

The antimicrobial peptide LL-37 enhances IL-8 release by human airway smooth muscle cells

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Background: Human airway smooth muscle (HASM) cells release various chemokines that are involved in recruitment of inflammatory cells, which can be found within or in the vicinity of the airway smooth muscle layer in patients with inflammatory lung diseases. Inflammatory cells contain antimicrobial peptides including the cathelicidin LL-37 and neutrophil defensins (HNP1-3).

Objective: The aim of the study was to determine the effects of antimicrobial peptides on IL-8 (CXC chemokine ligand 8) release by HASM cells, and to study the underlying mechanisms.

Methods: Human airway smooth muscle cells were stimulated with LL-37 and HNP1-3, and IL-8 protein and mRNA levels were determined by sandwich ELISA and PCR.

Phosphorylation of extracellular signal-regulated kinase (ERK) 1/2 was detected by using Western blot.

Results: LL-37 enhanced IL-8 release by HASM cells, which was dependent on ERK1/2 activation. Receptors known to be involved in LL-37-induced signaling, including the epidermal growth factor receptor and formyl peptide receptors, were not involved in LL-37 signaling in HASM cells. The purinergic receptor antagonist suramin did block LL-37-induced ERK1/2 phosphorylation and IL-8 release, and expression of mRNA for the purinergic receptor P2X₇ was detected in HASM cells. HNP1-3 did increase ERK1/2 phosphorylation, but did not enhance IL-8 release by HASM cells.

Conclusion: These data show that HASM cells respond to the antimicrobial peptide LL-37 by releasing IL-8, suggesting that LL-37 is a regulator of the inflammatory process in various inflammatory lung diseases by enhancing IL-8 production.

Clinical implications: LL-37 released by inflammatory cells may amplify inflammation through induction of IL-8 release by airway smooth muscle. (*J Allergy Clin Immunol* 2006;117:1328-35.)

Key words: Human airway smooth muscle cells, asthma, chronic obstructive pulmonary disease, inflammation, antimicrobial peptides, chemokines, IL-8, extracellular signal-regulated kinase

Human airway smooth muscle (HASM) cells are the main contractile elements in the airway wall, and are the functional basis for the bronchoconstriction observed in asthma. It is increasingly recognized that inflammatory cells colocalize with smooth muscle cells in the bronchial wall in several airway diseases. In asthma, increased numbers of mast cells are found in the airway smooth muscle layer, whereas in chronic obstructive pulmonary disease (COPD), this layer is infiltrated by neutrophils.¹⁻³ This colocalization suggests that these cells may interact with each other through release of mediators. Studies have shown that mediators released from mast cells affect HASM function, whereas HASM products may act in recruitment and retention of mast cells (see reviews^{4,5}). Tryptase, for instance, is mitogenic for HASM cells, whereas TNF- α and IL-1 β released by mast cells stimulate production of proinflammatory cytokines and chemokines by HASM cells. Supernatants from HASM cells stimulated with TNF- α , IL-1 β , and IFN- γ are chemotactic for mast cells through activation of the IP10 (CXC chemokine ligand [CXCL] 10)/CXC chemokine receptor (CXCR) 3 axis.⁶ Neutrophils and HASM cells may also interact; neutrophil elastase alters the survival of HASM cells by inducing apoptosis.⁷

Mast cells and neutrophils contain many mediators including antimicrobial peptides, which are involved in host defense of the airways. Both cell types express the human cationic antimicrobial protein (hCAP18)/LL-37, whereas neutrophils also contain neutrophil defensins.⁸⁻¹⁰ hCAP18/LL-37, the only human member of the family of cathelicidins,¹¹ is present in high concentrations in the specific granules of neutrophils, and is cleaved on release by proteinase 3 to form the active peptide LL-37.¹² Defensins are the other family of antimicrobial peptides important in host defense of the airways. Human defensins are small cationic peptides that are subdivided in 2 groups on the basis of their structure: the α -defensins (human neutrophil peptides [HNPs] 1-4, produced by neutrophils, and human defensins 5 and 6, expressed by Paneth cells and epithelial cells of the female reproductive tract), and the β -defensins (mainly expressed by epithelial cells).

In addition to their antimicrobial activity, LL-37 and defensins have been shown to display other activities. Defensins are involved in modulation of inflammatory responses, and regulation of wound repair.¹³⁻¹⁵ LL-37 can induce chemotaxis of inflammatory cells,^{16,17}

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Abbreviations used

COPD:	Chronic obstructive pulmonary disease
EGFR:	Epidermal growth factor receptor
ERK:	Extracellular signal-regulated kinase
FPRL-1:	Formyl peptide receptor-like 1
HASM:	Human airway smooth muscle
hCAP18:	Human cationic antimicrobial protein (18 kd)
HNP:	Human neutrophil peptide
MAPK:	Mitogen-activated protein kinase
tBoc-MLP:	Tert-butoxy-carbonyl-methionyl-leucyl-phenylalanine

stimulate angiogenesis,¹⁸ stimulate airway epithelial wound closure,¹⁹ and activate airway epithelial cells²⁰ through various receptors. LL-37–induced chemotaxis is dependent on the G protein–coupled formyl peptide receptors, whereas LL-37 induces processing and release of IL-1 β by monocytes through the purinergic P2X₇ receptor.²¹ Previously, we have shown that LL-37 activates mitogen-activated protein kinases (MAPKs) and enhances IL-8 (CXC ligand 8) release by epithelial cells.²⁰ This seems to be mediated through a receptor other than formyl peptide receptor-like 1 (FPRL-1), because inhibitors of formyl peptide receptors did not block extracellular signal-regulated kinase (ERK) 1/2 activation or chemokine release. Instead, LL-37 was suggested to transactivate the epidermal growth factor receptor (EGFR) by increasing metalloproteinase-mediated shedding of ligands for the EGFR from the cell membrane. A study with keratinocytes has shown that LL-37–induced migration of these cells is also dependent on transactivation of the EGFR.²² These data suggest that LL-37 may function through different mechanisms on different cell types. The receptors used by the neutrophil defensins to activate airway epithelial cells are not known.

