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The Human Antimicrobial Peptide LL-37 Is a Multifunctional Modulator of Innate Immune Responses[1]

Monisha G. Scott,* Donald J. Davidson,*[†] Michael R. Gold,* Dawn Bowdish,* and Robert E. W. Hancock[2]*

The role of LL-37, a human cationic antimicrobial peptide, in the immune system is not yet clearly understood. It is a widely expressed peptide that can be up-regulated during an immune response. In this report, we demonstrate that LL-37 is a potent antisepsis agent with the ability to inhibit macrophage stimulation by bacterial components such as LPS, lipoteichoic acid, and noncapped lipoarabinomannan. We also demonstrate that LL-37 protects mice against lethal endotoxemia. In addition to preventing macrophage activation by bacterial components, we hypothesized the LL-37 may also have direct effects on macrophage function. We therefore used gene expression profiling to identify macrophage functions that might be modulated by LL-37. These studies revealed that LL-37 directly up-regulates 29 genes and down-regulated another 20 genes. Among the genes predicted to be up-regulated by LL-37 were those encoding chemokines and chemokine receptors. Consistent with this, LL-37 up-regulated the expression of chemokines in macrophages and the mouse lung (monocyte chemoattractant protein 1), human A549 epithelial cells (IL-8), and whole human blood (monocyte chemoattractant protein 1 and IL-8), without stimulating the proinflammatory cytokine, TNFα. LL-37 also up-regulated the chemokine receptors CXCR-4, CCR2, and IL-8RB. These findings indicate that LL-37 may contribute to the immune response by limiting the damage caused by bacterial products and by recruiting immune cells to the site of infection so that they can clear the infection. *The Journal of Immunology,* 2002, 169: 3883–3891.

Cationic antimicrobial peptides, components of the innate host defenses of many species, have broad-spectrum activity against bacteria, fungi, parasites, and viruses (1, 2). They also have an affinity for LPS (3–6). Such cationic peptides can suppress cytokine production in response to endotoxic LPS and to varying extents can prevent lethal endotoxemia (7–9). It is becoming increasingly clear that antimicrobial peptides are a major player in local innate immunity, especially at mucosal and epithelial surfaces (2, 10). In the fruit fly *Drosophila*, peptides are recognized as the major form of defense against infection and are induced, in response to challenge by microbes or microbial signaling molecules like LPS, by a regulatory pathway similar to that used by the mammalian immune system (involving Toll receptors and the transcription factor NF-κB (11). Evidence for the key role of cationic antimicrobial peptides in innate immunity includes mutations affecting the induction of antibacterial peptides which reduce survival in response to bacterial challenge. Indeed mutations of the Toll pathway of *Drosophila* that lead to decreased antifungal peptide gene expression result in increased susceptibility to lethal

fungal infections (12). Although there are multiple defensins in mice, Wilson et al. (13) identified the single enzyme necessary for processing the preprodefensins to the active mature form. Genetic inactivation of this single gene (matrilysin) led to no production of active defensin in the small intestine and a consequent 10-fold increase in the susceptibility to infection by orally introduced virulent bacteria (13). Patients with specific granule deficiency syndrome, completely lacking in α defensins, suffer from frequent and severe bacterial infections (2, 14). Similarly, a group of HIV patients with lower salivary levels of histatin peptides showed a higher incidence of oral candidiasis and fungal infection (2). Other evidence includes the inducibility of some peptides by infectious agents and the very high concentrations that have been recorded at sites of inflammation (15–17).

The single known human cathelicidin, hCAP-18, is a major protein of the specific granules in neutrophils (18) and is also present in monocytes and certain lymphocyte populations (19), testis (20), human keratinocytes during inflammatory disorders (21), and airway epithelium (22). The characteristic feature of cathelicidin peptides is a high level of sequence identity at the N terminus prepro regions (23), termed the cathelin domain (24). Cathelicidin peptides are stored as inactive propeptide precursors that, upon stimulation, are processed into active peptides. hCAP-18 was found to be cleaved extracellularly by proteinase 3 to generate the peptide LL-37 (25). Overexpression of this peptide, by adenovirus-mediated gene transfer, of LL-37 in the mouse airway results in the increased ability to reduce bacterial load from *Pseudomonas aeruginosa* challenge and improved survival after administration of lethal doses of LPS (26). It is of interest to determine whether the natural role of peptides in the body involves direct bacterial killing or a combination of killing and stimulation of other mechanisms of innate immunity.

Cationic antimicrobial peptides may also regulate cell migration to promote the ability of leukocytes to combat bacterial infections. For example, two human α defensin peptides, HNP-1 and HNP-2,

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have been indicated to have direct chemotactic activity for murine and human T cells and monocytes (27, 28), and human β defensins appear to act as chemoattractants for immature dendritic cells and memory T cells through interaction with CCR6 (29). Similarly, the porcine cationic peptide PR-39 was found to be chemotactic for neutrophils (30). LL-37 has been shown to have chemotactic activity for monocytes, T cells, and neutrophils (31, 32) as well as mast cells (33).

The aim of our current study was to gain knowledge of the roles that the human peptide LL-37 may play in combating bacterial infection. We performed gene arrays on macrophage cells stimulated with LL-37 and discovered a large number of genes affected by the peptide. A number of LL-37-induced gene expression changes were confirmed by semiquantitative RT-PCR. We chose to follow-up on several genes up-regulated by LL-37 that are involved in chemotaxis. We report here the novel finding that LL-37 up-regulates the production of chemokines and the surface expression of chemokine receptors, and thus could promote cell migration, and that this occurs in vivo in the mouse lung. These properties are in addition to the ability of LL-37 to reduce the production of TNF-α by macrophages stimulated with LPS, lipoteichoic acid (LTA),[3] and *Mycobacterium* noncapped lipoarabinomannan (AraLAM) suggesting that LL-37 has a multifaceted role in controlling bacterial infection.

Materials and Methods

Reagents

Salmonella typhimurium LPS and *Escherichia coli* 0111:B4 LPS were purchased from Sigma-Aldrich (St. Louis, MO). LTA (Sigma) from *Staphylococcus aureus* was resuspended in endotoxin-free water (Sigma-Aldrich). The *Limulus* amebocyte lysate assay (Sigma-Aldrich) was performed on LTA preparations to confirm that lots were not significantly contaminated by endotoxin. Endotoxin contamination was <1 ng/ml, a concentration that did not cause significant cytokine production in the RAW 264.7 cells (34). AraLAM was a gift from Dr. J. T. Belisle of Colorado State University (Fort Collins, CO). The AraLAM from *Mycobacterium* was filter sterilized and the endotoxin contamination was found to be 3.75 ng per 1.0 mg of LAM as determined by the *Limulus* amebocyte assay. LL-37 (amino acid sequence: LLGDFFRKSKEKIGKEFKRIVQRIKDFLRNLVPRTES) was synthesized by F-moc chemistry at the Nucleic Acid/Protein Synthesis Unit at the University of British Columbia (UBC) as described previously (8).

