Leukotriene B_4 triggers release of the cathelicidin LL-37 from human neutrophils: novel lipid-peptide interactions in innate immune responses

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ABSTRACT **In humans, the antimicrobial peptide LL-37 and the potent chemotactic lipid leukotriene B_4 (LTB_4) are important mediators of innate immunity and host defense. Here we show that LTB_4, at very low (1 nM) concentrations, strongly promotes release of LL-37 peptides from human neutrophils (PMNs) in a time- and dose-dependent manner, as determined by Western blot, enzyme-linked immunoassay (ELISA), and antibacterial activity. The LTB_4-induced LL-37 release is mediated by the BLT1 receptor, and protein phosphatase-1 (PP-1) inhibits the release by suppressing the BLT1-mediated exocytosis of PMN granules. Conversely, LL-37 elicits translocation of 5-lipoxygenase (5-LO) from the cytosol to the perinuclear membrane in PMNs and promotes the synthesis and release of LTB_4, particularly from cells primed with LPS or GM-CSF. Furthermore, LL-37 stimulates PMN phagocytosis of *Escherichia coli* particles, a functional response that is enhanced by LTB_4, especially in GM-CSF pretreated cells. In these cells, LL-37 also enhances LTB_4-induced phagocytosis. Hence, in human PMNs, positive feedback circuits exist between LL-37 and LTB_4 that reciprocally stimulate the release of these mediators with the potential for synergistic bioactions and enhanced immune responses. Moreover, these novel lipid-peptide signaling pathways may offer new opportunities for pharmacological intervention and treatment of chronic inflammatory diseases.—Wan, M., Sabirsh, A., Wetterholm, A., Agerberth, B., Haeggström, J. Z. Leukotriene B_4 triggers release of the cathelicidin LL-37 from human neutrophils: novel lipid-peptide interactions in innate immune responses. *FASEB J.* 21, 2897–2905 (2007)**

Key Words: phagocytosis · inflammation

Leukotriene B_4 (LTB_4) is one of the most potent chemotactic agents known and is biosynthesized from arachidonic acid by the sequential action of 5-lipoxygenase (5-LO) and LTA_4 hydrolase, mainly in cells of myeloid lineage (1). Two G-protein-coupled LTB_4 receptors have been identified, BLT1 and BLT2, with high and low affinity for LTB_4, respectively (2, 3). Although LTB_4 is known to exert broad proinflammatory effects, evidence is accumulating regarding the antimicrobial functions of LTB_4 (4, 5). Furthermore, the LTB_4-BLT1 pathway was found to be important for linking early immune responses and the multiple classes of effector cells associated with acquired immunity (6).

Endogenous antimicrobial peptides in mammals comprising the defensins and cathelicidins are important effector molecules of host defense against invading microbes, and these peptides have a wide range of functions from direct antibacterial actions (7–9) to receptor-mediated immuno-modulation (10, 11) and angiogenesis (12). The only cathelicidin in humans, LL-37/hCAP18, is found at high concentrations in its unprocessed form (hCAP-18) in the secondary granules of neutrophils and is processed by proteinase 3 to LL-37 (13). Beyond its role as an antimicrobial agent, LL-37 exhibits additional functions important for early immune responses (*e.g.*, chemotaxis of neutrophils, monocytes, and T cells) (10). Furthermore, LL-37 has been linked to the pathophysiology of inflammatory diseases ranging from arthritis (14) and atherosclerosis (15) to dermatitis (16).

Innate immune responses are heavily dependent on neutrophils, and these cells are responsible for both leukotriene biosynthesis and the release of antimicrobial peptides. Although both LTB_4 and LL-37 have overlapping functions and play important roles during innate immune responses, the potential interactions between these two mediators have never been explored. Here we report that the formation and function of LL-37 are regulated by LTB_4 and, conversely, that LL-37 can affect the synthesis and function of LTB_4. Moreover, we provide evidence that positive feedback circuits exist between LL-37 and LTB_4, which in turn have important implications for neutrophil functions that are essential for innate immune responses and associated inflammatory diseases.

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MATERIALS AND METHODS

Reagents

Monoclonal LL-37 antibody was generated as described (17) and anti-mouse IgG conjugated with horseradish peroxidase was purchased from Bio-Rad Laboratories (Hercules, CA, USA); human 5-LO antiserum was a kind gift from Olof Rådmark (Karolinska Institutet, Stockholm, Sweden); a monoclonal antibody directed to T cell receptor was a kind gift from Hans Wigzell (Karolinska Institutet, Stockholm, Sweden); LTB_4, 20-OH-LTB_4, 20-COOH-LTB_4, 12(R)-HETE, and 5-HETE were purchased from BIOMOL (Plymouth Meeting, PA, USA); 5S,12S-DHETE was a kind gift from Hans-Erik Claesson (Karolinska Institutet, Stockholm, Sweden). *p*-Nitrophenylphosphate, lipopolysaccharide (LPS) from *E. coli* serotype O55:B5, adenosine deaminase (ADA), and granulocyte-macrophage colony-stimulating factor (GM-CSF) were from Sigma Chemical Co. (St. Louis, MO, USA); Compound A (4′-{[pentanoyl(phenyl) amino] methyl}-1, 1′-biphenyl-2carboxylic acid) was a kind gift from Dr. Shinji Nakade (ONO Pharmaceuticals Co., Ltd., Osaka, Japan). Streptavidin-AP was purchased from Dakocytomation (Glostrup, Denmark); polyclonal LL-37 antibody, synthetic LL-37 (NH_2-LLGDFFRKSKEKIGKEFKRIVQRIKDFFRNLV-PRTES-COOH) and sequence-scrambled LL-37 (NH_2GLK-LRFEFSKIKGEFLKTPEVRFRDIKLKDNRISVQR-COOH) peptides were obtained from Innovagen AB (Lund, Sweden).

