

Synergistic Induction of Inflammation by Bacterial Products Lipopolysaccharide and fMLP: An Important Microbial Pathogenic Mechanism¹

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A wide variety of stimuli have been shown to induce inflammation, but bacteria products/components are considered the major inducers during bacterial infections. We previously demonstrated that bacterial products/components such as LPS, a glycolipid component of the bacterial outer membrane, and formylated peptides (fMLP), a bacterial-derived peptide, induced proinflammatory cytokine gene expression in human peripheral blood monocytes. We now present evidence that mixtures of bacterial products/components LPS and fMLP behave synergistically in the induction of inflammation *in vitro* and *in vivo*. Furthermore, our results indicate that the TLR4 and the IKK β -I κ B α signaling pathways are involved in the synergistic induction of inflammatory cytokines. The mechanism of synergistic activation of NF- κ B is depended on nuclear translocation of p65 and phosphorylation of p65 at both Ser536 and Ser276 sites. These results demonstrate an important role for bacterial products/components from lysed bacteria in the pathogenesis of infectious diseases. We believe that this synergistic induction of inflammation by bacterial products LPS and fMLP represents an important pathogenic mechanism during bacterial infection, which may suggest novel therapeutic strategies or targets to minimize host injury following bacterial infection. *The Journal of Immunology*, 2009, 182: 2518–2524.

More people worldwide die of infectious diseases than of any other single cause. The bacteria induced release of proinflammatory cytokines by human peripheral blood monocytes and tissue macrophages are an important component of the inflammatory process. Despite the importance of understanding the host response to bacteria infection, many of the fundamental molecular mechanisms of host-pathogen interactions remain unknown. The proinflammatory cytokine such as TNF- α is considered one of the key inflammatory mediators during bacterial infection. This powerful protein, secreted mostly by human blood leukocytes such as monocytes/macrophages, acts as a host defense against bacterial infections. However, excessive elevations of TNF- α can lead to inflammatory disorders (1). A wide variety of stimuli have been shown to induce the production of TNF- α , but bacteria products/components are considered major inducers during bacterial infections. It has also become apparent that the pro-

duction of TNF- α in response to bacterial products is tightly regulated by NF- κ B, a nuclear transcription factor (2). In most cells, the DNA-binding activity of NF- κ B is suppressed by I κ B, a cytoplasmic inhibitory protein that binds to NF- κ B (3). Various signals can lead to the phosphorylation of I κ B, where the phosphorylated I κ B degrades and releases NF- κ B, which then translocates to the nucleus and enhances the expression of target genes such as TNF- α (4).

Previous work performed in our laboratory involved the use of single bacterial product such as LPS, a glycolipid component of the bacterial outer membrane, or formylated peptides (fMLP), a bacterial derived peptide, to induce proinflammatory cytokine gene expression (5–9). However, we did not address the significance of multiple bacteria products on proinflammatory cytokine production and inflammatory responses.

Previous works have shown that synergistic activation is achieved through the signaling pathways by different ligands (10–12), however, most research so far on the production of proinflammatory cytokines has been focused on understanding how cytokines are produced by a single bacterial product. This is unlike the situation *in vivo* where multiple bacterial products are present concurrently at the site of infection. Thus, an important question is raised: whether mixtures of bacterial products would have a significant impact on host-pathogen interactions. Our hypotheses are that inflammatory responses are induced by multiple inducers that operate synergistically by activating multiple signaling pathways, and that this synergy is likely to play a significant role in the induction of host defense to bacterial infections and in the pathogenesis of inflammatory disorders.

In this study, we report that bacterial products LPS and fMLP synergistically induce inflammatory response via multiple signaling pathways *in vitro* and *in vivo*. Moreover, our results indicate that the TLR4, IKK β -I κ B α , and NF- κ B signaling pathways are involved in the synergistic induction of TNF- α via p65 nuclear

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translocation-dependent mechanisms. Our data also suggest that phosphorylation of p65 at both Ser536 and Ser276 sites is critical for the synergistic activation of NF- κ B induced by LPS and fMLP in human peripheral blood monocytes. These results uncover a novel microbial pathogenic mechanism occurring during bacterial infection, which may suggest new therapeutic strategies or targets to minimize host injury following bacterial infection.

Materials and Methods

Reagents

Ultra pure LPS (*Escherichia coli*, 0111:B4) was obtained from InvivoGen. fMLP was purchased from Sigma-Aldrich. Recombinant human TNF- α was obtained from CP Biotech. Abs for phospho p65 (S536) and (S276), phospho I κ B α and I κ B α were purchased from Cell Signaling Technology. An Ab against p65 was obtained from Santa Cruz Biotechnology. Oligonucleotides and their complementary strands for electrophoretic mobility shift assay (EMSA)³ were from Promega and Santa Cruz Biotechnology. The sequences are: 5'-AGTTGAGGGGACTTT-CCCAGGC-3' (NF- κ B).

