The Human Cationic Peptide LL-37 Induces Activation of the Extracellular Signal-Regulated Kinase and p38 Kinase Pathways in Primary Human Monocytes

Dawn M. E. Bowdish, Donald J. Davidson, David P. Speert and Robert E. W. Hancock

J. Immunol. 2004;172;3758-3765
http://www.jimmunol.org/cgi/content/full/172/6/3758

The Journal of Immunology is published twice each month by The American Association of Immunologists, Inc., 9650 Rockville Pike, Bethesda, MD 20814-3994.

Print ISSN: 0022-1767 Online ISSN: 1550-6606.

The Human Cationic Peptide LL-37 Induces Activation of the Extracellular Signal-Regulated Kinase and p38 Kinase Pathways in Primary Human Monocytes[1]

Dawn M. E. Bowdish,* Donald J. Davidson,† David P. Speert,† and Robert E. W. Hancock[2]*

LL-37 is a cationic peptide that is found in the granules of neutrophils and is secreted by epithelial cells from a variety of tissues. Levels of LL-37 in vivo increase upon infection, and its production and secretion are increased upon stimulation with proinflammatory mediators. It has been postulated that LL-37 modulates the immune response by interacting with the effector cells of innate immunity; however, the mechanism of this interaction is unknown. LL-37 induced phosphorylation and activation of the mitogen-activated protein kinases, extracellular signal-regulated kinase 1/2 (ERK1/2) and p38, in human peripheral blood-derived monocytes and a human bronchial epithelial cell line, but not in B or T lymphocytes. Phosphorylation was not dependent on the G protein-coupled formyl peptide-like receptor 1, which was previously proposed to be the receptor for LL-37-induced chemotaxis on human monocytes and T cells. Activation of ERK1/2 and p38 was markedly increased by the presence of GM-CSF, but not M-CSF. Exposure to LL-37 also led to the activation of Elk-1, a transcription factor that is downstream of and activated by phosphorylated ERK1/2, the up-regulation of various Elk-1-controlled genes, and the transcription and secretion of IL-8. Inhibition of either p38 or ERK1/2 kinases led to a reduction in LL-37-induced IL-8 secretion and inhibition of the transcription of various chemokine genes. The ability of LL-37 to signal through these pathways has broad implications in immunity, monocyte activation, proliferation, and differentiation. ***The Journal of Immunology,*** **2004, 172: 3758–3765.**

Cationic host defense peptides are a primitive and conserved component of the innate immune response. These peptides can be expressed either constitutively or induced in response to pathogen-associated molecular patterns, such as bacterial LPS, or inflammatory mediators, such as IL-6 and TNF-α (1, 2). Although some of these peptides have the ability to kill microorganisms (3, 4), leading to their categorization as antimicrobial peptides, they also appear to have functions in modulating immune responses (5–7). The role of cationic peptides has been considered of primary significance to the innate immune response. However, it is becoming increasingly apparent that they provide a key link between the innate and adaptive responses (8, 9).

The cathelicidins are an evolutionarily conserved group of peptides, of which human cationic antimicrobial peptide 18 (hCAP18)/LL-37 is the only human member. In neutrophils, hCAP18 is found in its unprocessed form; however, upon release from cells the C-terminal domain, LL-37, is cleaved from the N-terminal cathelin domain (10). LL-37 is a potent modulator of the immune response, and to date a wide variety of interactions with the effector cells of the immune response have been described (11). Monocytes, the precursors of both macrophages and certain lineages of dendritic cells, have been shown to demonstrate chemotaxis toward LL-37 (12), which is also able to stimulate the production of chemokines and chemokine receptors (13). A variety of cationic peptides can also reduce proinflammatory cytokine production by macrophages to Toll-like receptor agonists, such as LPS (14). Consistent with this function, LL-37 can suppress endotoxin-mediated lethality in animals (15). Dendritic cells are also affected by LL-37 treatment. Recent studies have shown that the differentiation, function, and surface expression of a variety of cell surface markers of human monocyte-derived dendritic cells are altered by exposure to LL-37, which generates cells with enhanced Ag uptake and presentation capacity (50). LL-37 also has a variety of other functions, including promotion of histamine release from mast cells, inhibition of tissue proteases, stimulation of wound healing, and angiogenesis (16–19).

Despite burgeoning interest in the immunomodulatory properties of cationic peptides, little is known about their mechanism of stimulation of the effector cells of the innate immune response. The only receptor described for LL-37 to date is formyl peptide-like receptor 1 (FPRL-1),[3] a pertussis toxin-sensitive, G protein-coupled receptor that has been proposed to be the chemotactic receptor on monocytes, neutrophils, and subsets of T cells (12). The recently demonstrated ability of LL-37 to induce mast cell chemotaxis is also pertussis toxin sensitive and thus mediated by a G protein-coupled receptor; however, the FPRL-1 receptor was not found to be involved (20). These studies indicated that there may be two types of receptors for LL-37 on mast cells, a high affinity receptor that is responsible for LL-37-induced chemotaxis and a

*Department of Microbiology, University of British Columbia, and †British Columbia Research Institute for Children's and Women's Health, Vancouver, British Columbia, Canada

Received for publication July 7, 2003. Accepted for publication January 7, 2004.

The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked *advertisement* in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

[1] This work was supported by the Canadian Cystic Fibrosis Foundation, the Functional Pathogenomics of Mucosal Immunity Project funded by Genome BC, and Genome Prairie and their industry partners, Inimex Pharmaceuticals and Pyxis Genomics. R.E.W.H. holds a Canada Research Chair. D.M.E.B. was supported by a Canadian Institutes for Health Research studentship, D.J.D. was supported by a fellowship from the Canadian Cystic Fibrosis Foundation, and D.P.S. was supported by Canadian Institutes for Health Research Grant MOP-53269.

[2] Address correspondence and reprint requests to Dr. Robert E. W. Hancock, Department of Microbiology, University of British Columbia, Vancouver, British Columbia, Canada V6Y 1Z3. E-mail address: bob@cmdr.ubc.ca

[3] Abbreviations used in this paper: FPRL-1, formyl peptide-like receptor 1; ERK1/2, extracellular signal-regulated protein kinase; HBE, human bronchial epithelial cell; LDH-1, lactate dehydrogenase-1; MAPK, mitogen-activated protein kinase; MCP, monocyte chemoattractant protein; hCAP18, human cationic antimicrobial peptide 18.

0022-1767/04/$02.00

low affinity receptor with an undefined function (20). The downstream signaling and consequences of LL-37 binding to these receptors have not been characterized.

Although there is ample evidence that cationic host defense peptides interact directly with effector cells of the innate and adaptive immune responses, very little is known about eukaryotic cell signaling induced by peptide interaction. In this paper we demonstrate that LL-37 can signal via the induction of phosphorylation of the mitogen-activated protein kinases (MAPK), extracellular signal-regulated kinase 1/2 (ERK1/2) and p38, in peripheral blood-derived monocytes and in a human bronchial epithelial (HBE) cell line. We demonstrate that LL-37 promotes the activation of ERK1/2 and p38 at concentrations equivalent to those likely to be found at the site of acute inflammation (25–50 μg/ml). In addition, in the presence of the cytokine GM-CSF, activation of these kinases occurs at concentrations of LL-37 as low as 5–10 μg/ml, which would be predicted to be present at the onset of inflammation. Thus, we hypothesize that low levels of peptide are homeostatic and do not possess a major immunomodulatory effect unless they are expressed in the presence of other agonists, such as cytokines. Lastly, we demonstrate that at least two of the immunomodulatory properties of LL-37, increases in secretion of IL-8 and in the transcription of the chemokines, monocyte chemoattractant protein 1 (MCP-1), MCP-3, and IL-8, are dependent upon the activation of p38 and ERK1/2 kinases.

