Modulation of the TLR-Mediated Inflammatory Response by the Endogenous Human Host Defense Peptide LL-37


*J. Immunol.* 2006;176:2455-2464

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Modulation of the TLR-Mediated Inflammatory Response by the Endogenous Human Host Defense Peptide LL-37

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The sole human cathelicidin peptide, LL-37, has been demonstrated to protect animals against endotoxemia/sepsis. Low, physiological concentrations of LL-37 (≤1 μg/ml) were able to modulate inflammatory responses by inhibiting the release of the proinflammatory cytokine TNF-α in LPS-stimulated human monocytic cells. Microarray studies established a temporal transcriptional profile and identified differentially expressed genes in LPS-stimulated monocytes in the presence or absence of LL-37. LL-37 significantly inhibited the expression of specific proinflammatory genes up-regulated by NF-κB in the presence of LPS, including NFκB1 (p105/p50) and TNF-α-induced protein 2 (TNFIP2). In contrast, LL-37 did not significantly inhibit LPS-induced genes that antagonize inflammation, such as TNF-α-induced protein 3 (TNFIP3) and the NF-κB inhibitor, NFκBIA, or certain chemokine genes that are classically considered proinflammatory. Nuclear translocation, in LPS-treated cells, of the NF-κB pathway. LL-37 almost completely prevented the release of TNF-α and other cytokines by human PBMC following stimulation with LPS and other TLR2/4 and TLR9 agonists, but not with cytokines TNF-α or IL-1β. Biochemical and inhibitor studies were consistent with a model whereby LL-37 modulated the inflammatory response to LPS/endotoxin and other agonists of TLR by a complex mechanism involving multiple points of intervention. We propose that the natural human host defense peptide LL-37 plays roles in the delicate balancing of inflammatory responses in homeostasis as well as in combating sepsis induced by certain TLR agonists. The Journal of Immunology, 2006, 176: 2455–2464.

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Received for publication July 27, 2005. Accepted for publication November 8, 2005.

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1 This work was supported by Genome Prairie, Genome BC, and Inimex Pharmaceuticals for the “Functional Pathogenomics of Mucosal Immunity” research program. R.E.W.H. was supported by a Canada Research Chair Award, F.S.L.B. is a Canadian Institutes of Health Research (CIHR) New Investigator, F.S.L.B. and J.B. are Michael Smith Foundation for Health Research Scholars, J.B. was supported by Natural Sciences and Engineering Research Council, D.M.E.B. was supported by a CIHR studentship, and K.H. was supported by a postdoctoral award from the Michael Smith Foundation for Health Research.

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found at high concentrations in its unprocessed form (hCAP-18) in the granules of neutrophils and is processed upon degranulation and release (12). It is also produced by epithelial cells and keratinocytes, as the hCAP-18 precursor form, and is found as the processed 37-aa peptide LL-37 in a number of tissues and body fluids including gastric fluid, saliva, semen, sweat, plasma, airway surface liquid, and breast milk (13–17). Although LL-37 is often defined as an antimicrobial (direct killing) peptide (18), it has been demonstrated that, at physiological salt conditions, this peptide is not antimicrobial at the concentrations (1–5 µg/ml) normally found in adults at mucosal surfaces (19). Moreover, under these conditions and at these concentrations, LL-37 exhibits a variety of immunomodulatory functions (20–24). This could help to explain why LL-37 administration can protect mice against certain bacterial infections due to its ability to modulate immunity (19), LL-37 is also able to protect mice and rats against endotoxemia/sepsis induced by pure LPS (20, 25), indicating that LL-37 can suppress potentially harmful proinflammatory responses. Consistent with this, LL-37, like other cationic peptides (26) is able to reduce the LPS-stimulated production of proinflammatory cytokines in the presence of whole human blood (M. G. Scott and R. E. W. Hancock, unpublished results), as well as in the presence of 5% autologous human serum (N. Mookherjee and R. E. W. Hancock, unpublished results). The mechanism by which LL-37 confers protection at physiological concentrations merits further investigation.

This report provides evidence that human host defense peptide LL-37 has potent anti-endotoxin properties, at very low (≤1 µg/ml) concentrations and physiological salt conditions reflecting those found in vivo, e.g., at mucosal surfaces and in tissues. It is further demonstrated here that LL-37 had a general anti-inflammatory effect on TLR stimulation, inhibiting proinflammatory cytokine release from human monocytic cells stimulated with TLR2, TLR4, and TLR9 agonists. The suppression of inflammatory responses by LL-37 in LPS-stimulated cells was selective, because LL-37 did not block the expression of certain (proinflammatory) genes required for cell recruitment and movement, yet abrogated proinflammatory cytokine responses that can potentially lead to sepsis. The anti-inflammatory activity of LL-37 was apparently mediated through a diversity of mechanisms. This study suggests that the delicate balance of inflammatory genes involved in homeostasis, inflammation, and sepsis can be regulated by the human host defense peptide LL-37.