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 (13). The monocytic cell line THP-1 cells were differentiated by incubation with 1, 25(OH)₂D₃ for 3 days (14). THP1 cells were cultured in RPMI 1640 (Irvine Scientific) with 10% (v/v) heat-inactivated FBS, penicillin (100 U/ml), streptomycin (100 μ g/ml), and L-glutamine (2 mM; Irvine Scientific) and 2-ME (complete medium).

Quantitative real-time PCR analysis of TNF- α , IL-1 β , and IL-8

Human peripheral blood monocytes were stimulated with fMLP and LPS or fMLP/LPS for 2 h. 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 human TNF- α , IL-1 β , and IL-8) were used to detect expression of the gene. In brief, reactions were performed in duplicate containing 2 \times Universal Master Mix, 2 μ l of template cDNA, 200 nM primers, and 100 nM probe in a final volume of 25 μ l, and they were analyzed in a 96-well optical-reaction plate (Applied Biosystems). Probes include a fluorescent reporter dye, FAM, on the 5' end and labeled with a fluorescent quencher dye, 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 TNF- α , IL-1 β , and IL-8 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).

Western blot analysis

Western blot analysis was performed as described (15). Abs against phospho-I κ B α , total I κ B α , and phospho-Ser536 and -Ser276 of p65 were purchased from Cell Signaling Technology. Abs against p65 and TFIIB were purchased from Santa Cruz Biotechnology.

EMSA

Nuclear extracts were prepared from human peripheral blood monocytes using a modified method of Dignam et al. (16), and EMSA were performed using 2.5 μ g of the nuclear extract as described previously (17).

Luciferase activity assay

Expression plasmids I κ B α (S32/36A), IKK β (K49A) have been described previously (18). The reporter construct NF- κ B-LUC was generated as de-

scribed (18). Cells were cotransfected with either a dominant negative form of I κ B α (S32/36A) or a dominant negative form of IKK β (K49A) together with the NF- κ B-dependent luciferase reporter plasmid (pNF- κ B-LUC). The plasmid pCMV β (Clontech) was used as a control for transfection efficiency and this was monitored via the expression of β -galactosidase. Cells were transiently transfected with plasmids using DEAE-dextran. The transfected cells were cultivated for 48 h before a 6-h incubation in medium \pm fMLP, LPS, or fMLP/LPS. Luciferase activity was determined by using the luciferase assay kit (Promega) and a Monolight 3010 luminometer (Analytical Luminescence).

ELISA

Bronchoalveolar lavage (BAL) fluid (BALF) or the medium from stimulated human peripheral blood monocytes (fMLP, LPS, or fMLP/LPS for 6 h). The fluid or medium were collected and secreted TNF- α was measured by enzyme-linked immunosorbent assay (ELISA) using a commercial kit (Genzyme) according to the manufacturer's recommended protocol. The quantities of secreted TNF- α in the test samples were determined by using a standard curve generated with purified TNF- α .

Small interfering RNA (siRNA) approach

Custom designed siRNA duplexes for N-formyl peptide receptor (FPR), and siRNA controls (nontargeting and RISC-free) were purchased from Dharmacon. A final concentration of 100 nM of siRNA FPR or nontarget siRNA was transfected in to the THP-1 cells using DharmaFect 2 (Dharmacon). Thirty hours post transfection they were stimulated with fMLP, LPS, or fMLP plus LPS, TNF- α mRNA fold induction was analyzed by quantitative real-time PCR.

Mouse and animal experiments

C3H/HeOuJ or C3H/HeJ mice were purchased from The Jackson Laboratory, and all animal experiments were approved by the Institutional Animal Care and Use Committee at The Scripps Research Institute and Medical University of Ohio. 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 μ l of sterile PBS (control), BAL was performed by cannulating the trachea with sterilized PBS, and cells from BAL fluid were stained with Wright-Giemsa stain after cytocentrifuge. For TNF- α protein release, BAL fluid was collected and secreted TNF- α was measured by ELISA as described above.

Results

Bacterial products LPS and fMLP synergistically induce expression of TNF- α , IL-1 β , and IL-8 in human peripheral blood monocytes in vitro

Because inflammatory responses are mainly mediated by proinflammatory cytokines, we first studied whether the synergistic activity of leukocytes by fMLP and LPS was seen in the induction of proinflammatory cytokines. Using real time PCR to assay mRNA quantitatively (Fig. 1, A–C) and ELISA to detect protein level (Fig. 1D) in human peripheral blood monocytes, we tested the effect of LPS and fMLP on the expression of TNF- α (Fig. 1A) and IL-1 β (Fig. 1B) which are the two important cytokines for the initiation of inflammation. We also studied the expression of IL-8, an important proinflammatory mediator (Fig. 1C).

