treatment, and data was presented as fold induction. C. fMLP-induced TLR4 expressions in the lung of the mice. Wild-type or TLR4 KO mice were intranasally treated with fMLP (0.5 mg/kg) in 50 ml of sterile PBS (control). Total RNA was then isolated from the lung tissues of WT and TLR4 KO mice using TRIzol (Invitrogen) following the manufacturer's instruction. Data are expressed as mean ± SEM of three different experiments, and each had five mice per group. *, $P<0.05$ compared with control; **, $P<0.05$ compared with WT.

in cell lysates for comparison of Rho activity in different samples.

Western blot analysis

Antibody against TLR4 was purchased from eBioscience, antibody against b-actin was purchased from Sigma-Aldrich. Monocytes first incubated with fMLP and total protein were then extracted from the cells. Western blot analysis was performed as described previously [18]. Briefly, approximately 10 μg of cytoplasmic extracts were mixed with loading dye, boiled, electrophoresed on a 10% SDS gel and transferred to Hybond-ECL nitrocellulose membranes (Amersham). The membranes were blocked overnight at 4°C in PTS buffer (10 mM phosphate buffer, pH 7.0, 0.05% Tween 20, 0.9% NaCl, and 0.025% NaN_3) containing 2% bovine serum albumin. The protein was detected on the blots by incubation with an antibody to TLR4 (1:1000 dilution), followed by addition of peroxidase-conjugated goat anti-rabbit IgG at 1:10000 and analysis with enhanced chemiluminescence reagents (Du Pont-NEN).

Mouse and animal experiments

WT and TLR4 KO mice were purchased from the Jackson Lab, and all animal experiments were approved by the Institutional Animal Care and Use Committee at the Southern Medical

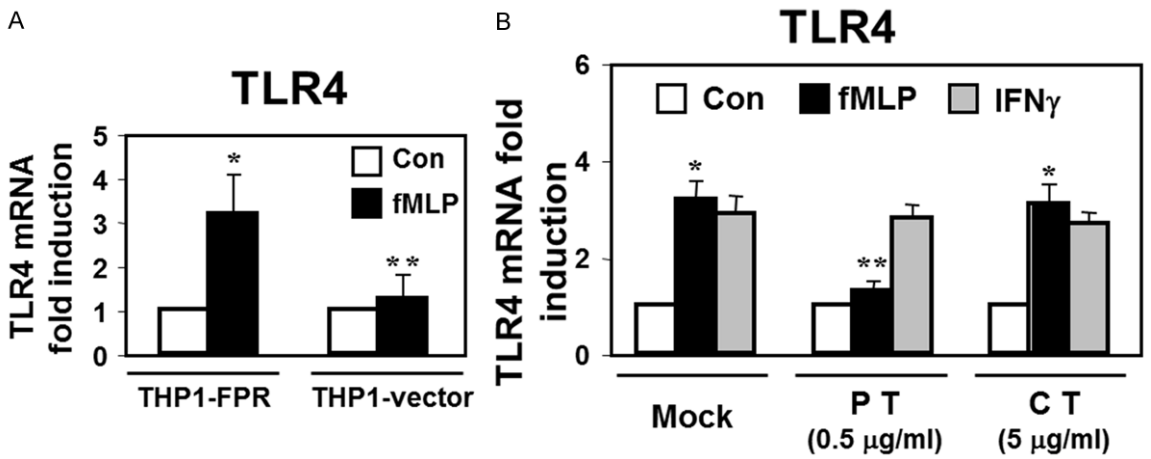

Figure 2. fMLP-induced TLR4 up-regulation involves fMLP receptor (FPR) coupled to Pertussis-sensitive heterotrimeric G proteins. A. THP1 cells were stably transfected with pcDNA (THP1-vector) or FPR (THP1-FPR). Cells were treated with fMLP (100 nM) and relative quantity of TLR4 mRNA expression was measured by Q-PCR analysis 5 hours after treatment. B. THP1-FPR cells were pre-incubated for 16 hours with either pertussis toxin (0.5 mg/ml) or cholera toxin (5 mg/ml) before stimulation with fMLP (100 nM) or IFNg (20 ng/ml). fMLP-induced TLR4 mRNA expression was determined by Q-PCR analysis 5 hours after fMLP treatment. These results are representative of three separate experiments. *, P<0.05 compared with control; **, P<0.05 compared with fMLP in THP1-FPR or MOCK.

