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# Host Defense Peptide LL-37 Selectively Reduces Proinflammatory Macrophage Responses

Kelly L. Brown,<sup>\*,†,1</sup> Grace F. T. Poon,<sup>†,1</sup> Darlene Birkenhead,<sup>†</sup> Olga M. Pena,<sup>†</sup> Reza Falsafi,<sup>†</sup> Claes Dahlgren,<sup>\*</sup> Anna Karlsson,<sup>\*</sup> Johan Bylund,<sup>\*</sup> Robert E. W. Hancock,<sup>†</sup> and Pauline Johnson<sup>†</sup>

The human cathelicidin peptide, LL-37, is a host defense peptide with a wide range of immunomodulatory activities and modest direct antimicrobial properties. LL-37 can exert both pro- and anti-inflammatory effects and can modulate the proinflammatory responses of human peripheral blood monocytes and epithelial cells. In this study, we evaluated the effect of LL-37 on mouse bone marrow-derived macrophages (BMDM) and tissue macrophages *in vitro* and *in vivo*. LL-37 dramatically reduced TNF- $\alpha$  and NO levels produced by LPS and IFN- $\gamma$ -polarized M1-BMDM and slightly reduced reactive oxygen species production by these cells. LL-37 did not affect the ability of IL-4-polarized M2-BMDM to upregulate arginase activity, although it did inhibit LPS-induced TNF- $\alpha$  secretion in these cells. LL-37 did not compromise the ability of M1-polarized BMDM to phagocytose and kill bacteria and did not affect the uptake of apoptotic neutrophils by M2-polarized BMDM. However, LL-37-treated M1-BMDM were more efficient at suppressing tumor growth *in vitro*. LL-37 significantly reduced LPS-induced TNF- $\alpha$  secretion in *ex vivo* alveolar macrophages, whereas its effect on peritoneal macrophages was much less dramatic. Effective inhibition of LPS-induced TNF- $\alpha$  secretion by alveolar macrophages also occurred *in vivo* when LL-37 was administered by intratracheal injection. This demonstrates a selective ability of LL-37 to decrease M1-BMDM, M2-BMDM, and tissue macrophage production of the proinflammatory cytokine TNF- $\alpha$  in response to LPS while leaving other crucial anti-inflammatory M1 and M2 macrophage functions unaltered. *The Journal of Immunology*, 2011, 186: 5497–5505.

Inflammation is a complex biological response to infection or injury that involves many different cell types, mediators, and stimuli. Macrophages recognize an array of stimuli from endogenous and exogenous sources and respond with remarkable phenotypic plasticity (1–5). Different inflammatory stimuli can temporarily induce distinct subsets of macrophages with polarized inflammatory phenotypes. The endogenous cytokine IFN- $\gamma$ , for example, in conjunction with microbial stimuli such as LPS, or other cytokines such as TNF- $\alpha$ , stimulates the classic polarization and activation of macrophages into potent, proinflammatory cells.

These macrophages are often referred to as classically activated M1 macrophages, as they mediate Th1-type immune responses. M1 macrophages are able to produce proinflammatory cytokines (TNF- $\alpha$ ), secrete reactive oxygen species (ROS) and NO, and suppress tumor cell growth. Macrophages can also be alternatively activated (e.g., by IL-4, IL-10, IL-13, immune complexes, and glucocorticoids), leading to the stimulation of anti-inflammatory processes and other functions that are important for the resolution of inflammation, wound healing, and repair. These alternatively activated macrophages are referred to as M2 macrophages and can be identified by a lack of M1-associated inflammatory cytokines, increased arginine metabolism, elevated expression of scavenger receptors, and the capacity to promote tissue repair and Th2-type immune responses (1, 5).

The M1/M2 nomenclature provides a basic classification system to distinguish proinflammatory macrophages from anti-inflammatory, wound-healing macrophages. This distinction in macrophage subtypes should not be confused with lineage specification, as the phenotypes can be influenced by the environment and are reversible. Further, *in vivo* macrophages often display a range of phenotypes and functions, and a clear demarcation between M1 and M2 macrophages is not always apparent (2, 4). M2 macrophages themselves can differ in their functions and be divided into different subsets that are linked to different activating stimuli (1, 6, 7). The ability to assume different phenotypes and functions, which reflect changes in the microenvironment, enables macrophages to participate in all stages of an inflammatory process, from development to resolution and the repair of tissue damage (8, 9). The precise nature of each acquired phenotype is directed by cues from the tissues and the immunological microenvironment (1). Endogenous molecules resulting from altered host homeostasis, infection, or damage provide environmental cues that activate macrophages and evoke profound physiological

<sup>\*</sup>Department of Rheumatology and Inflammation Research, University of Gothenburg, 40530 Gothenburg, Sweden; and <sup>†</sup>Department of Microbiology and Immunology, University of British Columbia, Vancouver, British Columbia V6T 1Z3, Canada

<sup>1</sup>K.L.B. and G.F.T.P. contributed equally to this work.

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Address correspondence and reprint requests to Dr. Pauline Johnson, Department of Microbiology and Immunology, University of British Columbia, 2350 Health Sciences Mall, Life Sciences Centre, Vancouver, BC V6T 1Z3, Canada. E-mail address: pauline@interchange.ubc.ca

Abbreviations used in this article: BAL, bronchoalveolar lavage; BMDM, bone marrow-derived macrophage; CFDA, carboxyfluorescein diacetate; Cy7, cyanine 7; iNOS2, inducible NO synthase 2; LBP, LPS binding protein; LTA, lipoteichoic acid; Pam3CSK4, tripalmitoylated lipopeptide; QRT-PCR, quantitative real-time PCR; ROS, reactive oxygen species; TCS, tissue culture supernatant; UBC, University of British Columbia.

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changes. Many of these activating molecules engage pattern recognition receptors, including TLRs, and trigger an inflammatory response.

Cathelicidins are a family of small cationic peptides that have diverse roles in the immune system. Humans have one cathelicidin, hCAP18, which is cleaved to yield the 37-aa bioactive peptide LL-37 that has a broad range of immunomodulatory functions and modest antimicrobial activity (10, 11). Mice have a cathelicidin related antimicrobial peptide, that is 67% identical with quite similar functions. LL-37 is primarily produced by neutrophils and mucosal epithelial cells. The expression of LL-37 can be regulated by vitamin D3 and hypoxia-inducing factor 1- $\alpha$ , and is enhanced under inflammatory conditions (10, 12–15). In vivo, the concentration of LL-37 is substantially elevated at sites of inflammation, particularly in the oral cavity, lungs, and skin (16–20).

LL-37 can interact with cell membranes, affect cell surface receptors, and enter cells. Previous work has demonstrated that LL-37 can exert both pro- and anti-inflammatory effects and can mediate these effects either directly by stimulating cells or indirectly by modulating the cellular response to a particular cytokine or signal. For example, LL-37 can promote the recruitment of inflammatory cells directly by stimulating leukocyte chemotaxis (21) or indirectly by inducing IL-8 release by bronchial epithelial cells and airway smooth muscle cells (22, 23). LL-37 induces the production of leukocyte chemoattractants such as the monocyte chemoattractant MCP1, as shown in a mouse macrophage cell line and when instilled into mice lungs (24). LL-37 can also augment proinflammatory cytokine IL-1 $\beta$ -induced secretion of IL-6, IL-10, MCP1, and MCP3 in myeloid cells (25). In contrast, LL-37 dramatically suppresses TNF- $\alpha$  production induced by LPS or lipoteichoic acid (LTA) stimulation of human monocytes or macrophage cell lines in vitro (26, 27). LL-37 also reduced LPS and IFN- $\gamma$  responses in human PBMCs and mouse splenic B cells (28). LL-37 reduced costimulatory molecule expression and the production of proinflammatory and Th1-polarizing cytokines (TNF- $\alpha$  and IL-12) on human monocytes and dendritic cells and reduced LPS/IFN- $\gamma$ -induced B cell proliferation and Ab production (28, 29). Consistent with these suppressive effects in vitro, LL-37 can protect in animal models of LPS or bacteria-induced sepsis (24, 30). In addition, LL-37 can promote wound healing (31) and angiogenesis (32).