As shown in Fig. 1D, lanes 5 and 6, either 10 nM fMLP or 10 ng/ml LPS induced the protein of TNF- α release in human peripheral blood monocytes. But they were ineffective when used at the lower concentrations of 0.1 nM and 0.1 ng/ml, respectively (Fig. 1, lanes 2 and 3). However, when human peripheral blood monocytes were stimulated with 0.1 nM fMLP and 0.1 ng/ml LPS together, significant cytokine production was seen (Fig. 1, lane 4). This experiment suggests that bacterial products LPS and fMLP synergistically stimulate inflammatory cytokine production in human peripheral blood monocytes. The effects of mixtures of bacterial products LPS and fMLP, each present at a low concentration, are more relevant to the in vivo situation occurring during a bacterial infection than the effects of a single inducer.

³ Abbreviations used in this paper: EMSA, electrophoretic mobility shift assay; BAL, bronchoalveolar lavage; BALF, BAL fluid; FPR, N-formyl peptide receptor; siRNA, small interfering RNA; WT, wild type.

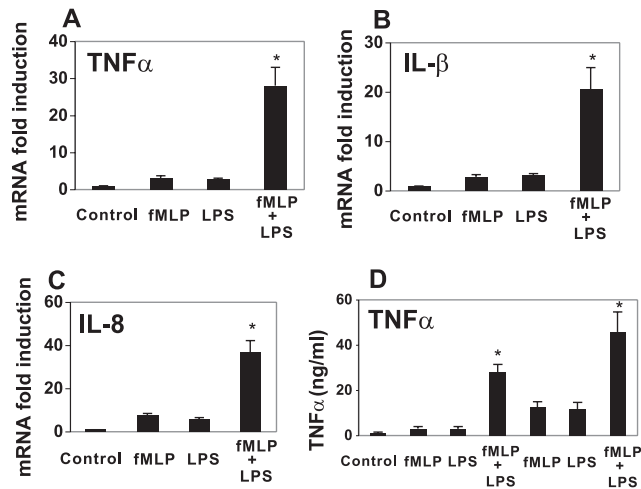


FIGURE 1. Bacterial products LPS and fMLP synergistically induce expression of TNF- α (A), IL-1 β (B), and IL-8 (C). Human peripheral blood monocytes were stimulated with medium (lane 1), 0.1 nM fMLP (lane 2), 0.1 ng/ml LPS (lane 3), both 0.1 nM fMLP and 0.1 ng/ml LPS (lane 4). Total-RNA was isolated and analyzed by quantitative real-time PCR analysis. RNA levels are normalized to levels of the housekeeping gene GAPDH and calculated as the mean of induction compared with control untreated cells. Data are representative of four independent experiments. D, Induction of immunoreactive TNF- α in supernatant from stimulated human monocytes. Monocytes were stimulated with medium (lane 1), 0.1 nM fMLP (lane 2), 0.1 ng/ml LPS (lane 3), both 0.1 nM fMLP and 0.1 ng/ml LPS (lane 4), 10 nM fMLP (lane 5), 10 ng/ml LPS (lane 6), and both 10 nM fMLP and 10 ng/ml LPS (lane 7). The supernatants were collected and the secreted TNF- α was measured by ELISA as described in *Materials and Methods*. Results shown are mean SEM from three separate measurements. Significance ($p < 0.001$), indicated by *, is fMLP plus LPS stimulated cells vs fMLP-stimulated cells plus LPS-stimulated cells.

Bacterial products LPS and fMLP synergistically induce inflammatory response in vivo

We next determined whether bacterial products LPS and fMLP synergistically induce lung inflammatory responses in mice by using C3H/HeOuJ mice. Mice were treated with LPS (0.3 mg/kg) and fMLP (0.5 mg/kg), which were administered intranasally in 50 μ l of sterile PBS. After 1 day (A, B, and C), BALF was assessed for infiltrating cells (Fig. 2, A and B) and proinflammatory cytokines (Fig. 2C) were examined by ELISA. Mice exposure to a mixture of fMLP and LPS had significantly more infiltrating cells in BALF than did the mice stimulated with either fMLP or LPS alone (Fig. 2, A and B). There was also a dramatic and significant increase in the amount of cytokines produced in BALF when mice received mixtures of fMLP and LPS rather than fMLP or LPS alone (Fig. 2C). Consistent with these in vivo results, it demonstrates that bacterial products LPS and fMLP synergistically induce inflammatory response in vitro and in the murine model of lung inflammation.

