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Synergistic Induction of CCL20 and Dendritic Cell Migration by Bacterial Chemoattractant fMLP and TLR2 Agonist: Roles of ERK/MAP Kinase

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Abstract

Background: Host-derived chemokines elicited by inflammatory stimuli are known to play an important role in leukocyte recruitment. CCL20, an important chemokine for dendritic cell recruitment, lymphocyte recruitment, and adaptive immune response initiation. Under clinical or *in vivo* situations such as bacterial infection, little is known about the host response to bacterial infection.

Aim: This study aimed to study the host response to bacterial infection *in vitro* and *in vivo*.

Methods and Results: By using real-time PCR, ELISA, small interfering RNA (siRNA), flow cytometry , and chemotaxis assays, we report that fMLP synergistically enhances Toll-like receptor 2 (TLR2) agonists' production of CCL20 in human monocytes and subsequent dendritic cell chemo attraction. Our data also suggest that activation of ERK/MAPK signalling is critical for this synergistic activation. These results uncover a novel mechanism in which this synergy between the bacterial product fMLP and TLR2 agonists closes the gap between the innate and adaptive immune responses.

Conclusions: Our results indicate the importance of bacterial chemoattractants, not only in the activation of the innate immune response, but also in the initiation of the adaptive immune response. Our findings also suggest that immune responses are induced by multiple molecules that operate synergistically through different signalling pathways.

Keywords: Cytokine; Immune cell; Bacterial chemoattractant; Signal transduction

Introduction

Chemokines constitute a family of small (5–20 kDa) proinflammatory chemotactic cytokines that regulate immune responses by binding to specific receptors on the surface of leukocytes [1,2]. So far, nearly 50 chemokines have been identified in humans. Based on the pattern of the conserved N-terminal cysteine motifs, chemokines can be classified into four subfamilies: C, CC, CXC and CX3C [3-5].

CCL20 is a recently described CC-chemokine produced by mucosal epithelial cells, macrophages, eosinophils, dendritic cells and several transformed cell lines. Similar to the immune response of other chemokines, CCL20 triggers the adaptive immune response primarily by attracting immature dendritic cells expressing CCR6 on their surface to the site of inflammation, allowing the dendritic cells to take up a foreign antigen and undergo maturation [6-8]. In this way, CCL20 can bridge the gap between innate and adaptive immune responses, and has been suggested to play a crucial role in the induction of host defence against bacterial infections. In addition to its chemotactic function, CCL20 has antimicrobial activity through its comparable structural, functional, and regulatory properties to beta-defensins.

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. Recent work performed in our laboratory provides evidence that pro-inflammatory cytokine gene expression induced by bacterial chemoattractant fMLP is a critical component of innate immunity [9-15]. Here we report that fMLP synergistically enhances Toll-like receptor 2 (TLR2) agonists' production of CCL20 in human monocytes and subsequent dendritic cell chemoattraction. Our data also suggest that activation of ERK/MAPK signalling is critical for this synergistic activation. These results uncover a novel mechanism where this synergy between bacterial product fMLP and TLR2 agonists closes the gap between the innate and adaptive immune responses.

Materials and Methods

Reagents

Pertussis Toxin (inhibitor of G-protein coupled receptors) and fMLP were purchased from Sigma-Aldrich. U0126 (ERK MAPK inhibitor) was purchased from Calbiochem. Functional Grade Purified anti-human TLR2 and TLR4 were obtained from eBioscience.

Dendritic cell preparation and cultures

Dendritic cells were generated from BM cells of C57BL/6 mice. Mouse BM cells were obtained by briefly flushing femurs. Red blood cells were removed by using red blood cell lysing buffer (Sigma-Aldrich). The cells were strained through a 70-μm filter and

resuspended in RPMI 1640 supplemented with 2.5 mM HEPES, 5.5 × 10^{-5} M 2-ME, 100 U/ml penicillin, 100 μg/ml streptomycin, 5 mM glutamine, 10% FBS, 10 ng/ml recombinant mouse GM-CSF (Sigma-Aldrich). Every 2 days, the floating cells were removed and fresh media was added. On day 6, the dendritic cells were collected and identified with surface expression of CD11c and MHC II by flow cytometry.