Despite this knowledge, very little is known about the function of LL-37 on tissue macrophages or the effect of LL-37 on the polarization and function of M1 and M2 macrophages. In this study, we investigated the effects of LL-37 on M1 and M2 macrophages and determined the effect of LL-37 on peritoneal and alveolar macrophages in vitro and in vivo. We found that LL-37 dramatically inhibited proinflammatory cytokine secretion in M1 and LPS-stimulated alveolar macrophages but only partially inhibited secretion by LPS-stimulated peritoneal macrophages and did not inhibit other M1 and M2 macrophage functions.

## Materials and Methods

### *Mice and cells*

C57BL/6J mice (B&K Universal, Sollentuna, Sweden, or The Jackson Laboratory, Bar Harbor, ME) were maintained under pathogen-free conditions in the Department of Rheumatology and Inflammation Research at the University of Gothenburg, Gothenburg, Sweden, or were bred and maintained in the animal facility at the University of British Columbia (UBC). Mice were used at 6–12 wk of age. All animal experiments were conducted in accordance with protocols approved by the University Animal Care Committee and Canadian Council of Animal Care guidelines at UBC or by the Ethical Committee of the University of Gothenburg in cooperation with Sahlgrenska Hospital, Gothenburg, Sweden, which approved work with primary human and murine cells. Cells were cultured at

37°C, 5% CO<sub>2</sub>, in phenol red-free RPMI 1640 media or DMEM media (as indicated) supplemented with 10% FCS, 2 mM L-glutamine, 1 mM sodium pyruvate, and 50 U/ml penicillin/streptomycin (all reagents from PAA Laboratories, Pasching, Austria; Invitrogen, Burlington, ON, Canada; or Sigma-Aldrich, St. Louis, MO).

### *Bone marrow isolation, differentiation, and polarization to M1 and M2 macrophages*

Mouse bone marrow cells were flushed from the femur and differentiated into bone marrow-derived macrophages (BMDM) in DMEM media with 20% FCS and 2.5–7% L929 cell conditioning media as a source of M-CSF, as described previously (33). BMDM were then cultured in BMDM media alone [DMEM or RPMI 1640 containing L929 cell conditioning media as described previously (33)] or in BMDM media with 100 ng/ml *Escherichia coli* 0111:B4 LPS (Invivogen, San Diego, CA) and 50 ng/ml mouse rIFN- $\gamma$  (R&D Systems, Minneapolis, MN) to generate M1-BMDM (polarizing M1-BMDM media) or with BMDM media containing 10 ng/ml mouse rIL-4 (R&D Systems) to generate alternatively activated M2-BMDM. The BMDM were incubated for 0.5–20 h (as specified in the experiment) in the presence or absence of 20  $\mu$ g/ml cationic human peptide LL-37. Alternatively, BMDM were stimulated with the TLR2 agonists: 100 ng/ml purified LTA from *Staphylococcus aureus* (Invivogen) or 1  $\mu$ g/ml synthetic tripalmitoylated lipopeptide (Pam3CSK4; Invivogen) for 20 h in BMDM media in the presence or absence of 20  $\mu$ g/ml LL-37. The LL-37 peptide (LLGDFFRKSKEKIGKEFKRIVQRIKDFRRNLYPRTES) was synthesized using F-moc chemistry at the Nucleic Acid/Protein Synthesis Unit, UBC. The peptide was resuspended in endotoxin-free water and deemed free of endotoxin contamination, as batches of the peptide were unable to stimulate TNF- $\alpha$  production by myeloid cells. BMDM incubated for 24 h in the absence or presence of 20  $\mu$ g/ml LL-37 displayed comparable viability (90  $\pm$  3% and 91  $\pm$  2%, respectively, determined by lack of propidium iodide uptake), indicating that the peptide was not cytotoxic to BMDM (data not shown).

Polarized M2-BMDM were also washed and resuspended in fresh media and incubated for a further 24 h in the absence or presence of either 100 ng/ml LPS, 20  $\mu$ g/ml LL-37, or a combination of LPS and LL-37. BMDM were also stimulated for 30 min with 100 ng/ml LPS and 50 ng/ml IFN- $\gamma$ , then washed and further cultured in the presence or absence of 20  $\mu$ g/ml LL-37 for 24 h.

### *Alveolar and peritoneal cell isolation and in vitro stimulation*

Alveolar cells were harvested from bronchoalveolar lavage (BAL) that was collected after catheterization of the trachea and washing three times, each with 1 ml PBS. Peritoneal cells were harvested by peritoneal lavage with 5 ml PBS. BAL and peritoneal lavage cells were resuspended and cultured in DMEM/10% FCS. Alveolar cells, 5  $\times$  10<sup>4</sup>/well, and peritoneal cells, 1  $\times$  10<sup>5</sup>/well, were cultured in a 96-well plate (Corning Costar, Corning, NY) in a final volume of 200  $\mu$ l. Cells from each mouse were unstimulated or stimulated with 100 ng/ml LPS (catalog No. L4391; Sigma-Aldrich), 20  $\mu$ g/ml LL-37, or a combination of LPS and LL-37 for 2 h.

### *Detection of TNF- $\alpha$ and NO*

Following M1 or M2 polarization of BMDM for 20 h in the presence or absence of LL-37 or in vitro stimulation of peritoneal and alveolar macrophages with LPS for 2 h, the tissue culture supernatants (TCS) or the BAL from in vivo stimulations were centrifuged and analyzed for TNF- $\alpha$  or NO. TNF- $\alpha$  was measured by a standard sandwich ELISA according to the manufacturer's directions (BD Biosciences, Franklin Lakes, NJ, or eBioscience, San Diego, CA). NO levels were determined by mixing an equal volume of TCS with Greiss reagent (Sigma-Aldrich) and comparing the OD at 540 nm against a sodium nitrite standard curve. OD was measured for both assays in either a PerkinElmer LS 55 (PerkinElmer, Waltham, MA) or a SpectraMax 190 (SpectraMax, Sunnyvale, CA) plate reader.

### *Quantitative real-time PCR*

RNA isolation was performed as described previously (27). Briefly, RNA was isolated from cell lysates using the Qiagen RNA Isolation Kit (RNeasyMini Kit; Qiagen, Valencia, CA), treated with RNase free DNase (Qiagen), and eluted in RNase-free water (Ambion, Austin, TX) as per the manufacturer's instructions. The RNA concentration was obtained using a NanoDrop spectrophotometer (Thermo Scientific, Wilmington, DE). Transcriptional changes were then identified by quantitative real-time PCR (QRT-PCR), which was performed using the SuperScript III Platinum Two-Step QRT-PCR kit with SYBR Green (Invitrogen) as per the manufacturer's instructions, and the ABI Prism 7000 sequence detection system (Applied Biosystems, Carlsbad, CA). Briefly, 500  $\mu$ g total RNA was re-

verse transcribed using qScript cDNA Synthesis Kit (Quanta Biosciences, Gaithersburg, MD). QRT-PCR was conducted in a 12.5  $\mu$ l reaction volume containing 2.5  $\mu$ l one fifth-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 normalizing the change in expression of the gene of interest to the housekeeping gene GAPDH, using the threshold cycle values. The primers sequences (Invitrogen) used for QRT-PCR are as follows: TNF- $\alpha$  forward, 5'-TCTTCTCATTCCTGCTTGTGG-3', TNF- $\alpha$  reverse, 5'-GAGGCCATTTGGGAACCTTCT-3'; inducible NO synthase 2 (iNOS2) forward, 5'-CTTTGCCACGGACGAGAC-3', iNOS2 reverse, 5'-TCATTGTACTCTGAGGGCTGAC-3'; and GAPDH forward, 5'-AGGTCGGTG TGAACGGATTG-3' and GAPDH reverse, 5'-TGTAGACCATGTAGTTGAGGTCA-3'.