University, the Medical University of Ohio and the Scripps Research Intitute. Under the anesthesia, mice were intranasally treated with LPS (0.3 mg/kg) or fMLP (0.5 mg/kg) or fMLP and LPS in 50 ml of sterile PBS (control). Total RNA was then isolated from the lung tissues of WT and TLR4 KO mice using TRIzol (Invitrogen) following the manufacturer's instruction. TLR4 and IL-1β mRNA were measured by Quantitative Real-Time PCR as described above.

Statistical analysis

Statistical significance between groups was determined by Student's t test. *p* values of less than 0.05 were considered statistically significant.

Results

fMLP induces TLR4 expression in vitro and in vivo

We initially investigated the effect of fMLP on TLR4 expression in human monocytes and THP1 cells. As shown in **Figure 1A**, TLR4 mRNA expression was enhanced by fMLP treatment in human monocytes in a time-dependent manner. TLR4 expression was markedly up-regulated at 3 h after treatment, peaked at 5 h and declined thereafter. Furthermore, Western blot analysis confirmed TLR4 up-regulation by fMLP at the protein level in human blood monocytes (**Figure 1B**). To further confirm if fMLP also induces TLR4 expression *in vivo*, we measured mRNA expression of TLR4 following treatment with fMLP in lung tissues of WT and TLR4 KO mice. As shown in **Figure 1C**, mRNA expressions of TLR4 by fMLP in the lung tissues of TLR4 KO mice were significantly decreased compared with those in WT mice. Consistent with our *in vitro* findings, our data showed that fMLP induces TLR4 up-regulation *in vitro* and *in vivo*.

FPR mediates fMLP-induced TLR4 up-regulation

FPR, as a pattern recoginition receptor, plays a critical role in recognizing extracellular pathogens and transducing the cell surface interaction between microbes and cells to intracellular components. Thus, to determine whether fMLP induced-TLR4 up-regulation is mediated by FPR, we examined TLR4 induction by fMLP in THP1-pcDNA (vector) and THP1-FPR cells, stably transfected with pcDNA or FPR, respectively. As expected, fMLP induced TLR4 mRNA up-regulation in THP1-FPR cells but not in THP1-pcDNA cells (**Figure 2A**). We previously demonstrated that fMLP stimulated NF-kB activation and was inhibited by pertussis toxin but not cholera toxin, suggesting that FPR-mediated NF-kB is the results of coupling to the G_i/G_o class of Ga proteins [8]. We therefore tested whether Gai or Gao class of heterotrimeric G

