Host Defense Peptide LL-37, in Synergy with Inflammatory Mediator IL-1β, Augments Immune Responses by Multiple Pathways[1]

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The human cathelicidin LL-37 is a cationic host defense peptide and serves as an important component of innate immunity. It has been demonstrated to be a multifunctional modulator of innate immune responses, although the mechanism(s) underlying this have not been well characterized. In this study, it was demonstrated that LL-37 synergistically enhanced the IL-1β-induced production of cytokines (IL-6, IL-10) and chemokines such as macrophage chemoattractant proteins (MCP-1, MCP-3) in human PBMC, indicating a role in enhancing certain innate immune responses. Similarly, LL-37 synergistically enhanced chemokine production in the presence of GM-CSF, but IFN-γ, IL-4, or IL-12 addition led to antagonism, indicating that the role of LL-37 in reinforcing specific immune responses is selective and restricted to particular endogenous immune mediators. The inhibition of G protein-coupled receptors and PI3K substantially suppressed the ability of IL-1β and LL-37 to synergistically enhance the production of chemokine MCP-3. Consistent with this, the combination of IL-1β and LL-37 enhanced the activation/phosphorylation of kinase Akt and the transcription factor CREB. The role of transcription factor NF-κB was revealed through the demonstration of enhanced phosphorylation of IκBα and the consequent nuclear translocation of NF-κB subunits p50 and p65, as well as the antagonistic effects of an inhibitor of IκBα phosphorylation. These results together indicate that the human host defense peptide LL-37 can work in synergy with the endogenous inflammatory mediator IL-1β to enhance the induction of specific inflammatory effectors by a complex mechanism involving multiple pathways, thus reinforcing certain innate immune responses. ***The Journal of Immunology,*** **2007, 179: 7684–7691.**

Cationic host defense peptides are evolutionarily ancient components of the innate immune system (1). Although some can directly kill microorganisms (2), recent studies have demonstrated that these peptides have immunomodulatory functions in that they can selectively boost innate immunity and/or link innate and adaptive immunity (3, 4). The two major families of host defense peptides in mammals are the cathelicidins and defensins. Cathelicidins are defined by a highly conserved N-terminal cathelin pro-domain and a structurally variable antimicrobial domain at the C terminus (5, 6), and they have been identified in various species, including cows, pigs, sheep, horses, mice, guinea pigs, and rabbits (7). The only known human cathelicidin is the 37-aa peptide LL-37 (8, 9). This peptide is produced by various cell types including neutrophils, lung epithelial cells, keratinocytes, monocytes, and mast cells (9–13). It is stored intracellularly as an inactive pro-peptide, hCAP18, which upon stimulation and secretion is extracellularly cleaved from the N-terminal cathelin domain by proteinase 3, leading to the generation of active LL-37 (14).

Recent evidence indicates that LL-37 can function as a potent modulator of innate immune responses. It can trigger mast cell degranulation and stimulate the production of chemokines, as well as acting as a direct chemoattractant for neutrophils, monocytes, and T cells, resulting in the recruitment of effector cells to local sites of infection (15, 16). In addition, LL-37 can suppress endotoxin-induced inflammatory responses by delicately balancing gene transcription involved in homeostasis, inflammation, and sepsis (16, 17), prolong neutrophil life span by suppressing neutrophil apoptosis (18), and influence the polarity of dendritic cells in the adaptive immune response (19). Several of these activities have been demonstrated in vivo, including the ability of LL-37 to protect against endotoxic shock in mice and rats (16, 20).

Previous studies have provided preliminary evidence that the production, by effector cells, of IL-6 and IL-8 is augmented by LL-37 in the presence of IL-1β (17) that would naturally be present at local inflammatory sites during the course of an infection, but not in the presence of TNF-α. However, the mechanism whereby LL-37 stimulates biological responses or enhances responses in synergy with other immune effectors has not yet been elucidated. This study focused on these physiologically relevant questions in human primary cells.

LL-37 is known to interact with and/or transactivate a variety of receptors (15, 21–25), for example, the direct chemoattractant activity of LL-37 is due, at least in part, to activation of the G

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protein-coupled receptor (GPCR)[3] FPRL1 (15), although this receptor is not involved in mediating other LL-37 activities (26). Cellular events important in the biological effects of LL-37 include the triggering of certain signaling pathways. LL-37 was shown to induce the phosphorylation of the MAPKs, ERK1/2 and p38, in human peripheral blood monocytes, a bronchial epithelial cell line, and skin mast cells (26, 27). Recent evidence indicates that LL-37 may also utilize PI3K in the inhibition of neutrophil apoptosis (18) and that it might act directly on the TLR4 to NF-κB pathway in inhibiting endotoxin-stimulated proinflammatory cytokine expression (17). However, the mechanism(s) by which LL-37 modulates the NF-κB pathway and its interaction with other signaling molecules that play a pivotal role in innate immune responses remain unclear.

In this study, we have demonstrated that LL-37 can selectively reinforce and/or enhance specific immune responses in the presence of immune mediators such as IL-1β and GM-CSF and that this property is restricted to only a subset of the endogenous immunomodulators present at the site of infection. In this study, we have further investigated the complex mode of action of this peptide in combination with the inflammatory mediator IL-1β. The results suggest that multiple signaling pathways are activated in human PBMCs, leading to the activation of pivotal transcriptional elements.

Materials and Methods

Cell isolation and cell culture

Primary human PBMCs were isolated as described previously (17). Briefly, 100 ml of human venous blood was collected from healthy volunteers in sodium heparin Vacutainer collection tubes (BD Biosciences) according to University of British Columbia Clinical Research Ethics Board guidelines and approval. The blood was mixed, at a 1:1 ratio, with RPMI 1640 medium (supplemented with 10% (v/v) FCS, 2 mM L-glutamine, and 1 mM sodium pyruvate) and separated by centrifugation over a Ficoll-Paque Plus (Amersham Biosciences). PBMCs were isolated from the buffy coat and the density was determined by trypan blue exclusion. PBMCs were seeded into tissue culture dishes (Falcon; BD Biosciences) at 37°C in 5% CO_2 and rested for 1–2 h before addition of various treatments.

