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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 (TNFAIP2). In contrast, LL-37 did not significantly inhibit LPS-induced genes that antagonize inflammation, such as TNF-α-induced protein 3 (TNFAIP3) 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 subunits p50 and p65 was reduced ≥50% in the presence of LL-37, demonstrating that the peptide altered gene expression in part by acting directly on the TLR-to-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.

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Our understanding of the early response to infection has broadened significantly since the discovery of Toll and TLR in the late 1990s. We now know that Toll/IL-1R domain-containing proteins play a pivotal role in initiating the inflammatory and immune responses (1–3). Responses to infection are substantially orchestrated as a result of the interaction of bacterial molecules with TLR. A breakdown in the appropriate regulation of the TLR pathway can cause common chronic inflammatory diseases. Alternatively, an exaggerated response to bacterial stimuli results in the initiation of systemic inflammatory response syndrome, or early sepsis (4) in which high levels of cytokines and inflammatory mediators become destructive, causing organ failure, cardiovascular shock, and/or death. TLR engagement by conserved microbial molecules results in the translocation of the pivotal transcription factor NF-κB and the transcription of "early response" genes encoding, for example, cytokines, chemokines, selected antimicrobial/host defense peptides, acute-phase proteins, cell adhesion molecules, costimulatory molecules, and proteins required for negative feedback to suppress these responses. In humans, inhalation of the Gram-negative bacterial component LPS (also termed endotoxin), a TLR4 agonist, results in increased cytokine and chemokine (TNF-α, IL-1-β, IL-6, IL-8) mRNA and protein expression within 4–6 h of inhalation (5, 6). Mutant mice lacking responsiveness to LPS (7) do not develop septic shock, demonstrating that the response to endotoxin is sufficient to promote sepsis. The nature, duration, and intensity of inflammatory/septic responses are considered to involve the interplay between TLR and other receptors, different adaptor molecules such as MyD88, TIRAP/Mal, and TRIF, and different signaling pathways (2, 8). An ideal therapeutic regulator of the inflammatory response would be antagonistic to potentially lethal conditions such as septic shock but maintain innate immune defenses against bacterial infections, thus sustaining a balance between the protective and destructive components of inflammation.

Cationic host defense peptides (also known as antimicrobial peptides) are important molecules in host defense against pathogenic microbe challenge demonstrating a wide range of functions from direct antimicrobial activity to a broad range of immunomodulatory functions (9–11). Mammals contain a variety of small, amphipathic peptides including the cathelicidins and defensins. The only endogenous cathelicidin in humans, hCAP-18/LL-37, is
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, 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 rested 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 injury 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)⁴ from *Staphylococcus aureus* and a synthetic tripalmitylated lipopeptide, PamCSK4, 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 a 1.25 endotoxin units (EU) per microgram of LTA. Polyoxin 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 (DFFRKSKEKIGKEFRVQRIKDFFRN), 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 p105/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 Amershams Biosciences, respectively.

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

THP-1 cells or PBMC were stimulated with LPS (10 or 100 ng/ml), LTA (1 μg/ml), PamCSK4 (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. Alternatively, cells were stimulated with LPS (20 ng/ml) for 30 min, washed with RPMI 1640 complete medium to remove the peptide and then stimulated with LPS (100 ng/ml). Polyoxin 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 stimulators.

**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 cytoplasmatic extracts (see Nuclear and cytoplasmatic 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 Luminex 100 StarStation software (Applied Cytometry Systems).

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

RNA was isolated from THP-1 cells with RNAeasy 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

⁴ Abbreviations used in this paper: LTA, lipoteichoic acid; qPCR, quantitative real-time PCR; PMA, phorbol 12-myristate 13-acetate; PBMC, polyvinylidene difluoride; LBP, 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 MessageAmpII 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). Yield 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 in 70% validation (14 of 20 selected genes).