Materials and Methods

Cell purification and culture

Blood monocytes were prepared using standard techniques (21). Briefly, 100 ml of fresh human venous blood was collected in sodium heparin Vacutainer collection tubes (BD Biosciences, Mississauga, Ontario, Canada) from volunteers according to University of British Columbia clinical research ethics board protocol. The blood was mixed, at a 1:1 ratio, with RPMI 1640 medium (supplemented with 10% (v/v) FCS, 1% L-glutamine, and 1 nM sodium pyruvate) in E-toxa-clean (Sigma-Aldrich, Oakville, Ontario, Canada)-washed, endotoxin-free bottle. PBMC were separated using Ficoll-Paque Plus (Amersham Pharmacia Biotech, Baie D'Urfé, Quebec, Canada) at room temperature and washed with PBS. Monocytes were enriched with the removal of T cells by rosetting with fresh sheep RBC (University of British Columbia animal care unit) pretreated with *Vibrio cholerae* neuraminidase (Calbiochem Biosciences, La Jolla, CA) and repeat separation by Ficoll-Paque Plus (22). The enriched monocytes were washed with PBS, then cultured ($\sim$2–3 $\times$ 10^6/well) for 1 h at 37°C, followed by the removal of nonadherent cells; monocytes were >95% pure, as determined by flow cytometry (data not shown). B lymphocytes were isolated by removing nonadherent cells and adding them to a new plate for 1 h at 37°C. This was repeated a total of three times. Any remaining monocytes adhered to the plates, and residual nonadherent cells were primarily B cells. Cells were cultured in Falcon tissue culture six-well plates (BD Biosciences). The adherent monocytes were cultured in 1 ml of medium at 37°C in which LL-37 and/or cytokines dissolved in endotoxin-free water (Sigma-Aldrich) were added. Endotoxin-free water was added as a vehicle control. For studies using pertussis toxin the medium was replaced with 1 ml of fresh medium containing 100 ng/ml of toxin and incubated for 60 min at 37°C. LL-37 and cytokines were added directly to the medium containing pertussis toxin.

For the isolation of T lymphocytes, rosetted T cells and SRBC were resuspended in 20 ml of PBS, and 10 ml of distilled water was added to lyse the latter. The cells were then centrifuged at 1000 rpm for 5 min, after which the supernatant was removed. The pelleted T cells were promptly washed in PBS, and increasing amounts of water were added until all sheep RBC had lysed. The remaining T cells were washed once in PBS, and viability was confirmed using a 0.4% trypan blue solution. Primary human blood monocytes and T cells were cultured in RPMI 1640 supplemented with 10% (v/v) heat-inactivated FBS, 1% (v/v) L-glutamine, and 1 nM sodium pyruvate (Life Technologies Invitrogen, Burlington, Ontario, Canada). For each experiment between two and eight donors were used.

The SV40-transformed, immortalized 16HBE4o- cell line was a gift from Dr. D. Gruenert (University of California, San Francisco, CA) (23). Cells were routinely cultured to confluence in 100% humidity and 5% CO_2 at 37°C. They were grown in MEM with Earle's salts (Life Technologies Invitrogen) containing 10% FBS (HyClone, Logan, UT) and 2 mM L-glutamine. For experiments, cells were seeded at 1 $\times$ 10^5 cells/ml medium in 24-well plates and cultured at 37°C and 5% CO_2 for 2 days. The cells were used between passages 8 and 20.

Media and reagents

LL-37 (sequence LLGDFFRKSKEKIGKEFKRIVQRIKDFLRNLVPRTES) and CP29 (sequence KWKSFIKKLTTAVKKVLTTGLPALIS) were synthesized by F-moc chemistry at the Nucleic Acid/Protein Synthesis Unit at University of British Columbia . Human recombinant GM-CSF, IL-4, and M-CSF were purchased from Research Diagnostics (Flanders, NJ). Pertussis toxin was supplied by List Biological Laboratories (Campbell, CA). SB 203580, a specific inhibitor of p38 kinase, was purchased from Sigma-Aldrich, and PD98059, a specific inhibitor of ERK1/2 kinase, was purchased from Cell Signaling Technology (Braintree, MA). Peptide WKYMVM was a gift from Dr. C. Dahlgren (University of Goteborg, Goteborg, Sweden).

Analysis of cytotoxicity and cell viability

Viability after cell isolation was determined by resuspending cells in a 0.4% trypan blue solution (Life Technologies, Gaithersburg, MD). Peptide cytotoxicity was assessed by collecting culture supernatants after stimulation with LL-37 or vehicle control. The concentration of lactate dehydrogenase-1 (LDH-1) in the supernatants was quantified using a cytotoxicity detection kit (Roche, Laval, Quebec, Canada) according to the manufacturer's instructions.

Analysis of endotoxin contamination

The media, peptide and chemokine stocks, and vehicle controls were tested for endotoxin contamination using a LAL Chromagenic Endpoint Assay (HyCult Biotechnology; Cedarlane Laboratories, Hornby, Ontario, Canada). Endotoxin levels were <0.3 EU/ml.

Western immunoblotting

After stimulation, cells were washed with ice-cold PBS containing 1 mM vanadate (Sigma-Aldrich). Next, 125 μl of RIPA buffer (50 mM Tris-HCl (pH 7.4); 1% Nonidet P-40; 0.25% sodium deoxycholate; 150 mM NaCl; 1 mM EDTA; 1 mM PMSF; 1 μg/ml each of aprotinin, leupeptin, and pepstatin; 1 mM sodium orthovanadate, and 1 mM NaF) was added, and the cells were incubated on ice until they were completely lysed, as assessed by visual inspection. The lysates were quantitated using a bicinchoninic acid assay (Pierce, Rockford, IL). Thirty micrograms of lysate was loaded onto 1.5-mm-thick gels, which were run at 100 V for $\sim$2 h. Proteins were transferred to nitrocellulose filters for 75 min at 70 V. The filters were blocked for 2 h at room temperature with 5% skim milk in TBST (10 mM Tris-HCl (pH 8), 150 mM NaCl, and 0.1% Tween 20). The filters were then incubated overnight at 4°C with the anti-ERK1/2-P or anti-p38-P (Cell Signaling Technology) mAbs. Immunoreactive bands were detected using HRP-conjugated sheep anti-mouse IgG Abs (Amersham Pharmacia Biotech, Piscataway, NJ) and chemiluminescence detection (Sigma-Aldrich). To quantify bands, the films were scanned and then quantified by densitometry using the software program ImageJ. The blots were reprobed with a β-actin Ab (ICN Biomedical, Irvine, CA), and densitometry was performed to allow correction for protein loading.

Kinase assay

An ERK1/2 activity assay was performed using a nonradioactive kit (Cell Signaling Technology). Briefly, cells were treated for 15 min and lysed in lysis buffer. Equal amounts of proteins were immunoprecipitated with an immobilized phospho-ERK1/2 Ab that reacts only with the phosphorylated (i.e., active) form of ERK1/2. The immobilized precipitated enzymes were then used for the kinase assay using Elk-1, followed by Western blot analysis with Abs that allow the detection and quantitation of phosphorylated substrates.

Quantification of IL-8

Human IL-8 from supernatants of 16HBE40- cells and blood-derived monocytes was measured using the commercially available ELISA kit (BioSource International, Camarillo, CA) according to the manufacturer's instructions.

Semiquantitative RT-PCR

Total RNA was isolated from donor blood-derived monocytes using an RNaqueous Microkit (Ambion, Austin, TX) as described by the manufacturer. The samples were DNase-treated, and then cDNA synthesis was

accomplished using a first-strand cDNA synthesis kit (Life Technologies, Invitrogen). The resultant cDNAs were used as a template in PCRs for various cytokine genes: MCP-1 (5′-TCATAGCAGCCACCTTCATTC-3′, 5′-TAGCGCAGATTCTTGGGTTG-3), MCP-3 (5′-TGTCCTTTCTCA GAGTGGTTCT-3′, 5′-TGCTTCCATAGGGACATCATA-3′), IL-6 (5′-ACCTGAACCTTCCAAAGATGG-3′, 5′-GCGCAGAATGAGATGAGT TG-3′), and IL-8 (5′-GTGCAGAGGGTTGTGGAGAAG-3′, 5′-TTCTC CCGTGCAATATCTAGG-3′), TNF-α (5′-AGGGAGCCTTTGGTTCT GG-3′, 5′-TCAGCAATGAGTGACAGTTGG-3′), and IL-β (5′-GGATA TGGAGCAACAAGTGG-3′, 5′-ATGTACCAGTTGGGGAACTG-3′). Each RT-PCR reaction was performed at least in duplicate. Results were analyzed in the linear phase of amplification and normalized to the housekeeping control, GAPDH. Reactions were verified by including controls generated without reverse transcriptase.