**Materials and Methods**

**Cell isolation and cell lines**

Human monocytic cells, THP-1 27), were obtained from American Type Culture Collection (ATCC) (TIB-202) and were grown in suspension in RPMI 1640 medium (Invitrogen Life Technologies), supplemented with 10% (v/v) heat-inactivated FBS, 2 mM l-glutamine, and 1 mM sodium pyruvate (all from Invitrogen Life Technologies). Cultures were maintained at 37°C in a humidified 5% (v/v) CO2 incubator up to a maximum of six passages. THP-1 cells at a density of 1 × 10⁶ cells/ml were treated with 0.3 µg/ml PMA (Sigma-Aldrich) for 24 h (28), inducing plastic-adherent cells that were further rest in complete RPMI 1640 medium for an additional 24 h before stimulations with various treatments. Venous blood (20 ml) from healthy volunteers was collected in Vacutainer collection tubes containing sodium heparin as an anticoagulant (BD Biosciences) in accordance with UBC ethical approval and guidelines. Blood was diluted 1/1 with complete RPMI 1640 medium and separated by centrifugation over a Ficoll-Paque Plus (Amersham Biosciences) density gradient. White blood cells were isolated from theuffy coat, washed twice in RPMI 1640 complete medium, and the number of PBMC was determined by trypan blue exclusion. PBMC (5 × 10⁶) were seeded into 12-well tissue culture dishes (Falcon; BD Biosciences) at 1 × 10⁶ cells/ml at 37°C in 5% CO2. All experiments using human THP-1 cells or PBMCs involved at least three biological replicates. The above conditions were chosen to mimic conditions for circulating blood monocytes entering tissues at the site of infection via extravasation.

**Stimulants, reagents, and Abs**

LPS from *Pseudomonas aeruginosa* strain H103 was highly purified free of proteins and lipids using the Darveau-Hancock method as described previously (29). Briefly, *P. aeruginosa* was grown overnight in Luria-Bertani broth at 37°C. Cells were collected and washed, and the isolated LPS pellets were extracted with a 2:1 chloroform:methanol solution to remove contaminating lipids. Purified LPS samples were quantitated using an assay for the specific sugar 2-keto-3-deoxyoctulosonic acid (KDO assay) and then resuspended in endotoxin-free water (Sigma-Aldrich).

TLR2 agonists lipoteichoic acid (LTA) from *Staphylococcus aureus* and a synthetic tripleteoioylated lipopeptide, Pam3CSK4, were purchased from InvivoGen. TLR9 agonist CpG oligodeoxynucleotide no. 2007 (30) was a gift from Dr. L. Babuik (Vaccine and Infectious Disease Organization, Saskatoon, SK, Canada). Recombinant human TNF-α and recombinant human IL-1β were obtained from Research Diagnostics (Flanders, NJ). All reagents were tested for endotoxin and reconstituted in endotoxin-free water. LTA from *S. aureus* used in this study had 1.25 endotoxin units (EU) per microgram of LTA. Polymyxin B was purchased from InvivoGen, actinomycin D (transcriptional inhibitor) was purchased from Calbiochem-Novabiochem, and monensin (inhibitor of protein secretion) was purchased from eBiosciences. The cationic peptide, human LL-37 (LLFRRSKESKEKIGKEFKRIVQRIKDFFRNLVPRTES), was synthesized using F-moc chemistry at the Nucleic Acid/Protein Synthesis Unit, University of British Columbia. The synthetic peptide was resuspended in endotoxin-free water and stored at −20°C until further use.

Rabbit polyclonal Abs against the NF-κB subunits p105/p50, p65 and RelB were purchased from Cell Signaling Technologies. Rabbit polyclonal Ab against the NF-κB subunit c-Rel was purchased from Chemicon International and mouse IgG2a mAb against NF-κB subunit p100/p52 was purchased from Upstate Cell Signaling Solutions. HRP-conjugated goat anti-rabbit and anti-mouse IgG Abs were purchased from Cell Signaling Technologies and Amersham Biosciences, respectively.

**Treatment with inflammatory stimuli, peptide, or inhibitors**

THP-1 cells or PBMCs were stimulated with LPS (10 or 100 ng/ml), LTA (1 µg/ml), Pam3CSK4 (100 ng/ml), CpG-ODN 2007 (2 µg/ml) (30), recombinant human TNF-α (50 ng/ml), or recombinant human IL-1β (50 ng/ml) for 1, 2, 4, or 24 h as indicated in Results. LL-37 (0.5–50 µg/ml) was added simultaneously or 30 min after addition of the stimulants as indicated in Results. Alternatively, cells were stimulated with LL-37 (20 µg/ml) for 30 min, washed with RPMI 1640 complete medium to remove the peptide and then stimulated with LPS (100 ng/ml). Polymyxin B (0.1 mg/ml), actinomycin D (4 µg/ml), or monensin (working concentration as per the manufacturer’s instructions) were added to the THP-1 cells 30 min before stimulants.

**Detection of cytokines**

Following incubation of the cells under various treatment regimens, the tissue culture supernatants were centrifuged at 1000 × g for 5 min, and then at 10,000 × g for 2 min to obtain cell-free samples. Supernatants were aliquoted and then stored at −20°C before assay for various cytokines. TNF-α and IL-8 secretion were detected with a capture ELISA (eBioScience and BioSource International, respectively) using either tissue culture supernatants or the nuclear and cytoplasmic extracts (see Nuclear and cytoplasmic extracts) as per the experimental design. All assays were performed in triplicate. The concentration of the cytokines in the culture medium was quantified by establishing a standard curve with serial dilutions of the recombinant human TNF-α or IL-8, respectively. Alternatively, five cytokines (GM-CSF, IL-1β, IL-6, IL-8, and TNF-α) were measured simultaneously using the Human Cytokine 5-Plex kit from BioSource International (Medicorp) per the manufacturer’s instructions. The multiplex bead immunoassays were analyzed using Luminox 100 StarStation software (Applied Cytometry Systems).