Isolation of polymorphonuclear neutrophils (PMNs)

Human PMNs were isolated from freshly prepared buffy coats (Karolinska Hospital Blood Bank, Stockholm, Sweden) by dextran sedimentation, hypotonic lysis of erythrocytes, and gradient centrifugation on Lymphoprep (Axis-Shield PoC AS, Oslo, Norway). PMNs were suspended at a density of 10×10^6/ml in Dulbecco's PBS [Life Technologies, Inc. (Invitrogen), Paisley, UK]. PMN purity (>95%) and viability (>98%) were determined using Hemacolor (J. T. Baker, Utrecht, Holland) and Trypan blue (Sigma Chemical Co.) staining, respectively.

Peptide/protein extraction

Frozen supernatants obtained from PMN cultures after various pretreatments were thawed and acidified by adding trifluoroacetic acid (TFA) to a final concentration of 0.1% (v/v). Proteins and peptides were then extracted using reverse phase chromatography (OASIS™cartridge, Waters®, Milford, MA, USA). The cartridge was activated with acetonitrile and equilibrated in 0.1% TFA before the application of the cell culture supernatant. Unbound material was removed using 0.1% TFA before the adsorbed proteins and peptides were eluted with 80% acetonitrile in 0.1% TFA. Finally, the eluate was frozen and lyophilized.

SDS-PAGE and Western blot analysis

After lyophilization, the protein- and peptide-enriched PMN supernatants were dissolved in 0.1% TFA, mixed with loading buffer, and denatured by incubation at 70°C for 10 min. Electrophoresis was performed using NuPAGE® Novex 4–12% Bis-Tris Gel (Invitrogen, Carlsbad, CA, USA). After separation, the peptides were transferred to polyvinylidene difluoride membranes according to the manufacturer's instructions (Invitrogen). Nonspecific binding sites were saturated by exposure to 5% fat-free milk in PBS with 0.25% Tween for 1 h before the membranes were incubated at room temperature with the monoclonal LL-37 antibody for 1 h, followed by anti-mouse IgG for 1 h. The enhanced chemiluminescence (ECL) Western blot detection system (Amersham Pharmacia Biotech, Uppsala, Sweden) was used to visualize the immunoreactive bands.

ELISA

ELISA plates (Maxisorp by Nunc, Naperville, IL, USA) were coated with 50 μl monoclonal LL-37 antibody (5 μg/ml in coating buffer: 1.59 g Na_2CO_3, 2.93 g $NaHCO_3$, 0.2 g NaN_3 in 1 l MilliQ water, pH=9.6) at 4°C overnight. After washing three times with PBS (200 μl/well), 0.1% gelatin in PBS was added (100 μl/well) to block unspecific binding for 1 h at room temperature. The plates were washed three times with PBS (0.05% Tween), then samples or standard LL-37 peptide, in a serial dilution (0.1–1000 ng/ml in PBS), were added in duplicate (100 μl/well) and incubated at 4°C overnight. After washing, the wells were incubated with 50 μl biotinylated polyclonal LL-37 antibody (20 μg/ml; 0.1% gelatin in PBS) at room temperature for 2 h, then incubated with 50 μl streptavidin-AP (1:2000; 0.1% gelatin in PBS) at room temperature for 2 h. Finally, *p*-nitrophenylphosphate (1 mg/ml) in diethanolamin buffer (pH=9.8) was added (100 μl/well), and the absorbance was measured in a microplate reader at 405 nm after 30–60 min.

Analysis of leukotriene biosynthesis by reverse-phase HPLC

For reverse-phase HPLC, the column (Nova-Pak C18, Waters®, Milford, MA, USA) was eluted with acetonitrile/methanol/water/acetic acid (30:35:35:0.01 by volume), at a flow rate of 1.0 ml/min and absorption was monitored at 270 nm. The data were quantified using area integrations (Baseline 810 computer software, Waters®, Milford, MA, USA) based on a standard curve of known amounts of prostaglandin B_2 and the compound to be measured.

Immunocytochemistry

Cytospin preparations of PMNs were fixed in acetone for 5 min at room temperature. After preincubation with 10% goat serum for 30 min, cells were incubated with human 5-LO antiserum at 4°C overnight. The cells were then rinsed and incubated with Cy3-conjugated goat anti-rabbit-IgG (Jackson ImmunoResearch, West Grove, PA, USA) at room temperature for 30 min. After staining, the slides were mounted using an antifading mounting medium (Vector Laboratories, Burlingame, CA, USA) and imaged using a confocal microscope (LSM 510, Zeiss, Oberkochen, Germany) equipped with plan-Apochromat 63×/1.4 and plan-Neofluor 40×/1.3 oil immersion lenses and LSM 3D image acquisition software.