Preparation and analysis of macrophages isolated from bone marrow of mice

Bone marrow macrophages were obtained from 8- to 10-wk-old BALB/c mice (Charles River Breeding Laboratories, Wilmington, MA) as previously described (35). The cells were cultured in 150-mm plates in DMEM (Life Technologies, Burlington, Ontario, Canada) supplemented with 20% FBS (Sigma-Aldrich) and 20% L cell-conditioned medium as a source of M-CSF. Once macrophages were 60–80% confluent, they were deprived of L cell-conditioned medium for 14–16 h to render the cells quiescent. The experiments were then conducted by adding 100 ng/ml *E. coli* O111:B4 LPS, LPS plus 20 μg/ml LL-37 (or CEMA), or medium alone (DMEM plus 20% FBS) to the cells for 24 h. The release of cytokines into the culture supernatant was determined by ELISA (R&D Systems, Minneapolis, MN).

Cytokine and chemokine production by RAW 264.7 macrophages and A549 epithelial cells

The murine macrophage cell line RAW 264.7 was obtained from American Type Culture Collection (Manassas, VA) and the human epithelial cell line A549 was obtained from Dr. D. Speert (Department of Pediatrics, UBC). Both cell lines were maintained in DMEM supplemented with 10% FCS. RAW 264.7 cells were seeded in 24-well plates at a density of 10^6 cells/well in DMEM (see above) and A549 cells were seeded in 24-well plates

at a density of 10^5 cells/well in DMEM (see above), and both were incubated at 37°C in 5% CO_2 overnight. DMEM was aspirated from cells grown overnight and replaced with fresh medium. LPS or other bacterial products were incubated with the cells for 6–24 h at 37°C in 5% CO_2. At the same time as LPS addition, cationic peptides were added at a range of concentrations. The supernatants were removed and tested for cytokine or chemokine production by ELISA (R&D Systems).

Chemokine production in whole blood

Blood from volunteer human donors was collected (according to procedures accepted by UBC Clinical Research Ethics Board, certificate C00-0537) by venipuncture into tubes (BD Labware, Franklin Lakes, NJ) containing 14.3 USP units heparin/ml blood. The blood was mixed with increasing concentrations of LL-37 in polypropylene tubes at 37°C for 6 h. The samples were centrifuged for 5 min at 2000 × *g*, and the plasma was collected and then stored at −20°C until analyzed for monocyte chemoattractant protein (MCP) 1, TNF-α, and IL-8 by ELISA (R&D Systems).

RNA isolation

RAW 264.7 cells were plated in 150-mm tissue culture dishes at 5.6×10^6 cells/dish, cultured overnight, and then incubated with 50 μg/ml LL-37 or medium alone for 4 h. After stimulation, the cells were washed once with diethyl pyrocarbonate-treated PBS, and detached from the dish using a cell scraper. Total RNA was isolated using TRIzol (Life Technologies) as described previously (1, 36). The quality of the RNA was assessed by gel electrophoresis on a 1% agarose gel. RNA samples were treated with DNase according to the manufacturer's instructions (DNA free; Ambion, Austin, TX) to remove any contaminating genomic DNA. Lack of genomic DNA contamination was confirmed by using the isolated RNA as a template for PCR amplification with β-actin-specific primers (5′-GTCCCTG TATGCCTCTGGTC-3′ and 5′-GATGTCACGCACGATTTCC-3′). Agarose gel electrophoresis and ethidium bromide staining confirmed the absence of an amplicon after 35 cycles.

Mouse cDNA expression arrays

Atlas cDNA Expression Arrays (Clontech Laboratories, Palo Alto, CA), which consist of 588 selected mouse cDNAs spotted in duplicate on positively charged membranes, were used for our gene array studies as described previously (1). Briefly, ^{32}P-radiolabeled cDNA probes were prepared from 5 μg total RNA that was incubated overnight at 71°C. The filters were washed extensively and then exposed to a PhosphorImager screen (Molecular Dynamics, Sunnyvale, CA) for 3 days at 4°C. The image was captured using a Molecular Dynamics PSI phosphoimager. The hybridization signals were analyzed using Atlas Image 1.0 Image Analysis software (Clontech Laboratories) and Excel (Microsoft, Redmond, WA). The intensities for each spot were corrected for background levels and normalized for differences in probe labeling using the average values for five genes observed to vary little among our stimulation conditions: β-actin, ubiquitin, ribosomal protein S29, GAPDH, and Ca^{2+}-binding protein. When the normalized hybridization intensity for a given cDNA was <20, it was assigned a value of 20 to calculate the ratios and relative expression (1, 36).

Semiquantitative RT-PCR

RNA was prepared as described above. The 1-μg RNA samples were incubated with 1 μl oligo(dT) (500 μg/ml) and 1 μl mixed dNTP stock at 1 mM in a 12-μl volume with diethyl pyrocarbonate-treated water at 65°C for 5 min in a thermocycler. Briefly, 4 μl 5× first-strand buffer, 2 μl 0.1 M DTT, and 1 μl RNaseOUT recombinant ribonuclease inhibitor (40 U/μl) were added and incubated at 42°C for 2 min, followed by the addition of 1 μl (200 U) of Superscript II (Invitrogen, Burlington, Ontario, Canada). Negative controls for each RNA source were generated using parallel reactions in the absence of Superscript II. cDNAs were amplified in the presence of 5′ and 3′ primers (1.0 μM), 0.2 mM dNTP mixture, 1.5 mM MgCl, 1 U *Taq* DNA polymerase (New England Biolabs, Mississauga, Ontario, Canada), and 1× PCR buffer. Each PCR was performed with a thermal cycler by using 30–40 cycles consisting of 30 s of denaturation at 94°C, 30 s of annealing at either 52 or 55°C and 40 s of extension at 72°C. The number of cycles of PCR was optimized to lie in the linear phase of the reaction for each primer and set of RNA samples. A housekeeping gene, β-actin, was amplified in each experiment to evaluate extraction procedure and to estimate the amount of RNA. The reaction product was visualized by electrophoresis and analyzed by densitometry, with relative starting RNA concentrations calculated with reference to β-actin amplification. The primers used are shown in Table I.