Quantitative Real-Time PCR (QRT-PCR)

After 4 h stimulation, total RNA was isolated using TRIzol Reagent (Invitrogen, Carlsbad, CA) according to the manufacturer's instructions. The mRNA was reverse transcribed using TaqMan reverse transcription reagents (Applied Biosystems). CCL20 mRNA was quantified by real-time quantitative PCR in an ABI PRISM R7000 sequence detection system using SYBR green buffer according to the manufacturer's instructions (Applied Biosystems). The sequences of the specific PCR primers were as follows (5' to 3'): CCL20, forward: GAG TTT GCT CCT GGC TGC TTT G; reverse: AAG TTG CTT GCT GCT TCT GAT TCG; GAPDH, forward: GGG AAG GTG AAG GTC GGA GT; reverse: TCC ACT TTA CCA GAG TTA AAA GCA G; Gene expression was normalized using the Ct of the housekeeping gene GAPDH. Relative quantity (fold induction) of the target gene mRNA was then calculated using the comparative Ct method.

Enzyme-Linked Immunosorbent Assay (ELISA)

CCL20 was measured in culture supernatants by ELISA using a commercial kit (R&D Systems, Minneapolis, MN) according to the manufacturer's recommended protocol.

Western blot analysis

Western blot analysis was performed as described [10]. Antibodies against phospho-p44/42 MAPK (Thr202/Tyr204), total p44/42 MAPK, phospho-p38 MAPK (Thr180/Tyr182) and phosphor-JNK were purchased from Cell Signaling Technology. Antibody against total p42 MAPK was purchased from Santa Cruz Biotechnology. Antibody against β-actin was purchased from Sigma-Aldrich.

Small interfering RNA (siRNA)

p44/42 MAPK (Erk1/2) siRNA and Control siRNA were purchased from Cell Signaling. A final concentration of 50 nM of siRNA was transfected into the THP1FPR cells using TransIT-TKO Transfection Reagent (Mirus, Madision, WI) according to the manufacturer's instructions. Further experiments were carried out after 48 hours siRNA transfection.

Flow cytometry

fMLP-eGFP expression in THP1 cells were measured in living cells by flow cytometry analysis (FACScan; Becton Dickinson, Mountain View, Calif). Data were collected for 10,000 events, stored in the list mode, and then analyzed with Lysis II software (Becton Dickinson).

Chemotaxis Assays

THP1FPR cells (1×10^6) were stimulated with 100 μM fMLP and/or 100 ng/ml Pam3 for 12h, then the culture mediums were replaced with fresh RPMI medium 1640 containing 10% (vol/vol) FBS, after another 12h incubation at 37°C, the supernatants were harvested for the following assays. Transwell chemotaxis assays were performed in 24-well transwells (6.5-mm diameter, 3 μm pore size, Corning, NY). The bottom chamber was filled with 0.6 ml of supernatants. Murine dendritic cells (1×10^6) in 0.1 ml of 10% FBS RPMI medium 1640 were added to the top chamber. After 4h of incubation at 37°C, the bottom chamber was harvested, and the transmigrated cells were counted on hemacytometer. Values were normalized to murine dendritic cells chemotaxis to supernate of non-stimulated THP1FPR cells.

Statistical analysis

Statistical significance between groups was determined by two tailed Student's t test (Excel; Microsoft). Differences were considered significant at $p<0.001$.

Results

Bacterial chemoattractant fMLP and TLR2 agonist Pam3CSK4 synergistically induce expression of CCL20 in human monocyte cell THP1 *in vitro* and *in vivo*