Results

LL-37 induces ERK1/2 and p38 phosphorylation in peripheral blood-derived monocytes.

To determine whether LL-37 induced activation of the MAPKs, ERK1/2 and/or p38, peripheral blood-derived monocytes were treated with 50 μg/ml LL-37 or water (as a vehicle control) for 15 min. To visualize the activated (phosphorylated) form of the kinases, Western blots were performed with Abs specific for the dually phosphorylated form of the kinases (phosphorylation on Thr^{202} and Tyr^{204} and on Thr^{180} and Tyr^{182} for ERK1/2 and p38, respectively). The gels were reprobed with an Ab for β-actin to normalize for loading differences. In all cases, increases in the phosphorylation of ERK1/2 ($n = 8$) and p38 ($n = 4$) were observed in response to LL-37 treatment (Fig. 1).

A serum component is required for LL-37-induced activation

It has been proposed that LL-37 binds to at least one serum protein, and that this binding modifies its activities (24). Thus, we tested the ability of LL-37 to activate ERK1/2 and p38 in the presence and the absence of serum. No cytotoxicity was observed in response to a 15-min LL-37 exposure in the presence or the absence of serum, as assessed by trypan blue exclusion or the LDH-1 assay (data not shown). In the absence of serum, no detectable activation of ERK1/2 or p38 was observed using Abs specific for the phosphorylated forms of the kinases, in contrast to the activation observed in the presence of FCS ($n = 3$; Fig. 2). In addition, a functional assay for ERK1/2 activation was performed. In this assay, activation of an ERK1/2-controlled transcription factor, Elk-1, was detected in medium containing serum, but not in serum-free medium ($n = 2$; Fig. 2). This indicates that LL-37 requires the addition of at least one serum component to induce activation of the MAPKs, ERK1/2 and p38.

LL-37 activation of MAPK pathways is specific for monocytes and epithelial cells

To assess whether LL-37-induced activation of the ERK1/2 and p38 kinases was specific to effector cells of the innate immune response, phosphorylation of p38 and ERK1/2 was studied in freshly isolated primary monocytes, B cells, T cells, and the human epithelial cell line 16HBE4o- (Fig. 3). No cytotoxicity was detected in any cell type under the assay conditions, as assessed by trypan blue exclusion or the LDH-1 assay (data not shown). Western blot analysis demonstrated that both the epithelial cell line ($n = 2$ experiments) and primary monocytes ($n = 8$ donors) showed substantial increases in ERK1/2 and p38 phosphorylation upon exposure to LL-37 for 15 min (as assessed after normalization for loading differences by reprobing with an Ab for β-actin). However, no phosphorylation of either ERK1/2 or p38 kinases was evident in human T cells or B cells ($n = 4$ donors and $n = 2$ donors, respectively). This cellular specificity indicates that activation of the MAPKs is specific to certain cell types and therefore may relate to similar receptors in these types of cells or to similarities in membrane organization.

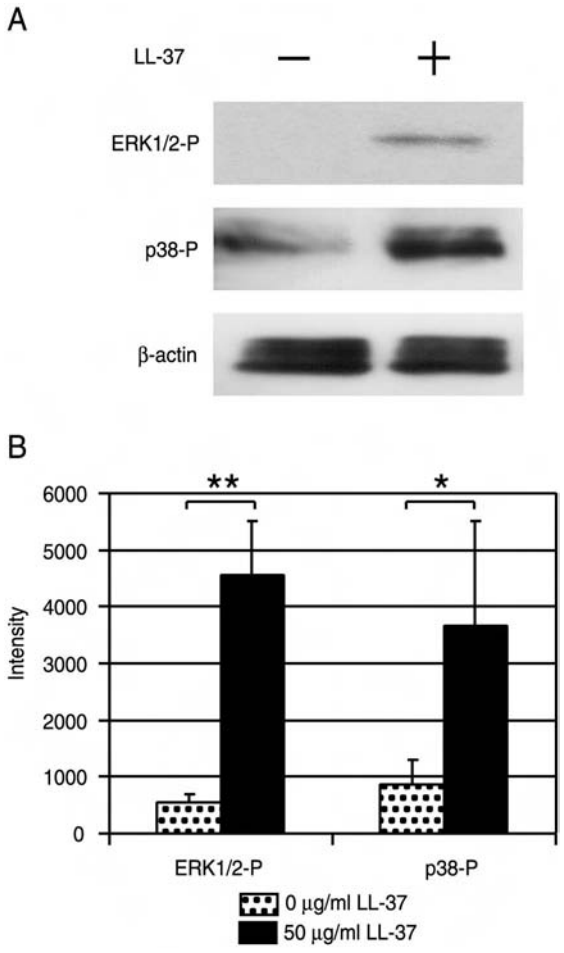

FIGURE 1. Exposure to LL-37 induces phosphorylation of ERK1/2 and p38. Human peripheral blood-derived monocytes were exposed to 50 μg/ml LL-37 (+) or endotoxin-free water (−) as a vehicle control for 15 min. Abs specific for the phosphorylated forms of ERK1/2 and p38 were used to detect activation of ERK1/2 and p38 in cell lysates. β-Actin was quantified to allow correction for protein loading. All donors tested showed increased phosphorylation of ERK1/2 and p38 in response to LL-37 treatment. *A*, One representative donor is shown. *B*, The phosphorylation of ERK1/2 ($n = 9$ donors) and p38 ($n = 4$ donors) was quantified by densitometry. Student's one-tailed t test was performed. **, $p < 0.01$; *, $p < 0.05$. The mean ± SE are shown.

G-coupled proteins are not involved in LL-37-induced activation of MAPK

FPRL-1, a pertussis toxin-sensitive, G-coupled protein has been proposed to be a receptor for LL-37 on monocytes, certain subsets of T cells, and neutrophils (12). To test whether FPRL-1 or other G protein-coupled receptors were involved in LL-37-induced signaling, sensitivity to inhibition with pertussis toxin was examined. No significant reduction of ERK1/2 phosphorylation was observed as a result of incubation with pertussis toxin. ERK1/2 phosphorylation upon stimulation with 50 μg/ml LL-37 was increased an average of 8.3-fold in the absence ($n = 9$) and 7.6-fold in the presence ($n = 3$) of pertussis toxin (Fig. 4*A*). A synthetic peptide activator/agonist (WKYMVM) of FPRL-1 and FPRL-2 was used as a positive control. The FPRL-1 agonist induced massive ERK1/2 phosphorylation, which was partially inhibited by the incubation with pertussis toxin (~6- and 9-fold inhibition was seen

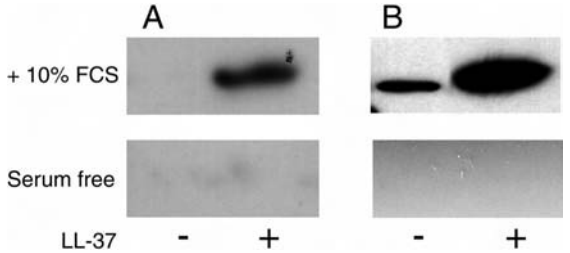

FIGURE 2. LL-37 is unable to induce MAPK activation in human monocytes under serum-free conditions. Cells were exposed to 50 μg/ml LL-37 (+) or endotoxin-free water (−) as a vehicle control for 15 min. *A*, After exposure to LL-37 in medium containing 10% FCS, phosphorylated ERK1/2 was detectable; however, no phosphorylation of ERK1/2 was detected in the absence of serum ($n = 3$). *B*, Elk-1, a transcription factor downstream of ERK1/2, was activated (phosphorylated) upon exposure to 50 μg/ml LL-37 in medium containing 10% FCS, but not in the absence of serum ($n = 2$).

in two donors; Fig. 4*B*), indicating that the incubation with pertussis toxin under the conditions studied did indeed inhibit G protein-coupled receptor signaling. As LL-37-induced signaling was not affected by pertussis toxin treatment, these data suggest that activation of ERK1/2 was not linked to a pertussis toxin-sensitive receptor and, in particular, was not linked to the FPRL-1 receptor.