#### Measurement of intracellular TNF- $\alpha$ in alveolar or peritoneal macrophages

Peritoneal cells were stimulated in vitro for 2 h as described above in the presence of 10  $\mu$ g/ml brefeldin A (Sigma-Aldrich) to suppress secretion and allow for the accumulation of intracellular TNF- $\alpha$ . Alveolar cells after in vitro or in vivo treatment (see below) did not require brefeldin A treatment to detect intracellular TNF- $\alpha$ . Alveolar ( $5 \times 10^4$ ) or peritoneal cells ( $1 \times 10^5$ ) were incubated with an Fc receptor blocking mAb, 2.4G2, for 20 min on ice, then labeled with FITC-conjugated anti-CD11c (0.5  $\mu$ g/ml; eBioscience) or PE-cyanine 7 (Cy7)-conjugated anti-F4/80 mAb (1  $\mu$ g/ml; eBioscience) diluted in PBS/2% BSA/2 mM EDTA for 20 min on ice. Cells were fixed with 1% paraformaldehyde (Canemco and Marivac, Quebec, Canada) and then permeabilized with 0.1% saponin (Calbiochem, La Jolla, CA) for 10 min. Intracellular labeling was performed by incubating with PE-conjugated anti-TNF- $\alpha$  mAb (0.5  $\mu$ g/ml; eBioscience) or PE-conjugated IgG1 isotype control Ab (0.5  $\mu$ g/ml; eBioscience) diluted in 0.1% saponin buffer for 45 min. The cells were washed and analyzed by flow cytometry (10,000 events on the LSRII; BD Biosciences) and the data analyzed using FlowJo (Tree Star, Ashland, OR) software. Intracellular levels of TNF- $\alpha$  were determined on F4/80<sup>+</sup> peritoneal macrophages and CD11c<sup>+</sup> alveolar macrophages. Typically, 50% of peritoneal cells were F4/80<sup>+</sup> macrophages (the other major cell population was B cells). In the BAL, 80–90% of the cells were CD11c<sup>+</sup> alveolar macrophages.

#### Arginase activity measured by urea production

Following polarization, M1 and M2 macrophages were lysed at  $5 \times 10^5$  cells/ml in a solution of 20 mM Tris (pH 7.5), 150 mM NaCl, 2mM EDTA, and 0.1% Triton X-100—containing 1 $\times$  protease inhibitor mixture (Pefabloc; Roche) for 30 min at room temperature. Lysates (100  $\mu$ l) were mixed with an equal volume of 10 mM Tris-HCL (pH 7.5) and one-tenth volume of 10 mM MgCl<sub>2</sub>, heated at 56°C for 10 min, then incubated with 100  $\mu$ l 0.5 M L-arginine at 37°C for 1 h. A total of 800  $\mu$ l acid solution (7:3:1, H<sub>2</sub>O/H<sub>3</sub>PO<sub>4</sub>/H<sub>2</sub>SO<sub>4</sub>) and 40  $\mu$ l 9%  $\alpha$ -isotonitrosopropiophenone (in 100% EtOH) were added to the samples as well as a dilution series of urea for the standards. Samples and standards were heated at 95°C for 30 min, placed in the dark (10 min), and transferred (200  $\mu$ l) to a 96-well flat-bottom plate, and the OD was read at 540 nm in a PerkinElmer LS 55 plate reader. All chemicals were purchased from Sigma-Aldrich.

#### Measurement of ROS

ROS was measured as described (34) using a luminal-ECL system with a Mithras LB940 plate reader (Berthold Technologies). In brief, BMDM, M1, and M2-BMDM polarized in the presence or absence of LL-37 were washed into Krebs-Ringer phosphate buffer (pH 7.3; KRG) at  $2 \times 10^6$  cells/ml. A minimum of  $2 \times 10^5$  BMDM were combined with 4 U/ml HRP (Boehringer-Mannheim, Ingelheim am Rhein, Germany) and 50  $\mu$ M luminol (Sigma-Aldrich) in 200  $\mu$ l/well of a 96-well, flat-bottom chemiluminescence plate. Cells were brought to 37°C, and light emission (ROS production) per 0.1 s was recorded every 5 s for a minimum of 200 readings.

#### Tumoricidal activity of BMDM

BMDM were polarized to M1 or M2-BMDM in 24-well TC plates ( $8 \times 10^4$  cells/well) in the absence or presence of LL-37 for 20 h, after which the media was replaced with 0.5 ml BMDM-polarizing media containing  $4 \times 10^5$  murine T-lymphoblast EL4 cells (European Cell Culture Collection No. 85023105) and incubated for a further 24 h. EL4 cells were then removed and counterstained with a one-fiftieth dilution of PE-Cy7-labeled anti-F4/80 Ab (eBioscience) for 1 h on ice. Samples were washed into 300  $\mu$ l ice-cold PBS, and EL4 cells were counted (excluding contaminating F4/80<sup>+</sup> BMDM) using an FACSCalibur flow cytometer (BD Biosciences) and

CellQuest software (BD Biosciences). To check for potential cytotoxic effects of LL-37 on EL4 cell growth, EL4 cells were cultured in polarizing media in the absence and presence of 20  $\mu$ g/ml LL-37 for 24 h.

#### Phagocytosis of human apoptotic neutrophils

Human neutrophils were isolated from 1-d-old buffy coats, carboxy-fluorescein diacetate (CFDA)-labeled according to the manufacturer's instructions (Invitrogen), and incubated in the presence of anti-CD95 Ab (Fas ligand; BD Biosciences) to induce apoptosis as described previously (33). Phagocytosis of apoptotic neutrophils was performed as described previously (33). Briefly, BMDM were polarized on 24-well TC plates, then incubated with CFDA-labeled neutrophils at a 1:4 (macrophage/neutrophil) ratio at 37°C for 2 h in 0.5 ml RPMI 1640/2% heat-inactivated FCS. Cytochalasin B (10  $\mu$ g/ml; Sigma-Aldrich) was used as a control to prevent phagocytosis. Apoptotic neutrophils in suspension (not phagocytosed) were discarded, and adherent macrophages were detached using Versene, fixed in ice-cold PBS/2% paraformaldehyde, and labeled for 1 h with PE-Cy7-anti-F4/80 Ab. A total of 10,000 events per sample were collected using an FACSCalibur flow cytometer (BD Biosciences), and the percentage of F4/80<sup>+</sup>CFDA<sup>+</sup> events (macrophages with phagocytosed neutrophils) was determined using CellQuest software (BD Biosciences).

#### Killing of bacteria

The concentration of *E. coli* MG1655 cultured overnight in Luria broth at 37°C was determined by OD at 600 nm. Bacteria were diluted in PBS to a final concentration of  $2 \times 10^8$ /ml. BMDM were polarized in 24-well TC plates in media without antibiotics ( $1 \times 10^5$  BMDM/well), washed into 0.45 ml RPMI 1640/2% heat-inactivated FCS, and incubated with 50  $\mu$ l bacteria ( $2 \times 10^7$ ) at 37°C for 2 h (1:50 ratio of effector macrophages/target bacteria). The supernatant, containing free bacteria, was transferred, and macrophages were lysed with 0.5 ml water. Supernatant and lysates were serially diluted in PBS, spread on Luria broth-agar plates, incubated overnight at 37°C, and the colonies enumerated.

#### In vivo LPS-induced lung inflammation

Anesthetized mice were injected by intratracheal instillation with 50  $\mu$ l PBS, 50  $\mu$ l PBS containing 100 ng LPS (catalog No. L4391; Sigma-Aldrich), or 60  $\mu$ l PBS containing 100 ng LPS and 20  $\mu$ g LL-37. After 1 h, mice were euthanized by isoflurane overdose. The supernatant from the first 1 ml BAL was assayed for TNF- $\alpha$  by ELISA. The cells pooled from 3 ml BAL were used for intracellular TNF- $\alpha$  analysis by flow cytometry.