Stimulants, reagents, and Abs

The cationic human host defense peptide, LL-37 (LLGDFFRKSKE KIGKEFKRIVQRIKDFLRNLVPRTES) was synthesized by F-moc chemistry at the Nucleic Acid/Protein Synthesis Unit at the University of British Columbia and was resuspended in endotoxin-free water. The concentration (w/v) of the peptide in solution was determined by amino acid analysis. Peptide WKYMVM was a gift from Dr. C. Dahlgren (University of Goteborg, Goteborg, Sweden). Recombinant human IL-1β, GM-CSF, IFN-γ, IL-4, and IL-12 were purchased from Research Diagnostics. The pharmacological inhibitors pertussis toxin and wortmannin were purchased from Merck Biosciences and BIOMOL, respectively. Inhibitors of IκBα (BAY 11-7085) and PI3K (LY294002 and wortmannin) were purchased from Cell Signaling Technology. Mouse mAb anti-phospho-IκBα and anti-phospho-CREB, rabbit anti-total-IκBα, anti-p105/p50, anti-p65, anti-phospho-Akt, and anti-total Akt were purchased from Cell Signaling Technology. HRP-conjugated goat anti-rabbit and anti-mouse IgG Abs were purchased from Cell Signaling Technology and Amersham Biosciences, respectively. All reagents were tested for endotoxin by *Limulus* amebocyte assay and reconstituted in endotoxin-free water. LPS from *Pseudomonas aeruginosa* strain H103 was purified free of proteins and lipids using the Darveau-Hancock method as previously described (28).

Treatment with various stimuli

Human PBMCs were stimulated with LL-37 (5–20 μg/ml) and/or recombinant human IL-1β, GM-CSF, IFN-γ, IL-4, or IL-12 (10 ng/ml) for the time periods indicated. When specified, cells were pretreated with various inhibitors or vehicle control (containing the same amount of DMSO, used to solubilize these inhibitors, as added with each of the inhibitors) 1 h before treatment with the various stimulants. For studies demonstrating synergistic mechanisms of the peptide LL-37 with inflammatory mediator IL-1β, both of the stimulants were added simultaneously.

[3] Abbreviations used in this paper: GPCR, G protein-coupled receptor; PK, protein kinase.

Western immunoblotting

Human PBMCs (5×10^6) seeded into 60-mm^2 petri dishes (VWR International) were stimulated with IL-1β, LL-37, and endotoxin-free water as a vehicle control for 30 or 60 min at 37°C in 5% CO_2. Cells were subsequently detached by gentle scraping, followed by washing and resuspension of the cells in ice-cold PBS containing 1 mM sodium vanadate. Nuclear and cytoplasmic extracts were isolated using the NE-PER Nuclear and Cytoplasmic Extraction Reagents Kit (Pierce) according to the manufacturer's instructions. The extracts were stored until further use at −80°C. Protein concentrations of the lysates were determined by the bicinchoninic acid assay (Pierce). Nuclear extracts (7.5–10 μg) and cytoplasmic extracts (15 μg) were resolved on an 8–10% SDS-PAGE, followed by subsequent transfer to Immunoblot polyvinylidene difluoride membranes (Bio-Rad). The membranes were probed with specific Abs at 1/1000 dilution in TBST (20 mM Tris-HCl (pH 7.4), 150 mM NaCl, and 0.1% Tween 20) containing 5% skimmed milk powder (TBST/milk) or 5% BSA (TBST/BSA) as blocking agents, followed by incubation with HRP-conjugated goat anti-mouse or anti-rabbit secondary Abs as required. The membranes were developed using chemiluminescence peroxidase substrate (Sigma-Aldrich) according to the manufacturer's instructions. The blots were reprobed with an anti-GAPDH Ab to ensure that equal amounts of protein had been loaded.

Detection of cytokines and chemokines

Human PBMCs were seeded at 8×10^5 cells/ml in complete RPMI 1640 medium. Cells were stimulated for 24 h with LL-37 and/or the indicated cytokines using endotoxin-free water in parallel as the vehicle control. Following stimulation, the tissue culture supernatants were centrifuged at $2500 \times g$ for 5 min to obtain cell-free samples. Supernatants were aliquoted and stored at −20°C before assay for various cytokines. The concentrations of MCP-3, MCP-1, IL-10, and IL-6 in the supernatants were measured using capture ELISA as per the manufacturer's suggestions (MCP-3: R&D Systems; MCP-1, IL-10, and IL-6: eBioscience).

RNA extraction and quantitative real-time PCR (qRT-PCR)

RNA was isolated using the RNeasy Mini Kit (Qiagen) and treated with RNase-Free DNase as per the manufacturer's instructions. The RNA was eluted in RNase-free water (Ambion). Gene expression was analyzed by qRT-PCR using a SuperScript III Platinum Two-Step qRT-PCR Kit with SYBR Green (Invitrogen Life Technologies), as per the manufacturer's instructions, in an Applied Biosystems Prism 7000 sequence detection system. Briefly, 100 ng of total RNA was reverse transcribed in a 20-μl reaction volume for 50 min at 42°C, and the reaction was terminated by incubating for 5 min at 85°C and then digested for 30 min at 37°C with RNase H. The PCR was conducted in a 12.5-μl reaction volume containing 2.5 μl of 1a /5 diluted cDNA template. A melting curve was performed to ensure that any product detected was specific to the desired amplicon. Fold changes were calculated after normalization to endogenous GAPDH and using the comparative Ct method (29). The sequences of forward and reverse primers used for qRT-PCR were, respectively: for GAPDH, GTCGCTGTTGAAGTCAGAGG and GAAACTGTGGCGTGATGG; for IL-6, AATTCGGTACATCCTCGACGG and GGTTGTTTTCTGCCAGTGCC; and for MCP-3 (CCL-7), TGTCCTTTCTCAGAGTGGTTCT and TGCTTCCATAGGGACATCATA.

