Cathelicidin LL-37 induces time-resolved release of LTB$_4$ and TXA$_2$ by human macrophages and triggers eicosanoid generation in vivo

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ABSTRACT In humans, LL-37 and eicosanoids are important mediators of inflammation and immune responses. Here we report that LL-37 promotes leukotriene B$_4$ (LTB$_4$) and thromboxane A$_2$ (TXA$_2$) generation by human monocye-derived macrophages (HMDMs). LL-37 evokes calcium mobilization apparently via the P2X$_7$ receptor (P2X$_7$R), activation of ERK1/2 and p38 MAPRs, as well as cytosolic phospholipase A$_2$ (cPLA$_2$) and 5-lipoxygenase in HMDMs, leading to an early (1 h) release of LTB$_4$. Similarly, TXA$_2$ production at an early time involved the same signaling sequence along an LL-37-P2X$_7$R-cPLA$_2$-cylooxygenase-1 (COX-1) axis. However, at later (6–8 h) time points, internalized LL-37 up-regulates COX-2 expression, promoting TXA$_2$ production. Furthermore, intraperitoneal injection of mice with murine cathelicidin-related antimicrobial peptide (mCRAMP) induces significantly higher levels of LTB$_4$ and TXA$_2$ in mouse ascites rich in macrophages. Conversely, cathelicidin-deficient (Cnlp$^{-/-}$) mice produce much less LTB$_4$ and TXB$_2$ in vivo in response to TNF-$\alpha$ compared with control mice. We conclude that LL-37 elicits a biphasic release of eicosanoids in macrophages with early, Ca$^{2+}$-dependent formation of LTB$_4$ and TXA$_2$ followed by a late peak of TXA$_2$, generated via induction of COX-2 by internalized LL-37, thus allowing eicosanoid production in a temporally controlled manner. Moreover, our findings provide evidence that LL-37 is an endogenous regulator of eicosanoid-dependent inflammatory responses in vivo.—Wan, M., Soehnlein, O., Tang, X., van der Does, A. M., Smedler, E., Uhlén, P., Lindbom, L., Ågerberth, B., Haeggström, J. Z. Cathelicidin LL-37 induces time-resolved release of LTB$_4$ and TXA$_2$ by human macrophages and triggers eicosanoid generation in vivo. FASEB J. 28, 3456–3467 (2014). www.fasebj.org

Key Words: inflammation • antimicrobial peptides • thromboxane • leukotriene • P2X$_7$R

Macrophages are important immune effector cells in various inflammatory diseases. They respond efficiently to danger signals by phagocytosis and secretion of inflammatory eicosanoids and cytokines to recruit and activate immune cells (1). Eicosanoids are a class of oxygenated hydrophobic compounds that largely function as paracrine mediators. Metabolism of arachidonic acid (AA) leads to several families of lipid mediators collectively known as eicosanoids, including prostaglandins (PGs), thromboxane, leukotrienes (LTs), and lipoxins, along 2 major metabolic pathways, the lipoxygenase (LOX) and the cyclooxygenase (COX) pathways. Eicosanoids possess potent biological activities and are involved in maintenance of normal hemostasis, regulation of blood pressure, renal function, and reproduction, as well as host defense (2).

Phospholipase A$_2$, in particular group IV cytosolic phospholipase A$_2$ (cPLA$_2$), mediates agonist-induced AA release, which is the rate-limiting step in the biosynthesis of eicosanoids (3). Activation of cPLA$_2$ requires...
phosphorylation by p38 MAPK and an increase in intracellular calcium (4, 5). 5-LOX catalyzes oxygenation of AA, resulting in LT biosynthesis. 5-LOX activity is regulated by several factors, including intracellular Ca$^{2+}$, translocation of 5-LOX from the cytosol to the nuclear membrane, and phosphorylation of 5-LOX (6). Alternatively, AA released by cPLA$_2$ is presented to COX and is then metabolized to an intermediate PG, PGH$_2$, coupled with metabolism by downstream enzymes into PGs, prostacyclin, and thromboxane A$_2$ (7). COX exists as 2 isoforms referred to as COX-1 and COX-2. COX-1 is expressed constitutively in most tissues whereas COX-2 is not detectable in most normal tissues or resting immune cells, but its expression can be induced by factors such as endotoxins, cytokines, growth factors, and carcinogens (8).