LL-37-induced activation of ERK1/2 and p38 is dose dependent and demonstrates synergy with GM-CSF

The precursor hCAP18 is found at low levels in blood and serum, whereas LL-37, the processed form of the peptide, is found at much higher concentrations in the context of infection (25–27). We hypothesized that LL-37-induced activation of the MAPKs may be more pronounced in the presence of cytokines that are up-regulated during the course of infection. To test this hypothesis we added GM-CSF, IL-4, or M-CSF (each at 100 ng/ml) concurrently with LL-37 and measured phosphorylation of ERK1/2 in freshly isolated human blood monocytes (Fig. 5). ERK1/2 phosphorylation was evident when cells were treated with 50 μg/ml LL-37 (8.3-fold increase over untreated; $n = 9$), but not at lower concentrations ($n = 2$). In the presence of 100 ng/ml GM-CSF, LL-37-induced ERK1/2 phosphorylation increased markedly (58-fold greater than untreated; $n = 5$). This synergistic activation did not occur in the presence of 100 ng/ml M-CSF or IL-4. Furthermore, in the presence of GM-CSF, activation of ERK1/2 occurred in response to concentrations of 5 and 10 μg/ml LL-37, respectively (as confirmed by densitometry), in the two donors tested (Fig. 5). This demonstrates that LL-37-induced activation of

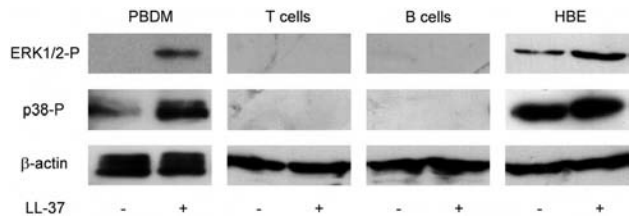

FIGURE 3. Activation of ERK1/2 and p38 phosphorylation by LL-37 is induced in cells of the innate immune system. Freshly isolated human peripheral blood-derived monocytes (PBDM), T cells, B cells, and HBE cell line were stimulated for 15 min with 50 μg/ml LL-37 (+) or endotoxin-free water as a vehicle control (−) in medium containing 10% FCS. Abs specific for the phosphorylated forms of ERK1/2 and p38 were used to detect their activation in cell lysates. Activation of ERK1/2 and p38 was evident in monocytes and the bronchial epithelial cell line, but not in T or B cells. For monocytes, T cells, and B cells, one representative donor is shown from eight, four, and two donors, respectively. For the 16HBE4o- cell line, one representative experiment of two is shown. β-Actin was quantified to allow correction for protein loading.

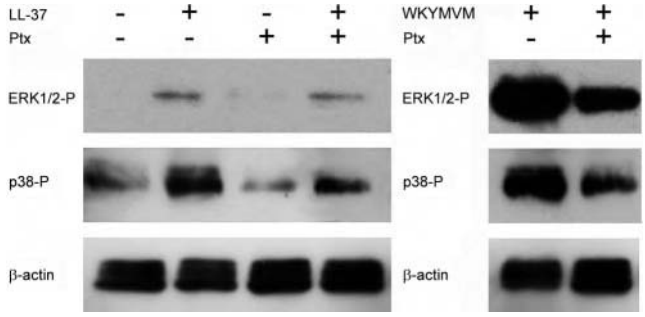

FIGURE 4. LL-37-induced phosphorylation of ERK1/2 and p38 kinases is not mediated by G protein-coupled receptors. Abs were used to detect the phosphorylated forms of ERK1/2 and p38 in cell lysates from human peripheral blood-derived monocytes. β-Actin was quantified to allow correction for protein loading. *A*, Cells were exposed to 50 μg/ml LL-37 (+) or endotoxin-free water (−) as a vehicle control for 15 min in medium containing 10% FCS. Phosphorylation induced by LL-37 was not inhibited by 100 ng/ml pertussis toxin (+) added 30 min before exposure to LL-37 and present during LL-37-induced stimulation. *B*, Cells were exposed to 100 ng/ml WKYMVM, a synthetic agonist of FPRL-1 (+). Phosphorylation of ERK induced by FPRL-1 was reduced ~9-fold by the addition of pertussis toxin (+). One representative experiment of two is shown.

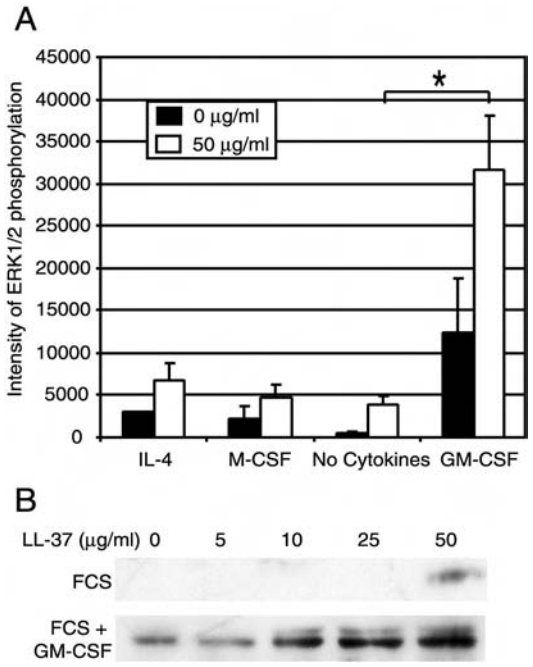

FIGURE 5. ERK1/2 activation is amplified and occurs at lower concentrations of LL-37 in the presence of GM-CSF. *A*, Freshly isolated monocytes were stimulated with LL-37 (50 μg/ml) or endotoxin-free water (0 μg/ml LL-37) as a vehicle control in medium containing 10% FCS and no cytokines, GM-CSF (100 ng/ml), M-CSF (100 ng/ml), or IL-4 (100 ng/ml). A minimum of three donors were used for each experiment. LL-37-induced phosphorylation of ERK1/2 was significantly enhanced in the presence of GM-CSF, but not IL-4 or M-CSF. Values are the mean ± SE. *, $p = 0.004$. *B*, The concentration of LL-37 required to induce phosphorylation of ERK1/2 was decreased from 50 to 5 μg/ml. One representative donor of two is shown.

ERK1/2 occurred at a lower threshold in the presence of GM-CSF, a cytokine found locally at sites of infection. The presence of GM-CSF also enhanced LL-37-induced phosphorylation of p38 (data not shown).

LL-37-induced MAPK activation is not related to cytotoxicity in human blood-derived monocytes

Some cationic peptides induce lysis of eukaryotic cells. Previous gene array experiments (13) demonstrated that 50 μg/ml LL-37 did not cause the up-regulation of any known stress- or cytotoxicity-regulated genes (e.g., heat shock proteins, apoptosis genes, etc.). To test whether LL-37 was cytotoxic in our model system over longer exposure periods, human blood-derived monocytes from two donors were incubated with LL-37 (10–50 μg/ml), the cytotoxic peptide CP29 (50 μg/ml), or a vehicle control for 0.5–4 h (Fig. 6*A*). The concentration of LDH-1 in the supernatants was quantified as a measure of cytotoxicity. The cytotoxic peptide, CP29, induced significant cell lysis beginning at 0.5 h. In contrast, cells exposed to up to 50 μg/ml LL-37 demonstrated no more lysis than the controls (Fig. 6*A*). These data demonstrate that MAPK phosphorylation induced by 15-min exposure to LL-37 in human blood-derived monocytes does not correlate with later cytotoxicity at the concentrations used in this study.