Human airway smooth muscle cells have been shown to secrete a variety of inflammatory mediators such as cytokines and chemokines in response to stimuli, including proinflammatory cytokines and T_H2 cytokines.^{23,24} Previously, we have shown that HASM cells release the eosinophil-selective chemokines eotaxin (CC chemokine ligand [CCL] 11) and eotaxin-3 (CCL26) after stimulation with T_H2 cytokines IL-4 and IL-13.²⁵ Release of these chemokines was differentially regulated by the fibrotic cytokine TGF- β . HASM cells also produce IL-8, a chemokine that attracts neutrophils, eosinophils, T lymphocytes, and monocytes, in response to inflammatory mediators.²⁶ Several studies have shown that chemokine release by HASM cells is dependent on activation of the MAPKs ERK1/2, p38, and stress-activated protein kinase (SAPK)/c-Jun N-terminal kinase.²⁷⁻²⁹

On the basis of our earlier observations on the effects of LL-37 and HNP1-3 on chemokine release by epithelial cells,^{14,20} the increasingly recognized role of HASM as a source of chemokines, and the colocalization of inflammatory cells and HASM cells, we hypothesized that LL-37 and HNP1-3 may activate airway smooth muscle function

with respect to chemokine production. Therefore, we investigated the effects of LL-37 and HNP1-3 on IL-8 release by HASM cells, and studied the involvement of receptors and intracellular signaling pathways.

METHODS

Reagents

Synthetic LL-37 and HNP1-3 isolated from neutrophil granules were prepared under LPS-free conditions and characterized as previously described.^{20,30} For reagents used to study signal transduction pathways and receptors, refer to this article's [Methods](#) section in the Online Repository at www.jacionline.org.

Cell culture and stimulation

Human airway smooth muscle cells were purchased from Stratagene (La Jolla, Calif). Cells were characterized by staining for α -smooth muscle actin. Cells were cultured in Dulbecco's Modified Eagle Medium: F-12 Nutrient Mixture (DMEM/F-12) 1:1 mixture (Invitrogen, Carlsbad, Calif) containing 25 mmol/L HEPES (Invitrogen), 10% (vol/vol) FCS (Invitrogen) supplemented with 2.5 mmol/L L-glutamine (Cambrex, East Rutherford, NJ), 1% (vol/vol) nonessential amino acids (Invitrogen), 50 U/mL penicillin, and 50 μ g/mL streptomycin (Cambrex). Cells were seeded at 5000 cells per cm², and medium was replaced every 72 hours. Confluent cells from passage 5 to 7 were used for experiments. Cells from 2 different donors were used in all experiments.

Before use, confluent cells were washed with PBS and cultured in serum-free Dulbecco's Modified Eagle Medium: F-12 Nutrient Mixture (D-MEM/F-12) 1:1 mixture containing 1% (vol/vol) Insulin-Transferrin-Selenium-A liquid media supplement (stock: 1 g/L insulin, 0.55 g/L transferrin, 0.67 mg/L sodium selenite; Invitrogen) for 24 hours. When inhibitors were used, cells were preincubated with these inhibitors 1 hour before stimulation.

Measurement of IL-8 release

Supernatants were harvested after 24 hours of stimulation. IL-8 protein levels were detected by using the hu-IL-8 cytoset from Biosource Europe SA (Nivelles, Belgium).

PCR

After 6 hours of stimulation, RNA was isolated by using the RNeasy Minikit (Qiagen, Valencia, Calif) according to manufacturer's instructions. The RNA concentration and purity were assessed by using OD measurements. Reverse-transcription of 1 μ g total RNA was performed by using Moloney-Murine Leukemia virus reverse transcriptase (Invitrogen) as described previously.²⁵

For primers used to detect IL-8, P2X₇, and β -actin mRNA and PCR conditions, refer to this article's Online Repository at www.jacionline.org. PCR products were visualized by electrophoresis on a 1.5% agarose gel containing ethidium bromide as a fluorescent dye and subsequent UV luminescence.

3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT) assay

For the protocol used for the mitochondrial metabolic activity assay, refer to this article's [Methods](#) section in the Online Repository at www.jacionline.org.

Gel electrophoresis and Western blotting

For detection of ERK1/2 phosphorylation, HASM cells were cultured until 90% confluence and subsequently starved for growth

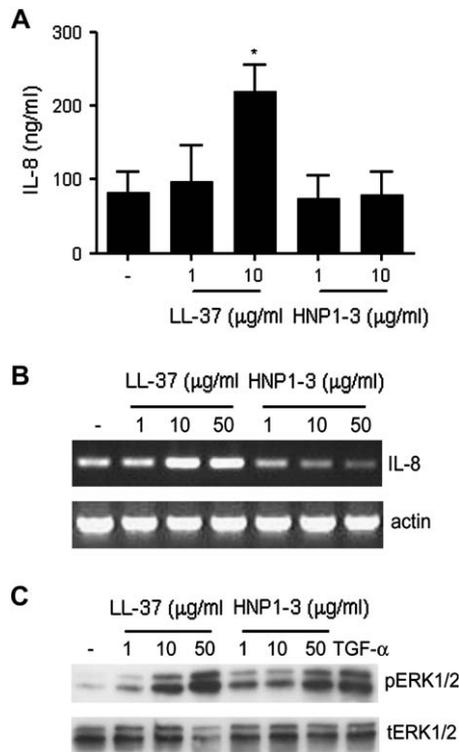


FIG 1. Cells were stimulated with various concentrations of LL-37 and HNP1-3. **A**, IL-8 protein levels, mean \pm SEM of 4 independent experiments in 2 donors, each experiment performed in triplicate. * $P < .05$, compared with medium-treated cells. **B**, Representative PCR for IL-8 and β -actin. **C**, representative blot for ERK1/2. Similar results were obtained in experiments using cells from another donor. pERK1/2, Phospho ERK1/2; tERK1/2, total ERK1/2.