Mammals express a variety of small, amphipathic antimicrobial peptides including defensins and cathelicidins. The only endogenous cathelicidin in humans, LL-37/hCAP-18, is expressed by various cell types, such as epithelial cells (9, 10), keratinocytes (11), and specific leukocytes (12). It is also present at high concentrations as the inactive proform hCAP-18 in the secondary granules of neutrophils (13, 14). Once hCAP18 is secreted from neutrophils, it is processed into the active LL-37 peptide by protease 3 that is present in the primary granules of neutrophils (15). Besides a broad range of direct antimicrobial activities, LL-37 also exhibits functions important for immune responses, which are mediated by several different receptors. For example, LL-37 induces angiogenesis (16) and serves as a chemoattractant for neutrophils, monocytes, T cells, and progenitor cells via FPR2/ALX (17, 18). In addition, LL-37 promotes IL-1β processing and release by activating the P2X$_7$ receptor (P2X$_7$-R; ref. 19) and induces keratinocyte migration via transactivation of epithelial growth factor receptor (EGFR; ref. 20).

In the present study, we show that LL-37 promotes LTB$_4$ and thromboxane A$_2$ (TXA$_2$) production by human monocyte-derived macrophages (HMDMs) via temporally and mechanistically distinct actions, suggesting that these 2 eicosanoids play separate roles in inflammatory responses elicited by macrophages. Finally, we demonstrate that the LL-37-eicosanoid signaling pathway indeed occurs during inflammatory responses in vivo.

MATERIALS AND METHODS

Reagents

Polyclonal antibodies against cPLA$_2$, phosphorylated cPLA$_2$, phosphorylated ERK1/2, phosphorylated p38, and COX-2 were from Cell Signaling Technology (Danvers, MA, USA). The antibodies against COX-1 and thromboxane synthase (TXAS) were purchased from Cayman Chemical (Ann Arbor, MI, USA). Human 5-LOX antiserum was a kind gift from Dr. Olof Rådmark (Karolinska Institutet, Stockholm, Sweden). RPMI 1640 cell culture medium, HEPES, phosphatase inhibitors, aspirin, SC-650, Celecoxib, KN-62, N-tosyl-L-phenylalanine chloromethyl ketone (TPCK), RIPA lysis buffer, and monoclonal anti-α-tubulin antibody were from Sigma-Aldrich (St. Louis, MO, USA). Fetal bovine serum (FBS), macrophage colony-stimulating factor (M-CSF), fura-2 acetoxymethyl ester (FURA-2AM), and Hoechst were from Life Technologies (Paisley, UK). U0126 and SB203580 were from Toecris Bioscience (Bristol, UK). Synthetic LL-37 (NH$_2$-LLGDFRRKSKIGKEFKRIVQRKDFPNLVPRTES-COOH), sequence-scrambled LL-37 (NH$_2$-GLKLREFSKIGKELKLPTEVRFRDIKLKDNRISVQR-COOH), cationic-related antimicrobial peptide (mCRAMP; NH$_2$-GLLLRGGEKIGEKLKIGQKIKNFQKLVQPQE-COOH), WKYMvm, WRW4 peptides, and panaxin-1 (Panx-1) antagonist peptide: 8Panx (WRQAFDSDY) were from Innoven (Lund, Sweden). Recombinant murine TNF-α was purchased from PeproTech (Rocky Hill, NJ, USA).

Cell culture

Human mononuclear cells were isolated from freshly prepared buffy coats (Karolinska Hospital Blood Bank, Stockholm, Sweden) by Ficoll-Paque Premium (GE Healthcare Bio-Sciences, Uppsala, Sweden) gradient centrifugation. Differentiation of human monocytes to macrophages was achieved by plating mononuclear cells in cell culture plates for 2 h, and then unbound cells were washed away with PBS, followed by cell culture over 7 d in RPMI 1640 supplemented with 10% FBS (heat-inactivated), 25 mM HEPES, penicillin (100 U/ml), streptomycin (100 μg/ml), and M-CSF (50 ng/ml).