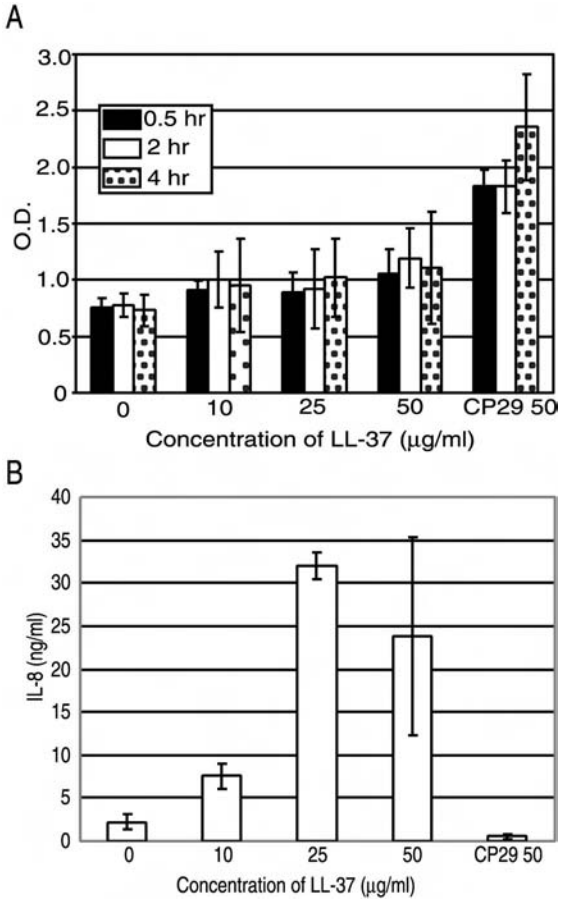

FIGURE 6. LL-37 induces IL-8 production in human blood-derived monocytes in the absence of cytotoxicity. *A*, Human peripheral blood-derived monocytes from two separate donors were exposed to 10–50 μg/ml LL-37 or the cytotoxic peptide CP-29 or to endotoxin-free water as a vehicle control for up to 4 h. The concentration of LDH-1 in the supernatants induced a colorimetric change that provided a measure of cytotoxicity. The average release of LDH by the two donors is shown. Bars represent the range of results for the two donors. *B*, After assaying for cytotoxicity, the supernatants were assayed for IL-8 by ELISA. The average of two donors is shown. Error bars represent the range of results for the two donors.

LL-37 exposure induces IL-8 secretion in human blood-derived monocytes

We have recently demonstrated that LL-37 induced the secretion of chemokines in the human A549 epithelial cell line (IL-8) and whole human blood (MCP-1 and IL-8) (13). In addition to testing for LDH levels, the supernatants from human blood-derived monocytes treated with 10–200 μg/ml, the cytotoxic peptide CP29 (50 μg/ml), or a vehicle control for up to 4 h were assayed for IL-8 secretion by ELISA. In both donors tested, the addition of LL-37 led to an increase in IL-8 secretion; however, the cytotoxic peptide CP29 did not induce IL-8 secretion (Fig. 6*B*). These data confirm that the immunomodulatory properties of LL-37 and cytotoxicity are independent.

Activation of ERK1/2 and p38 is necessary for transcription of Elk-1-controlled genes and secretion of IL-8

IL-8 release is known to be governed at least in part by activation of the ERK1/2 and p38 kinases (28). To determine whether activation of the MAPKs was required for IL-8 release, cells were incubated with a p38 kinase-specific inhibitor, SB 203580, and/or an ERK1/2-specific inhibitor, PD98059, for 1 h before a 4-h incubation with 50 μg/ml LL-37. IL-8 in the culture medium was assayed by ELISA. In both donors tested, LL-37-induced IL-8 secretion was reduced by the presence of either 10 μM ERK1/2 or p38 kinase inhibitors and was abrogated in the presence of the combination of both inhibitors (Fig. 7*A*).

When HBE cells were stimulated with 50 μg/ml LL-37 for 4 h, a significant increase in the amount of IL-8 released into the medium was detected (Fig. 7*B*). This LL-37-induced secretion of IL-8 was reduced in the presence of either the p38 inhibitor or the ERK1/2 inhibitor. In the presence of both inhibitors in combination, IL-8 secretion was not significantly different from the baseline level of IL-8 secretion (Fig. 7*B*). These data indicate that both p38 and ERK1/2 kinases are involved in LL-37-mediated IL-8 secretion in blood-derived monocytes and epithelial cells.

Activation of ERK1/2 and p38 is necessary for LL-37-induced transcription of certain chemokines

To determine the downstream transcriptional effects of LL-37-induced MAPK activation, the expression of genes known to be regulated by ERK1/2 or p38 was assessed. Semiquantitative RT-PCR was performed on RNA collected from monocytes isolated from two donors and pretreated with the ERK1/2 and p38 inhibitors or vehicle control before a 4-h exposure to 50 μg/ml LL-37 ($n = 2$). MCP-1 and IL-8 have been demonstrated to be under the transcriptional control of both ERK1/2 and p38 (29, 30). Consistent with these observations, the expression of these genes was up-regulated after exposure to LL-37, and this up-regulation was abolished in cells pretreated with either ERK1/2 or p38 inhibitors (Fig. 7*C*). Transcription of the gene encoding MCP-3 was also increased in the presence of LL-37 and was dependent on the activation of both p38 kinase and ERK1/2. Conversely, there were no increases in the transcription of the genes encoding the proinflammatory cytokines TNF-α, IL-1β, and IL-6 in either donor (data not shown). These data are consistent with the hypothesis that activation of the ERK1/2 and p38 signaling pathways has functional effects on the transcription of cytokine genes with immunomodulatory functions.