Synergistic induction of inflammation by bacterial products is TLR4- and FPR-dependent

Because synergistic induction of IL-8 by *Haemophilus influenzae* and *Streptococcus pneumoniae* does not require TLR4 in epithelial cells (19), we investigate the role of TLR4 in synergistic induction of inflammation in mice by comparing endotoxin-sensitive C3H/HeOuJ mice, which are wild type (WT) for TLR4 (the LPS receptor) with endotoxin-resistant C3H/HeJ mice, which have a defective TLR4 that cannot provide intra-

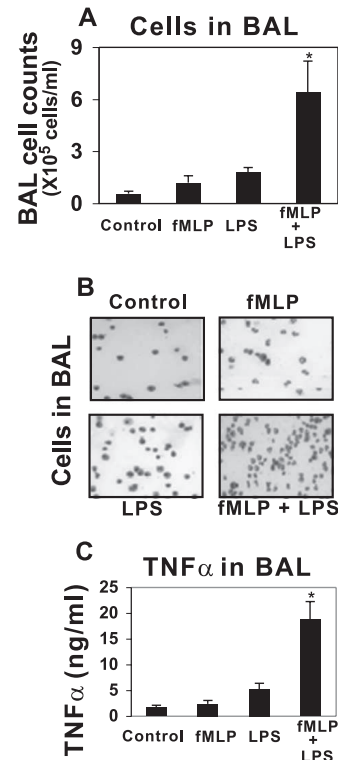


FIGURE 2. Synergistic induction of inflammation by bacterial products, LPS and fMLP in the murine model of lung inflammation. C3H/HeOuJ mice were treated intranasally with either fMLP (0.5 mg/kg) or LPS (0.3 mg/kg) or fMLP and LPS in 50 μ l of sterile PBS. Three hours (A and B) or 2 days (C) after the challenge, mice were sacrificed and the BALF was assessed for the presence of cellular infiltrates by cell counting (A), Wright-Giemsa stain (B), and TNF- α ELISA (C). Data are expressed as mean \pm SEM of three different experiments, and each had five mice per group. Significance ($p < 0.001$), indicated by *, is fMLP plus LPS challenged animals vs fMLP-challenged animals plus LPS-challenged animals.

cellular signaling. Mice were treated with LPS (0.3 mg/kg) and fMLP (0.5 mg/kg) administered intranasally in 50 μ l of sterile PBS. WT mice had a significant increase in the amount of infiltrating cells in BALF than did the TLR4-defective mice (Fig. 3A). There was four to five times more cytokines produced in WT mice than in the TLR4-defective mice (Fig. 3B). Consistent with these results, synergistic induction of TNF- α was not observed in FPR knock-down cells as compared with control cells (Fig. 3C). Thus, synergistic induction of inflammation by fMLP and LPS occurs in both the murine model of lung inflammation and in the human monocyte cells and appears to involve signaling via TLR4 and FPR.

The mechanisms of synergistic production of proinflammatory cytokine may involve in the activation of NF- κ B

More recent studies have shown that activation of the transcription factor NF- κ B is required to induce the expression of many genes encoded with inflammatory cytokines (20–24). In leukocytes, the activation of NF- κ B results in the transcription of immediate-early genes that encode TNF- α , IL-1, IL-2, IL-6, IL-8, MCP-1, GM-CSF, and several adhesion molecules (2). To determine mechanisms explaining how LPS and fMLP acted synergistically, the effects of LPS and fMLP on the activity of NF- κ B have been investigated.

We first determined whether there was evidence for the synergy in the activation of NF- κ B induced by LPS and fMLP. As