Monocyte cell line THP-1 is a well known model that has been used in studying the interaction of pathogen-associated molecular patterns and monocytoid cell-surface-expressed pattern recognition receptors. THP-1 cells were stably transfected with N-formyl peptide receptor (THP1 FPR). As shown in Figure 1A, nearly 100% of transfected cell express FPR tagged with eGFP. Using these cells, we tested the effect of bacterial chemoattractant fMLP and TLR2 agonist synthetic bacterial lipopeptide Pam3CSK4 on the expression of CCL20, an important chemokine for dendritic cell recruitment, lymphocyte recruitment, and adaptive immune response initiation. As shown in Figure 1B, fMLP alone (100 nM) has no effect on CCL20 expression; however, when cells were stimulated with fMLP and Pam3CSK4 (100 ng/ml) together, significant CCL20 mRNA was seen in THP1 cells (THP1 FPR). We next explored whether fMLP and Pam3CSK4 synergistically induce expression of CCL20 *in vivo*. Mice (C57BL/6) were treated with fMLP (10 μg/mouse) and Pam3CSK4 (10 μg/mouse) intranasally suspended in 50 μl of sterile PBS. After 2 days, bronchoalveolar lavage (BAL) fluid was assessed for chemokine CCL20 (Figure 1C) by ELISA. Mice exposed to a mixture of fMLP and Pam3CSK4 had a significantly larger amount of CCL20 found in broncho-alveolar lavage fluids (BALF) than mice stimulated with either fMLP or Pam3CSK4 alone (Figure 1C). Further *in vitro* experiments were consistent with these *in vivo* results and further demonstrated the synergistic activity of bacterial chemoattractant fMLP and TLR2 agonist Pam3CSK4.

Interestingly, as shown in Figures 2A and 2B, fMLP alone has no effect on expression of CCL20, whereas it was also ineffective when used at the lower concentration of 1 ng/ml Pam3CSK4 alone. However, when cells were stimulated with 1 nM fMLP and 1 ng/ml Pam3CSK4 together, significant CCL20 production was observed (Figure 2A). The synergistic expression of CCL20 followed a dose-dependent manner with a maximal response achieved using approximately 100 nM fMLP and 100 ng/ml Pam3CSK4 (Figure 2A). Our results also revealed the kinetics of this synergy with CCL20 production apparent in 3 hours and continued for at least 48 hours (Figure 2B). These experiments suggest that bacterial chemoattractant fMLP alone cannot stimulate chemokine CCL20 production directly, but it can synergistically activate Pam3CSK4 to produce CCL20. Moreover, the low concentration mixture of bacterial chemoattractant fMLP and synthetic bacterial lipopeptide Pam3CSK4b is more relevant to the in vivo situation and more tenable than the effects of a single inducer.