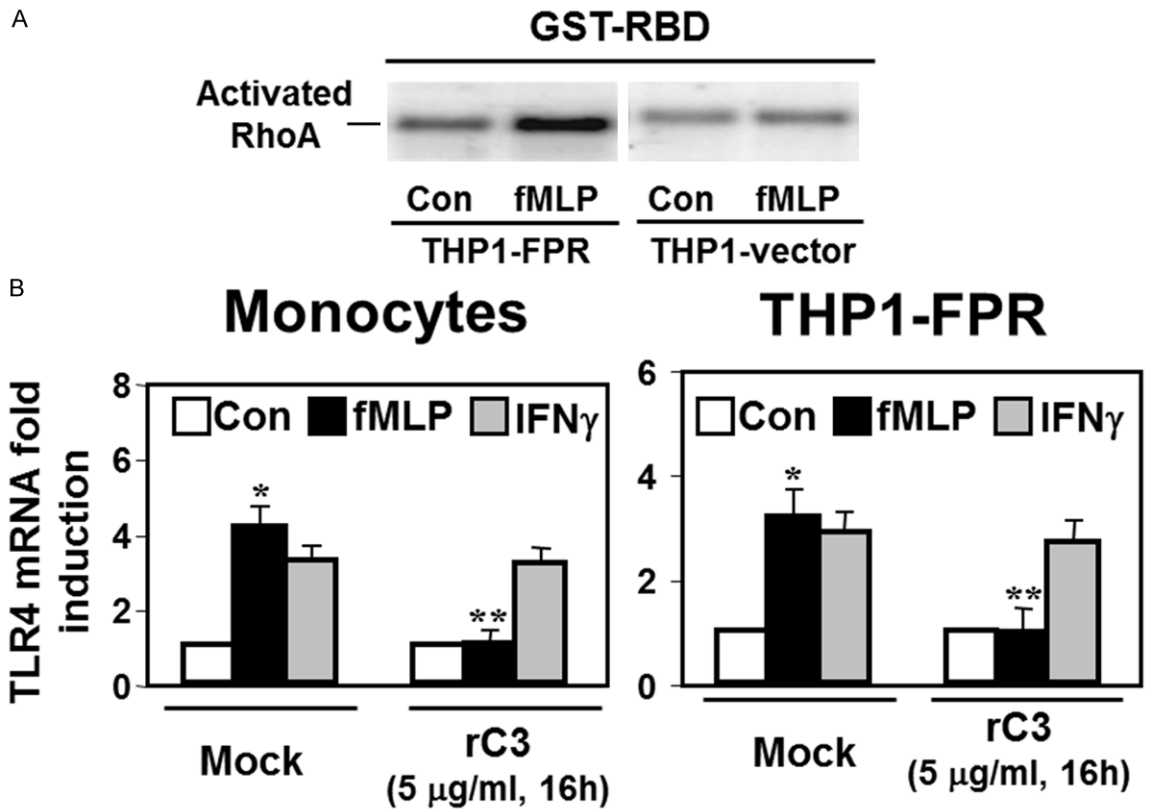

Figure 3. fMLP-induced TLR4 up-regulation is regulated by small GTPase Rho A. A. fMLP induces Rho A activity in THP1-FPR cells. Cells were stimulated with fMLP (100 nM) for 10 min. The whole cell lysates were incubated with GST-RBD (20 mg) beads at 4°C for 45 min. Rho activity is indicated by the amount of RBD-bound Rho A by Western blotting using a monoclonal antibody against Rho A (Santa Cruz, Inc) as described under "Experimental Procedures". Experiments were repeated 3 times with essentially identical results. B. The Rho A inhibitor, C3 transferase, abolishes fMLP-induced TLR4 up-regulation. Human peripheral blood monocytes (left panel) or THP1-FPR cells (right panel) were pre-incubated with media alone or recombinant C3 transferase (rC3 inhibitor; 5 mg/ml, 16 hours), stimulated with media alone, fMLP (100 nM), or IFNg (20 ng/ml) for 5 hours; and TLR4 mRNA expression was determined by Q-PCR analysis. These results are representative of three separate experiments. *, $P<0.05$ compared with control; **, $P<0.05$ compared with fMLP in MOCK.

proteins was important for this fMLP-induced TLR4 up-regulation by assessing the effect of the pertussis toxin and cholera toxin on fMLP-induced TLR4 up-regulation. **Figure 2B** shows that pertussis toxin inhibited fMLP-induced up-regulation of TLR4 but not cholera toxin in human blood monocytes and THP1-FPR cells. Taken together, these results provide evidence that FPR is required for fMLP-induced TLR4 up-regulation and Gi/Go class of heterotrimeric G proteins likely mediate these regulation in human peripheral blood monocytes.

fMLP-induced TLR4 up-regulation is regulated by small GTPase Rho A

We previously reported that fMLP stimulates NF-κB activation, and this function of fMLP requires small GTPase Rho A [8]. To determine the role of GTPase Rho A in fMLP-induced TLR4 up-regulation, we first evaluated activation of Rho A in monocytes and THP1-FPR cells by fMLP. As shown in **Figure 3A**, fMLP induced Rho A activity in THP1-FPR cells, but not in THP1-vector cells. To further investigate the role of Rho A in fMLP-induced TLR4 up-regulation, we examined the consequences of pre-incubating cells with a specific Rho inhibitor. The C3 transferase is an exotoxin produced by *Clostridium botulinum* that specifically inhibits the Rho small GTP binding proteins (Rho A, B, and C) but does not inhibit Rac or Cdc42 [19]. As shown in **Figure 3B**, fMLP-induced TLR4 up-regulation was greatly inhibited by C3 transferase. However, the C3 transferase only marginally affected IFNγ-induced TLR4 up-regulation (**Figure 3B**,