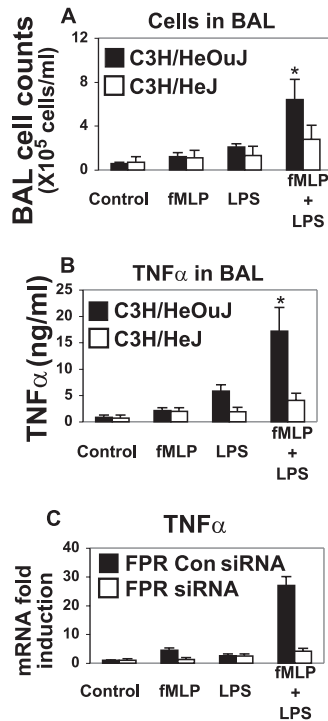


FIGURE 3. Synergistic induction of inflammation by LPS and fMLP is TLR4- and FPR-dependent. C3H/HeOuJ mice (■) or C3H/HeJ mice (□) were treated intranasally with either fMLP (0.5 mg/kg) or LPS (0.3 mg/kg) or fMLP plus LPS in 50 μ l of sterile PBS. Three hours (A) or 2 days (B), mice were sacrificed and the BALF was assessed for the presence of cellular infiltrates by cell counting (A) and TNF- α ELISA (B). C3H/HeJ mice: a strain that lacks functional TLR4 (LPS receptor); C3H/HeOuJ mice: wild type for TLR4 but are otherwise similar in genetic background with C3H/HeJ. Data are expressed as mean \pm SEM of three different experiments, and each had five mice per group. Significance ($p < 0.001$), indicated by *, is WT vs TLR4 defective mice (C3H/HeJ). C, FPR is required for synergistic induction of TNF- α by fMLP and LPS. THP1 cells were transfected with siRNA FPR or control siRNA. Thirty hours post transfection they were stimulated with fMLP, LPS, or fMLP plus LPS. Total RNA was extracted, reverse transcribed and the cDNA was amplified with human TNF- α primers by real time PCR. All experiments were repeated three times and in triplicates.

shown in Fig. 4A, lanes 5 and 6, both 10 nM fMLP and 10 ng/ml LPS induced the activation of NF- κ B in human peripheral blood monocytes, but they were ineffective when used at the lower concentrations of 0.1 nM and 0.1 ng/ml respectively (Fig. 4A, lanes 2 and 3). However, when human peripheral blood monocytes were stimulated with 0.1 nM fMLP and 0.1 ng/ml LPS together, significant NF- κ B activation was seen (Fig. 4, lane 4). This experiment suggests that synergistic induction of proinflammatory cytokine by fMLP and LPS acts on the level of transcriptional activation.

NF- κ B is normally present in the cytoplasm in an inactive state and bound to members of the I κ B inhibitor protein family, chiefly I κ B (25–27). In this complex, I κ B blocks the nuclear-localization signal, preventing nuclear translocation. To translocate NF- κ B into the nucleus, the cytoplasmic NF- κ B-I κ B complex needs to be disrupted (25). We then investigated the effects of LPS and fMLP on the phosphorylation of I κ B α . Our data indicate that LPS and fMLP synergistically induced degradation and phosphorylation of I κ B α (Fig. 4B) by using Abs against phosphorylated I κ B α and total I κ B α in human peripheral blood monocytes. These observations also implicate the involvement of I κ B α phosphorylation and degradation in the