**RNA extraction, amplification, and hybridization to DNA microarrays**

RNA was isolated from THP-1 cells with RNaseasy Mini kit, treated with RNase-Free DNase (Qiagen), and eluted in RNase-free water (Ambion) per the manufacturer’s instructions. RNA concentration, integrity, and purity

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4 Abbreviations used in this paper: LTA, lipoteichoic acid; qPCR, quantitative real-time PCR; PVDF, polyvinylidene difluoride; LB; LPS-binding protein.
were assessed by Agilent 2100 Bioanalyzer using RNA 6000 Nano kits (Agilent Technologies). RNA was (reverse) transcribed with incorporation of amino-allyl-UTP (aa-UTP) using the MessageAmp II amplification kit, according to the manufacturer’s instructions, and then column purified and eluted in nuclease-free water. Column-purified samples were labeled with monofunctional dyes, cyanine-3 and cyanine-5 (Amersham Biosciences), according to the manufacturer’s instructions, and then purified using the Mega Clear kit (Ambion). Yields and fluorophore incorporation was measured using a 35 UV/VIS fluorometer (PerkinElmer Life and Analytical Sciences). Microarray slides were printed with the human genome 21K array-Ready Oligo Set (Qiagen) at The Jack Bell Research Center (Vancouver, BC, Canada). The slides were prehybridized for 45 min at 48°C in prehybridization buffer containing 5X SSC (Ambion), 0.1% (w/v) SDS, and 0.2% (w/v) BSA. Equivalent (20 pmol) cyanine-labeled samples from control and treated cells were then mixed and hybridized on the array slides, in Ambion SlideHyb buffer no. 2 (Ambion) for 18 h at 37°C in a hybridization oven. Following hybridization, the slides were washed twice in 1X SSC/0.1% SDS for 5 min at 65°C, and then twice in 1X SSC and 0.1X SSC for 3 min each at 42°C. Slides were centrifuged for 5 min at 1000 × g, dried, and scanned using ScanArray Express software/scanner (scanner and software by Packard BioScience BioChip Technologies), and the images were quantified using ImaGene (BioDiscovery).

Analysis of DNA microarrays
Assessment of slide quality, normalization, detection of differential gene expression, and statistical analysis was conducted with ArrayPipe (version 1.6), a web-based, semiautomated software specifically designed for processing of microarray data (31) (www.pathogenomics.ca/arraypipe)). The following processing steps were applied: 1) flagging of markers and control spots, 2) subgrid-wise background correction, using the median of the lower 10% foreground intensity as an estimate for the background noise, 3) data-shifting, to rescue most of the negative spots, 4) printTip LOESS normalization, 5) merging of technical replicates, 6) two-sided one-sample Student’s t test on the log2 ratios within each treatment group, and 7) averaging of biological replicates to yield overall fold changes for each treatment group. Furthermore, the gene expression data was overlaid on molecular interaction networks using Cytoscape (32). Interactions networks were constructed from manually curated data and information contained within the Transpathway database (33). Differentially expressed genes with similar temporal expression profiles were clustered using the partitioning algorithm K-means procedure (as implemented in TIGR’s Multiple Experiment Viewer), with each cluster representing a set of potential coregulated genes (based on their similar expression profiles over time), although the number of time points used prevents highly precise predictions. Confirmation of array results was done using quantitative real-time PCR (qPCR) (described below), at four different time points, resulting in 70% validation (14 of 20 selected genes).

qPCR
Differential gene expression identified by microarray analysis was validated using qPCR using SuperScript III Platinum Two-Step qRT-PCR kit with SYBR Green (Invitrogen Life Technologies), per the manufacturer’s instructions, in the ABI Prism 7000 sequence detection system (Applied Biosystems). Briefly, 1 µg of total RNA was reverse transcribed in a 20-µl reaction volume for 50 min at 42°C, and the reaction was terminated by incubating for 5 min at 85°C and then digested for 30 min at 37°C with RNase H. The PCR was conducted in a 12.5-µl reaction volume containing 2.5 µl of 1/10 diluted cDNA template. A melting curve was performed to ensure that any product detected was specific to the desired amplicon. Fold changes were calculated after normalization to endogenous GAPDH and using the comparative Ct method (34). The primers used for qRT-PCR are reported in Table I.

Nuclear and cytoplasmic extracts
THP-1 cells (3 × 10^6) seeded into 60-mm² petri dishes (VWR International) were pretreated with inhibitors for 30 min, and then stimulated with agonists or peptide for 30 or 60 min. Cells were subsequently treated with Versene for 10 min at 37°C in 5% CO₂ (to detach adherent cells), and then washed twice with ice-cold PBS. Cytoplasmic and nuclear extracts were isolated using NE-PER Nuclear and Cytoplasmic Extraction Reagents kit (Pierce Biotechnology) according to the manufacturer’s instructions. The protein concentration of the extracts was quantified using a bichinonic acid (BCA) protein assay (Pierce Biotechnology), and the extracts were stored at −80°C until further use.

Translocation of NF-κB subunits
Equivalent nuclear extracts (5–10 µg) were resolved on a 7.5% SDSPAGE and transferred to polyvinylidene difluoride (PVDF) Immobilon-P membranes (Millipore). Equivalent protein loading was verified by staining PVDF membranes with Blot-Fast-Stain (Chemicon International) according to the manufacturer’s instructions. Subsequently, the PVDF membranes were incubated with anti-p105/p50, anti-p65, anti-c-REL, anti-NFκB, or anti-p100/p52 Abs at 1/1000 dilution in TBST (20 mM Tris (pH 7.5), 150 mM NaCl, 0.1% Tween 20) containing 5% skimmed milk powder (TBST/milk) for 1 h. Membranes were washed for 1 h in TBST and then incubated with a 1/5000 dilution of HRP-conjugated goat anti-mouse or anti-rabbit Ab (in TBST/milk) for 30 min. The membranes were incubated for 30 to 60 min in TBST and developed with chemiluminescence peroxidase substrate (Sigma-Aldrich), according to the manufacturer’s instructions. Alternatively, equivalent nuclear extracts (2.5–10 µg) were analyzed for NF-κB subunits p50 or p65 by StressXpress NF-κB p50 or p65 ELISA kits (Stressgen Bioreagents) according to the manufacturer’s instructions. Luminescence was detected with SpectraFluor Plus Multifunction Microplate Reader (Tecan Systems).