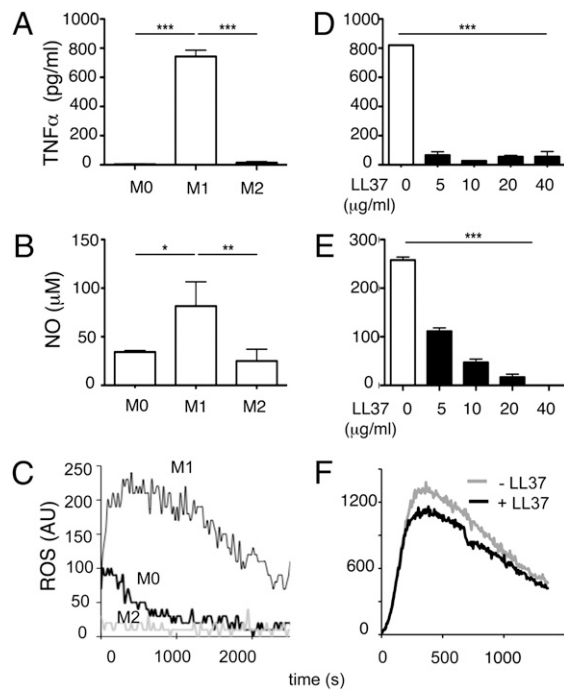
#### Statistical significance

Statistical significance was calculated by one-way ANOVA with post hoc Tukey's analysis or calculated by a two-tailed paired Student *t* test for experiments with BMDM. For experiments in vitro with alveolar and peritoneal macrophages, statistical significance was calculated by a two-tailed paired Student *t* test, whereas statistical significance for in vivo data obtained from individual mice was calculated by a two-tailed independent Student *t* test.

## Results

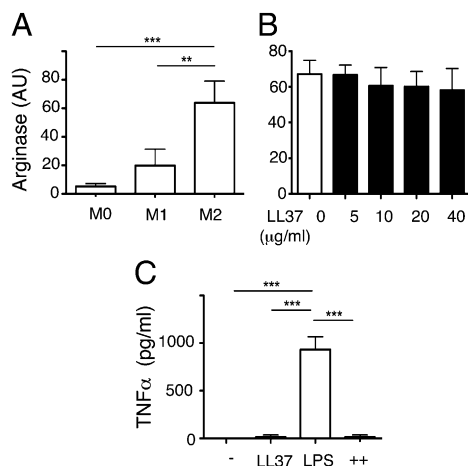
### LL-37 inhibited the production of proinflammatory mediators TNF- $\alpha$ and NO by M1-polarized BMDM

Proinflammatory M1-BMDM were induced by stimulation with LPS and IFN- $\gamma$ , and alternatively activated M2-BMDM were induced with IL-4. The M1-BMDM produced TNF- $\alpha$ , NO, and ROS in response to LPS and IFN- $\gamma$ , whereas nonpolarized (M0) and M2-polarized BMDM produced significantly lower levels or no TNF- $\alpha$ , ROS, and NO (Fig. 1A–C). The addition of as little as 5  $\mu$ g/ml LL-37 during the polarization of M1 macrophages by LPS and IFN- $\gamma$  resulted in a severe blockage of TNF- $\alpha$  production with >90% inhibition (Fig. 1D). There was a dose-dependent inhibition of NO production, but this was less sensitive to low concentrations of LL-37, as 5  $\mu$ g/ml LL-37 reduced NO production by  $57 \pm 3\%$  ( $n = 4$ ). However, NO production was significantly inhibited by  $86 \pm 3\%$  ( $n = 5$ ) with 20  $\mu$ g/ml LL-37 (Fig. 1E). LL-37 had a much lesser effect on ROS production, although it still significantly reduced peak production by  $23 \pm 7\%$  ( $n = 4$ ) (Fig. 1F).



**FIGURE 1.** TNF- $\alpha$ , NO, and ROS production by M1-BMDM. TNF- $\alpha$  (A), NO (B), and ROS (C) (y-axis) produced by nonpolarized (M0) or M1- and M2-BMDM polarized for 20 h. Macrophages were polarized to the M1 phenotype in the absence (white bars) and presence (black bars) of 5–40  $\mu$ g/ml LL-37 (x-axis), following which TNF- $\alpha$  (D) and NO (E) were measured in the TCS (y-axis). F, ROS production was measured as light units (arbitrary units [AU]; y-axis) detected over time (s; x-axis) in M1 macrophages polarized in the absence (gray line) or presence (black line) of 20  $\mu$ g/ml LL-37. The results are representative of five (A, B; average  $\pm$  SD), two (D, E; average  $\pm$  SD), and four (C, F) independent experiments. \* $p$  < 0.05, \*\* $p$  < 0.01, \*\*\* $p$  < 0.001.

The polarization of alternatively activated M2 macrophages was confirmed by an increase in arginase activity compared with nonpolarized and M1-polarized BMDM (Fig. 2A) and a lack of



**FIGURE 2.** M2 arginase activity. Relative arginase activity (arbitrary units [AU]) in M0-, M1-, and M2-BMDM (A) and M2-BMDM (B) polarized in the absence (white bars) and presence (black bars) of 5–40  $\mu$ g/ml LL-37. Results are the average  $\pm$  SD of three independent experiments. C, TNF- $\alpha$  in the TCS of M2-polarized BMDM following 24-h incubation in the absence (–) or presence of 100 ng/ml LPS, 20  $\mu$ g/ml LL-37, or LPS and LL-37 (++) . Results are the average  $\pm$  SD of two independent experiments. \*\* $p$  < 0.01, \*\*\* $p$  < 0.001.

TNF- $\alpha$ , NO, and ROS production (Fig. 1A–C). The addition of 5–40  $\mu$ g/ml LL-37 during M2 polarization did not significantly affect arginase activity (Fig. 2B). However, when polarized M2 macrophages were stimulated with LPS in the presence and absence of 20  $\mu$ g/ml LL-37, LL-37 effectively inhibited LPS-induced production of TNF- $\alpha$  (Fig. 2C).

Together, these data demonstrated that LL-37 dramatically inhibited the production of the LPS or LPS/IFN- $\gamma$ -induced proinflammatory mediator TNF- $\alpha$ . LL-37 also dramatically reduced NO production in M1-BMDM, but inhibited ROS production to a much lesser extent and did not affect arginase activity, a characteristic of M2-BMDM. This suggests that the host defense peptide LL-37 may selectively inhibit the proinflammatory responses of macrophages.

#### *The early and continued presence of LL-37 is required to inhibit TNF- $\alpha$ and NO production by M1-BMDM*

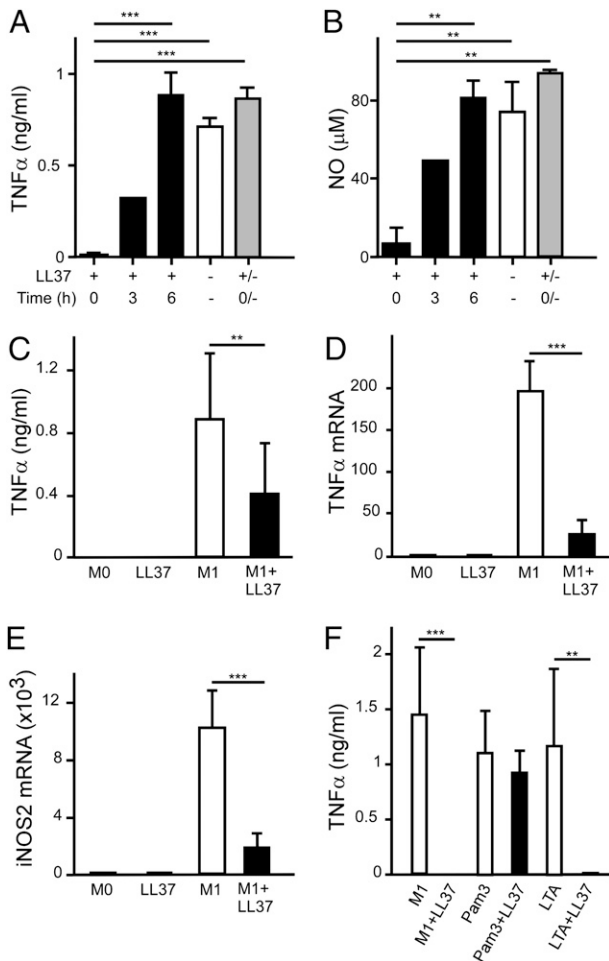
To determine at what stage LL-37 inhibited the production of TNF- $\alpha$  and NO, LL-37 was added concurrently with the polarizing stimuli (LPS and IFN- $\gamma$ ) or at various times afterward. The inhibitory effect of LL-37 on TNF- $\alpha$  and NO production was reduced by 30–55% if LL-37 was administered 3 h after M1 polarization, and no inhibition was seen if LL-37 was added 6 h after LPS and IFN- $\gamma$  (TNF- $\alpha$ , Fig. 3A; NO, Fig. 3B). BMDM were also polarized for 20 h in the presence of LL-37, then washed and incubated a subsequent 24 h in the absence of LL-37. Under these circumstances, TNF- $\alpha$  and NO produced by M1 macrophages were restored when LL-37 was removed from the media for the second 24-h period. These results demonstrated that the effect of LL-37 requires an early, continued presence to inhibit TNF- $\alpha$  and NO production by M1 macrophages.