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[3] Abbreviations used in this paper: LTA, lipoteichoic acid; AraLAM, lipoarabinomannan; MCP, monocyte chemoattractant protein; BAL, bronchoalveolar lavage; LBP, LPS-binding protein; BMP, bone morphogenetic protein; MIP, macrophage-inflammatory protein.

Table I. *Primer pairs for RT-PCR*

Gene	Primers (5′–3′)
β-actin	GTCCCTGTATGCCTCTGGTC
	GATGTCACGCACGAATTTCC
MCP-1	AGGTCCCTGTCATGCTTCTG
	GGTTCTGATCTCATTTGGTTCC
MCP-3	TAGGAATGTGAAATGGTCACG
	ACTTCCATGCCCTTCTTTGTC
IL-8RB	CAAGCTGATCAAGGAGACCTG
	GCCATGCTGAAAGACAAGAAG
IL-10	CTGCCTGCTCTTACTGACTGG
	CAATGCTCCTTGATTTCTGG
MIP-1β	CCAGCTGTGGTATTCCTGACC
	AATAGCAGAGTTTCAGCAATGG
CXCR4	ACTGCATCATCATCTCCAAGC
	CTCTCGAAGTCACATCCTTGC
Cyclin D1	CAGCTTAATGTGCCCTCTCC
	GGTAATGCCATCATGGTTCC
CD14	CTGATCTCAGCCCTCTGTCC
	CAGGAGGATGCAAATGTTCC
XRCC1	TCTTCTCAAGGCGGACACTTA
	CGGACTAACTTGTCAGGTCCAA

Flow cytometry

To analyze cell surface expression of IL-8RB, CXCR-4, and CCR2, RAW 264.7 macrophage cells were stained with 10 μg/ml of the appropriate primary Ab (Santa Cruz Biotechnology, Santa Cruz, CA) followed by FITC-conjugated goat anti-rabbit IgG (IL-8RB and CXCR-4; Jackson ImmunoResearch Laboratories, West Grove, PA) or FITC-conjugated donkey anti-goat IgG (Santa Cruz Biotechnology). The cells (10,000 live events were counted) were passed through a FACscan (BD Biosciences, Mountain View, CA) and forward and side scatter were used to gate on live cells.

Measurement of LL-37-induced chemokine production in the respiratory tract of mice

Animal studies were approved by the UBC Animal Care Committee (UBC ACC no. A01-0008). BALB/c mice were purchased from Charles River Breeding Laboratories and housed in standard animal facilities. Age-, sex-, and weight-matched adult mice were anesthetized with an i.p. injection of Avertin (4.4 mM 2,2,2-tribromoethanol, 2.5% 2-methyl-2-butanol, in distilled water) using 200 μl/10 g body weight. The instillation was performed using a nonsurgical, intratracheal instillation method adapted from Ho and Furst (37). Briefly, the anesthetized mouse was placed with its upper teeth hooked over a wire at the top of a support frame with its jaw held open and a spring pushing the thorax forward to position the pharynx, larynx, and trachea in a vertical straight line. The airway was illuminated externally and an intubation catheter was inserted into the clearly illuminated tracheal lumen. A 50-μg bolus of LL-37 suspended in 20 μl of sterile water, or sterile water alone, was placed in a well at the proximal end of the catheter and gently instilled into the trachea with 200 μl of air. The animal was maintained in an upright position for 2 min after instillation to allow the fluid to drain into the respiratory tree. After 4 h, the mice were euthanized by i.p. injection of 300 mg/kg pentobarbital. The trachea was exposed and an i.v. catheter was passed into the proximal trachea and tied in place with suture thread. Lavage was performed by introducing 0.75 ml sterile PBS into the lungs via the tracheal cannula and then after a few seconds, withdrawing the fluid. This was repeated three times with the same sample of PBS. The lavage fluid was placed in a tube on ice and the total recovery volume per mouse was ∼0.5 ml. The bronchoalveolar lavage (BAL) fluid was centrifuged at 1200 rpm for 10 min and the clear supernatant removed and tested for TNF-α and MCP-3 by ELISA.

Results

LL-37 neutralizes the activation of macrophages by LPS, LTA, and AraLAM

Cationic antimicrobial peptides have been shown to block many LPS-induced responses and are being considered as candidates for the treatment of sepsis. We have previously shown that CEMA (a synthetic peptide derived from the sequence of cecropin and melittin) blocks LPS-induced production of inflammatory cytokines and

selectively inhibits the ability of LPS to alter gene expression in the RAW 264.7 murine macrophage cell line (8, 36). Although CEMA reduced LPS-induced up-regulation of >40 genes, including many genes encoding inflammatory mediators, the regulation of 16 other genes by LPS was unaffected by CEMA. Thus, we proposed that cationic antimicrobial peptides selectively downregulate the proinflammatory responses of macrophages to LPS while leaving certain other responses intact. It appears possible that naturally occurring cationic antimicrobial peptides normally play such a role in either limiting or terminating inflammatory responses. Therefore, we wished to analyze the effects of a human cationic antimicrobial peptide, LL-37, on macrophage responses to LPS and other bacterial products.

The extensively studied RAW 264.7 macrophage cell line was used to determine whether the LL-37 could inhibit the production of TNF-α by macrophages stimulated with bacterial products. The RAW 264.7 cells were incubated with three types of LPS, with LTA, or with AraLAM and then the release of TNF-α into the culture supernatants was quantitated by ELISA. We used these bacterial products to represent products release by both Gram-negative and Gram-positive bacteria. LL-37 was able to significantly inhibit TNF-α production stimulated by *S. typhimurium, Burkholderia cepacia*, and *E. coli* O111:B4 LPS, with the former being affected to a somewhat lesser extent (Fig. 1*A*). At concentrations as low as 1 μg/ml LL-37 (0.25 nM), substantial inhibition of TNF-α production was observed in the latter two cases. These results were confirmed in primary cells, in that LL-37 and the positive control peptide CEMA significantly inhibited TNF-α production (>90%) by bone marrow-derived macrophages from BALB/c mice that had been stimulated with 100 ng/ml *E. coli* 0111:B4 LPS (Fig. 1*B*). These experiments were performed in the presence of serum, which contains LPS-binding protein (LBP), a protein that can mediate the rapid binding of LPS to CD14. Thus, we examined the kinetics of antagonism of LPS induction of TNF-α production (Fig. 1*C*). Delayed addition of LL-37 to the supernatants of macrophages 1 h after stimulation with 100 ng/ml *E. coli* LPS still resulted in substantial reduction of TNF-α production (70%).