Statistical analysis

Student's *t* test was performed to determine the statistical significance, with $p \leq 0.05$ being considered statistically significant. Values shown are expressed as mean ± SD or SE as indicated.

Results

Synergistic induction by LL-37 and IL-1β of MCP-3/CCL-7 and IL-6 transcription in human PBMCs

LL-37 has been previously demonstrated to be a robust antiendotoxin agent due to its ability to selectively modulate LPS-induced and lipoteichoic acid-induced proinflammatory cytokine production in human mononuclear cells (16, 17). In contrast, we previously demonstrated that LL-37 substantially enhanced the production of IL-8/CXCL8 and IL-6 in synergy with IL-1β (but did not significantly influence TNF-α) (17). Since IL-1β is an inflammatory cytokine involved in a variety of acute and chronic diseases, and similar to the TLR agonists LPS and lipoteichoic acid activates

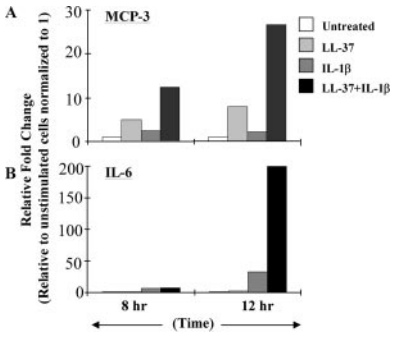

FIGURE 1. Enhanced *CCL-7* (*MCP-3*) and *IL-6* gene expression in response to costimulation with LL-37 and IL-1β in human PBMCs. Primary human PBMCs were treated with LL-37 (20 μg/ml) and/or IL-1β (10 ng/ml) for the indicated time periods. Transcriptional induction of *CCL-7* (*MCP-3*; *A*) and IL-6 (*B*) was analyzed by qRT-PCR. Fold changes (*y*-axis) for each gene were normalized to GAPDH and are relative to the gene expression in unstimulated cells (normalized to 1 using the comparative cycle threshold method). Typical results of an experiment with cells from one donor, representative of three separate donors, are shown. The biological variability between the three different donors at the 8-h time point was between ±2.4-fold for LL-37, ±2.7-fold for IL-1β, and ±20-fold for LL37 plus IL-1β-induced CCL-7gene expression; between ±0.17-fold for LL-37, ±0.19-fold for IL-1β, and ±108-fold for LL-37 plus IL-1β-induced IL-6 gene expression. Similarly, the biological variability between the three different donors at the 24-h time point was between ±122-fold for LL-37, ±4-fold for IL-1β, and ±219-fold for LL37 plus IL-1β-induced CCL-7 gene expression; between ±6.5-fold for LL-37, ±7.7-fold for IL-1β, and ±59-fold for LL-37 plus IL-1β-induced IL-6 gene expression.

a common TLR/IL-1R signaling pathway leading to IκBα phosphorylation and NF-κB nuclear translocation, the effects of LL-37 on IL-1β-mediated immune responses were further investigated.

The effect of LL-37 on IL-1β-mediated *MCP-3* and *IL-6* gene expression was analyzed by qRT-PCR. Primary human PBMCs were treated with IL-1β (10 ng/ml) and/or LL-37 (20 μg/ml) for 8 and 12 h. Treatment with IL-1β or LL-37 alone elicited modest (2- to 7-fold) up-regulation of *MCP-3* expression compared with that in untreated cells (Fig. 1*A*). In contrast, costimulation of the cells with IL-1β and LL-37 resulted in a substantially augmented *MCP-3* gene expression (to 12- to 27-fold) when compared with either IL-1β or LL-37 alone (Fig. 1*A*). Similarly, although IL-1β or LL-37 each caused modest up-regulation of *IL-6* mRNA accumulation in PBMCs from most donors, treatment with the two stimulants together caused substantial increases in the expression of *IL-6* at 8 h (19-fold) and 12 h (198-fold) (Fig. 1*B*). It should be noted that the level of *IL-6* gene expression by LL-37 alone appeared to be donor dependent (from 1- to 20-fold increased compared with untreated controls in PBMC preparations from five individuals after 12 h). These results confirmed that the host defense peptide LL-37 synergistically augmented the transcriptional responses induced by the inflammatory mediator IL-1β.

Synergistic induction of cytokine and chemokine protein production by LL-37 in the presence of IL-1β or GM-CSF, but not IFN-γ, IL-4, or IL-12

To further characterize the synergy between LL-37 and particular endogenous human immune mediators, human PBMCs were stimulated with LL-37 (5, 10, and 20 μg/ml) in the presence or absence of IL-1β, GM-CSF, IFN-γ, IL-4, or IL-12 (10 ng/ml each) for 6, 8, 12, 18, or 24 h as indicated. The amount of chemokines MCP-1/CCL2 and MCP-3 and cytokines IL-6 and IL-10 proteins secreted by the stimulated PBMCs were assessed in the tissue culture supernatants by ELISA.