factors for 24 hours. Cells were incubated with LL-37, HNP1-3, and TGF- α for 5 minutes. Inhibitors of several pathways were incubated 1 hour before stimulation. After stimulation, cells were washed with washing buffer (5 mmol/L Tris-HCL pH 7.4, 100 mmol/L NaCl, 1 mmol/L MgCl₂, 1 mmol/L CaCl₂) and lysed with lysis buffer (0.5% [vol/vol] Triton-X100, 1 mmol/L Na₃VO₄, and complete protease inhibitor cocktail [Roche, Basel, Switzerland] in washing buffer) on ice for 10 minutes. Cells were scraped and collected. After centrifugation for 5 minutes at 13,000 rpm, supernatants were collected and stored at -20°C until further use. Protein concentrations were measured by the bicinchoninic acid protein assay system (Pierce, Rockford, Ill). Three micrograms of total protein was separated on a 10% glycine-based gel using the Mini-protean 3 (Biorad, Hercules, Calif) SDS-PAGE system. Proteins were transferred to a polyvinylidene difluoride membrane (Biorad). Nonspecific binding sites were blocked (0.5% [wt/vol] casein, 0.05% Tween, PBS), and blots were incubated with rabbit polyclonal antibodies against phosphorylated and total ERK1/2. A secondary horseradish peroxidase-conjugated goat-antirabbit antibody together with the ECL Western Blotting Analysis system (Amersham Biosciences, Little Chalfont, United Kingdom) was used to visualize immunoreactivity.

Statistical analysis

Statistical analysis was performed by using a paired samples *t* test. Differences were considered to be significant when *P* values were smaller than .05.

RESULTS

LL-37 enhances IL-8 release and ERK1/2 phosphorylation

To study the effect of LL-37 and HNP1-3 on chemokine release, serum-deprived HASM cells were incubated with 1 and 10 $\mu\text{g}/\text{mL}$ LL-37 and HNP1-3. LL-37 enhanced IL-8 release by HASM cells, whereas HNP1-3 did not (Fig 1, A). Using a higher concentration of LL-37 or HNP1-3 (50 $\mu\text{g}/\text{mL}$), IL-8 release was not increased compared with the levels found with 10 $\mu\text{g}/\text{mL}$ peptide (data not shown). At the mRNA level, IL-8 was increased by LL-37 at 10 and 50 $\mu\text{g}/\text{mL}$, whereas HNP1-3 slightly reduced the IL-8 mRNA levels at 10 and 50 $\mu\text{g}/\text{mL}$ (Fig 1, B).

To study the effect of LL-37 on activation of the MAPK ERK1/2, HASM cells were stimulated with 1, 10, and 50 $\mu\text{g}/\text{mL}$ of LL-37 or HNP1-3 for 5 minutes. In a time course experiment, 5 minutes was found to be the most optimal time for detection of ERK1/2 phosphorylation (data not shown). TGF- α was used as a positive control for ERK1/2 phosphorylation. Both LL-37 and HNP1-3 induced ERK1/2 activation in a dose-dependent manner (Fig 1, C).

Cytotoxic activity of LL-37 and HNP1-3 on HASM cells

The effects of LL-37 and HNP1-3 on viability of HASM cells were determined by measuring mitochondrial metabolic activity using the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT) assay. Concentrations of LL-37 as high as 10 $\mu\text{g}/\text{mL}$ did not alter mitochondrial activity, whereas at the higher concentration of 50 $\mu\text{g}/\text{mL}$ activity was reduced. HNP1-3 did not reduce mitochondrial activity at any of the concentrations used (see this article's Fig E1 in the Online Repository at www.jacionline.org).

LL-37-induced ERK1/2 activation and IL-8 release are not dependent on transactivation of the EGFR

In airway epithelial cells, it has been shown that LL-37 activates ERK1/2 through metalloprotease-mediated transactivation of the EGFR.²⁰ Therefore, we studied the effect of inhibitors of this pathway on LL-37-induced ERK1/2 activation in HASM cells. Cells were preincubated with inhibitors for 1 hour and subsequently stimulated with LL-37 for 5 minutes. In 3 independent experiments, the tyrosine kinase inhibitor AG1478, which inhibits phosphorylation of the EGFR and subsequent signal transduction, did not markedly affect LL-37-induced ERK1/2 activation (Fig 2, A). In contrast, ERK1/2 activation induced by an excess of TGF- α was largely inhibited by AG1478, showing that the inhibitor was functional (Fig 2, B). The MAPK kinase (MEK) inhibitor U0126 did inhibit ERK1/2 activation by LL-37. GM6001, a broad-spectrum metalloprotease inhibitor that blocks metalloproteases involved in cleavage of ligands for the

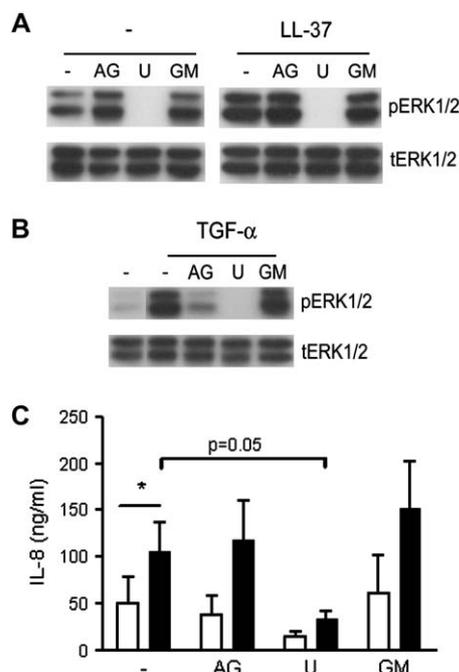


FIG 2. Cells were preincubated for 1 hour with inhibitors and stimulated with 10 μ g/mL LL-37. **A**, Representative blot of LL-37–induced ERK1/2 activation. Similar results were obtained by using cells from another donor. **B**, Blot of TGF- α –induced ERK1/2 activation. **C**, IL-8 protein levels, mean \pm SEM of 4 independent experiments performed in triplicate using cells from 2 donors. * $P < .05$, paired samples t test. AG, AG1478; U, U0126; GM, GM6001; pERK1/2, phospho ERK1/2; tERK1/2, total ERK1/2.