Results
Low, physiological concentrations of LL-37 suppress LPS-induced secretion of the proinflammatory cytokine TNF-α

LL-37 is found at mucosal surfaces at concentrations of ∼2.5 to 5 µg/ml in adults and up to 20 µg/ml in infants (35). Previous studies indicated that it has the ability to down-regulate proinflammatory cytokines in isolated mononuclear cells (20, 36). To determine the lowest dose of LL-37 that exhibited anti-endotoxin activity, THP-1 cells were stimulated with LPS (10 and 100 ng/ml) in the absence or presence of LL-37 added simultaneously at concentrations ranging from 0.5 to 50 µg/ml for a period of 4 h in complete RPMI 1640 cell culture medium (i.e., which contains physiological

### Table I. Sequence of primers (human) used for qPCR

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<th>Gene</th>
<th>Forward Primer (5’-3’)</th>
<th>Reverse Primer (5’-3’)</th>
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<td>GCCGCTTCACACCTGTCGAC</td>
</tr>
<tr>
<td>IL-8</td>
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</tr>
<tr>
<td>GAPDH</td>
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<td>GAAACTGTCGCGTGATAGG</td>
</tr>
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<tr>
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<td>AGGAAACCCGGAGAAGAAG</td>
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<tr>
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<td>TTGGTCCAGGATGCCGCT</td>
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</tr>
<tr>
<td>RELB</td>
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<td>TGGGTCCTGAAGAACATCGAGAATA</td>
</tr>
</tbody>
</table>
| NFκBIA | GTTCCAGAGGGGACCTGCTTAC | GTTCCAGCAGAATTTTCT }

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FIGURE 1. LL-37 suppresses LPS-induced secretion of TNF-α. The concentration of the proinflammatory cytokine TNF-α (y-axis) was monitored in the tissue culture supernatant or cytoplasmic extracts of cells by ELISA. The results are an average (±SD) of three independent experiments. A, THP-1 cells were stimulated with 10 or 100 ng/ml LPS in the presence of increasing concentrations of LL-37 (x-axis) for 4 h. B, PBMCs were stimulated with 100 ng/ml LPS in presence or absence of 20 μg/ml LL-37 for 4 h. The anti-endotoxin effect of LL-37 demonstrated in PBMC was statistically significant with a value of p < 0.05 (**). C, THP-1 cells were stimulated with 10 or 100 ng/ml LPS in the presence of increasing actinomycin D (inhibitor; Inh); the effect of actinomycin D on LPS-induced TNF-α secretion was statistically significant with a value of p < 0.001 (**). D, Cytoplasmic extracts of THP-1 cells treated with LPS, LL-37, or LPS plus LL-37 for 60 min in the absence or presence of monensin (inhibitor; Inh) were monitored by ELISA.

salt concentrations). Tissue culture supernatants were assayed by ELISA for the presence of the proinflammatory cytokine TNF-α (Fig. 1A). Very low concentrations (≤1 μg/ml) of LL-37 inhibited TNF-α release from LPS-induced cells, demonstrating that physiological concentrations of LL-37 exhibit anti-endotoxin activity. The anti-endotoxin effect of LL-37 was more pronounced when the cells were stimulated with 10 ng/ml LPS, a concentration at the lower level of concentrations used by investigators to mimic TLR signaling responses, but considerably higher than circulating endotoxin concentrations in septic patients (37). Under these conditions, 0.5 μg/ml LL-37 inhibited 50% of LPS-induced TNF-α release. This inhibitory effect increased to ≥80% with a dose of 1 μg/ml LL-37, and TNF-α was reduced to background levels with 2 μg/ml LL-37. In the presence of LPS at a higher concentration (100 ng/ml), 2 μg/ml LL-37 was required to inhibit 50% of TNF-α released into the tissue culture supernatant. Higher concentrations (20 μg/ml) of LL-37 caused ≥95% inhibition of TNF-α release. These results indicated that physiological concentrations of LL-37 exhibit an anti-endotoxin effect on LPS present at low and high concentrations. The anti-endotoxin effect of LL-37 was similarly observed in PBMCs (Fig. 1B), for which LL-37 (20 μg/ml) inhibited ≥91% of LPS (100 ng/ml)-induced TNF-α. Subsequent mechanistic studies used 100 ng/ml LPS, a concentration at which more robust transcriptional up-regulation responses were observed, and 20 μg/ml LL-37, which was not cytotoxic to primary cells (38) or THP-1 cells as determined by lactate dehydrogenase release and MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) assay (data not shown).

To gain further insight into the mode of inhibition exerted by LL-37, TNF-α production and release was monitored in the supernatants of LPS-stimulated THP-1 cells treated with the transcriptional inhibitor actinomycin D. A concentration of 4 μg/ml actinomycin D was used because this concentration was required for inhibition, by >96% within 1 h of treatment, of LPS-induced transcription of the genes for both the cytokine TNF-α and the proinflammatory TNF-α-inducible protein 2 (TNFAIP2) (monitored by qPCR; data not shown). Actinomycin D reduced the level of TNF-α release by 97.6% (Fig. 1C), indicating that LPS largely induced de novo expression of TNF-α as opposed to processing and release of intracellular pools of proform TNF-α. Moreover, the use of monensin as an inhibitor of TNF-α secretion led to accumulation of TNF-α within cells after LPS stimulation for 60 min (Fig. 1D). However, LL-37 by itself did not similarly lead to the accumulation of TNF-α inside cells, indicating that it also prevented TNF-α expression at the protein level rather than blocking secretion.