In human PBMCs, both LL-37 (20 μg/ml) and IL-1β (10 ng/ml) independently induced very modest production of chemokines MCP-1 and MCP-3 (between 2- and 7-fold increases compared with unstimulated cells) (Fig. 2, *A* and *B,* respectively). In contrast, stimulation of PBMCs with IL-1β and LL-37 in combination resulted in a 20-fold increase in the production of MCP-1 after 8 h of treatment (Fig. 2*A*), a 100-fold increase in the production of MCP-3 after 12 h of stimulation (Fig. 2*B*), a 10-fold increase in the release of IL-6 after 8 h of treatment (Fig. 2*C*), and an 8-fold increase in IL-10, a known anti-inflammatory cytokine, after 24 h of incubation (Fig. 2*D*) compared with unstimulated cells. However, this was not merely reinforcement of a proinflammatory response as there was no significant synergistic enhancement of TNF-α production (17), consistent with the notion that LL-37 is a selective immunomodulatory. Overall, there was a significant increase ($p < 0.05$) in the amount of MCP-1 as well as IL-6 production when cells were stimulated with both LL-37 and IL-1β together compared with the treatments alone. Similarly, the amount MCP-1 (Fig. 2*A*) or IL-6 (Fig. 2*C*) production induced by immune mediator GM-CSF was significantly ($p < 0.05$) enhanced in the presence of LL-37 (20 μg/ml).

In contrast, chemokine MCP-1 production (Fig. 2*A*) induced by the immune mediator IFN-γ was actually suppressed by 57 ± 11% after 8 h of treatment in the presence of LL-37 (20 μg/ml). Similarly, MCP-1 induced by LL-37 (20 μg/ml) was suppressed in the presence of IL-4 or IL-12 after 24 h of stimulation (Fig. 2*A*). Overall, these trends were observed despite the significant biological variability in the amount of chemokine production induced by immune mediators IFN-γ, IL-4, or IL-12 in the presence and absence of LL-37 between independent donors (Fig. 2*A*). These results thus indicate that the observed synergy between host defense peptide LL-37 and the endogenous immune mediators IL-1β or GM-CSF was quite specific, and certain other mediators actually lead to suppression.

The optimal concentration of LL-37 that enhanced IL-1β- or GM-CSF-induced responses in human PBMCs was 20 μg/ml, and no significant synergistic responses were observed in the induction of either MCP-1 or IL-6 production with 1, 5, or 10 μg/ml LL-37 (data not shown). MCP-1 production was actually enhanced upon pretreatment of the cells with IL-1β, if LL-37 was added 30 min after IL-1β stimulation (Fig. 3*A*). In contrast, the enhancement of MCP-1 production in the presence of the combination of LL-37 and IL-1β was abrogated when cells were pretreated with LL-37 30 min before IL-1β stimulation (Fig. 3*B*), indicating a sustained requirement for the presence of IL-1β for the observed synergistic effects. These results together suggests that LL-37 has the ability to synergistically enhance certain immune responses only during the presence of an infection or chronic inflammation when the peptide is found at a relatively high concentration (30) due to up-regulation or degranulation of neutrophils in which it is stored in the pro-form along with other inflammatory mediators such as IL-1β found naturally at the site of an infection or inflammatory response.

These results together confirm and extend the observation that LL-37 selectively enhances immune responses induced by certain endogenous immune mediators present during infection and inflammation such IL-1β and GM-CSF. This synergistic effect of the peptide in reinforcing specific immune responses did not extend to all endogenous immunomodulators.

Involvement of GPCR and the PI3K signaling pathway in the synergistic production of MCP-3 induced by LL-37 and IL-1β

To further understand and characterize the mechanism of synergism exhibited by LL-37 in the presence of endogenous immune mediators, chemokine induction by LL-37 in the presence of IL-1β