This suggested that LL-37 may need to be added concurrently with LPS and IFN- $\gamma$  to prevent TNF- $\alpha$  production. To directly address this, BMDM were stimulated with LPS and IFN- $\gamma$  for 30 min, washed, and then incubated in the presence or absence of LL-37 for an additional 24 h. Under these conditions, LL-37 significantly inhibited TNF- $\alpha$  production by M1-BMDM (Fig. 3C), albeit to a lesser extent than when LL-37 was added concurrently. Thus, LL-37 does not have to be added concurrently with LPS and IFN- $\gamma$  to have a significant inhibitory effect, but when it is, it has a stronger inhibitory effect.

#### *LL-37 reduces the transcription of LPS-induced TNF- $\alpha$ and NO*

To determine at what level LL-37 was inhibiting TNF- $\alpha$  and NO production, we used QRT-PCR to determine if LL-37 was affecting the transcription of TNF- $\alpha$  and NO, as this had been shown to be a major mechanism in human monocytic cells for LPS-induced TNF- $\alpha$  expression (27). Transcripts of mRNA that encode TNF- $\alpha$  and iNOS2, the enzyme required to generate NO, were increased >200- and 10,000-fold, respectively, when compared with GAPDH after 4-h polarization of BMDM with LPS and IFN- $\gamma$ . The presence of LL-37 (20  $\mu$ g/ml) significantly blocked the transcription of TNF- $\alpha$  mRNA by 87% and iNOS2 mRNA by 81% (Fig. 3D, 3E). Thus, a major mechanism by which LL-37 reduces TNF- $\alpha$  and NO secretion in M1-BMDM is by preventing the induced transcription of these genes.

We also monitored the inhibitory effect of LL-37 on TNF- $\alpha$  secretion in the presence of cytochalasin D, which disrupts actin polymerization and prevents internalization and phagocytosis. BMDM were stimulated for 1 h in M1-polarizing conditions in the presence or absence of LL-37 and were treated with a range of concentrations of cytochalasin D (1–20  $\mu$ g/ml). At no concentration tested did cytochalasin D block the inhibitory effect of LL-



**FIGURE 3.** Inhibitory effect of LL-37 on M1-polarized BMDM. Levels of TNF- $\alpha$  (A) and NO (B) produced by M1-BMDM polarized for 20 h with LPS/IFN- $\gamma$  in the absence (white bars, -) or presence (black and gray bars, +) of 20  $\mu$ g/ml LL-37. LL-37 was added at 0, 3, or 6 h after the addition of LPS/IFN- $\gamma$  (black bars). BMDM were also polarized for 20 h with LPS/IFN- $\gamma$  in the presence of 20  $\mu$ g/ml LL-37, then washed and incubated a subsequent 24 h in the absence of both LL-37 and LPS/IFN- $\gamma$  (gray bars), after which TNF- $\alpha$  was measured. Results are the average  $\pm$  SD of two independent experiments. C, BMDM were incubated in BMDM media (M0) or stimulated for 0.5 h with M1-polarizing media (100 ng/ml LPS and 50 ng/ml IFN- $\gamma$ ), then washed and incubated for 24 h in BMDM media alone (white bars) or with (black bars) 20  $\mu$ g/ml LL-37. The graph shows the average level of TNF- $\alpha$   $\pm$  SD in the TCS of BMDM from four mice over two independent experiments. D and E, Quantitative PCR analysis of the relative quantity of TNF- $\alpha$  and iNOS2 mRNA transcribed in BMDM after 4 h incubation with M1-polarizing media (M1) or BMDM media alone (M0) in the absence (white bars) or presence (black bars) of 20  $\mu$ g/ml LL-37 compared with the housekeeping gene GAPDH. The graphs show the average relative mRNA expression  $\pm$  SD of BMDM from six mice over two independent experiments. F, TNF- $\alpha$  produced by BMDM following 24 h incubation with M1-polarizing media, 1  $\mu$ g/ml Pam3CSK4, or 100 ng/ml LTA in the absence (white bars) or presence (black bars) of 20  $\mu$ g/ml LL-37. The graph shows the average TNF- $\alpha$   $\pm$  SD produced by BMDM from five to six mice over three independent experiments. \*\* $p$  < 0.01, \*\*\* $p$  < 0.001.

37 on TNF- $\alpha$  secretion, indicating that LL-37 did not require an intact actin cytoskeleton to mediate its inhibitory effect on TNF- $\alpha$  production (data not shown).

*LL-37 inhibits LTA- but not Pam3CSK4-induced TNF- $\alpha$*

To determine if LL-37 could inhibit proinflammatory cytokine production induced by other inflammatory stimuli, BMDM were

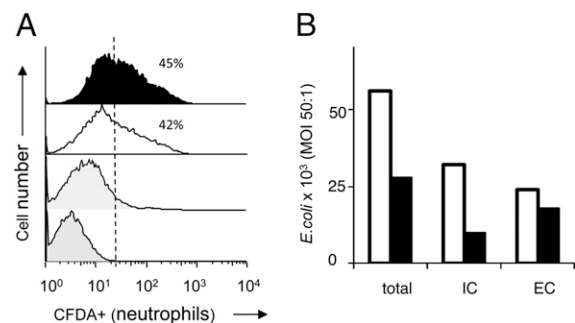
stimulated with the TLR2 agonists Pam3CSK4 or LTA in the absence and presence of LL-37 (20  $\mu$ g/ml). LTA shares some overall similarities with LPS, as it is amphiphilic, having both anionic and hydrophobic regions, and binds LPS binding proteins (LBP and CD14). LTA binds TLR2/6, whereas Pam3CSK4 binds to TLR2/1. LL-37 had no significant effect on the production of TNF- $\alpha$  when BMDM were stimulated by Pam3CSK4 but dramatically inhibited LTA induced TNF- $\alpha$  production (Fig. 3F).

*LL-37 did not inhibit M1- or M2-BMDM-mediated phagocytosis*

Resolving inflammation requires more than just the removal of proinflammatory cytokines; phagocytosis of bacteria and apoptotic neutrophils by macrophages are also important events in the resolution of an inflammatory response. To determine if LL-37 affected the phagocytosis of apoptotic neutrophils, an in vitro phagocytosis assay was performed. M2-macrophages phagocytosed apoptotic neutrophils within a 2-h time period, and no significant difference was observed with M2 macrophages polarized in the presence of LL-37 (Fig. 4A). We next determined if LL-37 reduced the killing or uptake of *E. coli* by M1-polarized macrophages and found that the LL-37-treated M1-BMDM were more efficient at killing *E. coli* bacteria over a 2-h period (Fig. 4B). Although this increase was not significant when averaged over multiple experiments, it demonstrates that the LL-37-treated macrophages were not defective in the clearance of bacteria by M1-BMDM. Therefore, LL-37 inhibits the production of the proinflammatory cytokine TNF- $\alpha$  in M1- and M2-BMDM without affecting other crucial macrophage functions such as the clearance of bacteria and dying cells.