The sustained presence of LL-37 inhibits TNF-α release

To determine the kinetics of the anti-endotoxin effect, the supernatant from THP-1 cells was monitored for TNF-α after 30 min of LPS treatment (Fig. 2A). When the peptide and LPS were added simultaneously, the release of TNF-α was substantially inhibited (90–97%) by LL-37 at all time points (Fig. 2A). When LL-37 was added 30 min after LPS addition, TNF-α secretion was reduced >50% at 2 and 4 h post-LPS treatment and by 80% after...
24 h (Fig. 2B), consistent with previous observations in mouse macrophages (20). In contrast, when the cells were pretreated with LL-37 for 30 min, washed, and stimulated with LPS, TNF-α secretion was substantially (64%) reduced after 1 h, but this declined to only 24–35% at subsequent time points (Fig. 2C). This indicated that a sustained presence of LL-37 was required to exhibit a maximal anti-endotoxin effect.

**LL-37 suppresses TLR-induced cytokine secretion by PBMC**

To determine whether LL-37 could suppress cytokine secretion induced by inflammatory stimuli such as LPS and other TLR agonists in primary cells, PBMC were treated with agonists of TLR2 (LTA, Pam3CSK4), TLR4 (LPS), TLR9 ( CpG), and the inflammatory cytokines TNF-α and IL-1β, in the presence or absence of LL-37. Cytokine production was analyzed by Luminex 100 StarSystem using the human multiplex cytokine kit to monitor IL-1β, IL-6, IL-8 and TNF-α in the culture supernatants. The cytokine profile of stimulated PBMC in the presence or absence of LL-37 was monitored after 4 or 24 h of treatment. The release of all four cytokines was significantly reduced by LL-37 in both LPS- and LTA-stimulated cells after 4 h of treatment, and this anti-inflammatory activity was sustained over 24 h (Fig. 3). Effects on IL-8 production were more modest, as anticipated, because LL-37 has the ability to induce IL-8 production (Ref. 20 and Fig. 3). In addition, LL-37 reduced IL-1β, IL-6, IL-8, and TNF-α production by TLR2 agonist Pam3CSK4-stimulated PBMC after 4 or 24 h of treatment, by ~30–50% (data not shown). These data show that LL-37 significantly reduced the production of proinflammatory cytokines resulting from activation of TLR2 or TLR4. LL-37 also reduced, by ~50%, IL-8 secretion by PBMC stimulated with the TLR9 agonist CpG for 24 h (Fig. 3).

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**FIGURE 3.** LL-37 modifies inflammatory agent-induced cytokine secretion by PBMC. PBMC were incubated alone or with TLR agonists (LPS, LTA, CpG) or inflammatory cytokines (TNF-α, IL-1β) for 4 or 24 h in the presence or absence of LL-37. See Materials and Methods for details. The concentration (y-axis) of IL-1β, IL-6, IL-8, and TNF-α (x-axis) were measured in the tissue culture supernatants by multiplex bead ELISA. The results are an average (± SD) of three independent experiments. The effect of LL-37 on agonist induced cytokine production was statistically significant with a value of p < 0.05 (***), p < 0.1 (**), or p < 0.15 (*).

In contrast, LL-37 enhanced TNF-α and IL-6 production by CpG-stimulated PBMC and IL-6, IL-8, and (modestly) TNF-α by PBMC stimulated with IL-1β (Fig. 3). Conversely, LL-37 had no effect on TNF-α-induced cytokine production. These results indicate that the LL-37 was anti-inflammatory in response to selected TLR ligands, and that it was likely modulating innate immune pathways rather than simply suppressing some step in the main TLR-to-NF-κB pathway.

**LPS-induced gene expression profile is altered by LL-37**

To determine whether LL-37 could suppress cytokine secretion induced by inflammatory stimuli such as LPS and other TLR agonists in primary cells, PBMC were treated with agonists of TLR2 (LTA, Pam3CSK4), TLR4 (LPS), TLR9 (CpG), and the inflammatory cytokines TNF-α and IL-1β, in the presence or absence of LL-37. Cytokine production was analyzed by Luminex 100 StarSystem using the human multiplex cytokine kit to monitor IL-1β, IL-6, IL-8 and TNF-α in the culture supernatants. The cytokine profile of stimulated PBMC in the presence or absence of LL-37 was monitored after 4 or 24 h of treatment. The release of all four cytokines was significantly reduced by LL-37 in both LPS- and LTA-stimulated cells after 4 h of treatment, and this anti-inflammatory activity was sustained over 24 h (Fig. 3). Effects on IL-8 production were more modest, as anticipated, because LL-37 has the ability to induce IL-8 production (Ref. 20 and Fig. 3). In addition, LL-37 reduced IL-1β, IL-6, IL-8, and TNF-α production by TLR2 agonist Pam3CSK4-stimulated PBMC after 4 or 24 h of treatment, by ~30–50% (data not shown). These data show that LL-37 significantly reduced the production of proinflammatory cytokines resulting from activation of TLR2 or TLR4. LL-37 also reduced, by ~50%, IL-8 secretion by PBMC stimulated with the TLR9 agonist CpG for 24 h (Fig. 3).