RNA extraction and quantitative real-time PCR

Extraction of total RNA was performed with the RNA Mini kit (Bioline, London, UK) including an on-column DNase digestion step. RNA concentration and quality were assessed by Nanodrop (Thermo Scientific, Wilmington, DE, USA). cDNA was synthesized from total RNA by SuperScript II reverse transcriptase (Life Technologies), and real-time PCR was performed in a TaqMan 7300 instrument (Life Technologies). Normalizations were made to β-actin. The primer/probe pairs were obtained by Assay-on-Demand (Life Technologies).

Western blot analysis

After treatments, HMDMs were lysed with RIPA buffer together with a complete protease inhibitor cocktail (Roche Diagnostics, Mannheim, Germany) and phosphatase inhibitor cocktail. The SDS-PAGE and Western blot analysis were carried out as described previously (21).

Analysis of biosynthesis of AA-derived lipids by enzyme immunoassay

The levels of leukotrienes and prostanoids were determined with enzyme immunoassay (Cayman Chemical), according to the manufacturer’s instructions. Formation of TXA$_2$ was also assessed by LC-MS and GC-MS analysis, essentially as described previously (22, 23).

Intracellular calcium mobilization

HMDMs were cultured and differentiated as described above. Measurements of intracellular calcium were carried out with fluorescence spectroscopy using FURA2-AM, essentially as described previously (21). Briefly, the cells were incubated in medium containing 4 μM FURA-2AM plus 2.5 mM probenecid.
cid or medium with 2.5 mM probenecid for 60 min in 5% CO₂ at 37°C. Cells were then washed 4 times with a buffer solution (135 mM NaCl, 4.6 mM KCl, 1.2 mM MgCl₂, 1.5 mM CaCl₂, 11 mM glucose, and 10 mM HEPES, pH 7.4), and 50 μl of buffer was added to each well. The plates were transferred to a fluorometer (Fluostar; BMG Technologies, Ortenburg, Germany), and 50 μl of different reagents or buffer solution (control) was injected into individual wells, and the cells were monitored for 120 s. Control wells containing cells that had not been exposed to FURA-2AM were used to subtract background autofluorescence. The results are given as the ratio of mean fluorescence intensities at 340 and 380 nm.

5-LOX translocation detected by immunohistochemistry

Human primary macrophages were cultured in Lab Tek II chamber slides (Thermo Fisher Scientific, Rochester, NY, USA). After incubation with 20 μg/ml of LL-37 for 30 min, cells were washed 3 times with PBS, followed by fixation with 2% paraformaldehyde (PFA) for 10 min and permeabilization with a mixture of 0.1% sodium citrate and 0.1% Triton X-100 for 5 min at room temperature. After preincubation with 10% goat serum for 30 min, cells were incubated with human 5-LOX 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 being stained, the slides were mounted using an antifading mounting medium (Vector Laboratories, Burlingame, CA, USA) and imaged with a confocal microscope (Olympus FV1000; Olympus Corp., Tokyo, Japan).

Flow cytometric analysis

HMDMs were cultured and differentiated in glass-bottomed microwell dishes (MatTek Corp., Ashland, MA, USA) for 7 d. After being washed with PBS, FITC-conjugated LL-37 (10 μg/ml) was added into the dishes and incubated at 37°C for 15 min, and then Heo cheat (0.5 μg/ml) was added for nuclear staining. After 5 min, the fluorescence in live cells was visualized by confocal microscopy (Olympus FV1000).

Statistical analysis

Results are presented as means ± sd. Differences between the means were evaluated using 1-way ANOVA or Student’s t test. A value of P < 0.05 was considered statistically significant.

RESULTS

LL-37 promotes the release of arachidonic acid-derived lipid mediators from HMDMs in a time- and dose-dependent manner

The levels of LTB₄, cysteinyl LTs (cysLT₁), PGI₂ (analyzed as 6-keto PGF₁α), PGE₂, and TXA₂ (analyzed as TXB₂) in the retrieved cell culture medium were measured after HMDMs were incubated with 20 μg/ml of LL-37 for different time periods. We found that LL-37 enhances the production of TXB₂, PGE₂, and LTB₄ from HMDMs, while the concentrations of 6-keto PGF₁α and cysLTs remain unchanged (Fig. 1A). Since HMDMs produced much higher amounts of TXB₂ than PGE₂ in response to LL-37 treatment, we next focused on LL-37-induced TXB₂ and LTB₄ production by HMDMs. Our results further show that LL-37-induced LTB₄ production from HMDMs peaked at ~1 h (Fig. 1B), while TXB₂ production steadily increased and peaked at a late time point at ~6–8 h (Fig. 1C). Furthermore, LL-37 promoted LTB₄ and TXB₂ production of HMDMs in a dose-dependent manner; while sequence-scrambled LL-37 (sLL-37) did not evoke any significant responses (Fig. 1D, E).