EGFR, did not affect LL-37–induced ERK1/2 activation (Fig 2, A).

To study the effect of the inhibitors of the EGFR pathway on IL-8 release, HASM cells were incubated with AG1478, U0126, and GM6001 for 1 hour. Subsequently, cells were stimulated with 10 μ g/mL LL-37 for 24 hours, supernatants were harvested, and IL-8 levels were measured. LL-37–induced IL-8 levels were not altered by AG1478 or GM6001, suggesting that transactivation of the EGFR by cleavage of ligands through metalloprotease activity is not involved in LL-37–induced IL-8 release. In contrast, the MEK inhibitor U0126 caused a marked inhibition ($P = .05$) of LL-37–induced IL-8 release, suggesting that ERK1/2 is involved (Fig 2, C).

LL-37–induced ERK1/2 activation and IL-8 release are not dependent on activation of formyl peptide receptors

The involvement of formyl peptide receptors in LL-37–induced ERK1/2 activation was studied by preincubating the cells with the antagonistic peptide tert-butoxy-carbonyl-methionyl-leucyl-phenylalanine (tBoc-MLP). Pretreatment of cells with tBoc-MLP did not affect LL-37–induced ERK1/2 activation, suggesting that formyl peptide receptors are not involved in LL-37–induced ERK1/2 activation (Fig 3, A). The inhibitory activity of the tBoc-MLP preparation used in the current study was confirmed in experiments on formyl-methionyl-leucyl-

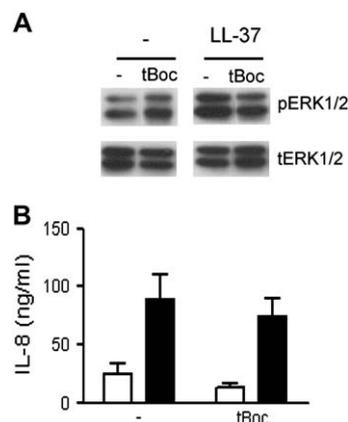


FIG 3. Cells were preincubated with tBoc-MLP for 1 hour and stimulated with 10 μ g/mL LL-37. **A**, Representative blot of ERK1/2 activation. Similar results were obtained by using cells from another donor. **B**, IL-8 protein levels, mean \pm SD of 2 independent experiments performed in triplicate using cells from 2 donors. pERK1/2, Phospho ERK1/2; tERK1/2, total ERK1/2.

phenylalanine (fMLP)–induced neutrophil chemotaxis (data not shown³¹). To study the involvement of formyl peptide receptors in IL-8 release, we incubated cells with tBoc-MLP for 1 hour; subsequently, cells were stimulated with 10 μ g/mL LL-37 for 24 hours. The antagonistic peptide tBoc-MLP did not affect LL-37–induced IL-8 release, suggesting that formyl peptide receptors are not involved (Fig 3, B).

Inhibitors of p38, Src, and purinergic receptors block LL-37–induced IL-8 release

We did not observe any effect of the inhibitors of p38 (SB230580), Src (PP1), and phosphoinositide 3-kinase (LY294002) pathways on LL-37–induced ERK1/2 activation. However, suramin (a P2 receptor antagonist) did inhibit basal and LL-37–induced ERK1/2 activation (Fig 4, A).

Both SB230580 and PP1 inhibited LL-37–induced IL-8 release, suggesting the involvement of p38 and Src in LL-37–induced IL-8 release (Fig 4, B). Suramin, the antagonist for purinergic receptors, also inhibited LL-37–induced IL-8 release.

To determine whether HASM cells express the purinergic receptor P2X₇ that has been implicated in LL-37–induced monocyte activation,²¹ mRNA expression of P2X₇ on HASM cells was studied by using PCR. This analysis, using monocyte cDNA as a positive control, demonstrated the presence of P2X₇ mRNA in HASM cells from 2 different donors (Fig 4, C).

DISCUSSION

In the current study, we explored whether LL-37 and neutrophil defensins activate HASM cells with respect to chemokine release. Our study shows for the first time that LL-37 enhances IL-8 release in HASM cells. LL-37–induced IL-8 release was not dependent on transactivation

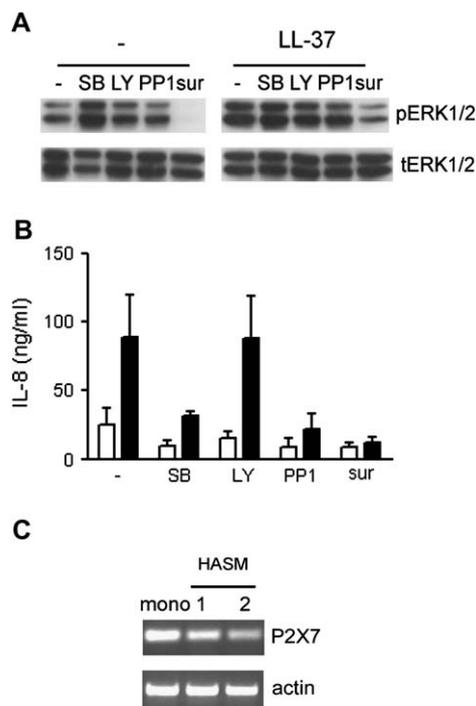


FIG 4. Cells were preincubated with inhibitors and stimulated with 10 $\mu\text{g}/\text{mL}$ LL-37. **A**, Representative blot of ERK1/2 activation. Similar results were obtained by using cells from another donor. **B**, IL-8 protein levels, mean \pm SD of 2 independent experiments performed in triplicate using cells from 2 donors. **C**, PCR for P2X₇ and β -actin on cDNA from monocytes (mono) and HASM cells from 2 donors. SB, SB230580; LY, LY294002; sur, suramin; pERK1/2, phospho ERK1/2; tERK1/2, total ERK1/2.