In contrast, LL-37 enhanced TNF-α and IL-6 production by CpG-stimulated PBMC and IL-6, IL-8, and (modestly) TNF-α by PBMC stimulated with IL-1β (Fig. 3). Conversely, LL-37 had no effect on TNF-α-induced cytokine production. These results indicate that the LL-37 was anti-inflammatory in response to selected TLR ligands, and that it was likely modulating innate immune pathways rather than simply suppressing some step in the main TLR-to-NF-κB pathway.
FIGURE 4. LPS-induced gene transcription profile in monocytes is altered by the presence of host defense peptide LL-37. A, THP-1 cells were stimulated with 100 ng/ml LPS in the absence (top panel) or presence (lower panel) of 20 μg/ml LL-37 for 1, 2, 4, or 24 h. Using microarray analysis, the gene expression in response to stimuli was calculated relative to that in unstimulated cells at each time point, selecting genes with a fold change of 1.5 and value of p < 0.05 (calculated using a two-sided one-sample Student t test on the log2 ratios within each treatment group). The relative gene expression is overlaid on the TLR4 protein network using the supervised clustering tool Cytoscape. The color code for the fold change and identification of proteins are in the left panel. B, Cluster analysis of the differentially expressed genes as measured using log ratio (y-axis) of microarray spot intensity, with NF-κB binding sites in response to 100 ng/ml LPS in the absence (top) or presence of 20 μg/ml LL-37 (bottom) based on similar temporal expression profiles over the time course of 1–24 h (x-axis). The table indicates the total number of differentially expressed genes, total number of clusters, number of clusters containing genes with NF-κB binding sites, and the NF-κB target genes found in the clusters.

To investigate further whether a defined portion of the LPS-responsive genes were likely coregulated by NF-κB, LPS-responsive, differentially expressed genes with similar temporal expression profiles were clustered using the K-means procedure (Fig. 4B). Each cluster thus represented a set of potential coregulated genes (based on their similar expression profiles over time). Based on this method, the LPS-induced genes were divided into 15 clusters. Three of these clusters, containing a total of 123 genes with peak expression at 2 h, 4 h, or both, contained 21 genes that are known from the literature to be NF-κB regulated (Fig. 4B). In contrast, the temporal expression patterns of the 410 genes induced by LPS in the presence of LL-37 fell into eight clusters, one of which contained 11 of the 12 differentially expressed NF-κB gene targets; six of these NF-κB target genes were also included in the subset of LPS-stimulated genes and demonstrated modestly to substantially decreased expression in the presence of LL-37. Many p50/p65 target genes (40) were found in the clusters containing the NF-κB genes. Thus, treatment with LL-37 clearly resulted in the suppression of LPS stimulation of a substantial number of known NF-κB target genes, and clustering data indicated that many other genes that might be NF-κB regulated were similarly suppressed. However, the data also suggested that the effect observed was selective in that some known NF-κB-regulated genes were still apparently differentially expressed in the presence of the combination of LPS and LL-37. To confirm these observations, genes with significant differential expression in response to LPS, and that were affected differentially (remained up-regulated or abrogated) by the presence of the peptide, were selected for validation by qPCR.

**LL-37 selectively modulates the transcription of specific LPS-induced inflammatory genes**

Using qPCR, the expression profiles were validated for 14 of 20 selected genes differentially expressed according to the microarray analysis (e.g., Fig. 5). Several known “proinflammatory” genes were up-regulated after 2 and 4 h of treatment with LPS, and this expression level invariably decreased by 24 h of stimulation. Furthermore, the expression of several LPS-induced genes was confirmed to be altered by the presence of LL-37. Even though the peptide had a dampening effect on selected LPS-induced expression of inflammatory genes, not all genes up-regulated by LPS were suppressed by the presence of LL-37, indicating that the effect of LL-37 on LPS-induced inflammation was selective (Fig. 5). The expression of proinflammatory genes such as *NFκB1* (p105/ p50) and *TNFAIP2* were substantially reduced (90–97%) in LPS-stimulated cells in the presence of LL-37 at all time points. Also, LPS-induced transcription of *TNF-α* was reduced in the presence of LL-37 by 87% after 1 h and ~80% at 2 and 4 h, but at 24 h, only 58% reduction was observed. Similarly, LPS-induced transcription of *IL-10* was reduced by >90% after 1 and 2 h in presence of LL-37, and this effect decreased to 77% after 4 h. In contrast, the expression of chemotactants such as *IL-8*, *CCL4*, and *CXCL1,*...
was reduced by LL-37 by only 30–40% after 2 h in LPS-stimulated cells but not completely eliminated. Likewise, the expressions of certain anti-inflammatory genes that are negative regulators of the TLR4-to-NF-κB pathway were only slightly reduced in the presence of LL-37. These genes included TNFAIP3 (TNF-α-inducible protein 3) and its interacting partner TNIP3 (TNFAIP3-interacting protein 3), as well as the NF-κB-inhibitor, NFκBIA. LPS-induced transcription of NF-κB inhibitor, NFκB1 (p105/p50), but not RELB, was completely abrogated by LL-37, whereas RELA (p65) did not show significant differential expression in response to LPS or LL-37.

From the temporal transcriptional profiling of LPS-induced genes, it was concluded that LL-37 did not substantially affect the LPS-induced expression of selected genes that are required for cell recruitment and movement (chemokines) or negative regulators of NF-κB. In contrast, LL-37 neutralized the expression of genes coding for inflammatory cytokines, NFκB1 (p105/p50), and TNF-α-induced proinflammatory genes such as TNFAIP2.

**LL-37 significantly inhibits LPS-induced translocation of the NF-κB subunits p50 and p65**

The above data indicated that, although LL-37 reduced TNF-α secretion by >95% at all time points, it had a lesser effect (58–87%) in reducing TNF-α transcription. To study this in more detail, we investigated the key transcription factor NF-κB. TLR activation results in nuclear translocation of NF-κB, the key transcription factor required for expression of many innate immunity and inflammatory genes (41, 42). Although NF-κB has a number of subunits with different primary transcriptional regulatory functions, the p50/p65 NF-κB heterodimer is most commonly implicated in the regulation of immunity genes. Nevertheless, transcriptionally active NF-κB heterodimers other than p50/p65 have important functions, because it has been shown that they can influence gene responses to bacterial molecules as well as susceptibility to a variety of infections (43, 44). To determine whether LL-37 suppressed LPS-induced changes in gene expression by affecting NF-κB translocation into the nucleus, the nuclear localization of five NF-κB subunits was assessed by Western blots. All monitored subunits of NF-κB (p105/50, p65, c-REL, RELB, and p100/p52) were detected in the nuclear extracts of THP-1 cells (Fig. 6A). The nuclear localization of p50, p65, c-REL, and RELB, and to a lesser extent p100/p52, was increased in THP-1 cells stimulated with LPS for 30 and 60 min (by 60 min, LPS had induced a 3.5-fold increase in nuclear p50, a 4.5-fold increase in p65, a 1.7-fold increase in RELB and c-REL, and a 1.2-fold increase in p100/p52 as assessed by densitometry). The LPS-induced translocation of p50, p65, and RELB was clearly suppressed in the presence of LL-37, because there was a ~35–70% decrease in subunit translocation after 60 min (Fig. 6A), whereas p100/p52 and c-REL did not appear to be affected.