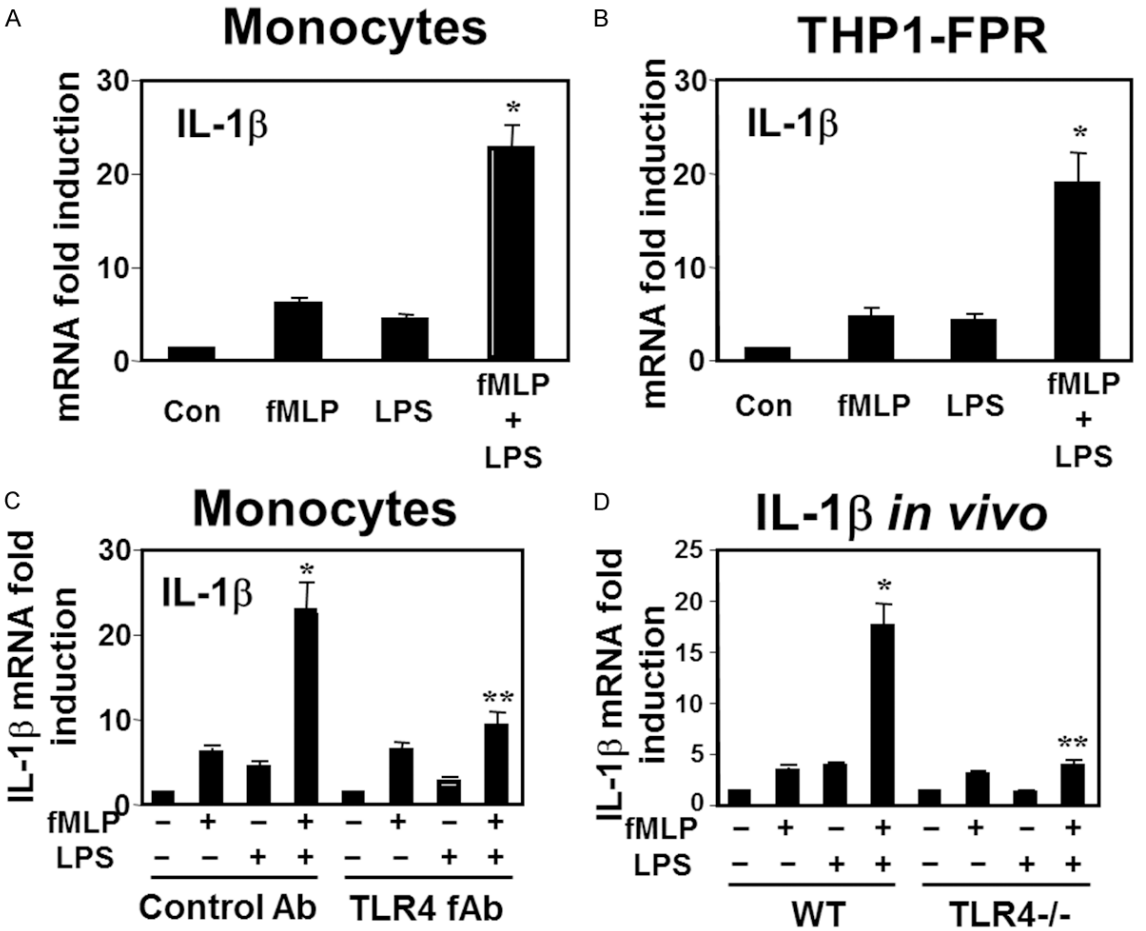

Figure 4. fMLP synergizes with LPS to induce inflammatory response by up-regulating TLR4. (A and B) Human blood monocytes (A) or THP1-FPR cells (B) were treated with fMLP with or without LPS, and IL-1β mRNA expression was measured 6 hours after treatment by Q-PCR analysis. (C) Human blood monocytes were pre-treated with functional antibody of TLR4 (TLR4fAb) for 1 hour and then stimulated with fMLP with or without LPS for 6 hours as assessed by performing real-time Q-PCR analysis. (D) IL-1β up-regulation in the lungs of WT and TLR4 KO mice following fMLP, LPS, or fMLP + LPS *in vivo*. Wild-type or TLR4 KO mice were intranasally treated with fMLP (0.5 mg/kg) or LPS (0.3 mg/kg) or fMLP and LPS in 50 ml of sterile PBS. Total RNA was then isolated from the lung tissues of WT and TLR4 KO mice using TRIzol (Invitrogen) following the manufacturer's instruction. Data are expressed as mean ± SEM of three different experiments, and each had five mice per group. Significance ($P<0.05$), indicated by *, is fMLP + LPS treatment *versus* fMLP treatment plus LPS treatment. Significance ($P<0.05$), indicated by **, is compared with fMLP + LPS in Control Ab or WT.

lane 6). These results suggest that Rho A is required for fMLP- but not for IFNγ-induced TLR4 up-regulation.

fMLP synergizes with LPS to induce inflammatory response via TLR4

To determine whether fMLP synergizes with LPS to induce inflammatory response, we investigated the effect of fMLP and LPS on the expression of IL-1β, a key NF-κB-dependent pro-inflammatory cytokine in human blood monocytes and THP1-FPR cells. As shown in **Figure 4A** and **4B**, fMLP and LPS synergistically enhances expression of IL-1β mRNA in human blood monocytes and THP1-FPR cells. Next we investigated the role of TLR4 in synergistic expression of IL-1β by fMLP and LPS using a TLR4 functional antibody (TLR4fAb) that blocks binding of LPS to TLR4. As shown in **Figure 4C**, pretreatment of human monocytes with the TLR4fAb markedly inhibits synergistic induction of IL-1β mRNA expression by fMLP and LPS, thereby providing evidence for the involvement of TLR4 in this synergistic induction.