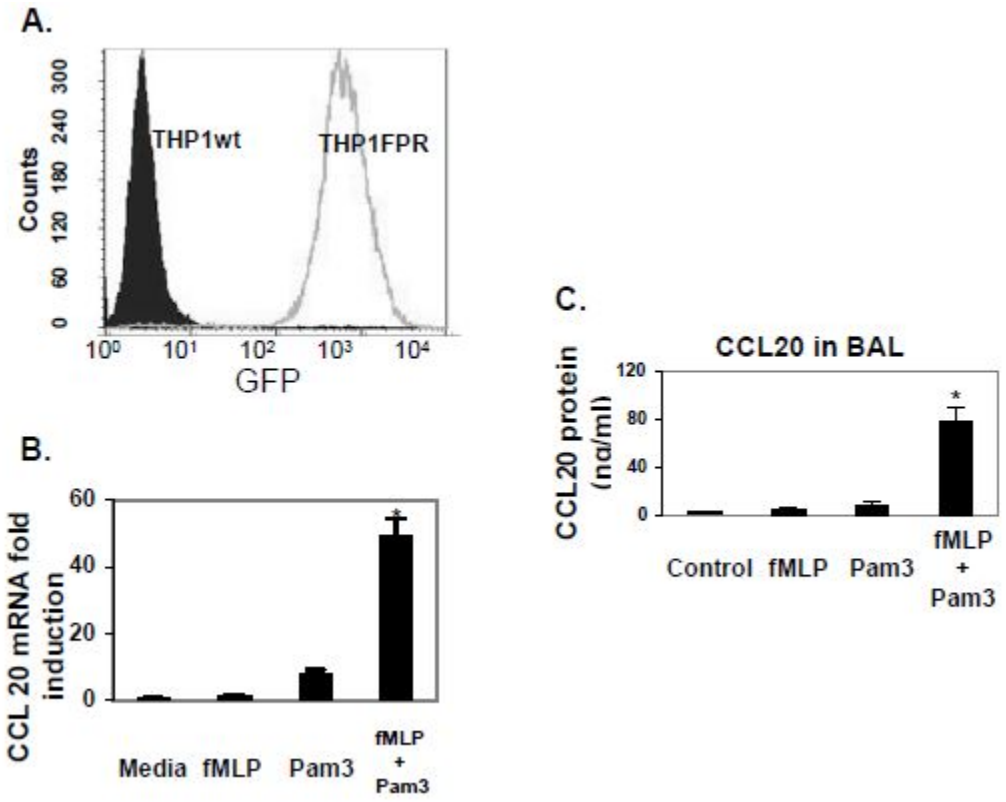

Figure 1: fMLP and TLR2 agonists synergistically induce expression of CCL20 *in vitro* and *in vivo*. (A) Transfection efficacy of FPR-eGFP in THP1 cells was determined by Flow Cytometry. (B) THP1FPR cells were stimulated with medium alone (lane 1), 0.1 M fMLP (lane 2), 0.1 g/ml Pam3CSK4 (lane 3), both 0.1 M fMLP and 0.1 g/ml Pam3CSK4 (lane 4) for 6 hours. CCL20 expression was analyzed by quantitative real-time PCR analysis. 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. Results shown are mean ± SEM from four independent experiments. Significance ($p<0.001$), indicated by *, is fMLP plus Pam3 stimulated cells *versus* fMLP-stimulated cells plus Pam3-stimulated cells. (C) C57BL/6 mice were treated intranasally with either fMLP (10 mg/mouse) or Pam3CSK4 (10 mg/mouse) or fMLP and Pam3 in 50 ml of sterile PBS. Two days after the challenge, mice were sacrificed and the BAL fluid was assessed for the presence of chemokine CCL20 by ELISA. Data are expressed as mean ± SEM of three different experiments, and each had five mice per group. Significance ($p<0.001$), indicated by *, is fMLP + Pam3 challenged animals *versus* fMLP-challenged animals plus Pam3- challenged animals.

Synergistic induction of CCL20 by bacterial products fMLP and Pam3CSK4 is FPR- and TLR2-dependent

Because synergistic induction of IL-8 by *H. influenzae* and *S. pneumoniae* does not require TLR4 in epithelial cells [16], we investigated the role of TLR2 in synergistic induction of CCL20 by fMLP and Pam3 by using anti-TLR2 functional antibody. As shown in Figure 3A, CCL20 was completely inhibited either in Pam3CSK4 stimulated or fMLP and Pam3CSK4 combinatory stimulated THP1FPR cells. However, anti-TLR4 functional antibody showed no changes compare with the control. Our previous works [17] have shown that FPR belongs to the G protein-coupled receptor (GPCR) family, whose signaling is regulated by Gi-containing heterotrimeric G protein [18,19]. Furthermore, THP1FPR cells pretreated with pertussis toxin (PTX), which blocks G protein coupling and activation, abolished the synergistic effects of fMLP and Pam3CSK4, but it does not affect expression from Pam3CSK4 alone. These results suggest that the synergistic effect of fMLP and Pam3CSK4 is FPR- and TLR2-dependent, with fMLP enhancing TLR2 signaling and chemokines production through a Gi-dependent PTX-sensitive pathway.

The mechanisms of synergistic production of pro-inflammatory cytokines may involve the activation of MAP kinase

The Mitogen-activated protein kinase (MAPK) pathway has been reported to mediate extracellular signals to the nucleus in several cell types and plays an important role in the expression of several cytokine and chemokine genes, including TNFα, IL-1, IL-8, and MCP-1. These functions are conducted by the activation of MAPKs (p38, JNK and ERK) and their downstream signaling. To determine mechanisms explaining how fMLP and Pam3CSK4 acted synergistically, the effects of fMLP and Pam3CSK4 on the activity of MAPKs were investigated using Western blot analysis. We first determined whether there was evidence for the synergy in the activation of MAPKs induced by fMLP and Pam3CSK4.