**FIGURE 6.** LL-37 suppresses LPS-induced translocation of NF-κB subunits p50 and p65. A, Western blot of NF-κB subunits (identified on the right) in the nuclear extract of THP-1 cells following incubation in the absence (−) or presence (+) of 100 ng/ml LPS or LPS and 20 μg/ml LL-37 for 60 min. Prestained molecular mass markers are indicated on the left. B, ELISA for NF-κB subunit p50 (upper panel) and NF-κB subunit p65 (lower panel) detected in the nuclear extracts of THP-1 cells stimulated for 60 min as described in A. The y-axis represents relative light units (luminescence), which reflects the amount of the assessed NFκB subunit in the nuclear extract. See Materials and Methods for details. Results are representative of three independent experiments.
To more accurately quantify the translocation of p50 or p65, the nuclear extracts were analyzed by ELISA-based immunoassays specific for these subunits (Fig. 6B). LL-37 partly suppressed LPS-induced p50 and p65 translocation at 30 and 60 min (80 ± 2 and 76 ± 5% inhibition of p50 at 30 and 60 min, respectively, and 75 ± 6 and 65 ± 3% inhibition of p65 at 30 and 60 min, respectively). As a control, it was demonstrated that polymyxin B, a known inhibitor of LPS-LPS-binding protein (LBP) engagement, more substantially inhibited the translocation of NF-κB subunits p50 and p65 by >98% at 60 min (data not shown), demonstrating that TLR4-to-NF-κB activation can be blocked significantly by agents acting at the cell surface. Although LL-37 has been reported to activate signal transduction pathways including MAPK in human monocytes and lung epithelial cells (38), LL-37 did not promote translocation of NF-κB subunits in human THP-1 cells. Together, these data demonstrate that LL-37 can moderately alter the LPS-induced translocation of NF-κB subunits, thereby providing one mechanism by which LL-37 suppressed proinflammatory cytokine production.

Discussion

The innate immune response is a dynamic system, because it can be triggered by TLR recognition of conserved bacterial components (2, 3), initiating a broad inflammatory response to infectious agents, but must be able to maintain homeostasis in the presence of commensal organisms, which contain many of these same conserved components. A delicate balance of pro- and anti-inflammatory mediators is vital for efficient functioning of the immune system under these disparate circumstances. In recent years, there has been speculation and some evidence implicating the sole human cathelicidin, LL-37, in maintaining homeostasis, combating pathogenic challenge, and protecting against endotoxia, an extreme inflammation-like condition (45–47). The data presented here and in previous papers. [1] LL-37 significantly inhibits LPS-induced translocation of the NF-κB subunits p50 and p65 (Fig. 6). [2] LL-37 selectively modulates gene transcription; completely inhibiting certain proinflammatory genes (p50, TNFαIP2) and reducing the expression of others (TNF-α) (Figs. 4 and 5). [3] LL-37 directly triggers MAPK pathways that can impact on proinflammatory pathways (38). [4] LL-37 can interact directly with LPS to reduce its binding to LBP, MD2, or another component of the TLR4 receptor complex, thus reducing activation of the downstream pathway (26). [5] Because LL-37 has a stronger effect on TNF-α protein production than on TNF-α gene expression (Figs. 1 and 5), it is hypothesized that it directly or indirectly influence protein translation, stabilization, or processing. Points of intervention by LL-37 are indicated by activation (⇒), inhibition (↓), or suppression (→→). Other abbreviations used are phosphorylation (P) and ubiquitination (U).

FIGURE 7. Model describing mechanisms of anti-endotoxins activity of LL-37. LL-37 is proposed to regulate LPS-induced gene transcription and cytokine production by several mechanisms, based on the data presented here and in previous papers. [1] LL-37 significantly inhibits LPS-induced translocation of the NF-κB subunits p50 and p65 (Fig. 6). [2] LL-37 selectively modulates gene transcription; completely inhibiting certain proinflammatory genes (p50, TNFαIP2) and reducing the expression of others (TNF-α) (Figs. 4 and 5). [3] LL-37 directly triggers MAPK pathways that can impact on proinflammatory pathways (38). [4] LL-37 can interact directly with LPS to reduce its binding to LBP, MD2, or another component of the TLR4 receptor complex, thus reducing activation of the downstream pathway (26). [5] Because LL-37 has a stronger effect on TNF-α protein production than on TNF-α gene expression (Figs. 1 and 5), it is hypothesized that it directly or indirectly influence protein translation, stabilization, or processing. Points of intervention by LL-37 are indicated by activation (⇒), inhibition (↓), or suppression (→→). Other abbreviations used are phosphorylation (P) and ubiquitination (U).
heterodimers that are associated with different transcriptional responses leading to specific biological effects (52–54). Many inflammatory stimuli trigger signal transduction pathways that result in nuclear localization of NF-κB (principally the NF-κB p50/p65 heterodimer (52, 53)) and subsequent transcription of inflammatory and immunity genes encoding for cytokines, chemokines, acute-phase reactants, and cell adhesion molecules, involved in innate immune responses to invading pathogens.