Consistent with the ability of LL-37 to prevent LPS-induced production of TNF-α in vitro, LL-37 partially protected mice against lethal endotoxic shock induced by a high concentration of endotoxin (LPS). CD-1 mice were sensitized to endotoxin with a prior injection of galactosamine. Mice that were injected with 3 μg of *E. coli* 0111:B4 LPS were all killed within 4–6 h. When 200 μg of LL-37 was injected 15 min after the LPS, 50% of the mice survived (Table II).

LL-37 could also reduce the ability of bacterial products to stimulate the production of inflammatory mediators by an epithelial cell line. In the A549 human lung epithelial cell line, LL-37 reduced the ability of LPS to stimulate the production of IL-8 and MCP-1 (Fig. 1*D*).

Since LL-37 was able to inhibit LPS-induced TNF-α production, we asked whether it could inhibit the ability of other bacterial products to induce TNF-α release. We therefore examined whether LL-37 would block the ability of *Mycobacterium* noncapped AraLAM and *S. aureus* LTA to activate RAW 264.7 cells. LL-37 and CEMA did indeed reduce induction of TNF-α in RAW 264.7 cells by AraLAM (Fig. 2). At a concentration of 1 μg/ml, LL-37 was able to substantially inhibit (>75%) the induction of TNF-α production by 1 μg/ml *S. aureus* LTA. At 20 μg/ml LL-37, there was >60% inhibition of AraLAM-induced TNF-α. Polymyxin B was included as a control to demonstrate that contaminating endotoxin was not a significant factor in the inhibition of AraLAM-induced TNF-α by LL-37. These studies demonstrated that the human peptide LL-37 can neutralize the effect of bacterial products
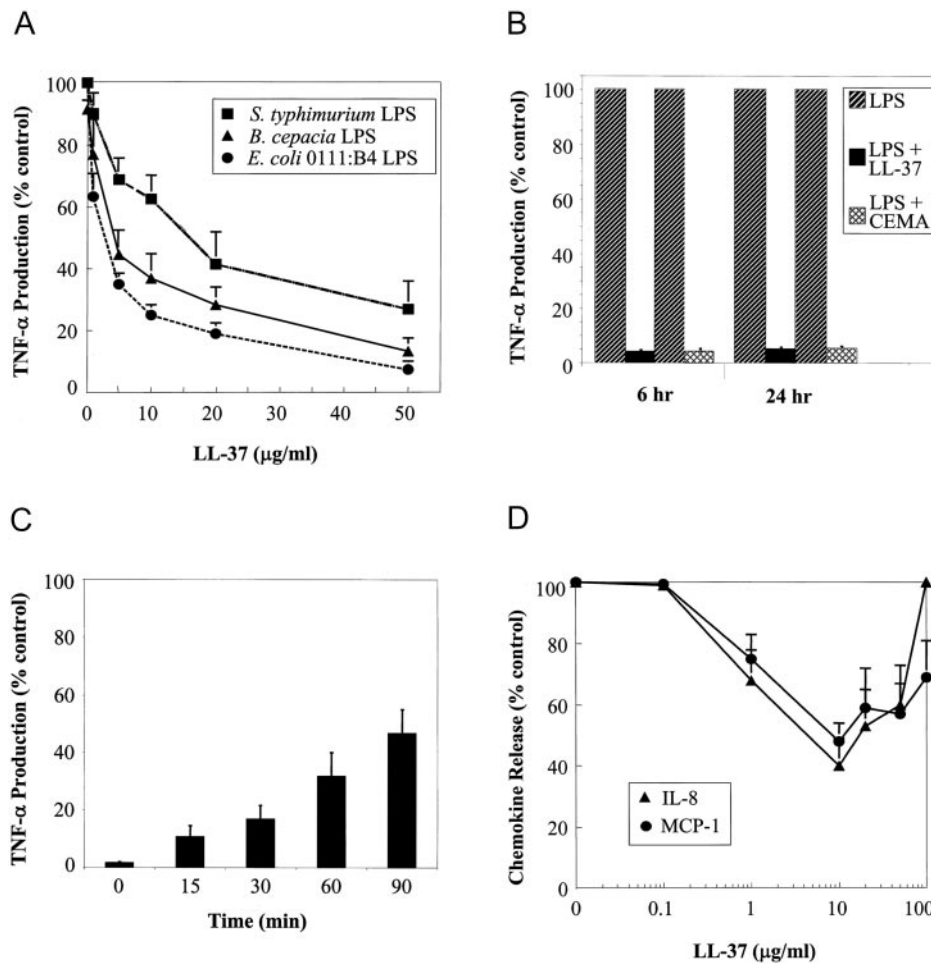
FIGURE 1. LL-37 reduces LPS-induced production of TNF-α. *A,* RAW 264.7 macrophage cells were stimulated with (■) 100 ng/ml *S. typhimurium* LPS, (▲) 100 ng/ml *B. cepacia* LPS, and (●) 100 ng/ml *E. coli* 0111:B4 LPS in the presence of the indicated concentrations of LL-37 for 6 h. The concentrations of TNF-α released into the culture supernatants were determined by ELISA. One hundred percent represents the amount of TNF-α resulting from RAW 264.7 cells incubated with LPS alone for 6 h (*S. typhimurium* LPS, 34.5 ± 3.2 ng/ml; *B. cepacia* LPS, 11.6 ± 2.9 ng/ml; and *E. coli* 0111:B4 LPS, 30.8 ± 2.4 ng/ml). Background levels of TNF-α production by the RAW 264.7 cells cultured with no stimuli for 6 h resulted in TNF-α levels ranging from 0.037 to 0.192 ng/ml. The data are from duplicate samples and presented as the mean of three experiments ± SE. *B,* Bone marrow-derived macrophages were cultured for either 6 or 24 h with 100 ng/ml *E. coli* 0111:B4 LPS in the presence or absence of 20 μg/ml LL-37 or CEMA. The supernatant was collected and tested for levels of TNF-α by ELISA. One hundred percent represents the amount of TNF-α resulting from duplicate wells of bone marrow-derived macrophages incubated with LPS alone for 6 h (1.1 ± 0.09 ng/ml) or 24 h (1.7 ± 0.2 ng/ml). Background levels of TNF-α were 0.038 ± 0.008 ng/ml for 6 h and 0.06 ± 0.012 ng/ml for 24 h. The data from duplicate samples are presented as the mean of three experiments + SE. *C,* LL-37 (20 μg/ml) was added at increasing time points to wells already containing 100 ng/ml *E. coli* 0111:B4 LPS. The supernatant was collected after 6 h and tested for levels of TNF-α by ELISA. The data are presented as the mean of three experiments + SE. *D,* A549 cells were stimulated with increasing concentrations of LL-37 in the presence of LPS (100 ng/ml *E. coli* O111:B4) for 24 h. The concentration of IL-8 (▲) and MCP-1 (●) in the culture supernatants was determined by ELISA. The background levels of IL-8 from cells alone was 0.172 ± 0.029 ng/ml and the background levels of human MCP-1 was 1.08 ± 0.12 ng/ml. The data are presented as the mean of three experiments + SE.

on the immune system and may aid the immune response to bacterial infection by modulating the immune response to bacterial products released upon infection.