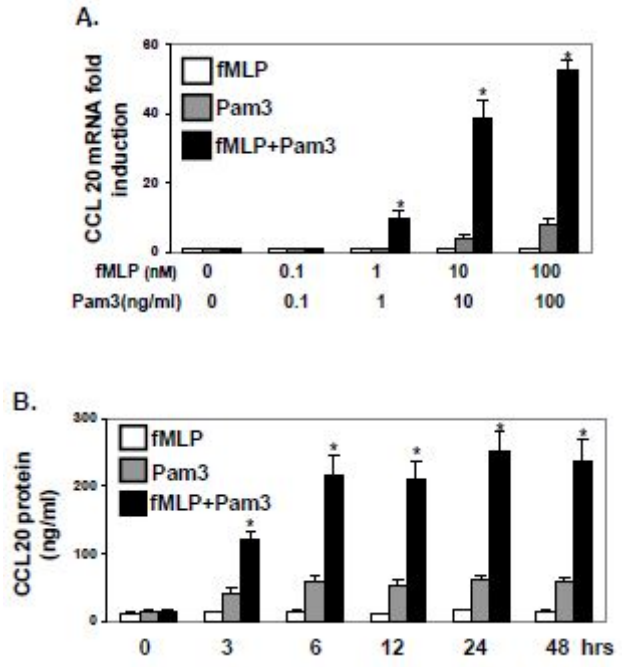

Figure 2: The dose-dependent and time-dependent synergistic effects of fMLP and Pam3. (A) THP1FPR cells were stimulated with different dose of fMLP (0.1~100 nM) and Pam3 (0.1~100 ng/ml), CCL20 mRNA was examined by quantitative real-time PCR analysis. (B) THP1FPR cells were stimulated with 100 nM fMLP and 100 ng/ml Pam3 for indicated time, the supernatants were collected and the secreted CCL20 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 + Pam3 stimulated cells *versus* fMLP-stimulated cells plus Pam3-stimulated cells.

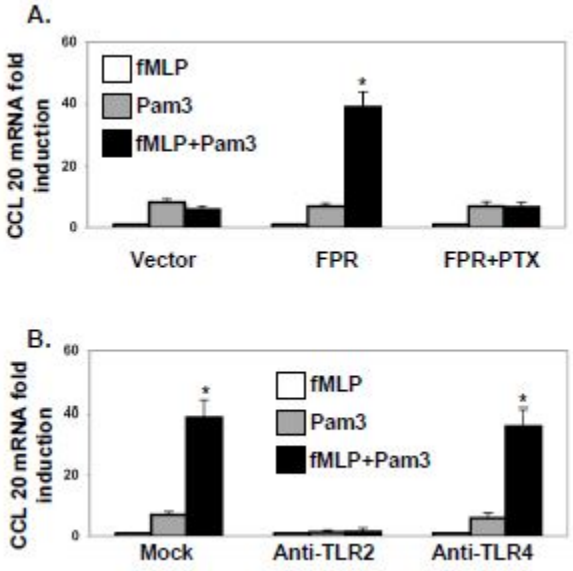

Figure 3: G-protein coupled receptors (GPCR) and TLR2 signaling pathways involved in the synergistic effects of fMLP and Pam3. (A) THP1FPR or THP1 vector cells pre-incubated for 4 h with vehicle (Lanes 1-6) or 100 ng/ml pertussis toxin (PTX) (Lanes 7-9), then cells were stimulated with 100 nM fMLP, 100 ng/ml Pam3 or fMLP plus Pam3. CCL20 mRNA was examined by RT-Q-PCR. (B) THP1FPR cells were pre-treated with isotype control antibody (lanes1-3), anti-TLR2 functional Ab (lanes 4-6), or anti-TLR4 functional Ab (lanes 7-9) for 2 h, and then the cells were stimulated with 100 nM fMLP and/or 100 ng/ml Pam3. CCL20 mRNA was examined by RT-Q-PCR. Results shown are mean SEM from three separate measurements. Significance ($p<0.001$), indicated by *, is fMLP + Pam3 stimulated cells *versus* PTX or anti-TLR2 functional Ab pre-incubated cells.