Inhibition zone assay

Thin plates (1 mm thick) were poured with 1% agarose in Luria-Bertani broth containing the test microbe (*Bacillus megaterium* strain Bm11) and the salt solution medium E (0.8 mM $MgSO_4$, 9.5 mM citric acid, 57.5 mM K_2HPO_4, 16.7 mM $NaNH_4HPO_4$). Small wells (diameter, 3 mm) were punched in the plates and 3 μl samples were applied in each well. After overnight incubation at 30°C, the diameters of inhibition zones were recorded by means of a magnification lens with an internal millimeter scale.

Neutralization of LL-37 antibacterial activity

Concentrated polypeptides, originating from supernatants of LTB_4-stimulated PMN, were incubated with an equal volume of PBS, monoclonal LL-37 antibody (4 mg/ml), or a monoclonal antibody directed to T cell receptor (4 mg/ml), serving as an unspecific monoclonal antibody in our assay for 4 h at room temperature. The mixture was then analyzed in the inhibition zone assay against *B. megaterium* Bm11, as described above.

Phagocytosis

PMN phagocytosis was evaluated using a commercial kit (Vybrant Phagocytosis Assay, Invitrogen, Paisley, UK) according to the manufacturer's instructions using a plate fluorometer (Polarstar™, BMG Technologies, Offenburg, Germany).

Statistical analysis

Data are presented as mean ± SD unless stated otherwise. Differences between the means were evaluated using 1-way ANOVA or Student's *t* tests. A value of $P < 0.05$ was considered statistically significant.

RESULTS

Release of LL-37 from PMNs activated with LTB_4

Challenge of isolated human PMNs with varying concentrations of LTB_4 elicited secretion of LL-37/hCAP18, as determined by Western blot. LTB_4 was a very potent secretagogue for LL-37/hCAP18 and caused a 7-fold increase in extracellular LL-37 levels even at concentrations as low as 1 nM (**Fig. 1*A***). Maximum responses corresponding to ~15-fold background levels of LL-37 were obtained at an LTB_4 dose of 100 nM. The LL-37 precursor, hCAP18, was also released from PMNs in response to LTB_4 but the increase was lower in magnitude. The time course for LTB_4-induced LL-37 release was rapid. Thus, when PMNs were exposed to 1 μM LTB_4 there was an immediate release of LL-37, with peak extracellular concentrations occurring after 60 s, followed by a gradual decline to background levels of >10 min of incubation (Fig. 1*B*). Release of LL-37/hCAP18 in response to LTB_4 was also measured with a specific ELISA, yielding a similar dose response (Fig. 1*C*). Furthermore, PMNs that had not been pretreated with cytochalasin B were challenged with LTB_4 and resulted in a response with a similar time course and dose response, but with significantly lower amounts of LL-37 released (data not shown).

Inhibition of LTB_4-induced release of LL-37/hCAP18 by protein phosphatase 1

The precursor of the LL-37 peptide hCAP18 is stored in PMN secondary granules whereas the protease responsible for cleavage of hCAP18 into LL-37, proteinase-3, is stored mainly in the primary granules (13, 18). LTB_4-induced release of LL-37 was completely blocked when human PMNs were pretreated with 30 μM protein phosphatase 1 (PP-1), an Src family tyrosine kinase inhibitor known to inhibit exocytosis of primary and secondary granules (19) (**Fig. 2**).

Effects of BLT1 and BLT2 selective agonists and antagonists on LL-37/hCAP18 release from human PMNs

LTB_4 is the preferred agonist for both BLT1 and BLT2, and several other structurally related eicosanoids bind