LL-37 alters macrophage transcriptional responses

We hypothesized that LL-37 could affect other macrophage functions as we had previously observed with CEMA (1). We therefore performed gene array studies to determine the transcriptional responses of macrophages to LL-37. RNA was extracted from RAW 264.7 cells that were cultured for 4 h with medium alone or 50 μg/ml LL-37 alone and converted into ^{32}P-labeled cDNA. The hybridization of the cDNA probes to each immobilized DNA was visualized by autoradiography and quantified using a PhosphorImager. Representative autoradiographic images of the gene arrays are shown in Fig. 3. Table III shows that LL-37 treatment of RAW

264.7 cells up-regulated the expression of at least 30 different genes. The genes up-regulated by LL-37 were mainly from two categories: one that includes receptors (growth, chemokine, IL, IFN, hormone, neurotransmitter), cell surface Ags, and cell adhesion and another one that includes cell-cell communication (growth factors, cytokines, chemokines, IL, IFN, hormones), cytoskeleton, motility, and protein turnover. The specific genes up-regulated included those encoding chemokine MCP-3, the anti-inflammatory cytokine IL-10, M-CSF, and receptors such as IL-1R2 (a putative antagonist of productive IL-1 binding to IL-1R1), platelet-derived growth factor receptor B, NOTCH4, LIF receptor, LFA-1, TGF-β receptor 1, G-CSF receptor, and IFN-γ receptor. Our gene array data suggested that LL-37 up-regulates the expression of the chemokine receptors IL-8RB, CXCR-4, and CCR2 by 10-, 4-, and 1.4-fold above unstimulated cells, respectively. To

Table II. *Protection against lethal endotoxemia in galactosamine-sensitized CD-1 mice by LL-37[a]*

D-Galactosamine Treatment (mg)	*E. coli* 0111: B4 LPS (μg)	LL-37 or Buffer	Total Mice	Survival Postendotoxin Shock
0	3	PBS	5	5 (100%)
20	3	PBS	12	0 (0%)
20	3	LL-37	12	6 (50%)

[a] CD-1 mice (9 wk old) were sensitized to endotoxin by i.p. injection of galactosamine (20 mg in 0.1 ml sterile PBS). Then endotoxic shock was induced by i.p. injection of *E. coli* 0111:B4 LPS (3 μg in 0.1 ml PBS). LL-37 (200 μg/mouse = 8 mg/kg) was injected at a separate i.p. site 15 min after injection of LPS. The mice were monitored for 48 h and the results were recorded.

confirm our gene array data, we examined, using flow cytometry, the surface expression of these receptors on RAW cells stimulated with peptide for 4 h. When 50 μg/ml LL-37 was incubated with RAW cells for 4 h, IL-8RB was up-regulated an average of 2.4-fold above unstimulated cells, CXCR-4 was up-regulated an average of 1.6-fold above unstimulated cells, and CCR2 was up-regulated 1.8-fold above unstimulated cells (data not shown). As a
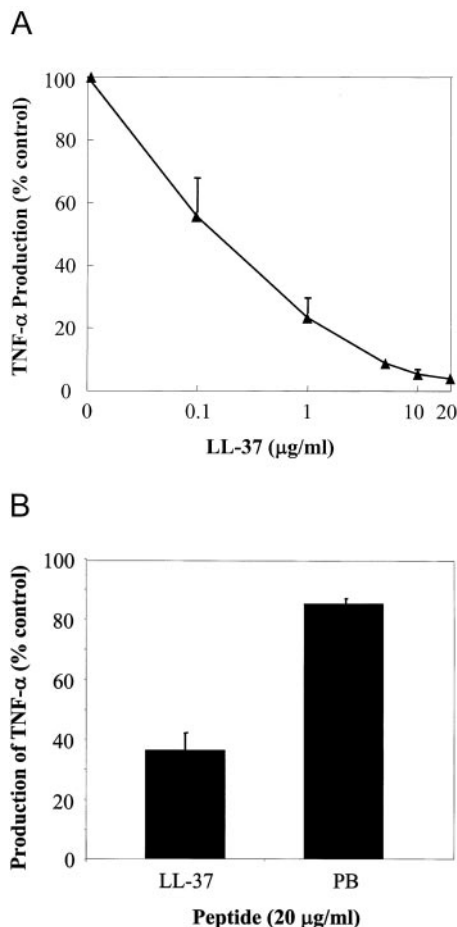

FIGURE 2. Inhibition of TNF-α production by RAW 264.7 macrophages stimulated with *S. aureus* LTA and *Mycobacterium* AraLAM by LL-37. RAW 264.7 macrophage cells were stimulated with 1 μg/ml *S. aureus* LTA (*A*) and 1 μg/ml AraLAM LPS (*B*) in the absence and presence of 20 μg/ml LL-37 or polymyxin B (PB). The supernatant was collected and tested for levels of TNF-α by ELISA. Background levels of TNF-α production by the RAW 264.7 cells cultured with no stimuli for 6 h resulted in TNF-α levels ranging from 0.037 to 0.192 ng/ml. The data are presented as the mean of three or more experiments + SE.
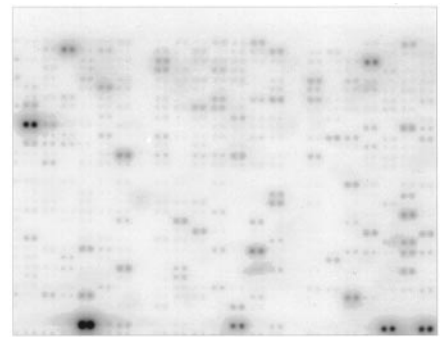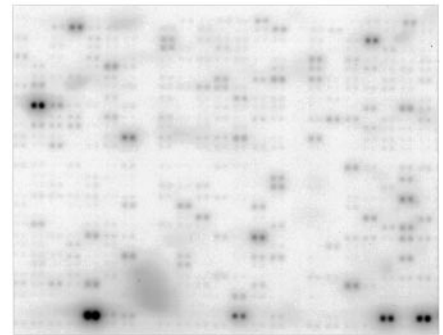