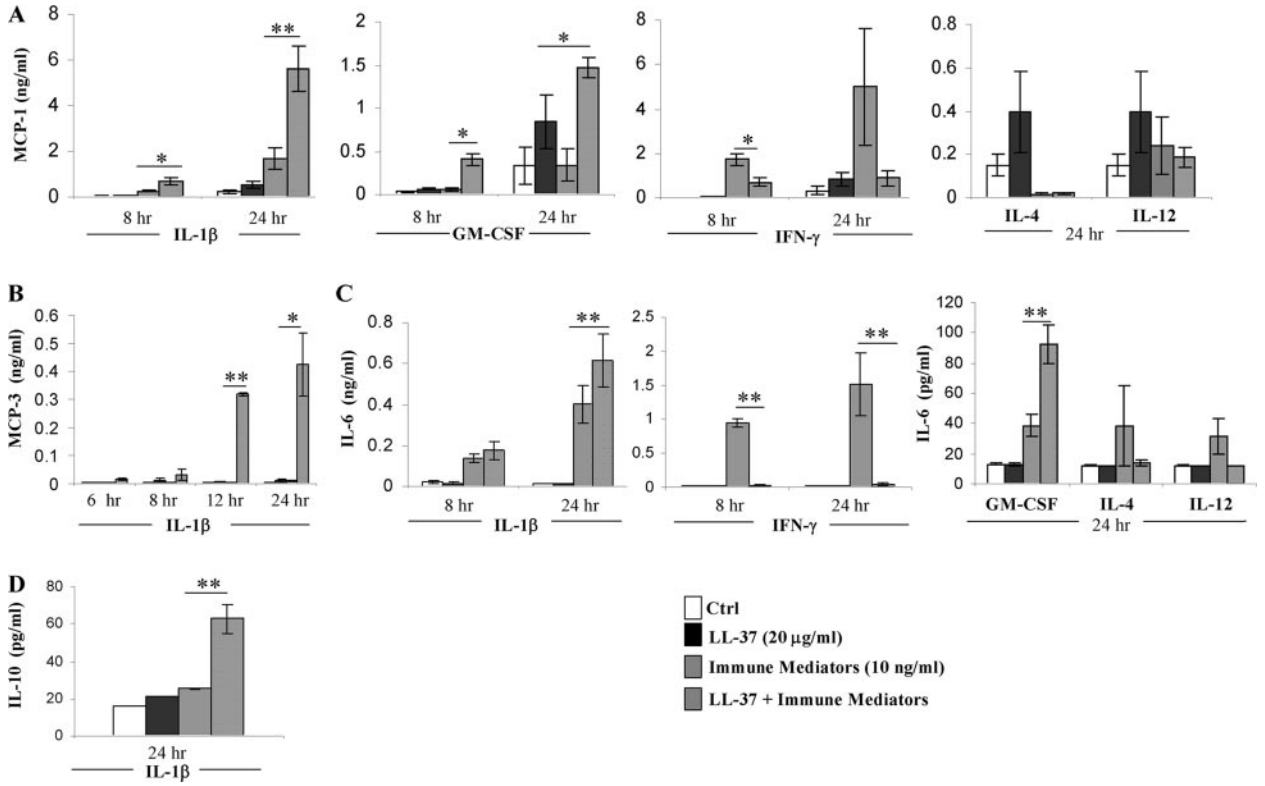

FIGURE 2. Assessment of cytokine and chemokine production upon stimulation of PBMCs with critical endogenous immune mediators IL-1β, GM-CSF, IFN-γ, IL-4, and IL-12 in the presence and absence of human cathelicidin peptide LL-37. Human PBMCs were incubated with LL-37 (20 μg/ml) and/or IL-1β, GM-CSF, IFN-γ, IL-4, and IL-12 (10 ng/ml each) for 6, 8, 12, 18, or 24 h as indicated. Tissue culture supernatants were analyzed by capture ELISA for the amount of secreted MCP-1 *(*A), MCP-3 *(*B), IL-6 (*C*), and IL-10 (*D*). Results are representative of the mean of at least three independent experiments from independent donors ± SE (*, $p < 0.05$; **, $p < 0.01$).

was studied in the presence of specific inhibitors of GPCRs and PI3K. The immunomodulatory effects of LL-37 have been proposed to be dependent on signaling through a variety of receptors, including GPCRs such as the chemokine receptor FPRL-1, specific transactivated receptors, and undetermined high- and low-affinity receptors (15, 31). To assess the possible significance of GPCRs, human PBMCs were preincubated with pertussis toxin (100 ng/ml), which is known to inhibit the activation of GPCRs, for 1 h before the addition of LL-37 (20 μg/ml) and/or IL-1β (10 ng/ml). The amount of MCP-3 released into the tissue culture supernatant was subsequently determined by capture ELISA after 24 h of incubation. In the presence of pertussis toxin, the production of MCP-3 induced by the combination of IL-1β and LL-37 was significantly suppressed (Fig. 4*A*). Similarly, the treatment of cells

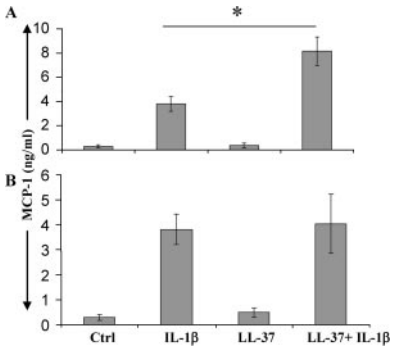

FIGURE 3. Effect of pretreatment of PBMCs with either LL-37 or IL-1β on the synergistic production of MCP-1. Human PBMCs were pretreated with either IL-1β (10 ng/ml; *A*) or LL-37 (20 μg/ml; *B*) for 30 min, followed by stimulation with either LL-37 or IL-1β, respectively, for 24 h. Tissue culture supernatants were assessed for the amount of MCP-1 secreted by capture ELISA. Results are representative of four independent experiments from independent donors ± SE (*, $p < 0.05$). Ctrl, Control.

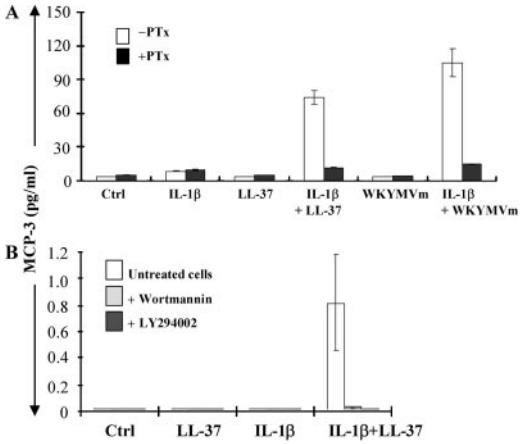

FIGURE 4. Effects of inhibition of GPCR and PI3K inhibition on the synergistic production of MCP-3 induced by LL-37 and IL-1β. Human PBMCs were pretreated with the inhibitors of GPCR (*A*) and pertussis toxin (100 ng/ml; *A*) or PI3K inhibitors LY294002 (10 μM; *B*) or wortmannin (1 μM; *B*) for 1 h. Subsequently, the cells were stimulated with LL-37 (20 μg/ml) and IL-1β (10 ng/ml) for 24 h. MCP-3 production in the culture supernatant was monitored by capture ELISA. The agonist of GPCR FPRL-1, peptide WKYMVm (5 μM), was used as a positive control. Results are representative of three independent experiments. Values are the mean ± SE. PTx, pertussin toxin.

with the FPRL-1-specific agonist WKYMVm (32) in the presence of IL-1β resulted in the synergistic induction of MCP-3 (Fig. 4*A*), and this effect was abrogated by pretreatment with pertussis toxin. These results together were consistent with the possibility that GPCRs were required for the synergistic production of MCP-3 induced by IL-1β and LL-37.