*LL-37 enhanced the tumoricidal activity of M1-BMDM*

Another characteristic of M1-BMDM, but not M2-BMDM, is the ability to inhibit tumor growth (9). We thus evaluated the ability of LL-37 to modulate this function. In accordance with previous reports, M2-BMDM did not affect tumor cell growth, whereas M1-BMDM suppressed it. M1-BMDM reduced the number of



**FIGURE 4.** Phagocytosis of apoptotic neutrophils and bacterial killing. A, Flow cytometry of F4/80<sup>+</sup> M2-BMDM. BMDM were polarized to an M2 phenotype in the absence (white) or presence (black) of 20  $\mu$ g/ml LL-37, washed, and incubated a subsequent 2 h with CFDA-labeled apoptotic neutrophils. M2-BMDM were also incubated in the absence of neutrophils (gray, bottom panel) and in the presence of neutrophils and cytochalasin B to prevent phagocytosis (gray, third panel down). F4/80<sup>+</sup> BMDM that phagocytosed neutrophils are CFDA<sup>+</sup> (x-axis). Results are a representative example of five to six independent experiments. B, BMDM polarized to an M1 phenotype in the absence (white bars) and presence (black bars) of 20  $\mu$ g/ml LL-37 were washed and incubated with *E. coli* at a ratio of 50:1 *E. coli*/BMDM. After 2 h, viable *E. coli* remaining associated with M1-BMDM (IC) or in the supernatant (EC) or both (total) were enumerated. The figure is one representative of three independent experiments.

EL4 tumor cells in culture, and macrophage polarization in the presence of LL-37 further enhanced their ability to suppress EL4 cell growth (Fig. 5). M2-BMDM had very little effect on the growth of EL4 tumor cells, and the effect of LL-37-treated M2-BMDM was not significantly different from that of M2-BMDM. These data demonstrated that, in contrast to the inhibitory effect of LL-37 on TNF- $\alpha$  and NO production, macrophage polarization in the presence of LL-37 significantly enhanced the ability of M1-BMDM to inhibit tumor cell growth.

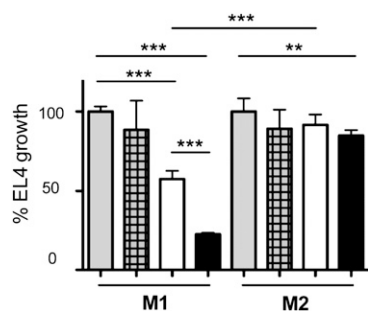
#### LL-37 reduced TNF- $\alpha$ production by in vitro-stimulated peritoneal macrophages

To determine whether LL-37 had similar effects on in vivo-derived macrophages, resident peritoneal cells were isolated from C57BL/6/J mice, plated, and stimulated in vitro with 100 ng/ml LPS in the presence or absence of 20  $\mu$ g/ml LL-37; ~50% of the isolated peritoneal cells were F4/80<sup>+</sup> macrophages (data not shown). Supernatants were collected after 2 h and analyzed by ELISA. LL-37 consistently reduced LPS-induced TNF- $\alpha$  secretion by ~40% (Fig. 6A, 6B), but this was not as dramatic as observed with M1-BMDM, in which >90% inhibition was observed after 20-h stimulation with LPS and IFN- $\gamma$ .

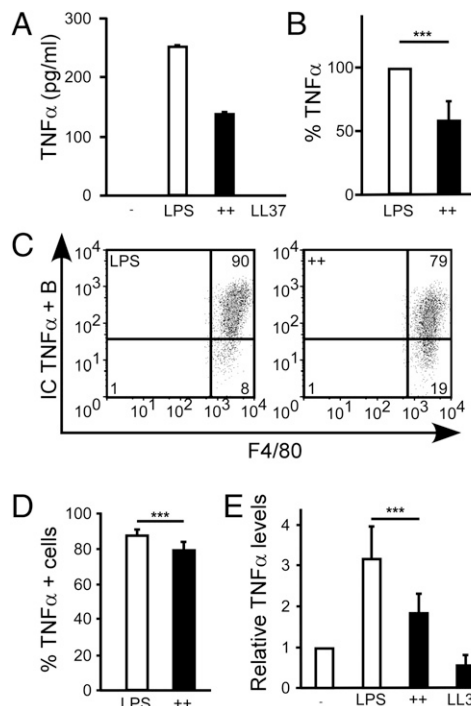
To specifically examine TNF- $\alpha$  production by the resident F4/80<sup>+</sup> peritoneal macrophages, peritoneal cells were plated in the presence of brefeldin A (to prevent secretion and capture intracellular TNF- $\alpha$ ) and cultured with LPS or LPS and LL-37 for 2 h. The cells were labeled with an F4/80 mAb, and intracellular TNF- $\alpha$  produced by F4/80<sup>+</sup> macrophages was determined by flow cytometry. Only the F4/80<sup>+</sup> macrophages produced TNF- $\alpha$  (data not shown). LL-37 caused a small but significant reduction of 8% in the percent of F4/80<sup>+</sup> peritoneal macrophages that produced TNF- $\alpha$  (Fig. 6C, 6D). In addition, LL-37 reduced the levels of LPS-induced intracellular TNF- $\alpha$  by ~40% (Fig. 6C, 6E). Thus, LL-37 modestly reduced the number of TNF- $\alpha$ -producing F4/80<sup>+</sup> peritoneal macrophages and the amount of LPS-induced TNF- $\alpha$  secreted by resident peritoneal F4/80<sup>+</sup> macrophages over a 2-h activation period.

#### LL-37 reduced TNF- $\alpha$ production by in vitro-stimulated alveolar macrophages

Alveolar macrophages are CD11c<sup>+</sup> and constitute the majority (~90%) of cells in the BAL of C57BL/6/J mice. In contrast to the

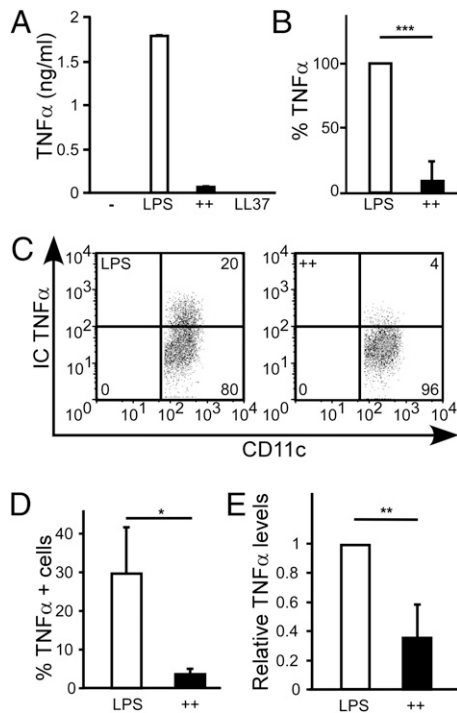


**FIGURE 5.** M1 tumoricidal activity. M1- and M2-BMDM were polarized in the absence (white bars) or presence (black bars) of 20  $\mu$ g/ml LL-37 for 20 h, then the media was replaced with EL4 murine T lymphocytes (at a ratio of 1:5 macrophages/EL4 cells) in polarization media and incubated for a further 24 h. The percent of EL4 cell growth after the 24-h period was calculated as a percent of EL4 cell growth in polarizing media alone (no macrophages, gray bars). The hatched bars represent the percent of EL4 cell growth in polarizing media plus 20  $\mu$ g/ml LL-37. The graph shows the average percent growth  $\pm$  SD from two to three independent experiments. \*\* $p$  < 0.01, \*\*\* $p$  < 0.001.