Over time PCR (qPCR) (described below), at four different time points, resulting in predictions. Confirmation of array results was done using quantitative real-time PCR, although the number of time points used prevents highly precise predictions. The gene expression data was overlaid on 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 foreground intensity as an estimate for the background noise, 3) merging of technical replicates, 4) normalization, 5) two-sided one-sample Student’s t test, 6) two-sided one-sample Student’s t test, 7) Pearson correlation, 8) ANOVA, 9) merging of technical replicates, and 10) ANOVA. The following processing steps were applied: 1) flagging of markers and control spots, 2) subgrid-wise background correction, using the median of the foreground intensity as an estimate for the background noise, 3) merging of technical replicates, 4) normalizing to the median of the foreground intensity, 5) two-sided one-sample Student’s t test, 6) Pearson correlation, 7) ANOVA, 8) merging of technical replicates, and 9) ANOVA. The following processing steps were applied: 1) flagging of markers and control spots, 2) subgrid-wise background correction, using the median of the foreground intensity as an estimate for the background noise, 3) merging of technical replicates, 4) normalizing to the median of the foreground intensity, 5) two-sided one-sample Student’s t test, 6) Pearson correlation, 7) ANOVA, 8) merging of technical replicates, and 9) ANOVA.

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

<table>
<thead>
<tr>
<th>Gene</th>
<th>Forward Primer (5’-3’)</th>
<th>Reverse Primer (5’-3’)</th>
</tr>
</thead>
<tbody>
<tr>
<td>CCL4</td>
<td>CTTTCTTCAACCGCGAGAA</td>
<td>GCAGAGGCTGCTGTCTCATT</td>
</tr>
<tr>
<td>CCL20</td>
<td>TGACGCTTCTGGATACAGA</td>
<td>TGTAGACTTTAGTCAAGCCT</td>
</tr>
<tr>
<td>CXCL</td>
<td>GCCAGTCGTTGCAAGACCT</td>
<td>GGCTGGTCAGAGCACAGGA</td>
</tr>
<tr>
<td>IL-8</td>
<td>GCCACACTCAGCCACACAC</td>
<td>CCTTCCAAACACTCTGCAC</td>
</tr>
<tr>
<td>GAPDH</td>
<td>GTGCCTGGTACTAGAGAGG</td>
<td>GAAACTGTGGCGTGATGG</td>
</tr>
<tr>
<td>IL-10</td>
<td>GTGGGCGAGGCTTTGCTGA</td>
<td>GGGCTGAGGCGGGCAGT</td>
</tr>
<tr>
<td>TNF-alpha</td>
<td>TGGAGAGGCTGAGAGAGCT</td>
<td>TCTTCAAGGAGCAAGTCCC</td>
</tr>
<tr>
<td>TNFAP2</td>
<td>CTAGCCGATGCTTTTTAATG</td>
<td>TCCGGAGAGCACGGAGTT</td>
</tr>
<tr>
<td>TNFAP3</td>
<td>CTAGCCGATGCTTTTTAATG</td>
<td>TCCGGAGAGCACGGAGTT</td>
</tr>
<tr>
<td>TIP3</td>
<td>TGAGAAGAAAGTGAGACAGTGAA</td>
<td>CCGCCGAGCAGGAGGTC</td>
</tr>
<tr>
<td>BIRC3</td>
<td>AAAGCCCCACAGGGTGGGA</td>
<td>AGGAAACCCAGCAGGAAAG</td>
</tr>
<tr>
<td>NF-kB1</td>
<td>CTTAGAGGAGAGGCCACCC</td>
<td>TTGGTCAAGGCTTTCAAAAT</td>
</tr>
<tr>
<td>RELA</td>
<td>TAGAGAAGGAGGTCCGAGGAT</td>
<td>CCGCTTCCATCAACACTGGA</td>
</tr>
<tr>
<td>RELB</td>
<td>TGGAGATGGAGGTCCACACAC</td>
<td>TGTCCTTGAGAAGGACACAT</td>
</tr>
<tr>
<td>NF-kBIA</td>
<td>GTGGAGAGAGGCCACCC</td>
<td>GTGCTCAGACAACTTCTGGC</td>
</tr>
</tbody>
</table>