Having shown that fMLP-induced TLR4 up-regulation is closely associated with synergistic

response by fMLP and LPS, we determined if TLR4-KO affects synergistic activation of IL-1β gene expression by fMLP and LPS *in vivo*. We measured mRNA expression of IL-1β following treatment with fMLP and LPS in lung tissues of WT and TLR4 KO mice. As shown in **Figure 4D**, mRNA expressions of IL-1β by fMLP with or without LPS in the lung tissues of TLR4 KO mice were also significantly decreased compared with those in WT mice. Taken together, these findings suggest that fMLP synergizes with LPS to induce IL-1β gene expression *via* TLR4 up-regulation.

Discussion

Under *in vivo* situations such as bacterial infections, the leukocytes are often exposed to multiple pathogens including bacteria and bacterial products/components. The recognition of pathogens is primarily mediated by a set of receptors on innate immune cells that are referred to as Pattern Recognition Receptors (PRRs). Recognition of Pathogen-Associate Molecular Patterns (PAMPs) by PRRs results in the activation of different intracellular signaling molecules that leads to the expression of various effector molecules such as proinflammatory cytokines, nitric oxide, and eichosaniods. These molecules mediate inflammatory responses and drive T cell development which leads to the activation of the adaptive immunity [20].

Recent studies revealed that mammalian Toll-like receptors (TLRs) are key molecules for recognizing microbial PAMPs to evoke the inflammatory response [21]. Among many innate immune components, TLRs are critical for initiating and mediating immune response. Thus, regulation of TLR expression plays an important role in modulating host response to microbes. However, little is known about the molecular mechanisms underlying regulation of TLRs expression in bacterial infection. As previously reported, TLR4 expression is relatively low in unstimulated human blood monocytes but is markedly up-regulated in response to invading microbes [22-24]. However, the link between TLR4 up-regulation and bacterial infection remains unknown. From what we have shown in the present data, it is evident that TLR4 expression is up-regulated by fMLP, a bacterial chemoattractant, via a FPR and Gi/Go class of heterotrimeric G proteins. We further show that fMLP and LPS synergize with each to induce IL-1β expression dependently of TLR4 up-regulation. In the present study, we provided evidence for the first time that fMLP synergizes with LPS to induce inflammatory response *via* TLR4 up-regulation *in vitro* and *in vivo*. Our findings may have important implications for host defense and immune response to bacterial infection. The increased TLR4 expression contributes to the accelerated immune response of leukocytes as well as to the resensitization of monocytes to invading pathogens. Therefore, regulation of TLR4 expression may be one of the immune regulatory mechanisms commonly involved in host defense against bacteria. The observation that TLR4 is up-regulated by fMLP also suggests that bacterial products can not only initiate the host immune response, but also modulate the eventual responsiveness of monocytes to the invading bacteria by regulating the TLR4 expression level. Interestingly, FPR has also been shown to be up-regulated in LPS treatment via transcriptional and posttranscriptional mechanisms [25, 26]. Therefore, these observations bring new insights to our understanding of the interaction between host and bacteria and the complex regulatory mechanisms underlying inflammation in bacterial infections.

Another interesting finding in this study is the experimental evidence for the regulation of fMLP-induced TLR4 up-regulation by small GTPase Rho A. Low molecular weight G proteins of the Rho family (consisting of Cdc42, Rac, and Rho A) have been shown to regulate actin cytoskeletons, focal adhesion complex formation, cell aggregation and cell motility [27-29]. The function of these small G proteins in leukocyte cytokine gene transcription, however, has recently been appreciated [30-34]. Our data suggest that fMLP-induced TLR4 up-regulation utilizes a signaling pathway that requires low molecular weight GTPase Rho A and is distinct from the signaling pathway utilized by IFNγ. The signaling molecules linking FPR-associated G_i/G_o proteins, the small GTPase Rho A and regulation of TLR4, however, remain to be determined.

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Disclosure of conflict of interest

None.

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