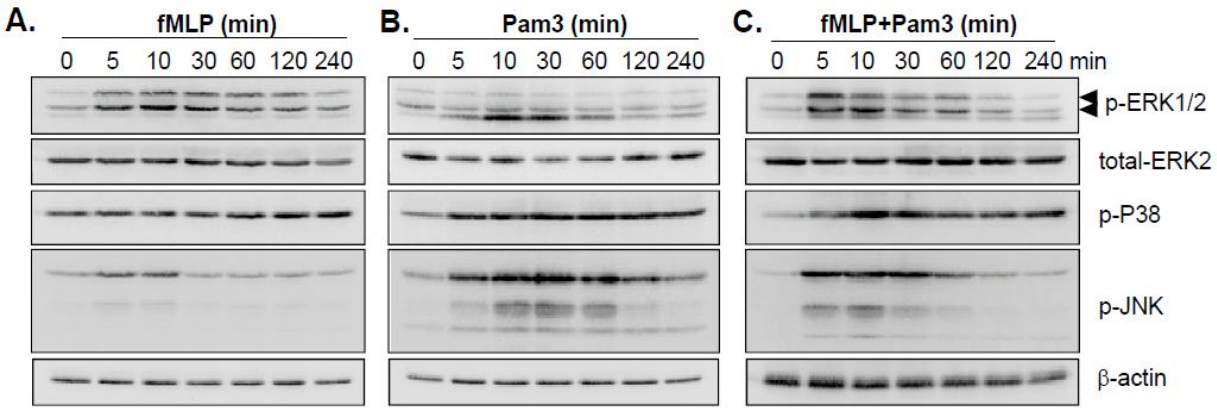

Figure 4: The mechanisms of synergistic production of pro-inflammatory cytokine may involve in the activation of MAP kinase. THP1FPR cells were stimulated with 100 nM fMLP (A), 100 ng/ml Pam3 (B), and fMLP plus Pam3 (C) for indicated time. Total protein was extracted and assessed by Western blot using phosphor-ERK1/2, total ERK2, phosphor-p38, phosphor-JNK and β-actin Abs. Results shown are mean SEM from three separate measurements.