Nuclear and cytoplasmic extracts
THP-1 cells (3 × 10⁶) seeded into 60-mm² petri dishes (VWR International) were pre-incubated 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 bicinchoninic 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% SDS-PAGE and transferred to polyvinylidene difluoride (PVDF) Immobilon-P membranes (Millipore). Equivalent protein loading was verified by staining PVDF membranes with Blot-Flast-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-RELB, or anti-p105/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 SpectraFlor 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 monocytes 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...
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 1, 2, 4, and 24 h of stimulation with LPS (100 ng/ml) in the absence or presence of LL-37 (20 μg/ml). 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. These results indicated that LL-37 on its anti-endotoxin activity. Tissue culture supernatants were screened for TNF-α by ELISA following stimulation of cells with LPS (100 ng/ml) in the absence or in the presence of 20 μg/ml LL-37 for 1, 2, 4, and 24 h of treatment. The x-axis represents TNF-α concentration, and the y-axis indicates time (in hours).

LL-37 (20 μg/ml) was added simultaneously with LPS (A), after 30 min of LPS treatment (B), or 30 min before LPS treatment (C). See Materials and Methods for details. The results are an average (±SD) of three independent experiments.
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).

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**

Human 21K oligo-based DNA microarrays were probed to elucidate the impact of LL-37 on LPS stimulation of gene responses in human monocyteic cells. Transcriptional responses were analyzed following 1, 2, 4, and 24 h of stimulation to provide a temporal profile of gene expression in monocytes equivalent to the early, intermediate, and late stages of innate immune responses. Microarray analyses were performed in duplicate from three independent biological replicates. Statistically significant, differentially expressed genes were defined as those with a fold change of at least 1.5, with a Student’s t-test p value ≤0.05 (the microarray data has been deposited into ArrayExpress under accession no. E-FPMI-4). The number of differentially expressed genes was greatest at the 2- and 4-h time points. Over the monitored time period, 561 and 410 genes were differentially regulated in the presence of LPS, without or with LL-37, respectively. Of the 561 genes that were differentially expressed in LPS-stimulated cells, only 36 (~7%) were identified as being up-regulated in cells stimulated with LPS in the presence of LL-37 (ZNF83, NFKBIA, Q9PB188, INVS, DIAPH1, IER3, Q9H640, GBP2, NANS, Q86XN7, TNFAIP3, Q96M88, Q97753, NTNG1, INHBE, BCL6, CXCL1, EHD1, RELB, HKR, CCL4, SESN2, NAB1, EB13, DDX21, XB1P, SLURP1, HADAC10, MEP1A, RAP2C, GYS1, RARRES3, PDP, MTL4, Q9H040, and Q9NUP6). More than 160 genes (many of which were well-characterized proinflammatory genes) that were up-regulated in cells stimulated with LPS were suppressed in the presence of LL-37 (data not shown). This indicates that LL-37 effectively suppressed the induction of a large subset of LPS-responsive genes, but permitted the maintenance of a subset of genes with a function in promoting some aspects of inflammation or an anti-inflammatory function.

Given that LPS is known to induce inflammatory responses primarily via the TLR4-to-NF-κB pathway (39) and the product of certain differentially expressed genes in the microarray analysis were associated with this pathway, we analyzed in more detail the NF-κB-regulated genes and the TLR4 pathway. This pathway was first mapped by integrating protein:protein interaction, signal transduction, and regulatory data from the literature into Cytoscape (www.cytoscape.org), an open-source bioinformatics software platform for visualizing molecular interaction networks and integrating these interactions with other data. The microarray expression data was then overlaid onto this signal transduction protein network by color coding the individual nodes (equivalent to specific genes/proteins) according to the extent of regulation (ranging from red to green, where the intensity of color demonstrated the extent of up- to down-regulation, respectively). This then provided a graphic illustration of the genes with altered expression in response to LPS in the absence or presence of LL-37 at each of the time points (Fig. 4A), and indicated that LPS generally up-regulated genes encoding elements of the TLR4-to-NF-κB pathway, with a peak response at 2–4 h, and that LL-37 generally dampened this up-regulation.
targets; six of these NF-κB gene binding sites in the presence of LL-37 fell into eight clusters, one of which contained 11 of the 12 differentially expressed NF-κB target genes found in the clusters. 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 chemoattractants 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 subunit 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 5.** LL-37 selectively modulates the transcription of LPS-induced proinflammatory genes. qPCR of gene expression in LPS-stimulated cells in presence or absence of LL-37 or the peptide alone, for 1, 2, 4, and 24 h (x-axis). Results shown are an average (±SE) of three independent experiments. Fold changes (y-axis, log scale) for each gene were normalized to GAPDH and are relative to the gene expression in unstimulated cells (normalized to 1) using the comparative Ct method (see Materials and Methods for details).