of the EGFR or activation of formyl peptide receptors. Experiments using inhibitors of several intracellular pathways showed that LL-37-induced IL-8 release is partly dependent on ERK1/2, p38, and Src. Furthermore, we found that suramin, an antagonist of purinergic receptors, inhibited IL-8 release, suggesting a role for these receptors in LL-37-induced IL-8 release. In line with this, we demonstrated by PCR analysis that HASM cells express the purinergic receptor P2X₇. In contrast with the effects of LL-37, HNP1-3 did not enhance IL-8 protein or mRNA levels.

To our knowledge, this is the first study showing an effect of an antimicrobial peptide on HASM cells. A study with vascular smooth muscle cells showed that α -defensin (HNP3) inhibits phenylephrine-induced contraction of rat aortic rings through the low-density lipoprotein receptor-related protein/ α_2 -macroglobulin receptor.³² Our study shows that the antimicrobial peptide LL-37, which is considered to be the major processing peptide product from hCAP-18, activates ERK1/2 in airway smooth muscle cells and enhances IL-8 release by these cells. It is likely that HASM cells are exposed to LL-37 in lung tissue, because cells that produce hCAP18/LL-37 (mast cells and neutrophils) are present in the vicinity of airway smooth muscle bundles as illustrated in Fig 5. At the concentration that induces activation (10 $\mu\text{g}/\text{mL}$), LL-37

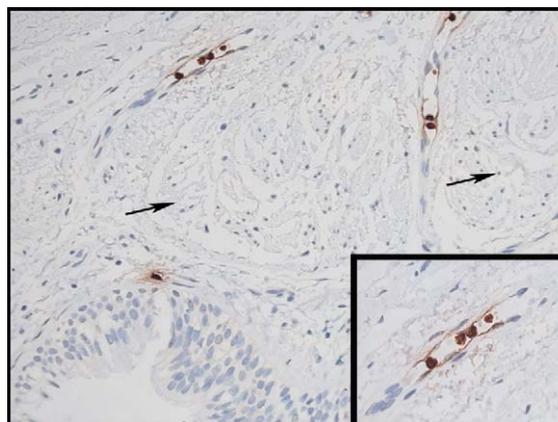


FIG 5. hCAP18/LL-37-expressing cells surrounding the airway smooth muscle bundles in bronchial tissue. A section of a bronchial ring was stained by using a monoclonal anti-LL-37 antibody (clone 1.1C12²⁰). Immunoreactive (red) cells surround the airway smooth muscle bundles (arrows). Inserted image shows high-power magnification of hCAP18/LL-37-expressing cells.

does not affect morphology or mitochondrial activity. However, at a higher concentration (50 $\mu\text{g}/\text{mL}$), cytotoxic effects were observed. As previously discussed, the concentrations used in the current study may be relevant to the *in vivo* situation in lung tissue.²⁰

In our study, in contrast with LL-37, HNP1-3 did not enhance IL-8 release by HASM cells, and IL-8 mRNA expression was slightly decreased after stimulation with HNP1-3. However, both LL-37 and HNP1-3 induced activation of ERK1/2. Because ERK1/2 activation is involved in a wide variety of cellular activities, we suggest that HNP1-3 may stimulate processes other than protein synthesis in HASM cells. In epithelial cells and fibroblasts, a role in proliferation, wound repair, and production of extracellular matrix has been established for HNP1-3,^{15,30,33} whereas in vascular smooth muscle cells, a role for these defensins in reducing phenylephrine-induced contraction has been established.³² Therefore, we speculate that HNP1-3 are involved in regulation of contraction, proliferation, and/or matrix synthesis of HASM cells. The receptors involved in the HNP1-3-induced ERK1/2 activation are unknown. Recently, it was shown that P2Y₆ is involved in defensin-induced IL-8 release by epithelial cells.³⁴ Interestingly, in HASM cells activation of purinergic receptors by UTP and ATP increases intracellular calcium concentrations, suggesting a role for these receptors in contraction.³⁵ Whether P2Y receptors are involved in defensin-induced ERK1/2 activation and regulate contraction and other cellular activities in HASM cells needs further investigation.

Several receptors have been implicated in the cellular response to LL-37, and we have addressed their role in LL-37-induced activation of HASM cells. The first receptor type identified in the chemotactic response of cells to LL-37 is the formyl peptide receptor FPRL-1.^{17,31} We observed that the formyl peptide receptor antagonistic peptide tBoc-MLP did not reduce LL-37-induced

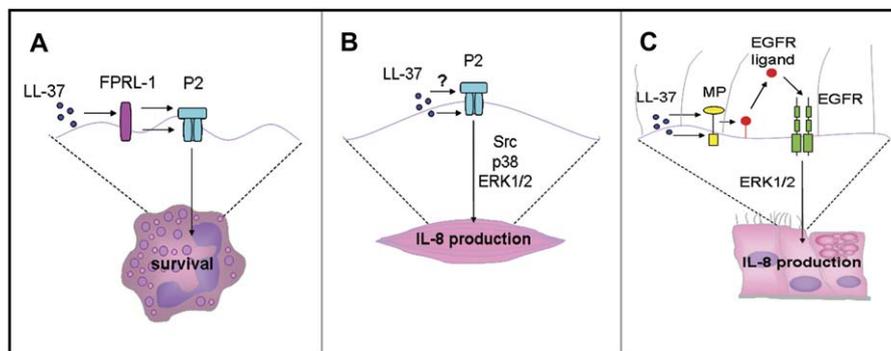


FIG 6. Comparison of putative mechanisms involved in activation of neutrophils, airway smooth muscle cells, and epithelial cells by LL-37. **A**, LL-37 activates FPRL-1, which subsequently activates P2₇ and promotes survival of neutrophils. **B**, LL-37 activates Src, p38, and ERK1/2 pathways via a P2 receptor leading to release of IL-8 by airway smooth muscle cells. **C**, LL-37 transactivates the EGFR via a metalloprotease (MP) leading to ERK1/2 activation and IL-8 release in epithelial cells.