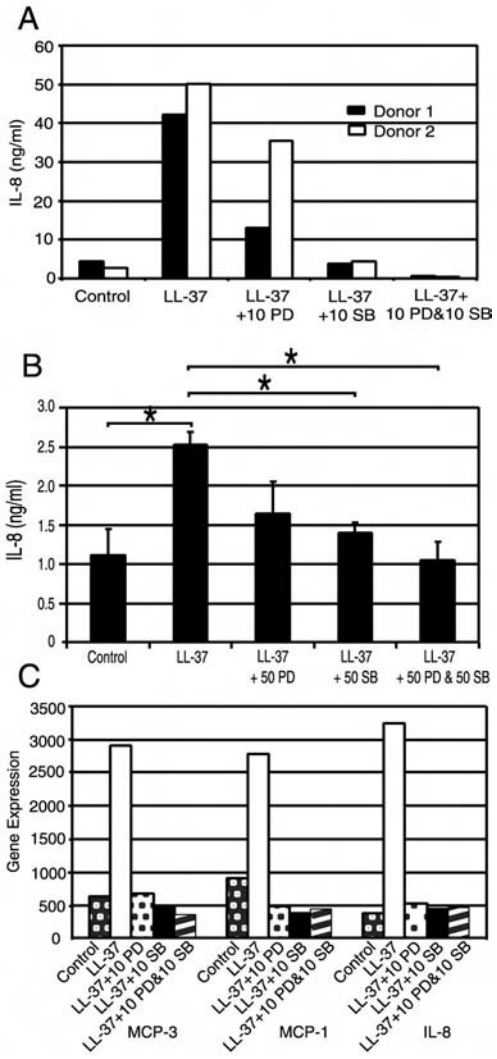

FIGURE 7. LL-37 induces IL-8 secretion and chemokine transcription in a p38 kinase- and ERK1/2-dependent manner. *A*, Human peripheral blood-derived monocytes from two donors were incubated with 10 μM p38 kinase-specific inhibitor SB 203580 (10 SB) and/or 10 μM ERK1/2-specific inhibitor PD98059 (10 PD) for 1 h, after which the cells were exposed for 4 h to 50 μg/ml LL-37 or endotoxin-free water as a vehicle control. IL-8 was assayed in the culture medium by ELISA. *B*, Approximately 1×10^5 cells of the HBE cell line 16HBE4o- were seeded per well of a 24-well plate and grown for 2 days. The cells were then incubated with 50 μM p38 kinase-specific inhibitor SB 203580 (50 SB) and/or 50 μM ERK1/2-specific inhibitor PD98059 (50 PD) for 1 h, after which the cells were exposed for 4 h to 50 μg/ml LL-37 or endotoxin free water as a vehicle control. IL-8 was assayed in the culture media by ELISA. Values are the mean $\pm$ SE of three independent experiments. $*$, $p < 0.05$. *C*, Human peripheral blood-derived monocytes were incubated with kinase inhibitors and LL-37 as described above. After 4 h, RNA was collected, and RT-PCR was performed. The mRNA expression of the chemokine genes was corrected for GAPDH expression. MCP-1, MCP-3, and IL-8 were up-regulated by LL-37 treatment, and this up-regulation was abrogated by treatment with inhibitors of either ERK1/2 or p38. Representative data for one of two donors tested are shown.

Discussion

There is a paucity of information on how antimicrobial peptides exert their effects on eukaryotic cells. Despite the fact that there have been hundreds, if not thousands, of antimicrobial peptides characterized to date, many of which have immunomodulatory properties, little or nothing is known about how they initiate signaling within eukaryotic cells. In this paper we demonstrate for the first time that the human cathelicidin, LL-37, induces activation of both the p38 and ERK1/2 kinases in monocytes and epithelial cells. Activation of these kinases has been demonstrated to have pleiotropic influences on the effector cells of the immune response, including cytokine production, cellular activation, proliferation, and differentiation (31). As LL-37 has been demonstrated to modulate all these processes, it is tempting to speculate that its immunomodulatory abilities may be regulated in part by its ability to signal through these pathways.

In a study by De Yang et al. (12), LL-37 was demonstrated to be a chemoattractant for monocytes, neutrophils, and T cells. This activity of LL-37 was proposed to be receptor mediated, functioning via the G protein-coupled receptor FPRL-1 and inhibited by an peptide agonist of FPRL-1 (12). Due to the insensitivity of LL-37-induced MAPK activation to both pertussis toxin (Fig. 4) and cholera toxin (data not shown), we believe that activation of ERK1/2 and p38 by LL-37 is not linked to a G protein-coupled receptor, such as FPRL-1. Indeed, although FPRL-1 is expressed on both monocytes and T cells, LL-37-induced activation of ERK1/2 and p38 was observed in monocytes and epithelial cells, but not on T or B cells, indicating that this receptor is not the mechanism by which the MAPKs are activated. Niyonsaba et al. (20) demonstrated that FPRL-1 was not the receptor on mast cells, which undergo both chemotaxis and degranulation in response to LL-37. In fact, these researchers suggest that LL-37 has two receptors in this cell type: one high affinity pertussis toxin-sensitive receptor that is linked to LL-37-induced chemotaxis, and one low affinity receptor with an as yet undefined function (20). Our study indicates that LL-37 induces activation of p38 kinase and ERK1/2, directly or indirectly, through a pertussis toxin-insensitive mechanism.

LL-37-induced signaling was not observed in the absence of serum. The properties of LL-37 have been demonstrated to be altered in the presence of serum (32). In fact, in the absence of serum, moderate concentrations (30–100 μg/ml) of LL-37 and related peptides were cytotoxic toward both prokaryotic and eukaryotic cells (24, 33, 34). However, in the presence of serum, cytotoxicity toward both cell types was substantially reduced (33, 35). In the current study it was demonstrated that in medium containing serum, LL-37 does not induce substantial cytotoxicity in human blood-derived monocytes. In other studies we have shown that in the absence of serum, 50 μg/ml LL-37 does result in increased cell lysis in as little as 2 h (data not shown). LL-37-induced activation of MAPK is not a result of cytotoxicity, as it does not occur in the absence of serum. When LL-37 is found in the blood or tissues during the course of infection, it may exist coupled to one or several proteins. The presence of apolipoprotein 1 has been proposed to reduce LL-37-induced cytotoxicity while also inhibiting its antimicrobial functions (24). It will be of interest to determine whether the presence of apolipoprotein can restore the ability of LL-37 to induce signaling in serum-free medium; however, it is not unreasonable to assume that there are multiple components in serum that may interact with the positively charged LL-37. These data illustrate the importance of studying the immunomodulatory effects of LL-37, and perhaps other cationic peptides, in the presence of serum.

LL-37 induces chemokine secretion in airway epithelial cell lines, human whole blood, and mouse models (13, 36, 37). In this study we demonstrated that the activation of ERK1/2 and p38 kinases is required for LL-37-induced transcription of the chemokines IL-8, MCP-1, and MCP-3 in human blood-derived monocytes. We also demonstrated that this transcription is specific for chemokine genes and not proinflammatory cytokines such as

TNF-α, IL-1β, and IL-6. Transcription of IL-8 and other chemokine genes was completely abrogated in the presence of inhibitors of either p38 kinase or ERK1/2. In addition, the secretion of IL-8 in both the epithelial cell line and the monocytes was only partially inhibited by the presence of either inhibitor and was only completely inhibited in the presence of both inhibitors, indicating that the kinases may affect chemokine production at both the transcriptional and post-transcriptional levels.

Circulating blood monocytes enter the tissues via the process of extravasation in response to gradients of chemokines (perhaps including LL-37) released at sites of infection. At the point at which they first encounter chemokines or other stimuli and begin the process of extravasation they are circulating monocytes similar to those used in this study. As they move to the site of inflammation or infection, these cells will be exposed to progressively greater concentrations of chemokines or other stimuli, such as LL-37 or GM-CSF, and the process of differentiation and activation begins (38). Upon detection of low concentrations of LL-37 and cytokines such as GM-CSF, or upon arrival at sites of high concentrations of LL-37, it is proposed that activation of the MAPKs would occur and that this activation would have important physiological outcomes.

Interestingly, LL-37-induced activation of ERK1/2 and p38 signaling is enhanced in the presence of GM-CSF, but not M-CSF or IL-4. Although initial characterization of GM-CSF focused on its ability to generate granulocytes and macrophage colonies from common precursor cells, it has more recently been proposed to have many roles in the inflammatory response. These include generating increased numbers of circulating neutrophils and peritoneal macrophages (39), promotion of PBMC survival (27), and potentiation of LPS-induced cytokine release and subsequent lethality in vivo (40). GM-CSF is not generally found in the serum of healthy adults (41); however, it is present in the serum after stimulation with LPS (42) and is produced by a variety of tissues, including upper airway epithelial cells, fibroblasts, endothelial cells, and monocytes themselves (43). This pattern of expression mimics that of LL-37 during the course of infection or stimulation with inflammatory mediators (26, 44–46). Hamilton et al. (47) propose that hemopoietic cells respond to infection by stimulating surrounding tissues to produce GM-CSF. This localized increase in the concentration of GM-CSF encourages survival and differentiation and alters the activation state of local monocytes and polymorphonuclear neutrophils, thus promoting clearance of infectious organisms (47). The production and action of GM-CSF occur locally at the site of inflammation, indicating that this activation and proliferation of hemopoietic cells would be a localized, rather than systemic, orchestration of the immune response. LL-37 alone activates cell signaling pathways only at high concentrations, which in vivo would be found only at sites of acute inflammation. The presence of GM-CSF lowers the threshold for LL-37-induced activation of the MAPKs to between 5–10 μg/ml, concentrations found in vivo at the onset of infection and that are modestly higher than the normal concentration of circulating hCAP-18 (25, 26, 48). We hypothesize that low concentrations of LL-37 (<5 μg/ml) are homeostatic and do not activate the effector cells of the innate immune response unless they exist in the presence of proinflammatory signals such as the presence of proinflammatory cytokines. The presence of both LL-37 and GM-CSF may thus lead to novel functional phenotypes of effector cells of the innate immune response.