**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 presence (−) 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 immunobioassays 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 endotoxemia, an extreme inflammation-like condition (45–47). The data presented in this paper are consistent with the hypothesis that LL-37 is an important component of human immunity that regulates the balance of pro- and anti-inflammatory molecules both under homeostatic conditions and during endotoxin challenge (i.e., infection situations). Overall, the data provided evidence that LL-37 can manipulate both pre- and posttranscriptional events (Fig. 7) to modulate the TLR-induced inflammatory response in monocytes. A model consistent with the data in this manuscript is outlined in Fig. 7.

This study conclusively demonstrates that endotoxin-induced inflammatory gene responses and cytokine secretion in monocytes were suppressed by low, physiological concentrations of LL-37, implicating LL-37 in the regulation and control of proinflammatory responses associated with pathogenic assault and, by extension, with homeostatic levels of TLR agonists secreted by commensals. This report further demonstrates that LL-37 can suppress LPS-induced NF-κB translocation and exert an anti-inflammatory effect that is not restricted to endotoxin-induced inflammation. In the human THP-1 monocytic cell line as well as in human PBMC, LL-37 suppressed proinflammatory cytokine production induced by LPS as well as other agonists of TLR2 (LTA, Pam3CSK4) and in part TLR9 (CpG), but selectively enhanced responses to the proinflammatory cytokines IL-1β and TNF-α. Selective enhancement of responses is consistent with the known complex interaction of LL-37 with cells (20, 38), including the induction of a mixture of pro- and anti-inflammatory gene products and MAPKs. In particular, it has been demonstrated that LL-37 induces the phosphorylation of Erk-1/2 and p38, which are known to be required for LPS induction of TNF-α production. It is possible therefore that MAPK induction was sufficient to selectively enhance the effects of certain ligands in inducing particular proinflammatory cytokines, but could not overcome other, pathway-specific inhibition events by LL-37. To gain mechanistic insight, transcriptional responses were profiled using microarrays and real-time PCR over the course of 1–24 h to study the effects of LL-37 on LPS-stimulated monocytes. Although the transcription of LPS-induced proinflammatory cytokines peaked at 2–4 h and waned by 24 h, a single, low dose of LL-37 suppressed proinflammatory cytokine secretion by 1 h, and this effect was sustained for 24 h. The model system used was chosen to reflect conditions for circulating blood monocytes entering tissues at the site of infection via extravasation and anti-endotoxic effects were observed at physiological salt and LL-37 concentrations and were observed at two different concentrations of LPS, in an attempt to reflect endotoxin concentrations ranging from the presumably low concentrations secreted by the normal flora (homeostatic conditions) and early in infection, to those observed in septic infections.

LPS-induced activation of the transcription factor NF-κB is mediated by a signal transduction pathway initiated by engagement of the surface receptor complex based on TLR4 (48). NF-κB is known to play a central role in pathogenesis resulting in sepsis (49, 50) as well as innate immunity to infections (50, 51). NF-κB is a dimeric complex of various subunits that belong to the REL family; p105/p50 (NFκB1), p100/p52 (NFκB2), p65 (REL), RELB, and c-REL, which can form different combinations of homo-
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 TNFAIP3 and NF-κBp105/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 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 modulate 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 FPR1-1, P2X7, and EGFR, 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.

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References
ROLE OF LL-37 IN MODULATION OF INFLAMMATION