ERK1/2 activation or IL-8 release by HASM cells, indicating that this receptor is not involved. We have previously demonstrated the involvement of the EGFR in activation of airway epithelial cells by LL-37.²⁰ In these cells, LL-37–induced ERK1/2 activation and IL-8 release were dependent on metalloprotease-dependent shedding of membrane-bound EGFR ligands, which subsequently activate the EGFR. However, in HASM cells, LL-37–induced ERK1/2 activation and IL-8 release were not affected by the EGFR tyrosine kinase inhibitor AG1478 or by the metalloprotease inhibitor GM6001, indicating that transactivation of the EGFR does not play a role in the activation of HASM cells by LL-37. Finally, purinergic receptors that mediate signaling by extracellular nucleotides such as ATP were reported to be involved in activation of monocytes and neutrophils,^{21,36} and of the lung epithelial cell line A549 by neutrophil defensins.³⁴ Our observations that suramin inhibits LL-37–induced activation of HASM cells, and that these cells express mRNA for the purinergic receptor P2X₇, suggest involvement of purinergic receptors in activation of HASM cells by LL-37. Activation of the FPRL-1 receptor is upstream of activation of P2X₇ receptor in the effect of LL-37 on neutrophil survival. Because FPRL-1 is not involved in the effect of LL-37 on HASM cells, the pathways upstream of P2 activation in HASM cells remain to be identified (Fig 6).

These results illustrate that the mechanism used by LL-37 to activate cells may differ between different cell types. Although the aforementioned receptors may mediate downstream signaling events after exposure to LL-37, it is not known whether the initial interaction between LL-37 and a target cell is receptor-dependent. Recently, it was shown that D-LL-37 also induces high levels of IL-8 protein release from keratinocytes, suggesting that at least in these cells, structure-dependent binding of LL-37 to a cell surface receptor is not likely to be involved in cell activation.³⁷

We have shown that the p38 inhibitor SB203580 and the Src inhibitor PP1 inhibited LL-37–enhanced IL-8 release. Studies with several cell types including human colonic

smooth muscle cells have shown that both Src and p38 are involved in cytokine-induced expression of IL-8 mRNA and protein,^{38,39} perhaps by stabilizing the IL-8 mRNA.⁴⁰ Although not much is known about the mechanisms involved in LL-37–induced IL-8 release, studies in airway epithelial cells have shown that LL-37 induces phosphorylation of p38.^{20,41} Furthermore, in lung epithelial cells, it has been shown that Src family kinases can regulate the MAPKs p38 and ERK1/2 and subsequent IL-8 expression.⁴² Our results show that inhibitors of ERK1/2, p38, and Src reduce LL-37–induced IL-8 release by HASM cells, suggesting that these 3 pathways act together to induce IL-8 release (Fig 6). Src may function upstream of p38 and ERK1/2 activation, although in our experiments, the Src inhibitor PP1 did not affect ERK1/2 activation. How LL-37 activates these 3 pathways and how they interconnect to mediate IL-8 production need further investigation.

Activation of P2X receptors induces ERK1/2 activation in several cell types, including rat astrocytes.⁴³ In human astrocytoma cells, ERK1/2 was activated by P2X₇ receptors through a cellular pathway that is dependent on c-Src, PI3K, and MEK1/2.⁴⁴ In human and rodent macrophages, P2X₇ activates the MAPK SAPK/c-Jun N-terminal kinase.⁴⁵ These studies suggest that the P2X₇ receptor is capable of activating multiple pathways leading to ERK1/2 activation. Our results using suramin suggest that P2 receptors are involved in LL-37–induced IL-8 release, which is dependent on p38, Src, and ERK1/2. How these pathways are connected in this process remains to be elucidated. In regard to the effect of suramin on LL-37–induced ERK1/2 activation and IL-8 release, whereas suramin is used as a P2 receptor inhibitor, it is not selective or very potent,⁴⁶ because it interacts with various proteins.⁴⁷ Suramin was shown to inhibit binding of growth factors (eg, epidermal growth factor, platelet-derived growth factor, and TGF- β) to their receptors.⁴⁸ Therefore, we cannot exclude the possibility that suramin inhibits other receptors than the P2 receptors in LL-37–induced IL-8 release.

Our results demonstrate that LL-37 activates neutrophils, epithelial cells, and HASM cells by common mechanisms using activation of P2 receptors and ERK1/2 pathways. However, whereas neutrophil activation by LL-37 is characterized by the involvement of FPRL-1, and epithelial cell activation by transactivation of the EGFR, a distinct mechanism appears to operate in HASM cells (Fig 6).

Recent studies and our own implicate LL-37 as a regulator of inflammation by activating chemokine release by airway epithelial cells,²⁰ keratinocytes,³⁷ and monocytes,⁴¹ and as a novel finding, HASM cells. Our results underline the role of LL-37 as a peptide important for activating inflammatory processes. LL-37 produced by mast cells and neutrophils present in the vicinity of the airway smooth muscle layer from patients with asthma and COPD may increase local IL-8 production and thereby enhance neutrophilia, eosinophilia, and mast cell numbers. We speculate that these events might contribute to exacerbations of asthma and COPD.