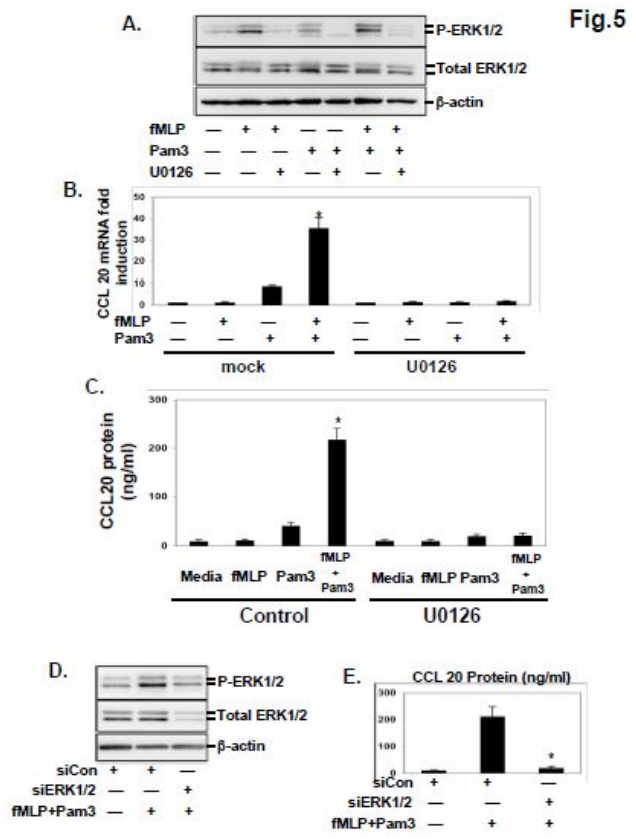

Figure 5: ERK MAPK signaling pathway is critical for the synergistic effects of fMLP and Pam3. (A) ERK MAPK inhibitor U0126 blocked phosphorylation of ERK1/2 stimulated by fMLP and/or Pam3. THP1FPR cells were pretreated with 10 μM U0126 for 1 h, and then stimulated with 100 nM fMLP and/or 100 ng/ml Pam3 for 10 minutes. Total protein was extracted and assessed by Western blot using phosphor-ERK1/2, total ERK1/2 and β-actin Abs. (B,C) MEK1/2 inhibitor U0126 abrogated the synergistic effects of CCL20 mRNA (B) and protein (C) induced by fMLP and Pam3. THP1FPR cells were pre-treated with 10 μM U0126 for 1 h, and then stimulated with 100 nM fMLP and/or 100 ng/ml Pam3 for 6 h. CCL20 mRNA was examined by RT-Q-PCR and protein was measured by ELISA. (D-E) ERK1/2 siRNA interfered the phosphorylation of ERK1/2 (D), and diminished the synergistic effects of fMLP and Pam3 on CCL20 expression (E). THP1FPR cells were transfected with non-silencing RNA (lane 1 and 2) or ERK1/2 siRNA (lane 3). The transfected cells were stimulated with fMLP + Pam3 for 10 minutes (D) or 6 hours (E). Total protein was extracted and assessed by Western blot using phosphor-ERK1/2, total ERK1/2 and β-actin Abs. CCL20 protein was measured by ELISA. Results shown are mean SEM from three separate measurements. Significance ($p<0.001$), indicated by *, is siRNA ERK1/2 cells *versus* control cells with same stimulation.

As shown in Figure 4, both fMLP or Pam3CSK4 can induce ERK, P38 and JNK activation in THP1FPR cells (Figures 4A and 4B). However, when THP1FPR cells were stimulated with 0.1 μM fMLP and 0.1 μM Pam3CSK4 together, synergistic activation of ERK, but not p38 or JNK, was seen (Figure 4C). This experiment suggests that the mechanism of synergistic production of pro-inflammatory cytokine may involve the activation of ERK.

To further test whether ERK MAPK is indeed functionally involved in mediating the synergistic induction of the chemokines, we employed two approaches: MEK1/2 inhibitor (U0126) and ERK1/2 specific siRNA to block the ERK MAPK pathway. Pretreatment with 10 μM U0126 completely inhibited the phosphorylation of ERK1/2 triggered by fMLP or fMLP plus Pam3CSK4 (Figure 5A); moreover, U0126 blocked the synergistic induction of CCL20 mRNA (Figure 5B) and protein (Figure 5C). Consistent with these findings, ERK1/2 specific siRNA knocked down the total ERK1/2 and reduced the phosphorylation of ERK1/2 (Figure 5D), inhibiting the synergy (Figure 5E). Taken together, these results demonstrate that bacterial products fMLP and Pam3CSK4 synergistically induce CCL20 by means of the phosphorylation of ERK1/2.

Chemotaxis of mouse dendritic cells triggered by supernatant of Pam3CSK4- stimulated THP1FPR cells is enhanced by fMLP

We next measured the chemotactic responses of mouse dendritic cells to the supernatants of THP1FPR cells by transwell migration assay. As shown in Figure 6, consistent with chemokines expression, the chemotactic response of mouse dendritic cells was significantly increased with the supernatant of both fMLP and Pam3CSK4-stimulated THP1FPR cells. Moreover, pretreatment of U0126 also blocked this synergistic chemotaxis effect (Figure 6).

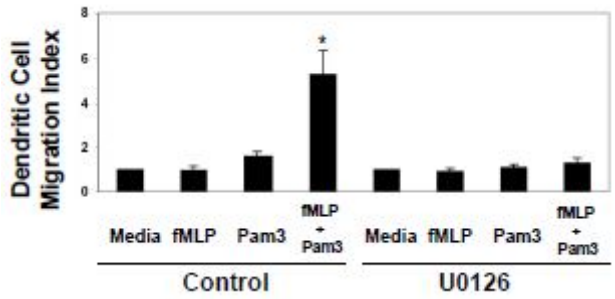

Figure 6: Chemotaxis of mouse dendritic cells triggered by supernatant of Pam3CSK4- stimulated THP1FPR cells is enhanced by fMLP. Directional migration of mouse dendritic cells toward the supernatants of fMLP and/or Pam3 stimulated THP1FPR cells was tested by standard transwell chemotaxis assay as described in Materials and Methods. Results shown are the mean SEM of three independent experiments. Significance ($p<0.05$), indicated by *, is fMLP + Pam3 stimulated cells *versus* U0126 pre-treated cells with same stimulation.