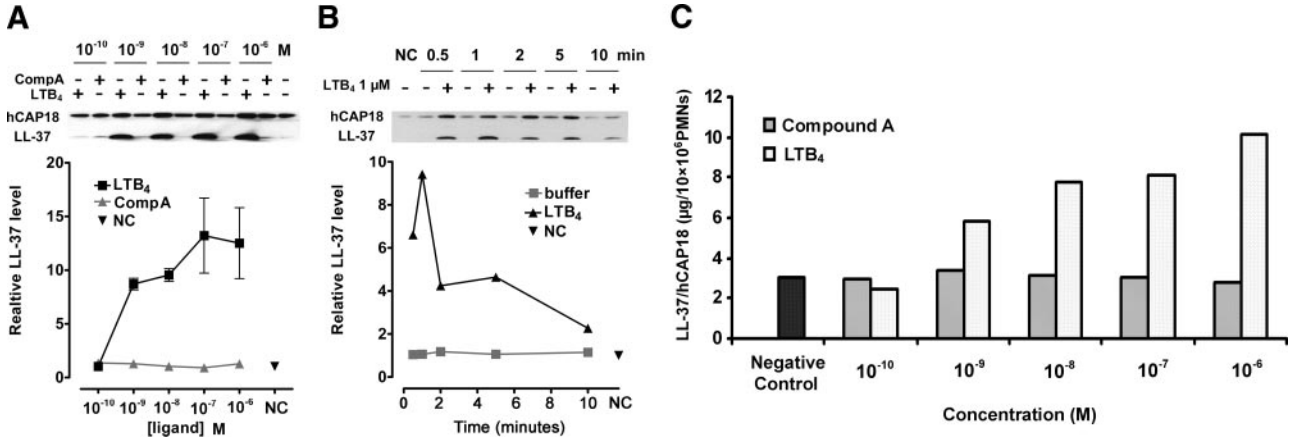

Figure 1. Release of LL-37/ hCAP18 from PMNs after exposure to LTB_4, in a time- and concentration-dependent manner. *A–C)* For each experiment, 10×10^6 PMNs were pretreated with 10 μM cytochalasin B plus 2 mM $CaCl_2$ for 5 min at 37°C. *A)* PMNs were exposed to LTB_4 (■) or Compound A (▲) at different concentrations for 1 min at 37°C and samples were analyzed by Western blot. *B)* PMNs were exposed to 1 μM LTB_4 (▲) or control solutions (■) at 37°C for up to 10 min. NC: negative control (▼), cells pretreated with only 10 μM cytochalasin B and 2 mM $CaCl_2$. *C)* PMNs were exposed to LTB_4 or Compound A at different concentrations for 1 min at 37°C and release of LL-37/hCAP18 was measured by ELISA. CompA, Compound A. Control solutions were made from buffer solutions by adding the appropriate solvent without any active substance. The gel image in panel *A* is representative of three separate experiments; error bars are ±SD. Results in panel *B* are representative of two experiments.

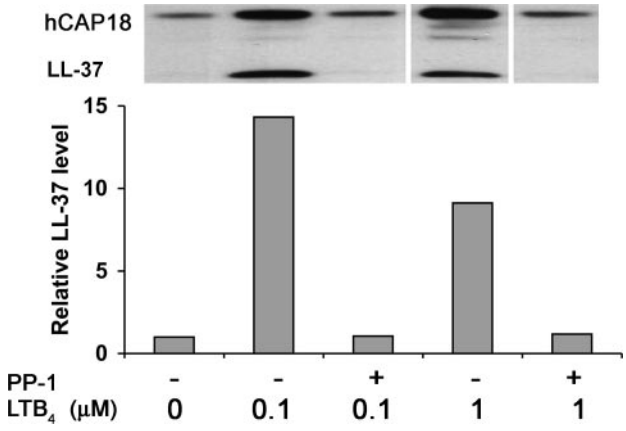

Figure 2. PP-1 inhibits LTB_4-induced LL-37/hCAP18 exocytosis from neutrophils. PMNs (10×10^6) were preincubated with a control solution or 30 μM PP-1 for 10 min at 37°C. After this pretreatment, 10 μM cytochalasin B was added and the cells were incubated another 5 min. Finally, 0.1 or 1 μM LTB_4 and 2 mM $CaCl_2$ were added to the reaction mixture and the cells were incubated for 1 additional min. The Western blot images shown are from noncontiguous sections of the same membrane and are representative of two experiments. Control solutions were made from buffer solutions by adding the appropriate solvent without any active substance.

the two receptors with different affinities (20). Recently, however, a synthetic compound, *viz.* 4′-{[pentanoyl(phenyl) amino] methyl}-1, 1′-biphenyl-2-carboxylic acid, was identified as a selective BLT2 agonist and was named Compound A (21). In our experiments, Compound A failed to induce LL-37 release from PMNs at any time point (1 μM, 0–15 min; data not shown) or at concentrations of up to 1 μM (Fig. 1*A*). The same result was obtained when release of LL-37 was assessed by ELISA (Fig. 1*C*). The BLT1 antagonist CP105696 and BLT2 antagonist LY255238 also had different effects on LTB_4-induced LL-37 release (**Fig. 3*A***). Thus, release of LL-37 induced by 100 nM LTB_4 (or 1 μM LTB_4; not shown) was almost completely inhibited by preincubation with 1 μM CP105696, whereas preincubation with the BLT2-specific antagonist LY255238 (1 μM) had no effect. We also tested additional BLT1 and BLT2 ligands and found that three other hydroxyeicosanoids were either less potent than LTB_4, with a rank order of LTB_4>20-OH-LTB_4>12R-HETE, or inactive, like 5-HETE; these relative ligand specificities did not vary significantly between 1 min, 5 min, and 10 min incubations (Fig. 3*B*). In a separate set of experiments, 5S,12S-DHETE was found to be a weak agonist for LL-37 release equipotent with 12R-HETE, whereas 20-COOH-LTB_4 was almost inactive in this respect (data not shown). Taken together, these relative potencies, especially those of LTB_4, 20-OH-LTB_4, 12R-HETE, and 5-HETE, agree well with reported ligand affinities for these compounds *vs.* BLT1 (20).

Impact of LL-37 on LTB_4 synthesis and release from human PMNs

Exposure of human PMNs to 30 μg/ml LL-37 triggered synthesis and release of low but detectable levels of LTB_4, as determined by HPLC coupled to an ELISA (data not shown). Furthermore, confocal microscopy of PMNs pretreated with 30 μg/ml LL-37 for 15 min revealed that the peptide promotes an early key step in leukotriene synthesis, namely, the translocation of 5-LO from the cytosol (**Fig. 4*A***) to the perinuclear membrane (Fig. 4*B*) (22).