**FIGURE 6.** Slight inhibitory effect of LL-37 on LPS-induced TNF- $\alpha$  production in peritoneal macrophages ex vivo. Cells were isolated from the peritoneum of C57BL/6/J mice, and  $1 \times 10^5$  cells, 50% of which were F4/80<sup>+</sup> peritoneal macrophages, were unstimulated (-) or stimulated with 100 ng/ml LPS, 20  $\mu$ g/ml LL-37, or LPS and LL-37 (++) in vitro for 2 h, then TNF- $\alpha$  levels were measured in the TCS by ELISA or in F4/80<sup>+</sup> macrophages by flow cytometry. **A**, Secreted TNF- $\alpha$  levels from peritoneal macrophages from one representative mouse. **B**, The average percent of TNF- $\alpha$  produced in the presence of LL-37 (++) relative to LPS stimulation alone, which was set to 100%. **C**, Representative flow cytometry dot plots showing the percentage of intracellular TNF- $\alpha$  (y-axis) produced in the presence of brefeldin A (IC TNF $\alpha$  + B) from LPS or LPS and LL-37-treated (++) F4/80<sup>+</sup> peritoneal macrophages (x-axis). **D**, The average percent of intracellular TNF- $\alpha$ -positive F4/80<sup>+</sup> macrophages. **E**, Relative intracellular TNF- $\alpha$  levels between unstimulated, LPS-stimulated, LPS and LL-37-treated (++) , and LL-37-treated cells. The intracellular levels of TNF- $\alpha$  in the absence of any stimuli were normalized to 1. The ELISA data were the average from seven mice over three experiments, and the flow cytometry data were from five mice over two experiments. Error bars represent  $\pm$  SD. \*\*\* $p$  < 0.001.

mild effect of LL-37 on resident peritoneal macrophages, there was a striking inhibition of TNF- $\alpha$  secretion by ex vivo alveolar macrophages stimulated for 2 h with LPS in the presence of LL-37. Fig. 7A shows TNF- $\alpha$  secreted from one representative mouse, and Fig. 7B shows the average percent inhibition of TNF- $\alpha$  (90  $\pm$  14%) by LL-37 from eight mice. This degree of inhibition on TNF- $\alpha$  production was comparable to that exerted by LL-37 on M1-BMDM and LPS-treated M2-BMDM after 20 h of stimulation. Determination of the intracellular levels of TNF- $\alpha$  in CD11c<sup>+</sup> alveolar macrophages by flow cytometry also indicated a reduction of >85% in the percent of cells that produced TNF- $\alpha$  (Fig. 7C, 7D), and only CD11c<sup>+</sup> macrophages made TNF- $\alpha$  (data not shown). The level of intracellular TNF- $\alpha$  produced by these cells was also reduced by 66% by LL-37 (Fig. 7E). Because alveolar macrophages generated nanogram amounts of TNF- $\alpha$ , the addition of brefeldin A was not necessary to detect intracellular TNF- $\alpha$  levels in these alveolar macrophages. These in vitro-stimulated alveolar macrophages produced >6-fold more TNF- $\alpha$  in a 2-h period than similarly stimulated peritoneal macrophages, yet LL-37 was much more effective at inhibiting alveolar mac-

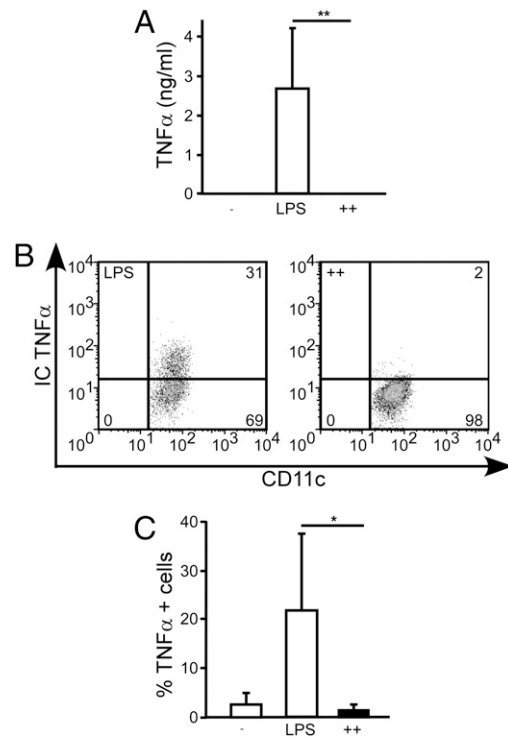


**FIGURE 7.** Inhibitory effect of LL-37 on LPS-induced TNF- $\alpha$  production in alveolar macrophages ex vivo. Alveolar cells were isolated from the BAL of C57BL/6J mice and  $5 \times 10^4$  cells incubated in vitro for 2 h in the absence of stimulation (-) or stimulated with 100 ng/ml LPS, 20  $\mu$ g/ml LL-37, or LPS and LL-37 (++). TNF- $\alpha$  was measured in the TCS by ELISA or in CD11c<sup>+</sup> alveolar macrophages by flow cytometry. *A*, TNF- $\alpha$  levels from alveolar macrophages from one representative mouse. *B*, The average percent of TNF- $\alpha$  produced in the presence of LL-37 (++) relative to LPS stimulation alone, which was set to 100%. *C*, Representative flow cytometry dot plots showing the percent of intracellular TNF- $\alpha$  (y-axis) produced in the absence of brefeldin A (IC TNF $\alpha$ ) in CD11c<sup>+</sup> alveolar macrophages (x-axis). *D*, The average percent of intracellular TNF- $\alpha$ -positive CD11c<sup>+</sup> macrophages. *E*, Relative intracellular TNF- $\alpha$  levels between LPS-stimulated and LL-37-treated cells in which the mean fluorescence intensity of IC TNF- $\alpha$  from LPS-stimulated cells was normalized to 1. The ELISA data were from eight mice over three experiments, and the flow cytometry data were from five mice over two experiments. Error bars represent  $\pm$  SD. \* $p$  < 0.05, \*\* $p$  < 0.01, \*\*\* $p$  < 0.001.

rophage TNF- $\alpha$  production, assessed by the percent of cells producing intracellular TNF- $\alpha$  (85 versus 8% reduction) and by the amount secreted (90 versus 40% reduction). Thus, LL-37 significantly reduced TNF- $\alpha$  production by LPS-stimulated alveolar macrophages in vitro.

*LL-37 inhibited LPS-induced TNF- $\alpha$  production in alveolar macrophages in vivo*

To determine if LL-37 also reduced TNF- $\alpha$  production of LPS-stimulated alveolar macrophages in vivo, LPS or LPS and LL-37 were administered by intratracheal instillation into the lungs of C57BL/6J mice. After 1 h, cells and supernatants were collected from the BAL and assayed for intracellular TNF- $\alpha$  by flow cytometry and TNF- $\alpha$  secretion by ELISA. LL-37 dramatically inhibited (by >90%) LPS-induced TNF- $\alpha$  secretion in the BAL (Fig. 8A). Only CD11c<sup>+</sup> alveolar macrophages made TNF- $\alpha$  (data not shown), and examination of the effect of LL-37 on the intracellular levels of TNF- $\alpha$  in the absence of brefeldin showed that LL-37 significantly reduced, by >90%, the percentage of CD11c<sup>+</sup> alveolar macrophages with intracellular TNF- $\alpha$  (Fig. 8B, 8C).



**FIGURE 8.** Inhibitory effect of LL-37 on LPS-induced TNF- $\alpha$  production in alveolar macrophages in vivo. C57BL/6J mice were treated with PBS (-), 100 ng LPS, or LPS and 20  $\mu$ g LL-37 (++) by intratracheal instillation for 1 h. *A*, The average amount of TNF- $\alpha$  present in the BAL from seven mice, measured by ELISA. *B*, Cells collected from the BAL were directly analyzed for intracellular (IC) TNF- $\alpha$  by flow cytometry. The percent of intracellular TNF- $\alpha$ <sup>+</sup> CD11c<sup>+</sup> alveolar macrophages is shown from a representative experiment. *C*, The average percent of TNF- $\alpha$ <sup>+</sup> alveolar macrophages from unstimulated (-), LPS-stimulated, or LPS- and LL-37 (++)-treated mice. ELISA and flow cytometry data were from seven mice over three experiments. Error bars represent  $\pm$  SD. \* $p$  < 0.05, \*\* $p$  < 0.01.