PI3Ks have been implicated in the effects of LL-37 in inhibiting neutrophil apoptosis but have not been implicated in other biological functions of LL-37. PI3K is downstream of the GPCR (33) and has also been proposed to act as an early intermediary of MAPK activation in response to inflammatory stimuli (34). In addition, it has been previously demonstrated that LL-37 induces the activation of the MAPKs, ERK1/2 and p38, in peripheral blood-derived monocytes and that GM-CSF synergistically enhances this activation (26). Therefore, the significance of PI3K in MCP-3 induction by LL-37 in conjunction with IL-1β was tested by preincubating PBMCs with the specific inhibitor LY294002 or the alternative PI3K inhibitor wortmannin for 1 h, before stimulation with LL-37 and/or IL-1β for 24 h. Pretreatment with either inhibitor significantly inhibited (≥95%) the augmented release of MCP-3 induced by the combination of LL-37 and IL-1β (Fig. 4*B*).

Protein kinase (PK) C (PKC) and PKA are also known to be downstream of GPCRs. Therefore, the PKC inhibitor GF109203x and PKA inhibitor 6-22 amide were used to assess the role of these two kinases in the induction of MCP-3 by the peptide in the presence of IL-1β. Neither PKA nor PKC inhibitors were able to suppress the production of MCP-3 induced by costimulation with IL-1β and LL-37 (data not shown), indicating that PKC and PKA are unlikely to be key mediators in the synergistic induction of MCP-3 by LL-37 and IL-1β.

To provide a more detailed understanding of the mechanism(s) influencing the observed synergy, the activation of relevant proteins in these pathways was assessed. Since the serine/threonine protein kinase Akt is known to be activated by phosphorylation through a PI3K-dependent pathway, the influence of stimulation with LL-37 and/or IL-1β on cellular Akt phosphorylation was analyzed by immunoblotting using an Ab directed at phosphorylated Ser^{473} of Akt. The results demonstrated that the phosphorylation of Akt was modestly increased after stimulation with LL-37 (20 μg/ml) or IL-1β (10 ng/ml) alone for 30 min. However, treatment with these two stimuli together induced an increase in phosphorylation of Akt after 30 min of stimulation of human PBMCs (Fig. 5*A*).

It has been conclusively established that LL-37 induces activation of the ERK1 MAPK pathway (26) and it is also known that ERK-1 phosphorylates and promotes nuclear translocation of the transcription factor CREB (35). Therefore, the effect of the peptide on CREB activation was assessed by immunoblotting using an Ab directed against Ser^{133}-phosphorylated CREB. Primary human PBMCs were stimulated with LL-37 and/or IL-1β for 60 min and nuclear extracts were obtained. Both LL-37 and IL-1β by themselves increased the amount of phosphorylated CREB, but this was substantially enhanced by the combination of LL-37 and IL-1β (Fig. 5*B*).

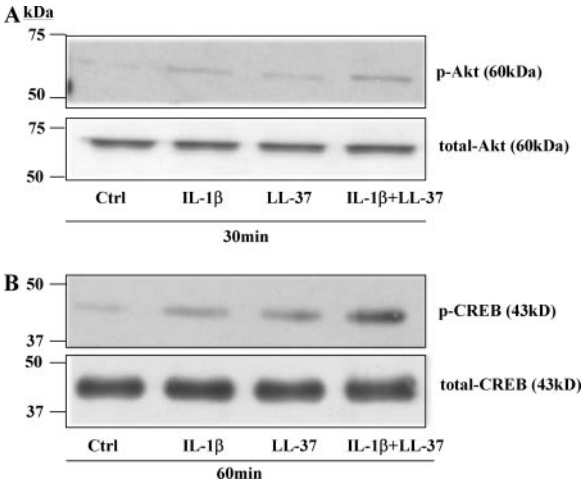

FIGURE 5. LL-37-induced phosphorylation of Akt and CREB in the presence of IL-1β. Human PBMCs were stimulated with LL-37 (20 μg/ml) and/or IL-1β (10 ng/ml). *A,* Cytoplasmic cell extracts were analyzed after 30 min of stimulation by immunoblotting for phosphorylation of Akt (*upper panel*) or total Akt (*middle panel*). *B,* Nuclear extracts were analyzed for phosphorylation of CREB (*lower panel*) or anti-total CREB (housekeeping gene) to allow correction for protein loading in the Western blot analysis. One gel, representative of three independent experiments, is shown for each panel. Ctrl, Control.

Involvement of NF-κB induction in the synergistic production of MCP-3 induced by LL-37 and IL-1β

The expression of MCP-3 is known to be regulated in part by the transcription factor NF-κB during innate immune responses (36). Therefore, primary human PBMCs were preincubated with IκBα inhibitor (BAY 11-7085) for 1 h before treatment with LL-37 (20 μg/ml) in the presence or absence of IL-1β (10 ng/ml) for 24 h, and the levels of secreted MCP-3 in tissue culture supernatants were monitored by capture ELISA. The inhibitor BAY 11-7085, which selectively inhibits phosphorylation of IκBα and subsequent nuclear translocation of transcription factor NF-κB, significantly inhibited the synergistic production of MCP-3 induced by the combination of IL-1β and LL-37 by >95% (Fig. 6).