Priming of PMNs with LPS or GM-CSF, a procedure that does not elicit leukotriene biosynthesis by itself, greatly increased the release of LTB_4 into the medium after stimulation with LL-37, and these amounts could be easily detected by direct HPLC analysis (Fig. 4*C–E*). In fact, the maximum levels of LTB_4 produced by GM-CSF primed PMNs in response to LL-37 (3.3 ng $LTB_4/10\times10^6$ PMNs) could even exceed those obtained with the classical agonist fMLP (2.4 ng $LTB_4/10\times10^6$ PMNs). For PMNs primed with GM-CSF, optimal release of LTB_4 occurred at a dose of 15 μg LL-37/ml and declined at a dose of 50 μg/ml, which suggests the presence of negative, dose-dependent feedback mechanisms. Priming with LPS promoted a

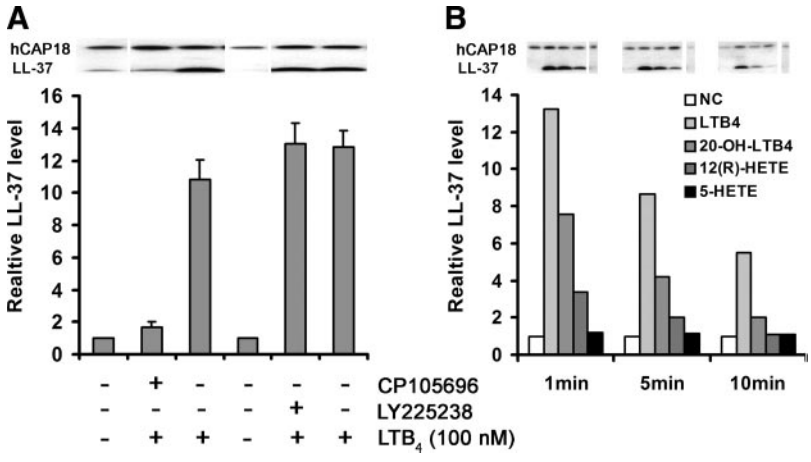

Figure 3. LTB_4-induced LL-37 release is mediated specifically by BLT1. *A)* PMNs (10×10^6) were pretreated with control solutions or with the BLT1 antagonist CP105696 or the BLT2 antagonist LY255238 for 20 min at 37°C. After this pretreatment, 10 μM cytochalasin B was added and the cells were incubated for 5 min. Finally, 100 nM LTB_4 and 2 mM $CaCl_2$ were added to the reaction mixture and the cells were incubated for another 1 min. *B)* 10×10^6 PMNs were pretreated with 10 μM cytochalasin B plus 2 mM $CaCl_2$ for 5 min at 37°C, then exposed to 1 μM LTB_4, 20-OH-LTB_4, 12(R)-HETE, 5-HETE, or control solutions at 37°C for 1, 5, or 10 min. *A, B)* Images from noncontiguous areas of the same membrane. The data in panel *A* are representative of three experiments and error bars are ±SD. The data in panel *B* are representative of two experiments. Control solutions were made from buffer solutions by adding the appropriate solvent without any active substance.