Intraperitoneal injection

In the first experiment, WT male C57BL/6 mice were intraperitoneally injected with 100 μl of PBS, mouse TNF-α (mTNFα, 50 ng/mouse), or mCRAMP (10 μg/mouse). In the second experiment, WT and Cnlp−/− male C57BL/6 mice were intraperitoneally injected with 100 μl mTNFα (50 ng/mouse). After 4 h, the peritoneal cavity was lavaged with 3 ml of PBS. The lavage fluid was centrifuged, and the resulting cell pellet was resuspended in PBS with 0.5% BSA and stained with anti-Gr-1 (Biolegend, San Diego, CA, USA) and anti-F4/80 (AbD Serotec, Düsseldorf, Germany) to discriminate between neutrophils, monocytes, and macrophages. Cell-surface molecule expression was assessed on a FACSort (BD Biosciences) and analyzed with BD Cellquest software to determine the percentage neutrophils (Gr-1⁺), monocytes (Gr-1⁻), and macrophages (F4/80⁻) in the recruited cell population. The remaining supernatants were saved at −80°C for subsequent detection of LTB₄ and TXB₂.

Further studies demonstrated that LL-37 but not sLL-37 induced intracellular calcium mobilization in HMDMs (Fig. 2A). In addition, two MAPKs, ERK1/2 and p38, were activated in LL-37-treated HMDMs in a time-dependent manner (Fig. 2B). Moreover, the specific ERK inhibitor U0126 totally abolished LL-37-induced LTB₄ production (Fig. 2C). The potent p38 MAPK inhibitor SB203580 also partly suppressed
LTB₄ production in LL-37-treated HMDMs (Fig. 2D). Lastly, cPLA₂ and 5-LOX, 2 key enzymes responsible for LTB₄ production, were activated in LL-37-treated HMDMs. Thus, LL-37 induced cPLA₂ phosphorylation in a time-dependent manner (Fig. 2B), and translocation of 5-LOX from cytosol into the nucleus was detected by confocal microscopy (Fig. 2E and Supplemental Fig. S1), although the gene and protein expression of 5-LOX was not significantly altered in LL-37-treated HMDMs (Fig. 3A, B). Moreover, our results showed that LL-37 (10 and 20 μg/ml) had no effects on cell viability of HMDMs (Supplemental Fig. S2), indicating that the LL-37-induced effects in our experimental settings were not due to cytotoxic activity of LL-37 on macrophages.

COX-1 and COX-2 are involved in LL-37-induced TXA₂ production by HMDMs with different temporal specificity

Additional experiments showed that LL-37 selectively upregulated the gene and protein expression of COX-2 in a time-dependent manner (Fig. 3A, B). Moreover, the ERK MAPK inhibitor U0126 significantly suppressed LL-37-induced COX-2 gene expression (Fig. 3C) and TXB₂ production at 6 h (Fig. 3D). However, the p38 MAPK inhibitor SB203580 and NF-κB inhibitor TPCK exhibited no significant effects on LL-37-induced COX-2 gene expression and TXB₂ production, indicating that these responses are independent of p38 MAPK and NF-κB pathways (Fig. 3C–E). Interestingly, the COX inhibitor aspirin and selective COX-1 inhibitor SC-560, but not the specific COX-2 inhibitor celecoxib, completely abolished LL-37-induced TXB₂ production after 1 h (Fig. 3F). In contrast, celecoxib completely inhibited LL-37-induced TXB₂ production by HMDMs at 6 h (Fig. 3G), demonstrating that LL-37-induced TXB₂ production by HMDMs at late time points is mainly derived from COX-2.