Although cationic peptides often have antimicrobial activity, the key to their therapeutic potential may lie among the myriad of other activities attributed to them, such as their ability to alter the inflammatory response (49). For example, under conditions analogous to those found in vivo, LL-37 is a weak antimicrobial agent (data not shown). Conversely, it is one of the most potent antiendotoxic agents among cationic host defense peptides, is directly chemotactic, and induces dramatic changes in the phenotype of monocyte-derived dendritic cells, indicating a substantial role in the innate immune response (13). Thus, it is becoming increasingly clear that peptides are more than simply nature's antibiotics, but, rather, play a complex role in resolving infection, attenuating inflammation, and, when this attempt at resolution is not sufficient, alerting the adaptive immune response. Characterization of the mechanism of action of these peptides is shedding light on the properties that make them potential therapeutic agents. We propose that LL-37-induced activation of ERK1/2 and p38 kinases is a pivotal function of this peptide, acting via an as yet undetermined G protein-coupled receptor-independent, but serum-dependent, mechanism. We demonstrate that this MAPK activation displays synergy with other modulators of the immune response and hypothesize that the direct downstream signaling consequences are fundamental to many of the immunomodulatory activities of LL-37 with regard to their physiological role and also as potential therapeutics.

Acknowledgments

We acknowledge Drs. Andrew Currie and Monisha Scott for helpful suggestions, materials, and guidance, and Sophie Tremblay-Paquet and Rebecca Ma for technical assistance.

References

1. Diamond, G., J. P. Russell, and C. L. Bevins. 1996. Inducible expression of an antibiotic peptide gene in lipopolysaccharide-challenged tracheal epithelial cells. *Proc. Natl. Acad. Sci. USA 93:5156.*
2. Liu, L., A. A. Roberts, and T. Ganz. 2003. By IL-1 signaling, monocyte-derived cells dramatically enhance the epidermal antimicrobial response to lipopolysaccharide. *J. Immunol. 170:575.*
3. Edgerton, M., S. E. Koshlukova, M. W. Araujo, R. C. Patel, J. Dong, and J. A. Bruenn. 2000. Salivary histatin 5 and human neutrophil defensin 1 kill Candida albicans via shared pathways. *Antimicrob. Agents Chemother. 44:3310.*
4. Giacometti, A., O. Cirioni, F. Barchiesi, M. Fortuna, and G. Scalise. 1999. In-vitro activity of cationic peptides alone and in combination with clinically used antimicrobial agents against *Pseudomonas aeruginosa*. *J. Antimicrob. Chemother. 44:641.*
5. Chaly, Y. V., E. M. Paleolog, T. S. Kolesnikova, Tikhonov, I. I., E. V. Petratchenko, and N. N. Voitenok. 2000. Neutrophil α-defensin human neutrophil peptide modulates cytokine production in human monocytes and adhesion molecule expression in endothelial cells. *Eur. Cytokine Network. 11:257.*
6. Chertov, O., D. F. Michiel, L. Xu, J. M. Wang, K. Tani, W. J. Murphy, D. L. Longo, D. D. Taub, and J. J. Oppenheim. 1996. Identification of defensin-1, defensin-2, and CAP37/azurocidin as T-cell chemoattractant proteins released from interleukin-8-stimulated neutrophils. *J. Biol. Chem. 271:2935.*
7. Kirikae, T., M. Hirata, H. Yamasu, F. Kirikae, H. Tamura, F. Kayama, K. Nakatsuka, T. Yokochi, and M. Nakano. 1998. Protective effects of a human 18-kilodalton cationic antimicrobial protein (CAP18)-derived peptide against murine endotoxemia. *Infect. Immun. 66:1861.*
8. Tani, K., W. J. Murphy, O. Chertov, R. Salcedo, C. Y. Koh, I. Utsunomiya, S. Funakoshi, O. Asai, S. H. Herrmann, J. M. Wang, et al. 2000. Defensins act as potent adjuvants that promote cellular and humoral immune responses in mice to a lymphoma idiotype and carrier antigens. *Int. Immunol. 12:691.*
9. Lillard, J. W., Jr., P. N. Boyaka, O. Chertov, J. J. Oppenheim, and J. R. McGhee. 1999. Mechanisms for induction of acquired host immunity by neutrophil peptide defensins. *Proc. Natl. Acad. Sci. USA 96:651.*
10. Lehrer, R. I., and T. Ganz. 2002. Cathelicidins: a family of endogenous antimicrobial peptides. *Curr. Opin. Hematol. 9:18.*
11. Hancock, R. E. W., and G. Diamond. 2000. The role of cationic antimicrobial peptides in innate host defences. *Trends Microbiol. 8:402.*
12. De, Y., Q. Chen, A. P. Schmidt, G. M. Anderson, J. M. Wang, J. Wooters, J. J. Oppenheim, and O. Chertov. 2000. LL-37, the neutrophil granule- and epithelial cell-derived cathelicidin, utilizes formyl peptide receptor-like 1 (FPRL1) as a receptor to chemoattract human peripheral blood neutrophils, monocytes, and T cells. *J. Exp. Med. 192:1069.*
13. Scott, M. G., D. J. Davidson, M. R. Gold, D. Bowdish, and R. E. W. Hancock. 2002. The human antimicrobial peptide LL-37 is a multifunctional modulator of innate immune responses. *J. Immunol. 169:3883.*
14. Nagaoka, I., S. Hirota, F. Niyonsaba, M. Hirata, Y. Adachi, H. Tamura, and D. Heumann. 2001. Cathelicidin family of antibacterial peptides CAP18 and CAP11 inhibit the expression of TNF-α by blocking the binding of LPS to $CD14^+$ cells. *J. Immunol. 167:3329.*