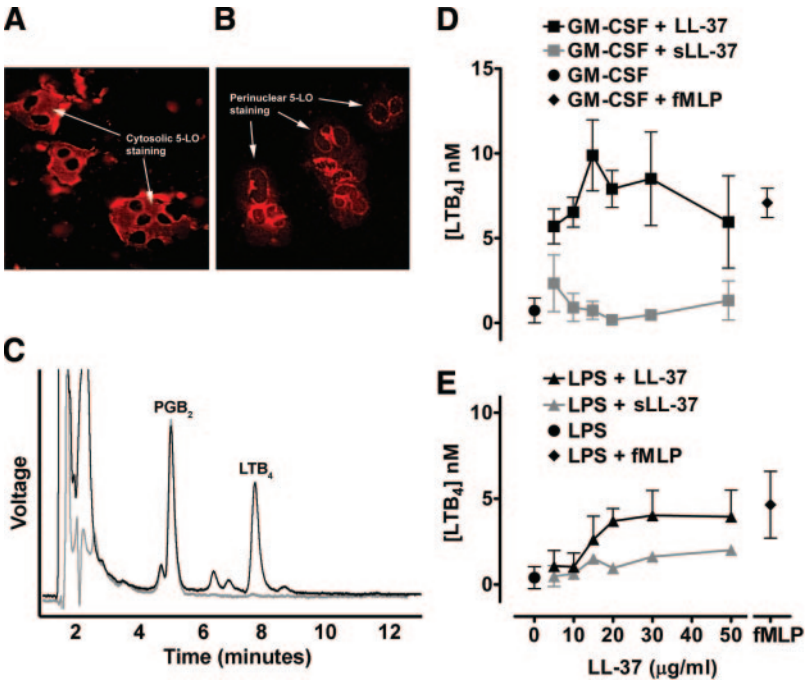

Figure 4. LL-37-induced LTB_4 release from LPS- or GM-CSF-primed PMNs. *A, B)* Confocal microscopic images of fixed PMNs stained with 5-LO primary antibody and Cy3-labeled secondary antibody and imaged at 600× magnification. *A)* Cells exposed to a control solution plus 2 mM $CaCl_2$ for 15 min at 37°C. *B)* Cells exposed to 30 μg/ml LL-37 plus 2 mM $CaCl_2$ for 15 min at 37°C. *C)* Representative HPLC chromatograms showing robust LTB_4 production by PMNs (10×10^6) preincubated for 30 min with 1 nM GM-CSF, then exposed either to 20 μg/ml LL-37 (black trace) for 5 min or to buffer alone (light gray trace). Cells were pelleted by centrifugation, and LTB_4 content was measured in supernatants. Prostaglandin B_2 (PGB_2) was added prior to analysis as an internal standard. Only cells exposed to both GM-CSF and LL-37 produced LTB_4. *D, E)* PMNs were preincubated with 1 nM GM-CSF, $n = 3$ donors (*D*), or 5 ng/ml LPS, $n = 4$ donors (*E*) for 30 min at 37°C. The cells were then treated with ADA (0.1 U/ml) for 5 min before 2 mM $CaCl_2$, then different concentrations (0–50 μg/ml) of LL-37 or the scrambled LL-37 peptide (sLL-37) were added and incubated with the cells for 5 min at 37°C. Responses to stimulation with 0.1 μM fMLP are shown for comparison.

somewhat lower production of LTB_4 compared to GM-CSF, and the release reached a maximum plateau at concentrations ≥20 μg LL-37/ml. Moreover, LL-37-induced stimulation of LTB_4 production in primed PMNs appeared to be specific since a scrambled peptide (sLL-37) did not evoke this response (Fig. 4*D, E*).

Presence of functionally active LL-37 from LTB_4-treated PMNs

The secreted material from LTB_4-treated PMNs exhibited antibacterial activity against the $Gram^+$ bacterium *B. megaterium* strain Bm11, as demonstrated by an inhibition zone assay. When the secreted material was incubated with a specific monoclonal LL-37 antibody, the antibacterial activity decreased by 92% compared to a 45% decrease observed with a nonspecific monoclonal antibody (**Fig. 5**). Hence, most of the antibacterial activity observed in the material secreted from LTB_4-stimulated PMN originated from functionally active LL-37.

Effects of LL-37 and LTB_4 interactions on PMN functions

LL-37 has, by itself, small but clearly detectable effects on PMN phagocytic activity (**Fig. 6*A***). If, however, 10 nM LTB_4 is given at the same time, there is a marked increase in the ability of LL-37 to stimulate phagocytic uptake of *E. coli* particles. When compared with the phagocytic effect elicited by 10 nM LTB_4 alone, the increased effect of LL-37 seemed to be additive rather than synergistic (Fig. 6*A*). We also tested whether priming could enhance the additive effects of LL-37 and LTB_4 on phagocytosis. Thus, in one set of experiments PMNs were pretreated with 1 nM GM-CSF for 30 min, then exposed to 10 μg/ml LL-37. A subsequent addition of 10 nM LTB_4 enhanced the phagocytic response significantly (Fig. 6*B*). In another set of experiments, phagocytic stimuli were added in an opposite order. Primed PMNs (1 nM GM-CSF; 30 min) were first exposed to LTB_4 (10 nM), then challenged with LL-37 (10 μg/ml), a combination that also led to significant enhancement of the phagocytic response (Fig. 6*B*). A similar additive pattern for LTB_4 and LL-37 was also observed using control cells that had not been

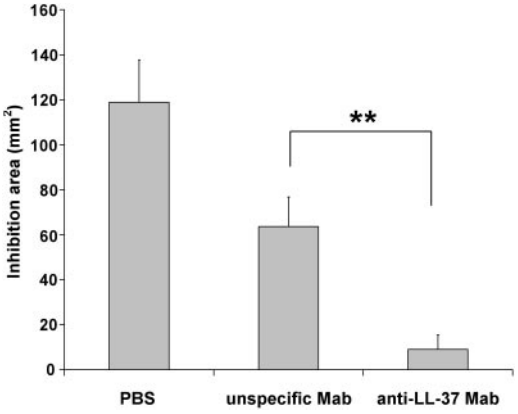

Figure 5. Antibacterial activity of LTB_4-induced LL-37 from PMNs. 10×10^6 PMNs were treated with 0.1 μM LTB_4, and the supernatant was lyophilized and resuspended in 20 μl PBS. Then the sample was incubated with the same amount of PBS, LL-37-specific monoclonal antibody (anti-LL-37 Mab), or a monoclonal antibody directed to T cell receptor (unspecific Mab, used as negative control). The antibacterial activity was demonstrated as the area of the inhibition zone. Error bars are mean ± SD, $n = 3$. $^{**}P$ value <0.01.