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REFERENCES

- Brightling CE, Bradding P, Symon FA, Holgate ST, Wardlaw AJ, Pavord ID. Mast-cell infiltration of airway smooth muscle in asthma. *N Engl J Med* 2002;346:1699-705.
- Carroll NG, Mutavdzic S, James AL. Distribution and degranulation of airway mast cells in normal and asthmatic subjects. *Eur Respir J* 2002;19:879-85.
- Baraldo S, Turato G, Badin C, Bazzan E, Beghe B, Zuin R, et al. Neutrophilic infiltration within the airway smooth muscle in patients with COPD. *Thorax* 2004;59:308-12.
- Page S, Ammit AJ, Black JL, Armour CL. Human mast cell and airway smooth muscle cell interactions: implications for asthma. *Am J Physiol Lung Cell Mol Physiol* 2001;281:L1313-23.
- Robinson DS. The role of the mast cell in asthma: induction of airway hyperresponsiveness by interaction with smooth muscle? *J Allergy Clin Immunol* 2004;114:58-65.
- Brightling CE, Ammit AJ, Kaur D, Black JL, Wardlaw AJ, Hughes JM, et al. The CXCL10/CXCR3 axis mediates human lung mast cell migration to asthmatic airway smooth muscle. *Am J Respir Crit Care Med* 2005;171:1103-8.
- Oltmanns U, Sukkar MB, Xie S, John M, Chung KF. Induction of human airway smooth muscle apoptosis by neutrophils and neutrophil elastase. *Am J Respir Cell Mol Biol* 2005;32:334-41.
- Di Nardo A, Vitiello A, Gallo RL. Cutting edge: mast cell antimicrobial activity is mediated by expression of cathelicidin antimicrobial peptide. *J Immunol* 2003;170:2274-8.
- Sorensen O, Amljots K, Cowland JB, Bainton DF, Borregaard N. The human antibacterial cathelicidin, hCAP-18, is synthesized in myelocytes and metamyelocytes and localized to specific granules in neutrophils. *Blood* 1997;90:2796-803.
- Lehrer RI, Lichtenstein AK, Ganz T. Defensins: antimicrobial and cytotoxic peptides of mammalian cells. *Annu Rev Immunol* 1993;11:105-28.
- Tjabringa GS, Rabe KF, Hiemstra PS. The human cathelicidin LL-37: a multifunctional peptide involved in infection and inflammation in the lung. *Pulm Pharmacol Ther* 2005;18:321-7.
- Sorensen OE, Follin P, Johnsen AH, Calafat J, Tjabringa GS, Hiemstra PS, et al. Human cathelicidin, hCAP-18, is processed to the antimicrobial peptide LL-37 by extracellular cleavage with proteinase 3. *Blood* 2001;97:3951-9.
- Van Wetering S, Tjabringa GS, Hiemstra PS. Interactions between neutrophil-derived antimicrobial peptides and airway epithelial cells. *J Leukoc Biol* 2005;77:444-50.
- Van Wetering S, Manesse-Lazeroms SP, van Sterkenburg MA, Daha MR, Dijkman JH, Hiemstra PS. Effect of defensins on interleukin-8 synthesis in airway epithelial cells. *Am J Physiol* 1997;272:L888-96.
- Aarbiou J, Verhoosel RM, Van Wetering S, de Boer WI, van Krieken JH, Litvinov SV, et al. Neutrophil defensins enhance lung epithelial wound closure and mucin gene expression in vitro. *Am J Respir Cell Mol Biol* 2004;30:193-201.
- Zanetti M. Cathelicidins, multifunctional peptides of the innate immunity. *J Leukoc Biol* 2004;75:39-48.
- Yang D, Chen Q, Schmidt AP, Anderson GM, Wang JM, Wooters J, et al. 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* 2000;192:1069-74.
- Koczulla R, von Degenfeld G, Kupatt C, Krotz F, Zahler S, Gloe T, et al. An angiogenic role for the human peptide antibiotic LL-37/hCAP-18. *J Clin Invest* 2003;111:1665-72.
- Shaykhiyev R, Beisswenger C, Kandler K, Senske J, Puchner A, Damm T, et al. Human endogenous antibiotic LL-37 stimulates airway epithelial cell proliferation and wound closure. *Am J Physiol Lung Cell Mol Physiol* 2005;289:L842-8.
- Tjabringa GS, Aarbiou J, Ninaber DK, Drijfhout JW, Sorensen OE, Borregaard N, et al. The antimicrobial peptide LL-37 activates innate immunity at the airway epithelial surface by transactivation of the epidermal growth factor receptor. *J Immunol* 2003;171:6690-6.
- Elssner A, Duncan M, Gavrilin M, Wewers MD. A novel P2X7 receptor activator, the human cathelicidin-derived peptide LL37, induces IL-1 beta processing and release. *J Immunol* 2004;172:4987-94.
- Tokumaru S, Sayama K, Shirakata Y, Komatsuzawa H, Ouhara K, Hanakawa Y, et al. Induction of keratinocyte migration via transactivation of the epidermal growth factor receptor by the antimicrobial peptide LL-37. *J Immunol* 2005;175:4662-8.
- Hirst SJ. Regulation of airway smooth muscle cell immunomodulatory function: role in asthma. *Respir Physiol Neurobiol* 2003;137:309-26.
- Lazaar AL, Panettieri RA Jr. Airway smooth muscle as an immunomodulatory cell: a new target for pharmacotherapy? *Curr Opin Pharmacol* 2001;1:259-64.
- Zuyderduyn S, Hiemstra PS, Rabe KF. TGF-beta differentially regulates TH2 cytokine-induced eotaxin and eotaxin-3 release by human airway smooth muscle cells. *J Allergy Clin Immunol* 2004;114:791-8.
- Vanaudenaerde BM, Wuys WA, Dupont LJ, Van Raemdonck DE, Demedts MM, Verleden GM. Interleukin-17 stimulates release of interleukin-8 by human airway smooth muscle cells in vitro: a potential role for interleukin-17 and airway smooth muscle cells in bronchiolitis obliterans syndrome. *J Heart Lung Transplant* 2003;22:1280-3.
- Moore PE, Church TL, Chism DD, Panettieri RA Jr, Shore SA. IL-13 and IL-4 cause eotaxin release in human airway smooth muscle cells: a role for ERK. *Am J Physiol Lung Cell Mol Physiol* 2002;282:L847-53.
- Oltmanns U, Issa R, Sukkar MB, John M, Chung KF. Role of c-jun N-terminal kinase in the induced release of GM-CSF, RANTES and IL-8 from human airway smooth muscle cells. *Br J Pharmacol* 2003;139:1228-34.
- Wuys WA, Vanaudenaerde BM, Dupont LJ, Demedts MG, Verleden GM. Involvement of p38 MAPK, JNK, p42/p44 ERK and NF-kappaB in IL-1beta-induced chemokine release in human airway smooth muscle cells. *Respir Med* 2003;97:811-7.
- Aarbiou J, Ertmann M, Van Wetering S, van Noort P, Rook D, Rabe KF, et al. Human neutrophil defensins induce lung epithelial cell proliferation in vitro. *J Leukoc Biol* 2002;72:167-74.
- Tjabringa GS, Ninaber DK, Drijfhout JW, Rabe KF, Hiemstra PS. The human cathelicidin LL-37 is a chemoattractant for eosinophils and neutrophils that acts via formyl-peptide receptors. *Int Arch Allergy Immunol* 2006;140:103-12.
- Nassar T, Akkawi S, Bar-Shavit R, Haj-Yehia A, Bdeir K, Al Mehdi AB, et al. Human alpha-defensin regulates smooth muscle cell contraction:

- a role for low-density lipoprotein receptor-related protein/alpha 2-macroglobulin receptor. *Blood* 2002;100:4026-32.
33. Oono T, Shirafuji Y, Huh WK, Akiyama H, Iwatsuki K. Effects of human neutrophil peptide-1 on the expression of interstitial collagenase and type I collagen in human dermal fibroblasts. *Arch Dermatol Res* 2002;294:185-9.
 34. Khine AA, Del Sorbo L, Vaschetto R, Voglis S, Tullis E, Slutsky AS, et al. Human neutrophil peptides induce interleukin-8 production via P2Y6 signaling pathway. *Blood* 2006;107:2936-42.
 35. Govindaraju V, Martin JG, Maghni K, Ferraro P, Michoud MC. The effects of extracellular purines and pyrimidines on human airway smooth muscle cells. *J Pharmacol Exp Ther* 2005;315:941-8.
 36. Nagaoka I, Tamura H, Hirata M. An antimicrobial cathelicidin peptide, human CAP18/LL-37, suppresses neutrophil apoptosis via the activation of formyl-peptide receptor-like 1 and P2X7. *J Immunol* 2006;176:3044-52.
 37. Braff MH, Hawkins MA, Nardo AD, Lopez-Garcia B, Howell MD, Wong C, et al. Structure-function relationships among human cathelicidin peptides: dissociation of antimicrobial properties from host immunostimulatory activities. *J Immunol* 2005;174:4271-8.
 38. Salinthon S, Singer CA, Gerthoffer WT. Inflammatory gene expression by human colonic smooth muscle cells. *Am J Physiol Gastrointest Liver Physiol* 2004;287:G627-37.
 39. Jijon HB, Panenka WJ, Madsen KL, Parsons HG. MAP kinases contribute to IL-8 secretion by intestinal epithelial cells via a posttranscriptional mechanism. *Am J Physiol Cell Physiol* 2002;283:C31-41.
 40. Holtmann H, Winzen R, Holland P, Eickemeier S, Hoffmann E, Wallach D, et al. Induction of interleukin-8 synthesis integrates effects on transcription and mRNA degradation from at least three different cytokine- or stress-activated signal transduction pathways. *Mol Cell Biol* 1999;19:6742-53.
 41. Bowdish DM, Davidson DJ, Speert DP, Hancock RE. The human cationic peptide LL-37 induces activation of the extracellular signal-regulated kinase and p38 kinase pathways in primary human monocytes. *J Immunol* 2004;172:3758-65.
 42. Ovreik J, Lag M, Schwarze P, Refsnes M. p38 and Src-ERK1/2 pathways regulate crystalline silica-induced chemokine release in pulmonary epithelial cells. *Toxicol Sci* 2004;81:480-90.
 43. Panenka W, Jijon H, Herx LM, Armstrong JN, Feighan D, Wei T, et al. P2X7-like receptor activation in astrocytes increases chemokine monocyte chemoattractant protein-1 expression via mitogen-activated protein kinase. *J Neurosci* 2001;21:7135-42.
 44. Gendron FP, Neary JT, Theiss PM, Sun GY, Gonzalez FA, Weisman GA. Mechanisms of P2X7 receptor-mediated ERK1/2 phosphorylation in human astrocytoma cells. *Am J Physiol Cell Physiol* 2003;284:C571-81.
 45. Humphreys BD, Rice J, Kertesz SB, Dubyak GR. Stress-activated protein kinase/JNK activation and apoptotic induction by the macrophage P2X7 nucleotide receptor. *J Biol Chem* 2000;275:26792-8.
 46. Ullmann H, Meis S, Hongwiset D, Marzian C, Wiese M, Nickel P, et al. Synthesis and structure-activity relationships of suramin-derived P2Y11 receptor antagonists with nanomolar potency. *J Med Chem* 2005;48:7040-8.
 47. Lambrecht G, Braun K, Damer M, Ganso M, Hildebrandt C, Ullmann H, et al. Structure-activity relationships of suramin and pyridoxal-5'-phosphate derivatives as P2 receptor antagonists. *Curr Pharm Des* 2002;8:2371-99.
 48. Stein CA, LaRocca RV, Thomas R, McAtee N, Myers CE. Suramin: an anticancer drug with a unique mechanism of action. *J Clin Oncol* 1989;7:499-508.