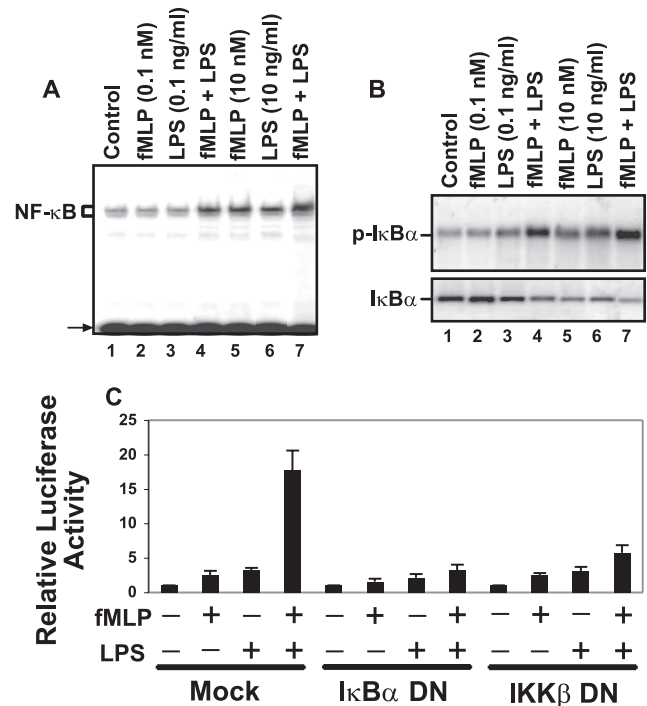


FIGURE 4. The mechanisms of synergistic induction of inflammation may involve the activation of NF- κ B. Bacterial products LPS and fMLP synergistically activate of NF- κ B in human peripheral blood monocytes (A and B) and NF- κ B-regulated luciferase reporter gene in THP1 cells (C). Monocytes were stimulated with medium (lane 1), 0.1 nM fMLP (lane 2), 0.1 ng/ml LPS (lane 3), both 0.1 nM fMLP and 0.1 ng/ml LPS (lane 4), 10 nM fMLP (lane 5), 10 ng/ml LPS (lane 6), and both 10 nM fMLP and 10 ng/ml LPS (lane 7) for 15 min (A and B). Nuclear extracts were prepared and synergistic activation of NF- κ B binding activity was measured by EMSA as described in *Materials and Methods*. A, The EMSA autoradiograph is shown with the DNA-protein complex marked with a bracket and the unbound probe indicated by an arrow. B, The synergistic induction of phosphorylation and degradation of I κ B α was observed by Western blot using phospho I κ B α (upper panel) and I κ B α Abs (lower panel) in human peripheral blood monocytes treated with LPS plus fMLP. C, Cotransfection of a dominant-negative mutant form of IKK β with NF- κ B-regulated luciferase reporter gene markedly inhibited synergistic NF- κ B activation in THP1 cells. FMLP, 0.1 nM; LPS, 0.1 ng/ml.

synergistic activation of NF- κ B by LPS and fMLP. To further confirm whether I κ B α is indeed involved, we transfected THP1 cells with a transdominant mutant (S32A, S36A) of I κ B α in which two critical serine residues required for inducer-mediated phosphorylation were mutated (28) or a dominant-negative mutant form of IKK β , IKK β (K49A). As shown in Fig. 4C, the synergistic activation of NF- κ B was markedly inhibited by both treatments. Together, these findings clearly demonstrate that bacterial products LPS and fMLP synergistically induce NF- κ B by means of the regulation of IKK β -I κ B α , which, in turn, leads to NF- κ B activation.

LPS and fMLP synergistic activation of NF- κ B via a p65 nuclear translocation-dependent mechanism in human peripheral blood monocytes

Because nuclear translocation is a key step for NF- κ B to exert its transcriptional activity (25), we next sought to explore the possibility that LPS and fMLP synergistically activate NF- κ B by inducing its nuclear translocation of p65, a key subunit of NF- κ B, by performing Western blot analysis using nuclear protein in human peripheral blood monocytes. As shown in Fig. 5A, when the cells

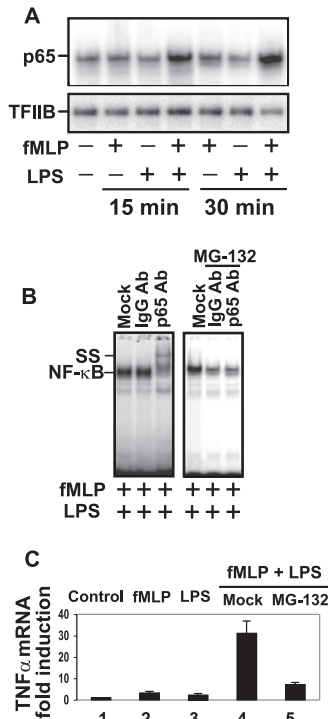


FIGURE 5. Bacterial products LPS and fMLP synergistically activate NF- κ B via a p65 nuclear translocation-dependent mechanism. Nuclear translocation of p65 by LPS and fMLP is synergistically induced in human peripheral blood monocytes. Monocytes were stimulated with medium, 0.1 nM fMLP, 0.1 ng/ml LPS, and both 0.1 nM fMLP and 0.1 ng/ml LPS for 15 min (A and B), and nuclear protein was assessed by Western blot using p65 Abs (A). B, p65 appears to be the major subunit of the NF- κ B band that was synergistically induced by LPS and fMLP, as assessed by supershift assays. IgG was used as a control. C, The nuclear translocation inhibitor MG-132 abolished MG-132 abrogated the synergistic induction of nuclear translocation of p65 (B, lane 6) and TNF- α expression (C, lane 5) by LPS and fMLP in human peripheral blood monocytes. Monocytes were: preincubated with medium alone (B, lane 1 and C, lane 4), 2 μ M MG-132 (B, lanes 2 and 3, and lane 5) for 30 min, then stimulated with 0.1 nM fMLP (C, lanes 2), 0.1 ng/ml LPS (C, lanes 3), and both 0.1 nM fMLP and 0.1 ng/ml LPS (B, lanes 4–6, and C, lanes 4 and 5) for 15 min (B) and 6 h (C). Nuclear extracts were prepared and NF- κ B binding activity was assessed by EMSA. These results are representative of three separate experiments.

were stimulated with LPS (0.1 ng/ml) or fMLP (0.1 nM) alone, p65 was not translocated to the nucleus (Fig. 5A, lanes 2 and 3 and lanes 5 and 6), whereas simultaneous treatment with both LPS and fMLP resulted in synergistic induction of p65 translocation in 15 min (Fig. 5A, lane 4 and lane 7). Consistent with this result, we performed gel supershift assays to determine whether the LPS and fMLP-induced DNA-protein complexes contain p65.