Conclusion

Human peripheral blood monocytes are known to play an important role in the inflammatory process. In addition to their unique combination of cellular functions, including phagocytosis, generation of lipid mediators, and highly reactive superoxide radicals, monocytes are capable of producing a variety of chemokines. These molecules promote leukocyte migration to inflammatory sites and trigger

adaptive immune responses [20]. Previous work has shown that bacterial products/components can induce chemokines through specific pattern-recognition molecules, such as TLRs and NODs [21-23]. However, most research has been focused on the induction of chemokines and inflammatory responses by a single bacterial product. This is not same as the situation *in vivo*, where different bacterial products are simultaneously present at the site of infection. In current studies, we provide evidence that the bacterial product fMLP, in conjunction with a TLR2 agonist, induce chemokine expression and chemotaxis in a synergistic manner. These results extend our understanding of how bacterial products and/or components behave in the pathogenesis of infectious diseases. The nature of infections with bacteria, which contain multiple antigens, means that the immune system will never be stimulated with a sole product *in vivo*. Where many previous experiments in this vein have focused solely on one bacterial product or synthetic agonist, we have provided a model that more closely approximates bacterial infections in a complex mammalian organism.

The bacterial product fMLP, which was classically described as a chemoattractant has been found to conduct multiple functions. Here, our results show that although fMLP alone had no effect, it synergistically activated TLR2 agonist-stimulated CCL20 production in monocyte THP1FPR cells (Figures 1 and 2). While the TLR2 functional antibodies completely inhibited CCL20 production, pertussis toxin blocked only the synergy between fMLP and Pam3CSK4 (Figure 3), which suggests that although the production of chemokines is TLR2-dependent, the synergistic effect is mediated primarily by fMLP and its receptor FPR, which belongs to GPCR. To investigate the downstream signaling, we employed a set of specific inhibitors for different signal pathways. The results showed that the MEK1/2 inhibitor U0126 abolished the synergistic effect of CCL20 gene expression (Figure 4B), while the other inhibitors for PKCs, p38 MAPK, JNK MAPK, PI3K, and Rho-associated protein kinase (ROCK) had very little or no effect (data not shown). These results suggest that bacterial chemoattractant fMLP and Pam3CSK4 synergistically induce CCL20 by means of the activation of ERK1/2.

Another interesting finding in our study is that bacterial chemoattractants not only activate innate immune cells, but also contribute directly to the adaptive immune response *via* dendritic cell activation. Previous studies performed in our laboratory indicate that release of the proinflammatory cytokines induced by bacterial chemoattractants in human peripheral blood monocytes, neutrophils, and tissue macrophages are an important component of innate immunity. We now present evidence that a single bacterial chemoattractant (fMLP) has no effect on the expression of CCL20, an important chemokine to initiate adaptive immune response through the recruitment of dendritic cells and lymphocytes. However, a mixture of fMLP and TLR2 agonist Pam3CSK4 behave synergistically in the induction of CCL20 *in vitro* and *in vivo*, resulting in the subsequent recruitment of dendritic cells. The mechanism of synergy may involve the phosphorylation of ERK-MAP kinase. U0126, an inhibitor of ERK MAP kinase, greatly inhibits the synergistic induction of CCL20 and dendritic cell recruitment. Our results suggest an important role for bacterial chemoattractants in the immune responses, not only to activate the innate immune response, but also the direct initiation of the adaptive immune response. Immune responses then are induced by multiple factors that operate synergistically through multiple signaling pathways.

Crosstalk between the innate and adaptive immune systems, which have previously been considered discrete components of the mammalian immune response, is an important and fairly recent area of immune-directed research. This paper shows that native cytokines and chemokines play an important role in the recruitment of dendritic cells, which play an important role in antigen presentation to the adaptive immune system as well as recognition of potentially harmful antigens by the innate immune system. Future experiments in this vein may help to clarify the role of different chemokines in the recruitment of these and other antigen-presenting cells, such as cells of the monocyte line.

Ultimately, the role of multiple antigens in immune cell recruitment, as explored in this paper, may help lead to the development of treatments that target multiple inflammatory antigens in host-based defense against bacterial infections. Conversely, the role of CCL20 and other important pro-inflammatory cytokines may aid in stimulation of the adaptive immune system and promote its beneficial memory effects. Treatments to this end may potentially be developed, using the results from this paper and others as a base.

Acknowledgments

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