P2X₇R is a candidate receptor involved in LL-37-induced early production of LTB₄ and TXA₂

Our finding that sLL-37 does not promote LTB₄ or TXB₂ production from HMDMs (Fig. 1D, E) indicates that LL-37-induced responses are mediated by specific receptor(s). Therefore, we tested several inhibitors of receptors known to transduce LL-37 signaling (the specific P2X₇R inhibitor KN-62, FPR2/ALX antagonist WRW4, P2X receptor inhibitor suramin, GPCR inhibitor pertussis toxin, and EGFR inhibitor AG-1478). The specific P2X₇R inhibitor KN-62 totally abolished LTB₄ production from LL-37-treated HMDMs at 1 h (Fig. 4A), while the FPR2/ALX antagonist WRW4 had no effect (Fig. 4B). KN-62 pretreatment also blocked LL-37-induced intracellular calcium mobilization (Fig. 4C), as did a specific P2X₇R antibody (Fig. 4D). KN-62 also partly inhibited phosphorylation of ERK1/2 in LL-37-treated HMDMs (Fig. 4E). Accordingly, WRW4 did not inhibit ERK1/2 phosphorylation in LL-37-treated HMDMs (Fig. 4E). Surprisingly, KN-62 pretreatment had no effect on LL-37-induced TXB₂ production at 6 h.
or LL-37-induced up-regulation of COX-2 gene expression at 4 h (Fig. 4H), although it totally abolished LL-37-induced TXB₂ production at 1 h (Fig. 4F). We also tested WRW4, suramin, pertussis toxin, and AG-1478, but none of them exhibited inhibitory effects on LL-37-induced COX-2 expression or TXB₂ production at late time points (Supplemental Fig. S3).

It has been reported that Panx-1 is a component of the P2X₇R signaling pathways and Panx1 channels can be activated by prolonged stimulation of P2X₇R (25). However, blocking of Panx-1 by a specific antagonist, viz., peptide ¹⁰Panx, did not show any inhibitory effects on LL-37-induced LTB₄ release at 1 h (Fig. 4I) or TXB₂ production 6 h (Fig. 4J).

**P < 0.01, ***P < 0.001.
Internalization of LL-37 by HMDMs contributes to LL-37-induced late COX-2 activation and TXA2 production

Next, we searched for the molecular process that is responsible for LL-37-induced COX-2 expression and TXA2 production at late time points. Intriguingly, we found that LL-37 was internalized by HMDMs and located in the cytosolic compartment (Fig. 5A, B, cf. Supplemental Video S1). Two inhibitors of endocytosis, cytochalasin B and dynasore, significantly
blocked LL-37 internalization by HMDMs (Fig. 5C) as well as COX-2 induction (Fig. 5D, E) and TXB₂ production (Fig. 5F, G) at late time points, suggesting that COX-2 expression is enhanced via internalized LL-37.

Cathelicidin elicits LTB₄ and TXA₂ production in vivo

To find evidence for the involvement of cathelicidin in the production of eicosanoids in vivo, we designed 2 experiments. First, C57BL/6 WT mice were injected intraperitoneally with mCRAMP (mouse LL-37 orthologue), using PBS and mTNFα/H9251 as negative and positive controls, respectively. Thus, injection of mCRAMP significantly enhanced levels of LTB₄ (Fig. 6A) and TXA₂ (Fig. 6B) in mouse ascites. Meanwhile, the amount of leukocytes in ascites from mice injected with PBS was 2.14 ± 0.87 x 10⁵ cells/ml (~40% macrophages, ~37% neutrophils, and ~23% monocytes), and similar amounts and composition of cells were observed in ascites from mice injected with mCRAMP (2.14 ± 0.77 x 10⁵ cells/ml, ~35% macrophages, ~37% neutrophils, and ~28% monocytes). In mice injected with mTNFα, the total amount of cells and relative amounts of neutrophils were increased (3.94 ± 1.24 x 10⁵ cells/ml, ~24% macrophages, ~50% neutrophils, and ~26% monocytes). In the second experiment, mTNFα was injected intraperitoneally into WT and Cnlp⁻/⁻ mice. After 4 h, significantly less LTB₄ and TXB₂ were detected in mouse ascites from Cnlp⁻/⁻ mice than WT mice (Fig. 6C, D). Compared with WT mice (6.24 ± 5.66 x 10⁵ cells/ml, ~40% macrophages, ~56% neutrophils, and ~4% monocytes), the total amount of...
cells and macrophages were slightly increased in ascites from Cnlp−/− mice (8.54±4.73×10⁵ cells/ml, ~52% macrophages, ~43% neutrophils, and ~5% monocytes).