The activation of transcription factor NF-κB is a major downstream target of IL-1β (37). In resting cells, NF-κB (particularly p65/p50) is bound to inhibitory IκB proteins and is sequestered in the cytoplasm. Stimulation and subsequent phosphorylation of IκB proteins leads to the degradation of the complex, which allows the liberated NF-κB subunits to translocate to the nucleus and enhance the transcription of several target genes, including those encoding for cytokines, chemokines, and adhesion molecules (38, 39). Since IL-6 and MCP-3 are partially controlled by NF-κB, the involvement of IκBα and NF-κB subunits was also investigated in human PBMCs. LL-37 by itself induced transient phosphorylation of IκBα after 30 min of stimulation. IL-1β by itself led to more sustained phosphorylation of IκBα, but the peptide in the presence of IL-1β enhanced this, particularly at the 30-min time point (Fig. 7*A*). Moreover, nuclear translocation of NF-κB subunits p50 and p65 was assessed by Western blots. We found that increased levels of p65 and p50 were detected in the nuclear extract of PBMCs stimulated with LL-37 alone after 30–60 min dependent on donor variation (Fig. 7*B*). Consistent with the activation of IκBα, the

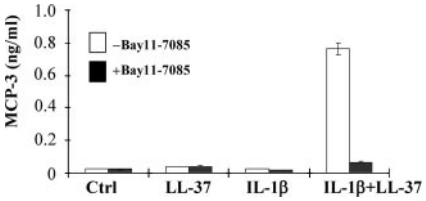

FIGURE 6. Influence of IκBα inhibition on the synergistic production of MCP-3 induced by LL-37 and IL-1β. Human PBMCs were pretreated with IκBα inhibitor BAY 11-7085 (10 μM) for 1 h. Subsequently, the cells were stimulated with LL-37 (20 μg/ml) and IL-1β (10 ng/ml) for 24 h. MCP-3 production in the tissue supernatant was monitored by capture ELISA. Results are representative of three independent experiments. Values are the mean ± SE. Ctrl, Control.

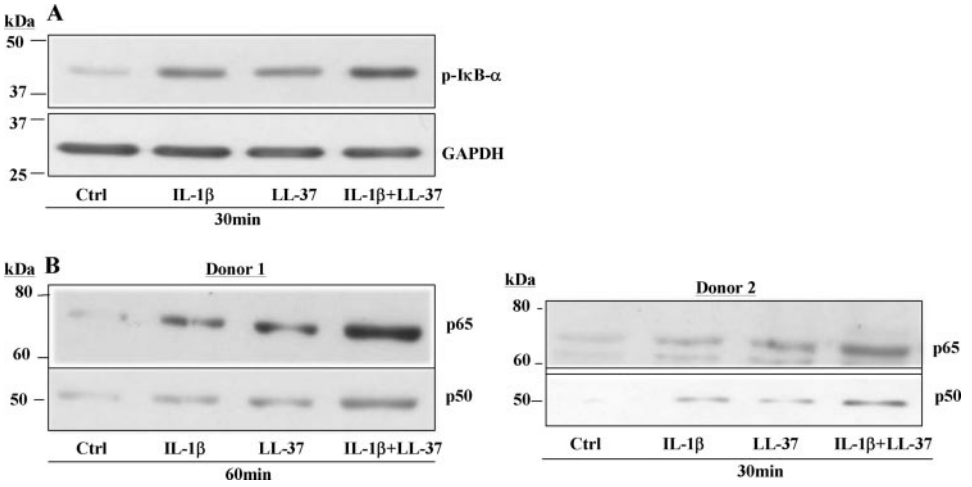

FIGURE 7. LL-37-induced IκBα phosphorylation and subsequent translocation of NF-κB subunits (p50 and p65) in the presence of IL-1β. Human PBMCs were stimulated with LL-37 (20 μg/ml) and/or IL-1β (10 ng/ml). *A,* Cytoplasmic extracts were analyzed by immunoblotting after 30 min of stimulation for phosphorylation of IκBα (*upper panel*). Immunoblotting using anti-*GAPDH* (housekeeping gene) was performed to allow correction for protein loading in the Western blot analysis. *B,* Nuclear extracts of cells stimulated for 30 or 60 min were analyzed for nuclear translocation of NF-κB subunits p50 and p65 by Western blots using anti-p105/50 and anti-p65 Abs. One gel, representative of three independent experiments, is shown for each panel. Ctrl, Control.

combination of IL-1β and LL-37 led to enhanced nuclear translocation of p65 and p50 at the same time points (Fig. 7*B*).

Discussion

The innate immune system is a multicomponent host defense system, the functioning of which is balanced by complex interactions between its various effector and regulatory molecules. The sole human cathelicidin LL-37 has been increasingly described as a robust immunomodulatory molecule that enhances certain inflammatory responses (e.g., the up-regulation of certain chemokines while generally suppressing the production of certain proinflammatory cytokines such as TNF-α). It has been shown that LL-37 expression is up-regulated during infection and inflammation, e.g., in bronchoalveolar lavage of infants with pulmonary infections, in individuals with cystic fibrosis, and in psoriatic skin lesions (30, 40, 41). In addition, LL-37 can also be locally induced at sites of inflammation within epithelial cells (42). This led us to speculate that raised levels of LL-37 could enhance, sustain, or even amplify certain immune responses in the presence of other endogenous signals. The most interesting of these would appear to be IL-1β since it can induce LL-37 expression in vitro in keratinocytes (43), while LL-37 is known to promote IL-1 processing through transactivation of the receptor P_2X7 (25). Previous data (17) indicted that LL-37 had quite different effects on LPS- and IL-1β-induced proinflammatory cytokine responses, suppressing the LPS-mediated TNF-α, IL-6,and IL-8 production but substantially enhancing the production of IL-1β-induced IL-6 and IL-8 (while only slightly influencing TNF-α). Similarly, previous studies indicated that LL-37-induced MAPK p38 phosphorylation was enhanced in the presence of immune mediator GM-CSF that is present at local sites of infection (26). In this study, we have extended these initial observations and demonstrated that host defense peptide LL-37 enhanced cytokine responses in human PBMCs in the presence of certain endogenous immune mediators, primarily IL-1β and GM-CSF, but the presence of IFN-γ, IL-4, or IL-12 caused the opposite (antagonistic) response. In addition, this study demonstrated that host defense peptide LL-37 can enhance and/or reinforce certain immune responses, such as chemokine production, at relatively high concentrations and only in the sustained presence of the appropriate endogenous inflammatory mediator, such as IL-1β, indicating that this phenomenon is selective and restricted to conditions obtainable in the presence of an infection and/or acute or chronic inflammatory response. We have further demonstrated that LL-37 interacts in a mechanistically complex fashion with the endogenous immune mediator IL-1β to modulate the production of important effectors of inflammation. This effect was somewhat specific to LL-37 as a synthetic immunomodulator IDR-1 did not demonstrate any significant synergy with IL-1β (N. Mookherjee and R. E. W. Hancock, unpublished data).