Anti-p65 Abs induced a shift of the DNA-protein complexes with nuclear extracts from LPS and fMLP-stimulated cells (Fig. 5B, lane 3), while control IgG Ab did not cause a supershift of the DNA-protein complex (Fig. 5B, lane 2). Furthermore, we used MG-132, a proteasome inhibitor that prevents the nuclear translocation of NF- κ B, to block nuclear translocation of p65 (Fig. 5B, lane 6), greatly inhibiting the production of TNF- α in human peripheral blood monocytes (Fig. 5C, lane 5). These results indicate that the NF- κ B signaling pathway is involved in synergistic activation of TNF- α by bacterial products LPS and fMLP via a p65 nuclear translocation-dependent mechanism in human peripheral blood monocytes.

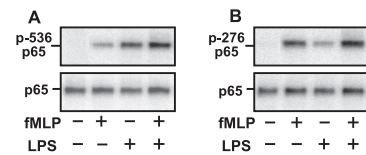


FIGURE 6. Bacterial products LPS and fMLP synergistically induced phosphorylation of p65 at both Ser536 and Ser276 sites in human peripheral blood monocytes. Monocytes were stimulated with medium, 0.1 nM fMLP, 0.1 ng/ml LPS, and both 0.1 nM fMLP and 0.1 ng/ml LPS for 15 min, and nuclear protein was assessed by Western blot using phospho-Ser536 (A), phospho-Ser276 (B) or p65 Abs. These results are representative of three separate experiments.

Phosphorylation of p65 at both Ser536 and Ser276 sites is required for synergistic activation of NF- κ B by LPS and fMLP

Previous studies identify serine 536 as the major phosphorylation site of p65 in response to LPS treatment (29, 30). We sought to determine whether phosphorylation of p65 at S536 is involved in mediating this synergistic activation of NF- κ B. As shown in Fig. 6, serine 536 is the major phosphorylation site of p65 in response to LPS treatment (Fig. 6A), while serine 276 is the major phosphorylation site of p65 in response to fMLP stimulation (Fig. 6B). MG-132, an inhibitor of NF- κ B, blocked phosphorylation of p65 at both S536 and S276 residues (Fig. 7A) in human peripheral blood monocytes. To further confirm whether the Ser536 and Ser276 sites of p65 are indeed functionally involved in mediating the synergistic induction of NF- κ B, we assessed the effects of co-expressing the dominant-negative mutant forms of p65 (S536A or S276A) on synergistic induction of NF- κ B. As shown in Fig. 7B, coexpressing both mutant forms inhibits the synergistic induction of NF- κ B. Together, these data indicate that phosphorylation of p65 at both Ser536 and Ser276 sites is critical for the synergistic

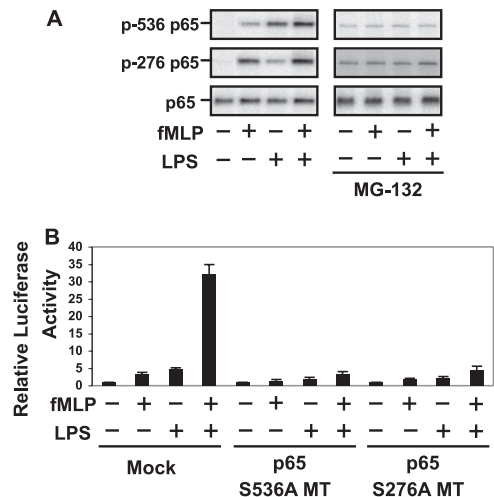


FIGURE 7. Phosphorylation of p65 at both Ser536 and Ser276 sites is required for synergistic activation of NF- κ B by LPS and fMLP. A, The nuclear translocation inhibitor MG-132 abolished the phosphorylation of p65 at both Ser536 and Ser276 sites (lane 8) induced by LPS and fMLP in human peripheral blood monocytes. Monocytes were: preincubated with medium alone (lanes 1–4), 2 μ M MG-132 (lanes 5–8) for 30 min, then stimulated with 0.1 nM fMLP, 0.1 ng/ml LPS, and both 0.1 nM fMLP and 0.1 ng/ml LPS for 15 min, and nuclear protein was assessed by Western blot using phospho-Ser536, phospho-Ser276 or p65 Abs. B, Overexpressing the mutant forms p65 (S536A) or (S276A) with NF- κ B-regulated luciferase reporter gene markedly inhibited synergistic activation of NF- κ B by fMLP and LPS in THP1 cells. fMLP, 0.1 nM; LPS, 0.1 ng/ml.

activation of NF- κ B induced by LPS and fMLP in human peripheral blood monocytes.