FIGURE 3. Effect of LL-37 on gene expression in RAW 264.7 macrophage cells. RAW 264.7 macrophage cells were stimulated with medium alone (*A*) for 4 h or 50 μg/ml LL-37 (*B*). The RNA was isolated from the cells with TRIzol, DNase treated, and used to make ^{32}P-labeled cDNA probes, which were hybridized to the Clontech Atlas arrays. After a 3-day exposure, they were analyzed using a PhosphorImager and Clontech Atlas software. These data are representative of three experiments.

control we demonstrated that CEMA caused similar up-regulation. LL-37 also up-regulated genes encoding several metalloproteinases and inhibitors thereof, including the bone morphogenetic proteins bone morphogenetic protein (BMP) 1, BMP-2, BMP-8a, tissue inhibitor of matrix metalloproteinase 2, and tissue inhibitor of matrix metalloproteinase 3. As well, LL-37 up-regulated specific transcription factors, including JunD, and the YY and LIM-1 transcription factors, and kinases such as Etk1 and Csk, demonstrating its widespread effects. We also discovered from our gene array studies that LL-37 down-regulated at least 20 genes in RAW macrophage cells (Table IV). The genes down-regulated by LL-37 included DNA repair proteins and several inflammatory mediators such as macrophage-inflammatory protein (MIP) 1α, oncostatin M, and IL-12.

Confirmation of LL-37-altered macrophage transcriptional responses

The changes in gene expression in RAW 264.7 cells in response to LL-37 compared with medium-only controls were also confirmed for a number of genes by semiquantitative RT-PCR (Fig. 4). These included genes that were up- and down-regulated or unchanged. The chemokine receptors CXCR-4 and IL-8RB were up-regulated to similar extents by gene arrays and RT-PCR. CXCR-4 was up-regulated by LL-37 relative to medium-only controls, 4 ± 1.7-fold by gene arrays, and 4.1 ± 0.9-fold by RT-PCR, and IL-8RB was up-regulated 9.5 ± 7.6-fold (average ± SE) by gene arrays and 7.1 ± 1.4-fold by RT-PCR. The relative expression of CD14 was 0.9 ± 0.1-fold and 0.8 ± 0.3-fold by gene arrays and RT-PCR, respectively. MCP-1, which was not on the gene arrays but whose expression was followed up at the protein level (Fig. 5), was found to be up-regulated 3.5 ± 1.4-fold by RT-PCR. However, there was a disparity in the recorded levels of expression of IL-10, MCP-3,

Table III.   *Genes up-regulated by LL-37 treatment of RAW 264.7 macrophage cells[a]*

Gene or Protein	Gene[b]	Gene Function	Unstimulated Intensity	Ratio LL-37: Unstimulated[c]	Accession Number
Etk1	B2l	Tyrosine protein kinase receptor	20	43	M68513
PDGFRB	E2i	Growth factor receptor	24	25	X04367
NOTCH4	A5h	Protooncogene	48	18	M80456
IL-1R2	E2n	IL receptor	20	16	X59769
MCP-3	E1k	Chemokine	56	14	S71251
BMP-1	F1b	BMP (growth factor)	20	14	L24755
LIFR	E1l	LIF (cytokine) receptor	20	12	D26177
BMP-8a	F1f	BMP (growth factor)	20	12	M97017
MCSF	A5g	M-CSF1	85	11	X05010
GCSFR	E1j	G-CSF receptor	20	11	M58288
IL-8RB	E3h	Chemokine receptor	112	10	D17630
C5AR	E1g	Chemokine receptor	300	4	S46665
L-myc	A3h	Oncogene	208	4	X13945
IL-10	F4l	IL	168	4	M37897
CXCR-4	B3d	Chemokine receptor	36	4	D87747

[a] Total RNA was isolated from unstimulated RAW 264.7 cells and cells treated for 4 h with 50 μg/ml LL-37. After reverse transcription, ^{32}P-labeled cDNA was used to probe Clontech Atlas gene array filters. Hybridization was analyzed using Atlas Image (Clontech) software. The changes in the normalized intensities of the housekeeping genes ranged from 0.8- to 1.2-fold, validating the use of these genes for normalization. When the normalized hybridization intensity for a given cDNA was <20, it was assigned a value of 20 (36) to calculate the ratios and relative expression. The array experiments were repeated three times with different RNA preparations and the average fold change is shown above. Genes with a 2-fold or greater change in relative expression levels are presented.

[b] The gene classes (given by the first letter of the gene name) include: class A: oncogenes, tumor suppressors, and cell cycle regulators; class B: stress response, ion channels, transport, modulators, effectors, and intracellular transducers; class C: apoptosis, DNA synthesis, and repair; class D: transcription factors and DNA-binding proteins; class E: receptors (growth, chemokine, IL, IFN, hormone, neurotransmitter), cell surface Ags, and cell adhesion; class F: cell-cell communication (growth factors, cytokines, chemokines, IL, IFNs, hormones), cytoskeleton, motility, and protein turnover.

[c] The ratio was calculated by dividing the intensities for cells treated with 50 μg/ml LL-37 by the intensities for unstimulated cells.

cyclin D1, and MIP-1β (Fig. 4) after LL-37 treatment relative to medium control in gene array and RT-PCR experiments indicating that the array methodology provides qualitative rather than quantitative data. Although the changes in RNA levels for a number of genes in RAW 264.7 cells treated with LL-37 were confirmed, it should be noted that this could be the result of either an increase/decrease in transcription or change in RNA stability.

LL-37 induces chemokine secretion

Our gene array studies indicated that LL-37 increased the expression of chemokine genes in RAW 264.7 cells. This suggests that LL-37 may induce macrophages to produce chemokines, which could in turn recruit additional immune cells to the sites of infection. We went on to confirm the up-regulation of chemokines in several different systems. First, we examined the effect of LL-37 on RAW 264.7 cell production of MCP-1 (MCP-1 was not represented on our arrays but has similar activities to MCP-3 for which no ELISA is available). The murine MCP-1, a homologue of the human MCP-1, is a member of the β(C-C) chemokine family. MCP-1 has been demonstrated to recruit monocytes (38), NK cells (39), and some T lymphocytes (40). When RAW macrophages were stimulated with increasing concentrations of LL-37, they pro-

duced significant levels of MCP-1 in their supernatant, as judged by ELISA (Fig. 4). RAW264.7 cells stimulated with peptide concentrations ranging from 20 to 50 μg/ml for 24 h produced significant levels of MCP-1 (200–400 pg/ml above background). When the cells were stimulated with 100 μg/ml LL-37, very high levels of MCP-1 (>1000 pg/ml above background) were produced. The ability of LL-37 to induce human MCP-1 in blood was also tested. Human blood from three separate donors was incubated with LL-37 for 4 h, the samples were centrifuged, and the serum was removed and tested for human MCP-1 by ELISA. Although there was significant production of human MCP-1 in response to LL-37 by all three donors, there was substantial variation of donor response to the peptide, as indicated by the large SE in Fig. 5.