This report provides evidence that the host defense peptide, LL-37, can reduce LPS-induced p50/p65 translocation to the nucleus, indicating that this is one mechanism whereby LL-37 suppressed LPS-induced gene transcription and exerted an anti-endotoxin effect. However, if LL-37 were merely blocking the binding of LPS to the TLR4 receptor through inhibiting its interaction with LBP and/or the LPS receptor complex (26), it would be expected that NF-κB translocation, and all NF-κB-dependent transcriptional events would be inhibited to the same extent as TNF-α release, that is, >95%; however, this was not observed here (cf. the known antagonist polymyxin B). Instead, the effects of LL-37 on NF-κB subunit translocation were selective and intermediate, and effects on LPS-stimulated transcription of NF-κB-regulated genes ranged from very high, e.g., >95% for TNFAIP2 and NF-κB1 p105/p50, to moderate (~80%) for TNF-α itself, through to almost no inhibition for other NF-κB-regulated genes like TNFAIP3. Similarly, LL-37 can protect against sepsis in animal models when administered shortly after endotoxin (38).

In mouse model experiments, it LL-37 can protect against sepsis in animal models when administered shortly after endotoxin (38). Mouse model experiments, it was demonstrated that 200 μg of LL-37 could protect against an 80% lethal dose (400 μg) of *Escherichia coli* LPS administered peritoneally (K. Lee, M. G. Scott, and R. E. W. Hancock, unpublished results). Under such circumstances, the LPS would be in 5-fold molar excess, and it seems unlikely that in this situation LPS neutralization alone could explain the protection exhibited by LL-37. It seemed possible that a lower effective concentration of LPS in presence of LL-37, due to the neutralization by binding of a portion of the LPS, might be responsible for the gene expression responses observed. However, measurement of TNF-α responses and examination of global gene expression in response to, for example, 5 ng/ml LPS produced data (not shown) that were inconsistent with this notion.

This study indicates that the host defense peptide LL-37 can selectively regulate genes that mediate inflammatory responses by suppressing NF-κB translocation leading to dysregulation (modulation) of TLR-triggered transcriptional responses. LL-37 caused inhibition of LPS-triggered proinflammatory gene TNFAIP2 but did not neutralize the LPS-induced expression of some of the known negative regulators of NF-κB such as TNFAIP3, TNIP3, and NFκBIA (IκBα). Conversely, the transcription of known LPS-induced genes that are regulated by p50/p65 (Fig. 4B) were also inhibited >90% in the presence of LL-37. However, although NF-κB transcription factor activity is influenced by changes in nuclear concentration and subunit composition, the observed partial inhibition of p50/p65 translocation in LPS-induced cells by LL-37 (Fig. 6), seems unlikely to completely account for the >95% reduction in TNF-α protein production and release. Rather, this nearly complete inhibition of proinflammatory cytokine release, without an equivalent abrogation of gene transcription, implies that mechanisms other than inhibition of NF-κB are also required for LL-37 to regulate TLR-induced inflammation. Such anomalies are consistent with the hypothesis that LL-37 can influence posttranscriptional events to modulate the inflammatory response. We hypothesize that LL-37 can affect components of protein translation, maturation, or secretion directly and/or indirectly via LL-37-activated effectors or LL-37-induced gene transcription (Fig. 7). It is known that LL-37 can activate components of the MAPK pathway, in particular, p38 (which can influence posttranscriptional events) and ERK, and can promote the activity of the transcription factor, Elk-1 (38). The putative receptors for LL-37, including FPRL-1, P2X7, and EGRFR, do not appear to be responsible for LL-37-induced activation of the MAPK pathway in monocytes (38). Neither of the G-protein–coupled receptors appeared to be responsible for the effects observed, because treatment with pertussis toxin had no effect on TNF-α production in response to the combination of LL-37 and LPS (data not shown).

In *Drosophila*, the peptidoglycan-mediated up-regulation of expression of NF-κB-dependent genes is reported to be suppressed by a MAPK-regulated transcription factor, AP-1 (55). LL-37 also demonstrates synergy with inflammatory stimuli such as GM-CSF (38) and IL-1β (Fig. 3), which likely reflect activation of cooperative signal transduction pathways or transcription of genes whose products contribute to a stabilized, enhanced, or prolonged response. Thus, LL-37 probably works alone or synergistically with other effector molecules of innate immunity, potentially via the MAPK pathway, to modulate TLR activation and enhance host defense mechanisms.

In conclusion, this report demonstrates that LL-37 selectively suppresses the proinflammatory response in monocytes, particularly the TLR-induced secretion of proinflammatory cytokines. The ability of LL-37 to dampen proinflammatory (septic) responses would be valuable for maintaining homeostasis in the face of natural shedding of microflora-associated TLR agonist molecules, as well as limiting the induction of systemic inflammatory syndrome/septic shock in response to moderate pathogen challenge. The anti-inflammatory effects of LL-37 were observed at physiologically relevant concentrations of the peptide, and small changes in peptide concentration led to substantial impact on the cellular response to bacterial components such as LPS. LL-37 thus appears to manifest multiple, complex mechanisms of action, including direct and indirect inhibition of TLR activation and transcription. Our improving understanding of the mechanism(s) used by LL-37 to selectively modulate inflammation, thereby balancing the TLR response to commensal or pathogenic bacteria, indicates that endogenous cationic host defense peptides may be important players in limiting overactive inflammation.

**Acknowledgments**

We acknowledge Ms. Shuyu Fan for assistance with statistical evaluation.

**Disclosures**

R. E. W. H. is a consultant with Inimex Pharmaceuticals, which provided a modest portion (~10%) of the funding for this project.

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