15. Bals, R., D. J. Weiner, A. D. Moscioni, R. L. Meegalla, and J. M. Wilson. 1999. Augmentation of innate host defense by expression of a cathelicidin antimicrobial peptide. *Infect. Immun. 67:6084*.
16. Koczulla, R., G. von Degenfeld, C. Kupatt, F. Krotz, S. Zahler, T. Gloe, K. Issbrucker, P. Unterberger, M. Zaiou, C. Lebherz, et al. 2003. An angiogenic role for the human peptide antibiotic LL-37/hCAP-18. *J. Clin. Invest. 111:1665*.
17. Heilborn, J. D., M. F. Nilsson, G. Kratz, G. Weber, O. Sorensen, N. Borregaard, and M. Stahle-Backdahl. 2003. The cathelicidin anti-microbial peptide LL-37 is involved in re-epithelialization of human skin wounds and is lacking in chronic ulcer epithelium. *J. Invest. Dermatol. 120:379*.
18. Niyonsaba, F., A. Someya, M. Hirata, H. Ogawa, and I. Nagaoka. 2001. Evaluation of the effects of peptide antibiotics human β-defensins-1/-2 and LL-37 on histamine release and prostaglandin D_2 production from mast cells. *Eur. J. Immunol. 31:1066*.
19. Zaiou, M., V. Nizet, and R. L. Gallo. 2003. Antimicrobial and protease inhibitory functions of the human cathelicidin (hCAP18/LL-37) prosequence. *J. Invest. Dermatol. 120:810*.
20. Niyonsaba, F., K. Iwabuchi, A. Someya, M. Hirata, H. Matsuda, H. Ogawa, and I. Nagaoka. 2002. A cathelicidin family of human antibacterial peptide LL-37 induces mast cell chemotaxis. *Immunology 106:20*.
21. Sallusto, F., and A. Lanzavecchia. 1994. Efficient presentation of soluble antigen by cultured human dendritic cells is maintained by granulocyte/macrophage colony-stimulating factor plus interleukin 4 and downregulated by tumor necrosis factor α. *J. Exp. Med. 179:1109*.
22. Indiveri, F., J. Huddlestone, M. A. Pellegrino, and S. Ferrone. 1980. Isolation of human T lymphocytes: comparison between nylon wool filtration and rosetting with neuraminidase (VCN) and 2-aminoethylisothiouronium bromide (AET)-treated sheep red blood cells (SRBC). *J. Immunol. Methods 34:107*.
23. Cozens, A. L., M. J. Yezzi, K. Kunzelmann, T. Ohrui, L. Chin, K. Eng, W. E. Finkbeiner, J. H. Widdicombe, and D. C. Gruenert. 1994. CFTR expression and chloride secretion in polarized immortal human bronchial epithelial cells. *Am. J. Respir. Cell Mol. Biol. 10:38*.
24. Wang, Y., B. Agerberth, A. Lothgren, A. Almstedt, and J. Johansson. 1998. Apolipoprotein A-I binds and inhibits the human antibacterial/cytotoxic peptide LL-37. *J. Biol. Chem. 273:33115*.
25. Sorensen, O., J. B. Cowland, J. Askaa, and N. Borregaard. 1997. An ELISA for hCAP-18, the cathelicidin present in human neutrophils and plasma. *J. Immunol. Methods 206:53*.
26. Schaller-Bals, S., A. Schulze, and R. Bals. 2002. Increased levels of antimicrobial peptides in tracheal aspirates of newborn infants during infection. *Am. J. Respir. Crit. Care Med. 165:992*.
27. Xing, Z., T. Ohtoshi, P. Ralph, J. Gauldie, and M. Jordana. 1992. Human upper airway structural cell-derived cytokines support human peripheral blood monocyte survival: a potential mechanism for monocyte/macrophage accumulation in the tissue. *Am. J. Respir. Cell Mol. Biol. 6:212*.
28. O'Brien, D., T. O'Connor, F. Shanahan, and J. O'Connell. 2002. Activation of the p38 MAPK and ERK1/2 pathways is required for Fas-induced IL-8 production in colonic epithelial cells. *Ann. NY Acad. Sci. 973:161*.
29. Kumar, A., A. J. Knox, and A. M. Boriek. 2003. CCAAT/enhancer-binding protein and activator protein-1 transcription factors regulate the expression of interleukin-8 through the mitogen-activated protein kinase pathways in response to mechanical stretch of human airway smooth muscle cells. *J. Biol. Chem. 278:18868*.
30. Parhar, K., A. Ray, U. Steinbrecher, C. Nelson, and B. Salh. 2003. The p38 mitogen-activated protein kinase regulates interleukin-1β-induced IL-8 expression via an effect on the IL-8 promoter in intestinal epithelial cells. *Immunology 108:502*.
31. Dong, C., R. J. Davis, and R. A. Flavell. 2002. MAP kinases in the immune response. *Annu. Rev. Immunol. 20:55*.
32. Johansson, J., G. H. Gudmundsson, M. E. Rottenberg, K. D. Berndt, and B. Agerberth. 1998. Conformation-dependent antibacterial activity of the naturally occurring human peptide LL-37. *J. Biol. Chem. 273:3718*.
33. Oren, Z., J. C. Lerman, G. H. Gudmundsson, B. Agerberth, and Y. Shai. 1999. Structure and organization of the human antimicrobial peptide LL-37 in phospholipid membranes: relevance to the molecular basis for its non-cell-selective activity. *Biochem. J. 341:501*.
34. Ciornei, C. D., A. Egesten, and M. Bodelsson. 2003. Effects of human cathelicidin antimicrobial peptide LL-37 on lipopolysaccharide-induced nitric oxide release from rat aorta in vitro. *Acta Anaesthesiol. Scand. 47:213*.
35. Wang, Y., B. Agerberth, and J. Johansson. 1998. Structure and activity of cathelicidin antibacterial proteins. *J. Protein Chem. 17:522*.
36. Van Wetering, S., S. P. Mannesse-Lazeroms, J. H. Dijkman, and P. S. Hiemstra. 1997. Effect of neutrophil serine proteinases and defensins on lung epithelial cells: modulation of cytotoxicity and IL-8 production. *J. Leukocyte Biol. 62:217*.
37. Van Wetering, S., S. P. Mannesse-Lazeroms, M. A. Van Sterkenburg, M. R. Daha, J. H. Dijkman, and P. S. Hiemstra. 1997. Effect of defensins on interleukin-8 synthesis in airway epithelial cells. *Am. J. Physiol. 272:L888*.
38. Muller, W. A., and G. J. Randolph. 1999. Migration of leukocytes across endothelium and beyond: molecules involved in the transmigration and fate of monocytes. *J. Leukocyte Biol. 66:698*.
39. Metcalf, D., C. G. Begley, D. J. Williamson, E. C. Nice, J. De Lamarter, J. J. Mermod, D. Thatcher, and A. Schmidt. 1987. Hemopoietic responses in mice injected with purified recombinant murine GM-CSF. *Exp Hematol. 15:1*.
40. Tiegs, G., J. Barsig, B. Matiba, S. Uhlig, and A. Wendel. 1994. Potentiation by granulocyte macrophage colony-stimulating factor of lipopolysaccharide toxicity in mice. *J. Clin. Invest. 93:2616*.
41. Cebon, J., J. E. Layton, D. Maher, and G. Morstyn. 1994. Endogenous haemopoietic growth factors in neutropenia and infection. *Br. J. Haematol. 86:265*.
42. Sheridan, J. W., and D. Metcalf. 1972. Studies on the bone marrow colony stimulating factor (CSF): relation of tissue CSF to serum CSF. *J. Cell Physiol. 80:129*.
43. Hamilton, J. A. 2002. GM-CSF in inflammation and autoimmunity. *Trends Immunol. 23:403*.
44. Agerberth, B., J. Charo, J. Werr, B. Olsson, F. Idali, L. Lindbom, R. Kiessling, H. Jornvall, H. Wigzell, and G. H. Gudmundsson. 2000. The human antimicrobial and chemotactic peptides LL-37 and α-defensins are expressed by specific lymphocyte and monocyte populations. *Blood 96:3086*.
45. Agerberth, B., J. Grunewald, E. Castanos-Velez, B. Olsson, H. Jornvall, H. Wigzell, A. Eklund, and G. H. Gudmundsson. 1999. Antibacterial components in bronchoalveolar lavage fluid from healthy individuals and sarcoidosis patients. *Am. J. Respir. Crit. Care Med. 160:283*.
46. Frohm, M., B. Agerberth, G. Ahangari, M. Stahle-Backdahl, S. Liden, H. Wigzell, and G. H. Gudmundsson. 1997. The expression of the gene coding for the antibacterial peptide LL-37 is induced in human keratinocytes during inflammatory disorders. *J. Biol. Chem. 272:15258*.
47. Hamilton, J. A. 1993. Colony stimulating factors, cytokines and monocyte-macrophages: some controversies. *Immunol. Today 14:18*.
48. Murakami, M., T. Ohtake, R. A. Dorschner, B. Schittek, C. Garbe, and R. L. Gallo. 2002. Cathelicidin anti-microbial peptide expression in sweat, an innate defense system for the skin. *J. Invest. Dermatol. 119:1090*.
49. Scott, M. G., and R. E. W. Hancock. 2000. Cationic antimicrobial peptides and their multifunctional role in the immune system. *Crit. Rev. Immunol. 20:407*.
50. Davidson, D. J., A. J. Currie, G. S. Reid, D. M. Bowdish, K. L. MacDonald, R. C. Ma, R. E. Hancock, and D. P. Speert. 2004. The cationic antimicrobial peptide LL-37 modulates dendritic cell differentiation and dendritic cell-induced T cell polarization. *J. Immunol. 172:1146*.