We also examined the effect of LL-37 on chemokine induction in a completely different cell system, A549 human epithelial cells. Interestingly, although these cells produce MCP-1 in response to LPS (Fig. 1*D*) and this response could be antagonized by LL-37, there was no production of MCP-1 in direct response to LL-37. LL-37 did however induce production of IL-8, a neutrophil-specific chemokine (Fig. 6*A*). Thus, LL-37 can induce a different spectrum of responses from different cell types. Significant but low

Table IV.   *Genes down-regulated by LL-37 treatment of RAW 264.7 macrophage cells[a]*

Gene or Protein	Gene	Gene Function	Unstimulated Intensity	Ratio LL-37: Unstimulated	Accession Number
XRCC1	C7n	DNA repair protein	227	0.12	U02887
XPAC	C7m	DNA repair protein	485	0.17	X74351
Voltage-gated sodium channel	B2f	Voltage-gated ion channel	257	0.24	L36179
PMS2 DNA	C7d	DNA repair protein	200	0.3	U28724
MIP-1β	F3f	Cytokine	327	0.42	M23503

[a] RNA was isolated from unstimulated RAW 264.7 macrophage cells and RAW 264.7 cells treated for 4 h with 50 μg/ml LL-37. The array experiments were repeated three times with different RNA preparations and the average fold change is shown above. Genes with an approximately 2-fold or greater change in relative expression levels are presented (refer to Table III for details).
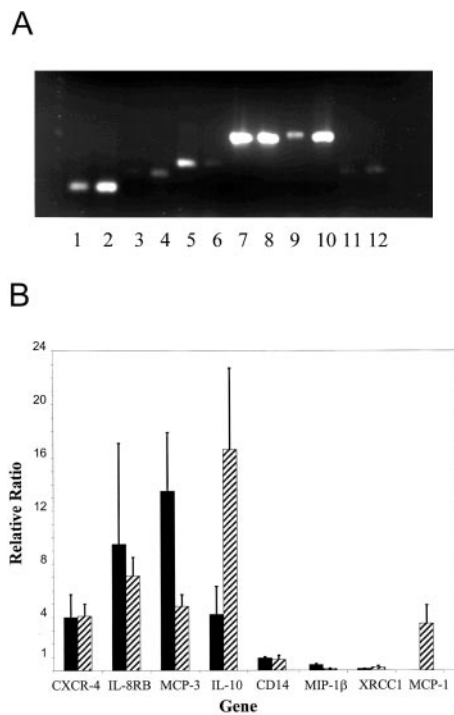
A

B

A

B

FIGURE 4. Confirmation of LL-37 induced macrophage gene expression changes by RT-PCR. RAW 264.7 macrophage cells were incubated with 50 μg/ml LL-37 or medium only for 4 h. Semiquantitative RT-PCR was performed on total RNA isolated from the RAW 264.7 cells. Specific primer pairs for each gene (Table I) were used for amplification of RNA. Amplification of β-actin was used as a positive control and for standardization. *A,* The products for medium-only-treated cells and LL-37-treated cells are shown in the *first* and *second lane* for each gene. *Lanes 1* and *2,* β-Actin; *lanes 3* and *4,* XRCC1; *lanes 5* and *6,* MIP-1β; *lanes 7* and *8,* CD14; *lanes 9* and *10,* cyclin D1; and *lanes 11* and *12,* IL-8RB. *B,* Densitometric analysis of each gene was performed. The relative fold change refers to the change in gene expression of LL-37-treated cells compared with cells incubated with medium alone. ■, The results from gene array experiments; ▨, results from RT-PCR experiments. The data are presented as the mean ± SE of three experiments.
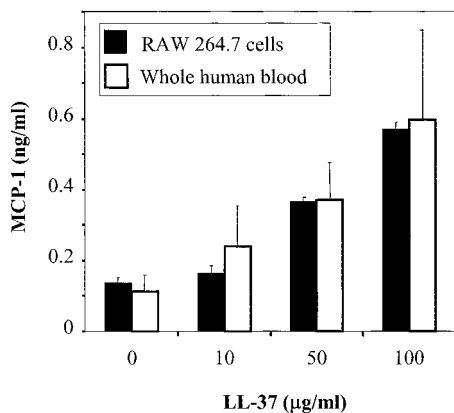

FIGURE 5. Induction of MCP-1 in RAW 264.7 macrophage cells and whole human blood. RAW 264.7 macrophage cells or whole human blood were stimulated with increasing concentrations of LL-37 for 4 h. The human blood samples were centrifuged and the serum was removed and tested for MCP-1 by ELISA along with the supernatants from the RAW cells. The RAW cell data presented are the mean of three or more experiments ± SE and the human blood data represent the mean ± SE from three separate donors.
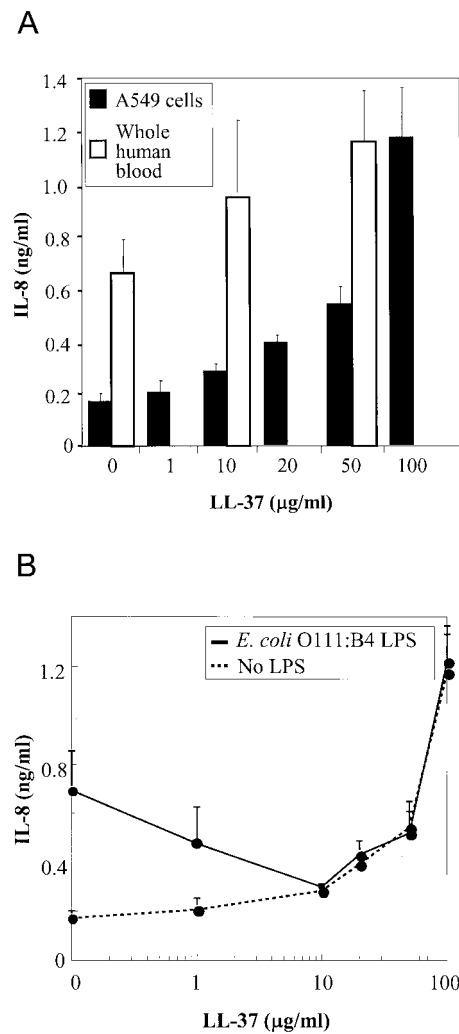
FIGURE 6. Induction of IL-8 by A549 epithelial cells and whole human blood. *A,* A549 cells or whole human blood were stimulated with increasing concentrations of LL-37 for 24 and 4 h, respectively. The human blood samples were centrifuged and the serum was removed and tested for IL-8 by ELISA along with the supernatants from the A549 cells. The A549 cell data presented are the mean of three or more experiments ± SE and the human blood data represent the mean ± SE from three separate donors. *B,* A549 cells were stimulated with 100 ng/ml *E. coli* O111:B4 LPS (—) and LPS plus increasing concentrations of LL-37 (- - -) for 24 h. The supernatant was removed and tested for IL-8 by ELISA. This experiment was repeated four times and one representative experiment is shown.