This demonstrated that LL-37 significantly inhibited LPS-induced TNF- $\alpha$  production by alveolar macrophages in vivo.

**Discussion**

Inflammatory and healing macrophages play key roles in the induction and resolution of inflammation and are a key target population for treating common inflammatory disorders such as inflammatory bowel disease, rheumatoid arthritis, and atherosclerosis. The possibility of dampening inflammation and enhancing resolution by regulating macrophage functions is an attractive one. LL-37 has been a template for the development of synthetic innate defense regulator peptides that are effective at controlling infection and inflammation and promote macrophage recruitment to sites of inflammation in vivo (35, 36).

In this study, we examined the effect of the host defense peptide LL-37 on M1- and M2-polarized macrophages and on primary alveolar and peritoneal macrophages in vitro and in vivo. We found that LL-37 dramatically inhibited the production of the proinflammatory cytokine TNF- $\alpha$  and NO in LPS/IFN- $\gamma$ -stimulated M1-BMDM and inhibited TNF- $\alpha$  production after LTA stimulation of BMDM, consistent with experiments on human peripheral blood monocytes and macrophage cell lines (26, 27, 37). LL-37 also had a strong suppressive effect on TNF- $\alpha$  production by LPS-stimulated alveolar macrophages both in vitro and in vivo after only 1 to 2 h stimulation, but had a much milder inhibition on



resident peritoneal macrophages, suggesting that the effect of LL-37 can differ with the type of tissue macrophage examined. The potent inhibitory effect of LL-37 was restricted to specific functions of classically activated (LPS or LPS/IFN- $\gamma$ ) macrophages, namely TNF- $\alpha$  and NO production, as it did not affect the up-regulation of arginase activity occurring in alternatively activated macrophages and did not affect the ability of M1-BMDM to phagocytose and kill bacteria or the ability of M2-BMDM to phagocytose apoptotic neutrophils, events that are imperative for wound healing and inflammatory resolution. Furthermore, LL-37 had only a slight reducing effect on ROS production and enhanced the ability of M1-BMDM to suppress tumor growth. Thus, LL-37 selectively inhibited proinflammatory cytokine and NO secretion without affecting other important macrophage functions.

The selective nature of LL-37, inhibiting TNF- $\alpha$  and NO production by M1-BMDM but not other M1- or M2-BMDM functions, is consistent with data from the human monocytic cell line THP-1, showing that LL-37 affects a subset of LPS-induced genes (27). Both these data from mouse BMDM and data from human THP-1 cells support the idea that some signaling does occur in LL-37-treated M1-BMDM that enables them to produce ROS and kill tumor cells and enables human THP-1 cells to produce chemokines (27). The fact that maximal LL-37 inhibition of LPS induced TNF- $\alpha$  production was obtained when LL-37 was added simultaneously with LPS suggests that LL-37 must act early in the LPS activation pathway. Indeed, data from human THP-1 monocytes have shown that LL-37 reduces the LPS-induced nuclear translocation of NF- $\kappa$ B (p50/p65) and substantially inhibits TNF- $\alpha$  production by blocking its transcription (27). In this study, we also find that LL-37 substantially prevents the transcription of both TNF- $\alpha$  and NO in M1-BMDM, indicating that a major effect of LL-37 is to prevent LPS-induced activation of these genes. It remains to be established exactly how LL-37 functions to regulate this pathway, but it has been shown that LL-37 can bind to LPS and prevent its interaction with LBP, thereby reducing its ability to stimulate macrophages (26, 38, 39). Thus, one mode of action of LL-37 may be to neutralize LPS. Consistent with this, LL-37 also inhibited TNF- $\alpha$  production induced by the TLR-2 agonist LTA (an amphiphilic molecule similar to LPS that also binds to LBP) but not by another TLR-2 agonist, Pam3CSK4, that binds directly to the TLR1/2 complex. However, this is unlikely to be the only mode of action, as LL-37 still had a significant inhibitory effect when added 30 min after LPS and IFN- $\gamma$  had been added and washed away. In addition, the differential inhibitory effect of LL-37 on ex vivo peritoneal and alveolar macrophages is not easily explained by LPS neutralization.

In another study with human M1 and M2 macrophages, LL-37 was added concurrently with M-CSF or GM-CSF to human monocytes during the 6-d differentiation process, and then these macrophages were stimulated with LPS in the absence of LL-37. In this case, proinflammatory cytokine production was enhanced and IL-10 secretion reduced in LL-37-pretreated cells, particularly in the M2 subset (40). LL-37 also augmented the responses of GM-CSF-treated human PBMCs (25), suggesting that LL-37 has a positive effect on GM-CSF signaling. In our study, the optimal inhibitory effect of LL-37 on TNF- $\alpha$  and NO production was observed when it was added at the initiation of M1 polarization. This effect of LL-37 was decreased when LL-37 was added at a later time point and was reversed after removal of LL-37 by washing the cells and replacing the media. Together, these data show that the effect of LL-37 on cells of the myeloid lineage may depend on the time LL-37 is added, the stimulus, and the maturation state of the monocyte or macrophage. This was also reflected in the ability of LL-37 to exert potent inhibitory effects

on LPS-treated alveolar macrophages but not peritoneal macrophages. This may relate to the phenotypic and functionally distinct nature of these two macrophage populations (41). Resident peritoneal macrophages are F4/80<sup>+</sup>, CD11b<sup>+</sup>, CD11c<sup>-</sup>, whereas alveolar macrophages are F4/80<sup>lo</sup>, CD11b<sup>-</sup>, CD11c<sup>+</sup> (data not shown). In addition, the amount of TNF- $\alpha$  produced by the two types of macrophages in response to LPS was very different; alveolar macrophages produced  $\sim$ 1.5 ng/ml TNF- $\alpha$  after a 2-h stimulation in vitro with 100 ng/ml LPS, whereas peritoneal macrophages produced  $\sim$ 250 pg/ml. Despite this, TNF- $\alpha$  production by alveolar macrophages was highly sensitive to treatment with LL-37 and implies that tissue macrophages have different sensitivities to LL-37.

LL-37 is expressed at high levels in the lung, particularly during an inflammatory response. Although 1–10  $\mu$ g/ml LL-37 may be typical under noninflammatory conditions, this can rise to 20  $\mu$ g/ml or more under inflammatory conditions (42). To allow the lung to function effectively, it is important to be able to rapidly clear invading bacteria with a minimal amount of inflammation. Both epithelial cells and alveolar macrophages have antimicrobial mechanisms to effectively remove invading bacteria. Epithelial cells secrete antimicrobial peptides, and alveolar macrophages are highly phagocytic. Given the effective inhibition of LL-37 on TNF- $\alpha$  production by alveolar macrophages both in vitro and in vivo, it is possible that elevated concentrations of LL-37 in the inflamed lung will suppress the inflammatory response. However, LL-37 is also known to act on airway epithelial cells. In a lung infection model, LL-37 promoted the apoptosis of airway epithelium and provided protection from *Pseudomonas aeruginosa* (42). LL-37 can also stimulate IL-8 production in bronchial epithelial cells (22), which recruits neutrophils and promotes an inflammatory response, so the overall outcome of LL-37 on lung inflammation is not clear.

Overall, we have demonstrated that LL-37 selectively suppressed LPS, and LPS/IFN- $\gamma$  induced proinflammatory responses (TNF- $\alpha$  and NO production) while preserving other functions, such as ROS production and the ability to suppress tumor growth and actively remove microbial products and inflammatory cells by phagocytosis. The results also suggest that inflammatory concentrations of LL-37 in the lung can dampen the proinflammatory properties of LPS on alveolar macrophages. These data are consistent with an overall role for LL-37 as a modulator of inflammatory responses.

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## Disclosures

The authors have no financial conflicts of interest.

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