**DISCUSSION**

Previous work has shown that various antimicrobial peptides, including LL-37, are released by human polymorphonuclear neutrophils, epithelial cells, and additional inflammatory cells during infection and certain inflammatory responses. The released peptides interact with the surrounding macrophages to enhance clearance of bacteria (26), promote adhesion of classical monocytes (27) and regulate the production of inflammatory mediators in different cell types (28–30). Therefore, we attempted to investigate if LL-37 can regulate the production of eicosanoids by HMDMs. Results from initial LC-MS and GC-MS analyses indicated that the level of TXB₂ in the medium from LL-37-treated HMDMs was significantly elevated. This result was confirmed by screening the production of a series of AA-derived lipid mediators [LTB₄, CysLTs, PGI₂ (as 6-keto PGF₁α), PGE₂, and TXA₂ (as TXB₂)] by HMDMs treated with LL-37 for different time points and was found that the amounts of LTB₄, TXA₂, and PGE₂ released from HMDMs were significantly increased after treatment with LL-37.

**COX-1 and COX-2 mediate LL-37-induced early and late TXB₂ production by HMDMs**

Several lines of evidence indicate that the 2 COX isoforms can regulate different phases of prostanoid biosynthesis in activated cells (31–33). In line with this notion, we found that intact HMDMs contain abundant COX-1 whereas COX-2 is expressed at low levels (Fig.
At early time points, before COX-2 expression is upregulated (~1 h), free AA is metabolized by existing COX-1 and TXAS into TXA2. However, COX-2 expression is gradually elevated and both COX-1 and COX-2 exist in HMDMs at late time points (~6 h). Although COX-2 appears to couple preferentially to PGE synthase or PGI2 synthase in various cell types (7), our pharmacological data show that COX-2 couples to TXAS to produce TXA2 in LL-37-stimulated HMDMs at late time points.

LL-37-induced early LTB4 and TXB2 production appears to be mediated via P2X7R

We next asked whether any of the previously described cell surface receptors for LL-37, viz. FPR2/ALX (16, 17) P2X7R, (19), EGFR (20), and CXCR2 (34), are involved the biphasic profile of eicosanoid release from HMDMs. Since LL-37-induced LTB4 production in human neutrophils is mediated via FPR2/ALX, we were surprised that our pharmacological data, as well as blocking effects of a P2X7R antibody, suggested that LL-37-induced early lipid production is signaled via P2X7R, rather than the expected FPR2/ALX (Fig. 4).

P2X7R is a ligand-gated ion channel and shows remarkable functional plasticity (35). It has been reported that P2X7R is highly expressed by cells of the hemopoietic lineage and mediates cell death, killing of infectious organisms, and regulation of inflammatory responses (36). In our hands, HMDMs also demonstrated high expression of P2X7R (Supplemental Fig. S4). We also ascertained that effects of LL-37 are not an indirect effect of the P2X7R ligand ATP or involve the channel protein Panx-1 that can be activated by prolonged stimulation of P2X7R (25) leading to formation of pore-like structures permeable to large organic molecules up to 900 Da (ref. 37, Supplemental Fig. S5, and Fig. 4I, J).

Internalization of LL-37 by HMDMs contributes to COX-2 up-regulation

We next used pharmacological tools to test whether TXA2 production by HMDMs at late time points is receptor dependent. We could not demonstrate involvement of P2X7R, although it has been reported that this receptor signals LL-37-induced COX-2 expression and PGE2 production in human gingival fibroblasts (38). Furthermore, suramin (P2X), WRW4 (FPR2/ALX), AG-1478 (EGF), and pertussis toxin (GPCR) all failed to affect LL-37-induced COX-2 expression and TXA2 production (Supplemental Fig. S3). Instead, we found that LL-37, with time, was internalized by HMDMs, gradually localizing in the cytosolic compartment (Fig. 5A, B, and Supplemental Video S1). Interestingly, previous studies have also indicated that endocytosis of LL-37 is associated with several functional responses of the recipient cell. For example, in this way, LL-37 induced IL-8 expression in epithelial cells, stimulated maturation/differentiation of human dendritic cells and monocytes, and changed adhesive properties of endothelial cells (10, 27, 39, 40). However, in all these instances it was not clarified how internalized LL-37 couples to intracellular second messengers, in our case activated ERK MAPK, to elicit a biological response. A mechanistic model for LL-37-induced early and late eicosanoid generation in macrophages is presented in Fig. 7.