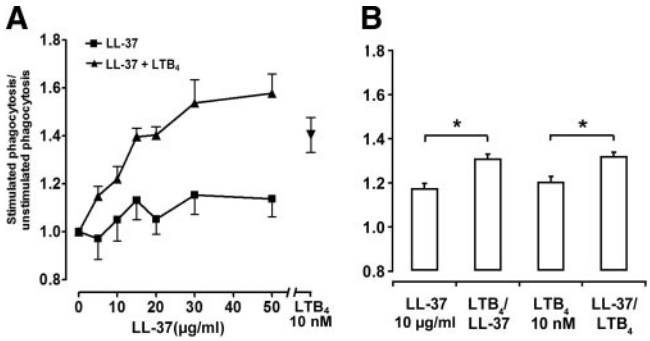

Figure 6. Functional effects of LL-37 and LTB_4 interactions. *A)* PMN phagocytosis of *E. coli* particles stimulated by LL-37 (■). LL-37-induced PMN phagocytosis enhanced by concurrent treatment with 10 nM LTB_4 (▲). As a reference, phagocytosis elicited by preincubation with 10 nM LTB_4 alone, is also shown (▼). The results (mean±SE) are presented as a ratio to baseline phagocytosis (polymorphonuclear cells exposed to control solutions), and represent data from triplicate wells in at least three separate experiments using PMNs from different donors. *B)* PMNs were pretreated with 1 nM GM-CSF for 30 min. Exposure to either 10 μg/ml LL-37 or 10 nM LTB_4 enhanced the subsequent response to 10 nM LTB_4 or 10 μg/ml LL-37, respectively. The results (mean±SE) are presented as a ratio to baseline phagocytosis (PMNs pretreated with GM-CSF but not exposed to LL-37 or LTB_4), and represent data from triplicate wells in at least three separate experiments using PMNs from different donors. Control solutions were made from buffer solutions by adding the appropriate solvent without any active substance. *P value <0.05.

exposed to GM-CSF, but the differences were not significant.

DISCUSSION

Leukotriene B_4 is a powerful lipid mediator of inflammation. LL-37, on the other hand, is recognized mostly as a peptide with antibacterial activities, but several reports indicate that it also has broader functions related to innate immunity (10, 12), some of which overlap with those of LTB_4 (10). Since both LTB_4 and the host defense peptide LL-37 have been implicated in certain chronic inflammatory diseases—in particular, rheumatoid arthritis, dermatitis, and atherosclerosis—we searched for potential cross-talk and synergy between these two mediators.

LTB_4 is a potent stimulus of LL-37 release from human PMNs: potential contribution to proinflammatory and antimicrobial effects of LTB_4

Exposure of human PMNs treated with cytochalasin B to as little as 1 nM LTB_4 led to an almost instantaneous (within 60 s) degranulation and release of LL-37, with a maximum response at 100 nM, generating levels by almost 13-fold the background (Fig. 1*A, B*). Furthermore, the secreted LL-37 was functionally active since material secreted from LTB_4-stimulated PMNs killed the Gram$^+$ bacterium *B. megaterium*, and this activity could be neutralized by an LL-37-specific monoclonal antibody (Fig. 5).

Exocytosis from different neutrophil granules occurs in a specific order: secretory vesicles, gelatinase granules, secondary (specific) granules, and primary (azurophil) granules (23). The precursor of the LL-37 peptide, hCAP18, is stored in PMN secondary granules, while the protease responsible for the cleavage of hCAP18 into LL-37, proteinase-3, is mainly stored in the primary granules (18). Our results therefore imply that LTB_4 induces exocytosis of both primary and secondary PMN granules. Many reports have shown that the Src family tyrosine kinases are associated with the release of primary and secondary granules from neutrophils on fMLP stimulation (24), and protein phosphatase 1 (PP-1) is a highly potent and selective inhibitor of Src family tyrosine kinases (25). In our hands, pretreatment with 30 μM PP-1 completely blocked the LTB_4-induced release of LL-37 from human neutrophils (Fig. 2), indicating that this response is also dependent on Src family tyrosine kinases.

LTB_4 is a potent proinflammatory mediator, but LL-37 exhibits proinflammatory effects of its own, hence the increased release of this peptide may result in an even more robust inflammatory response. Furthermore, since LL-37 exhibits a wide range of antimicrobial effects (8, 9), release of this mediator most likely contributes to the previously described antimicrobial activities of LTB_4 (5).

The LTB_4-induced release of LL-37 from PMNs occurs through activation of BLT1

It has been described that LTB_4 is a complete secretagogue in human neutrophils and can stimulate lysosomal enzyme release from human PMNs at 10^{-8} M (26, 27), which agrees quite well with our results. Other investigators reported that μM concentrations of LTB_4 are required to stimulate secretion (28), discrepancies that may be explained by differences in purity of the LTB_4 preparations or other experimental conditions. Further studies revealed that bioactions of LTB_4 seemed to be mediated *via* high- and low-affinity classes of LTB_4 receptors, with the latter class being responsible for LTB_4-induced secretion (29, 30). Now we know that there are two molecularly distinct receptors, BLT1 and BLT2, both of which are expressed on human leukocytes and potentially able to transduce the secretory effects of LTB_4 (3). In keeping with previous data, one would expect that secretory effects of LTB_4, such as release of LL-37, are primarily mediated *via* BLT2 (*i.e.*, the receptor exhibiting the lower affinity for LTB_4). It was therefore interesting to find that the unique BLT2-specific agonist, Compound A (21), failed to induce LL-37 release even at 1 μM concentrations (Fig. 1*A*). Furthermore, the LTB_4-induced release of LL-37/hCAP18 from PMNs could be efficiently inhibited by the BLT1-specific antagonist CP105696, whereas the BLT2 antagonist LY255283 was ineffective (Fig. 3*A*). In

addition, the effects of other eicosanoid BLT1 agonists on LL-37 release were consistent with their affinity for BLT1 rather than BLT2. Taken together, our results indicate that exocytosis of LL-37/hCAP18 from human PMNs after LTB_4 stimulation is primarily mediated by BLT1.