levels of IL-8 were produced at LL-37 concentrations of 20 μg/ml. At 100 μg/ml LL-37, there were high levels of IL-8 produced (>1 ng/ml). This is in contrast to another cationic α helical peptide that was tested and found not to cause induction of IL-8 (data not shown), suggesting that the effect of LL-37 is specific. LL-37 also induced significant levels of IL-8 in whole human blood (Fig. 6*A*). Since LPS is a known potent stimulus of IL-8 production and LL-37 neutralized the responses of LPS (Fig. 1*D*), we studied the direct effect of LL-37 on LPS-induced IL-8 production in A549 cells. We confirmed that at low concentrations of LL-37 (1–20 μg/ml), the peptide inhibited the LPS-induced IL-8 production, but that at high concentrations of LL-37 (50–100 μg/ml), there was stimulation of IL-8 production independent of LPS. This indicates that LL-37 may have differential roles in the immune system depending on the concentration found at the site of infection.
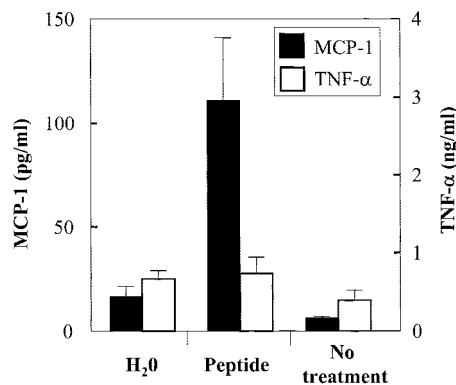
FIGURE 7. Production of MCP-1 and TNF-α in murine BAL fluid in response to LL-37. BALB/c mice were anesthetized with avertin and given intratracheal instillation of LL-37 or water or no instillation (no treatment). The mice were monitored for 4 h, anesthetized, and the BAL fluid was isolated and analyzed for MCP-1 and TNF-α concentrations by ELISA. The data shown are the mean of four or five mice for each condition $\pm$ SE.

LL-37 up-regulates the production of MCP-1 but not TNF-α in the BAL fluid of mice given LL-37 by intratracheal instillation

To examine the effects of LL-37 in an in vivo situation, BALB/c mice were given LL-37 or endotoxin-free water by intratracheal instillation and the levels of MCP-1 and TNF-α were examined in the BAL fluid after 3–4 h. We found that the mice treated with an intratracheal bolus of LL-37 produced significantly increased levels of MCP-1 over mice given water or anesthetic alone (Fig. 7). This was not a general proinflammatory response to LL-37 since no significant difference in BAL TNF-α was observed in peptide-treated mice when compared with mice given water or anesthetic alone. Furthermore, no significant induction of TNF-α production was observed in RAW 264.7 cells or bone marrow-derived macrophages treated with LL-37 (100 μg/ml LL-37 resulted in 0.033 $\pm$ 0.001 ng/ml TNF-α, medium alone resulted in 0.038 $\pm$ 0.008 ng/ml TNF-α). No decrease in the viability of RAW 264.7 cells was observed in the presence of serum with up to 125 μg/ml LL-37 as measured by the MTT assay (data not shown). Thus, LL-37 selectively induces the production of chemokines without inducing the production of inflammatory mediators such as TNF-α. This illustrates the dual role of LL-37 as a factor that can block bacterial product-induced inflammation while helping to recruit phagocytes that can augment clearance of bacterial infections.

Discussion

The role of cationic antimicrobial peptides in antimicrobial defenses has become increasingly apparent. For example, in a mouse endotoxemia model, treatment with the insect-derived peptide CEMA dramatically suppressed the LPS-stimulated induction of the important sepsis-mediating cytokine TNF-α (8). The antiendotoxin activity of CEMA could be partially attributed to its ability to block the interaction of LPS with LBP (41) and its ability to selectively suppress LPS-induced macrophage gene expression (36). We found here that the human peptide LL-37, when added to macrophages stimulated with bacterial products, was able to reduce the production of the proinflammatory cytokine TNF-α. By binding bacterial products and reducing their ability to stimulate macrophages, LL-37 could reduce the development of sepsis in moderate infections. We have previously shown that other cationic antimicrobial peptides bind to LPS and inhibit its binding to LBP (41). Thus, cationic peptides provide not only a natural response to bacterial infection, as seen with LL-37, but also a possible therapeutic intervention. LL-37 could help not only limit bacterial in-

fection but also prevent an overwhelming immune response that can lead to sepsis and even death. Their ability to block macrophage activation suggests that they have a role in terminating immune responses.

The results presented here indicate that before acting as feedback inhibitors of the immune response, cationic peptides may also have a role in promoting the ability of leukocytes to combat bacterial infections. This could be done in part by up-regulating the chemokines IL-8 and MCP-1 and possibly also by up-regulating the surface expression of chemokine receptors such as IL-8RB, CXCR-4, and CCR2. The function of LL-37 could depend on the concentration of the peptide. For instance, at low peptide concentrations (i.e., 2 μg/ml LL-37; Ref. 42) found in normal human epithelia, LL-37 could function as an immune watchdog and, at high concentrations found when LL-37 is induced by bacteria or bacterial products, LL-37 could function to promote migration of immune cells to help control the infection. This feature appears to involve the regulation of a large number of genes, some of which are known to have anti-inflammatory and some proinflammatory roles. Importantly, we have discovered from our studies the novel finding that LL-37 induces chemokine production and surface expression of chemokine receptors and appears to do this when instilled into the lungs of mice. Thus, LL-37 may act indirectly to promote the migration of immune cells, and this may be partially responsible for its ability to protect against infection.

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