Discussion

Under in vivo situations such as bacterial infection, little is known about the host response to bacterial infection. Many of the fundamental molecular mechanisms of host-pathogen interactions remain unknown. In the present study, we showed that mixtures of bacterial products LPS and fMLP behave synergistically in the induction of inflammation in vitro and in vivo. These results demonstrate an important role for bacterial products/components from lysed bacteria in the pathogenesis of bacterial infections. The most current studies on inflammatory regulation in bacterial infections have focused primarily on the induction of inflammation by a single bacterial product/component and not by mixtures. Although studies with single bacterial product/component are indeed critical for our understanding of the molecular basis of the relevant signaling pathway, the information derived may be insufficient for a full understanding of how inflammation is induced in vivo where a mixture of bacterial products/components, like fMLP and LPS, is present.

Another interesting finding in our study is the evidence that synergistic induction of NF- κ B is dependent on phosphorylation of p65 at both Ser536 and Ser276 sites. Previous studies identify serine 536 as the major phosphorylation site of p65 in response to LPS treatment (29, 30), however, the phosphorylation of p65 at Ser 276 was also been reported by phosphotungstic acid and TNF (31, 32). Joo and Jetten (33) showed that the S276A mutation significantly reduced the induction of NF- κ B transcription activation by farnesol, and that inhibition of MEK1/2 by U0126 or knockdown of MEK1/2 expression greatly diminished the phosphorylation of p65 at Ser276 site (but not that of Ser536), suggesting that phosphorylation of p65 at Ser276 is dependent on the activation of the MEK1/2-ERK1/2 pathway. Our unpublished data indicate that the fMLP stimulate a time-dependent increase in phosphorylation of ERK1/2 in human peripheral blood monocytes. These results suggest that the MEK1/2-ERK1/2 pathway may play a role in the synergistic activation of NF- κ B induced by fMLP and LPS. In the present study, we showed that serine 536 is the major phosphorylation site of p65 in response to LPS treatment, while serine 276 is the major phosphorylation site of p65 in response to fMLP stimulation. Moreover, MG-132, an inhibitor of NF- κ B, greatly inhibits the synergistic induction of p65 phosphorylation at both Ser536 and Ser276 sites. Thus, our data provide evidence that the phosphorylation of p65 at both Ser536 and Ser276 sites is critical for the synergistic activation of NF- κ B induced by LPS and fMLP in human peripheral blood monocytes (Fig. 8). All of our findings are consistent with the notion that posttranslational modifications, particularly phosphorylation of p65, play critical roles in synergistic induction of inflammation.

These experiments provide information that is both unique and potentially important in light of increasingly common medical interventions, such as antibiotic therapy or vaccination. Because synergistic induction of inflammation is important and LPS has been targeted in the past, focusing on signaling molecules that are synergistically activated by bacterial products/components may be a worthwhile endeavor. For instance, the FPR or receptor-mediated signaling molecules could be targeted to subdue or abbreviate the inflammatory response (34). A specific example may involve the targeting of mesenchymal cells, which are susceptible to activation by tissue injury (35). PCR and cytometric flow analysis reveal that several formyl peptide receptors are expressed on the transcriptional and protein levels, suggesting that they may be amenable to interventions (35). In the case of our study, targeting the p65 nuclear translocation-dependent mechanism may be of value.

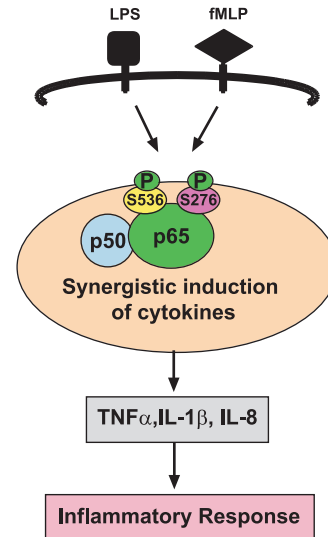


FIGURE 8. Schematic representation of synergistic activation of NF- κ B by bacterial products LPS and fMLP and the subsequent inflammatory responses in vitro and in vivo.

In this study, we found that the bacterial products LPS and fMLP regulate the induction of inflammation in a synergistic manner both in vitro and in vivo. The control of inflammation is likely to best be understood on the level of this synergistic regulation. These results represent an important pathogenic phenomenon, which is not fully understood in bacterial infection. This study has significantly expanded and enhanced our understanding of how inflammation is induced in vivo when low concentrations of bacterial products LPS and fMLP are present simultaneously. There is very limited information currently available concerning the synergistic activation of inflammation. A better understanding could bring new insights into the regulation of inflammation and may suggest novel therapeutic strategies or targets to minimize host injury following bacterial infection.

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Disclosures

The authors have no financial conflict of interest.

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