LL-37 induces the synthesis and release of LTB_4 from human PMNs: a positive feedback loop

While LTB_4 could induce the release of LL-37, we have also shown that LL-37 can stimulate the production and release of LTB_4. Using HPLC coupled to EIA, we could detect LTB_4 production at very low levels by PMNs, after exposure to LL-37 alone. Confocal microscopy of PMNs pretreated with LL-37 revealed that this peptide promotes an early key step in leukotriene synthesis, namely, the translocation of 5-lipoxygenase from the cytosol to the perinuclear membrane (22). It is well recognized that translocation of 5-lipoxygenase depends on influx of extracellular Ca^{2+} (31–33), but redistribution of the enzyme from cytosol to membrane can also occur in a Ca^{2+}-independent manner that involves activation of the p38 MAP kinase pathway and phosphorylation of the protein (34). It has been reported that LL-37 can induce chemotaxis and elicit Ca^{2+} mobilization in human monocytes (10), effects also observed by us in PMN (data not shown). Thus, it appears likely that LL-37-induced translocation of 5-lipoxygenase is a Ca^{2+}-dependent event, although other mechanisms cannot be excluded.

Pretreatment or "priming" of circulating neutrophils with proinflammatory stimuli, such as LPS or certain cytokines like GM-CSF, enhances neutrophil responses to subsequent stimulation and results in an increased potential to generate LTB_4 (35, 36). Priming of effector cells, including neutrophils, is important for modulating inflammation, since the direct activation of neutrophils at inappropriate times or distances from inflammatory sites would be potentially deleterious to the host. When PMNs were primed with LPS or GM-CSF, essentially no LTB_4 was produced. This priming process did, however, result in greater and more prompt production of LTB_4 (~5 min to reach peak concentrations) after secondary LL-37 stimulation, and these amounts could easily be detected by direct HPLC analysis (Fig. 4*C–E*). PMNs primed with GM-CSF produced much more LTB_4 when stimulated with $\geq$10 μg/ml LL-37, whereas sequence-scrambled LL-37 peptide was almost inactive, indicating that the effects of LL-37 are specific and presumably receptor mediated (*e.g.*, *via* formyl peptide receptor like 1) (10). Similar results were observed after LPS priming, although in this context LL-37 was a less potent stimulus for LTB_4 production relative to sLL-37. The lower potency of LPS might be explained by the fact that LL-37 has been shown to bind LPS, preventing LPS binding to cells (37).

Our results show that the endogenous host defense peptide LL-37 can stimulate LPS or GM-CSF-primed PMNs to release large quantities of LTB_4, which in turn may lead to more generation of LL-37. Thus, positive feedback loops appear to exist between LL-37 and LTB_4, and the resulting proinflammatory escalation may promote the resolution of infection.

LTB_4 promotes LL-37-induced phagocytosis: a positive feedback loop for an important functional response

Cross-talk between LTB_4 and LL-37 opens for several potential functional consequences and effects in the microenvironment. One of the most important functions of PMNs and the innate immune system is the phagocytosis of invading microorganisms. LL-37 exhibits a direct, broad-spectrum bactericidal activity (8, 9, 38, 39). On the other hand, LTB_4 has also been shown to augment the ability of leukocytes to kill a variety of microorganisms (4, 5, 40, 41) and to modulate innate immune responses (6, 42). We found that LL-37 can also induce phagocytosis of *E. coli* particles and that this function is significantly enhanced by concurrent exposure to 10 nM LTB_4 (Fig. 6*A*). When PMNs were primed with GM-CSF, we observed reciprocal additive effects of both LTB_4 and LL-37 on phagocytosis evoked by the respective mediator (Fig. 6*B*). Thus, the effects of cross-talk between LL-37 and LTB_4 are not limited to their formation and signaling, but extend to functional behaviors like phagocytosis, an activity important for the efficacy and guidance of innate immune responses.

CONCLUSIONS

Here we present novel interactions between LTB_4 and the human cathelicidin LL-37 that can enhance PMN activities critical to the function of innate immune responses. It is well established that LTB_4 is involved in many inflammatory diseases, including arthritis (43) and atherosclerosis (44, 45), and that the expression of LL-37 is up-regulated in similar inflammatory diseases (15). Given the results of the present work, further investigations into the mechanisms and consequences of LTB_4 and LL-37 signaling circuits may provide new insight into leukotriene- and cathelicidin-dependent immune responses, and identify novel targets for medical treatments of the associated inflammatory conditions. FJ

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