Cathelicidin mCRAMP can regulate eicosanoid production in vivo

To determine whether LL-37 can regulate eicosanoid production in vivo, we used the acute peritonitis model...
Pathophysiological implications of LL-37-induced eicosanoid production by HMDMs

An inflammatory response can be divided into separate phases, *i.e.*, initiation, progression, and resolution, involving an array of cells and mediators. Recently, different classes of lipid mediators have been shown to promote discrete steps in this process, with LTB₄ as an early initiating mediator, followed by PGE₂, and finally an array of proresolving lipid mediators, including lipoxins, resolvins, protectins, and maresins (42). Levy *et al.* (43) demonstrated that during the course of a self-resolving acute inflammatory response there is a clear temporal staging and functional interconnections between distinct classes of eicosanoids. At early time points, 5-LOX–dependent LTB₄ generation prevails and contributes to the initiation phase. Then appears COX-derived PGE₂, which dampens 5-LOX and promotes 15-LO expression and LXA₄ production, resulting in resolution. In our experimental setting, LTB₄ and TXA₂ production by LL-37-treated macrophages reached the highest levels at ~1 and 6 h, respectively. These temporal relationships suggest that LTB₄ will contribute to the initiation phase with recruitment of leukocytes to the inflammatory site, whereas TXA₂ will appear later during the progression phase with a role yet to be determined. We also observed that LL-37 induced PGE₂ formation, which *in vivo* may dampen the 5-LOX pathway and promote resolution (Fig. 1).

Inasmuch as LL-37 and eicosanoids are both important mediators of the innate immune responses, the potential physiological or pathological consequences of LL-37-induced eicosanoid generation by macrophages are vast. If one considers the most predominant product downstream of LL-37, viz. TXA₂, this prothrombotic mediator plays a role in endothelial migration and angiogenesis (44) and is an established atherogenic mediator (45–47). Interestingly, elevated levels of both LL-37 and TXAS have been detected in human atherosclerotic plaque, associated with macrophages (23, 48, 49), and mice deficient in the mouse cathelicidin mCRAMP are protected against atherosclerosis (50).

Thus, it seems possible that macrophage-dependent LL-37-TXA₂ signaling may contribute to the pathogenesis of atherosclerosis and the thrombotic state characterizing plaque instability. Further studies are required to delineate the role of LL-37, TXA₂, and LTB₄ in macrophage-driven disease processes, and whether this signaling pathway may offer opportunities for therapeutic interventions.

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**Figure 7.** Schematic model for activation of 5-LOX and COX pathways in human macrophages by LL-37. *A* Resting HMDMs express high levels of P2X₇, cPLA₂, 5-LOX, and COX-1 while COX-2 expression is hardly detected. *B* Once HMDMs are exposed to LL-37, P2X₇ is activated to induce calcium influx and rapid activation of ERK and p38 MAPKs, resulting in cPLA₂ phosphorylation and 5-LOX translocation from cytosol to the nuclear compartment. Activated cPLA₂ releases free AA, which is further metabolized into LTB₄ and TXB₂ in the early phase (~1 h) via 5-LOX and COX-1, respectively. On the other hand, in the late phase, LL-37 is internalized by HMDMs which is associated with ERK MAPK activation, resulting in enhanced COX-2 expression and increased TXB₂ production.

in the mouse in a dual approach. Thus, we injected murine cathelicidin (mCRAMP) into the peritoneal cavity of WT mice, which resulted in significantly increased levels of both LTB₄ and TXB₂ in the peritoneal lavage fluid. Conversely, injection of murine TNF-α into the peritoneal cavity of WT mice and mice deficient in mCRAMP (*Cnlp−/−* mice) revealed significantly reduced levels of LTB₄ and TXB₂ in lavage fluids from mCRAMP-deficient mice. We have previously shown that LL-37 also affects LTB₄ production from human neutrophils (21, 41). Therefore, we cannot distinguish the cell source(s) of eicosanoid production in our mice experiments. Taken together, our *in vivo* results corroborate our *in vitro* results and provide convincing evidence that mCRAMP can regulate eicosanoid generation from leukocytes *in vivo*. 

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