A variety of transcription factors has been separately implicated previously in the biological activities of LL-37, e.g., LL-37 induces activation of the MAPKs ERK1/2 and p38 and transcription factor Elk-1 that control the transcription and secretion of IL-8 in primary human monocytes (26); LL-37 indirectly stimulates of epidermal growth factor receptor and downstream transcription factor STAT-3 in keratinocytes (44); and LL-37 appears to act in part through the PI3K pathway in inhibiting neutrophil apoptosis (18). This is the first study that has demonstrated that multiple transcription factors are involved in an immunomodulatory function of LL-37, including the pivotal transcription factor NF-κB that is involved in the inflammatory and immune responses. Although it was previously demonstrated that LL-37 could suppress LPS-induced NF-κB activation at later time points (17), by itself it was able to transiently (at 15–30 min) activate IκBα and initiate nuclear translocation of NF-κB subunits in primary human PBMCs. There is recent evidence suggesting that the NF-κB p50 subunit specifically associates with endogenous IκBζ in a manner that is essential for the transcription of IL-6 (45). In addition to NF-κB transcription factor binding sites, the 5′-flanking sequence upstream of the IL-6 gene contains several response elements for the transcription factors AP-1, CREB, and C/EBP, indicating that its transcription may require the activity of a complex of transcription factors (46). Similarly, a transcription factor complex containing NF-κB, CREB, and AP-1 is essential for the production of CC family chemokines such as IL-8, MCP-1, and MCP-3 (47, 48), as well as the anti-inflammatory cytokine IL-10 (49–53). Alterations in the relative amounts or activities of transcription factors have generally been thought to control the regulation of gene expression at the transcriptional level (54). This leads us to speculate that a complex of multiple transcriptional elements such as NF-κB,

CREB, and Elk-1, etc., could be essential for the delicate balancing of immunomodulatory activities by LL-37. The outcome of action of this presumed complex would be the selective up-regulation of several key components involved in innate immunity.

We previously demonstrated in preliminary studies that LL-37 augmented the ability of the TLR/IL-1R agonist IL-1β in human PBMCs to induce production of IL-8 and IL-6, but not TNF-α (17). Also LL-37 enhanced the release of IL-8 from primary neutrophils after stimulation with IL-1β (18). In this study, we have confirmed and considerably extended these observations to investigate the specificity, kinetics, and mechanism of this enhancement. Under inflammatory conditions, effector cells of the innate immune system are exposed to a broad range of cytokines and inflammatory mediators in vivo. This study showed that IL-1β and LL-37 induced the synergistic production of IL-10, MCP-1, MCP-3, and IL-6, although it did not induce significant production of TNF-α (17); similarly, LL-37 and GM-CSF in combination induced enhanced production of chemokine MCP-1 and cytokine IL-6, indicating that the synergistic effect between LL-37 and immune mediators (IL-1β, GM-CSF) is selective with respect to the inflammatory mediators induced. In contrast, in the presence of LL-37, the production of chemokine MCP-1 and cytokine IL-6 was suppressed, rather than enhanced, by the immune mediators IFN-γ, IL-4, or IL-12. We speculate that the ability of the host defense peptide LL-37 to boost certain immune responses during infection or inflammation can be selectively reinforced by certain endogenous immune mediators, and that this would have a significant impact on the efficient modulation of inflammation by LL-37. Consistent with this, we previously demonstrated that LL-37 antagonized proinflammatory responses mediated by TLR4 agonist LPS and TLR2 agonists lipoteichoic acid and the synthetic ligand Palmitoyl-3-cysteine-serine-lysine-4, but demonstrated mixed results with the TLR9 ligand CpG (e.g., antagonizing IL-8 production but enhancing IL-6 and TNF-α (17)).

To date, a number of putative binding and transactivated receptors, including GPCRs, have been described as influencing the activity of LL-37 (15). Interestingly, the involvement of GPCRs is quite selective. For example, they were found to be involved in the chemoattractant property of LL-37 (15, 21) and in LL-37-mediated inhibition of neutrophil apoptosis (18), but not in the activation of p38 and ERK1/2 MAPK pathways induced by LL-37 (26). This report demonstrated that a GPCR was involved in IL-1β-mediated enhancement of LL-37-induced MCP-3 production in PBMCs. To gain insight into the intracellular transduction events involved in this synergistic enhancement of immunomodulation activity, the underlying mechanism(s) of the synergistic effect between IL-1β and LL-37 was further investigated in this study. MCP-3 production induced by IL-1β and LL-37 was significantly attenuated by inhibitors of PI3K and IκBα phosphorylation (marking the latter protein for degradation and release of NF-κB), and the presence of IL-1β reinforced LL-37-induced activation of multiple molecules, including Akt, CREB, IκB-α, and NF-κB subunits p50 and p65,. Therefore, one possible interpretation for the observation that the modest responses induced by the host defense peptide were enhanced in the presence of the inflammatory mediator IL-1β may be the reinforcement by IL-1β of the activation of multiple transcriptional elements involved in the biological activity of LL-37 and vice versa. In addition, IL-1β and/or LL-37 may induce alternative signaling pathways that either supplement or feed into the activation of the transcription factor complex(es) mediating the biological responses of each other, thereby enhancing specific transcriptional events and leading to selective immune responses.

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Disclosures

The authors have no financial conflict of interest.

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