THE UNIVERSITY *of* EDINBURGH

Edinburgh Research Explorer

The human cathelicidin LL-37 preferentially promotes apoptosis of infected airway epithelium

Citation for published version:

Barlow, PG, Beaumont, PE, Cosseau, C, Mackellar, A, Wilkinson, TS, Hancock, REW, Haslett, C, Govan, JRW, Simpson, AJ & Davidson, DJ 2010, 'The human cathelicidin LL-37 preferentially promotes apoptosis of infected airway epithelium', *American Journal of Respiratory Cell and Molecular Biology*, vol. 43, no. 6, pp. 692-702. <https://doi.org/10.1165/rcmb.2009-0250OC>

Digital Object Identifier (DOI):

[10.1165/rcmb.2009-0250OC](https://doi.org/10.1165/rcmb.2009-0250OC)

Link:

[Link to publication record in Edinburgh Research Explorer](#)

Document Version:

Publisher's PDF, also known as Version of record

Published In:

American Journal of Respiratory Cell and Molecular Biology

Publisher Rights Statement:

© 2010 The American Thoracic Society

General rights

Copyright for the publications made accessible via the Edinburgh Research Explorer is retained by the author(s) and / or other copyright owners and it is a condition of accessing these publications that users recognise and abide by the legal requirements associated with these rights.

Take down policy

The University of Edinburgh has made every reasonable effort to ensure that Edinburgh Research Explorer content complies with UK legislation. If you believe that the public display of this file breaches copyright please contact openaccess@ed.ac.uk providing details, and we will remove access to the work immediately and investigate your claim.

The Human Cathelicidin LL-37 Preferentially Promotes Apoptosis of Infected Airway Epithelium

Peter G. Barlow¹, Paula E. Beaumont¹, Celine Cosseau², Annie Mackellar¹, Thomas S. Wilkinson³, Robert E. W. Hancock², Chris Haslett¹, John R. W. Govan⁴, A. John Simpson¹, and Donald J. Davidson¹

¹Medical Research Council/University of Edinburgh Centre for Inflammation Research, Queen's Medical Research Institute, Edinburgh, Scotland, United Kingdom; ²Centre for Microbial Diseases and Immunity Research, University of British Columbia, Vancouver, British Columbia, Canada; ³Institute of Life Science, Microbiology, and Infection, School of Medicine, Swansea University, Swansea, Wales, United Kingdom; and ⁴Centre for Infectious Diseases, New Royal Infirmary, University of Edinburgh, Edinburgh, Scotland, United Kingdom

Cationic host defense peptides are key, evolutionarily conserved components of the innate immune system. The human cathelicidin LL-37 is an important cationic host defense peptide up-regulated in infection and inflammation, specifically in the human lung, and was shown to enhance the pulmonary clearance of the opportunistic pathogen *Pseudomonas aeruginosa* *in vivo* by as yet undefined mechanisms. In addition to its direct microbicidal potential, LL-37 can modulate inflammation and immune mechanisms in host defense against infection, including the capacity to modulate cell death pathways. We demonstrate that at physiologically relevant concentrations of LL-37, this peptide preferentially promoted the apoptosis of infected airway epithelium, via enhanced LL-37-induced mitochondrial membrane depolarization and release of cytochrome c, with activation of caspase-9 and caspase-3 and induction of apoptosis, which only occurred in the presence of both peptide and bacteria, but not with either stimulus alone. This synergistic induction of apoptosis in infected cells was caspase-dependent, contrasting with the caspase-independent cell death induced by supraphysiologic levels of peptide alone. We demonstrate that the synergistic induction of apoptosis by LL-37 and *Pseudomonas aeruginosa* required specific bacteria–epithelial cell interactions with whole, live bacteria, and bacterial invasion of the epithelial cell. We propose that the LL-37-mediated apoptosis of infected, compromised airway epithelial cells may represent a novel inflammomodulatory role for this peptide in innate host defense, promoting the clearance of respiratory pathogens.

Keywords: cationic host defense peptide; antimicrobial peptide; innate immunity; *Pseudomonas*; apoptosis

Cationic host defense peptides (CHDPs; also known as antimicrobial peptides) are key, conserved components of innate host defenses. The broad-spectrum, direct microbicidal potential of CHDPs has made these peptides attractive therapeutic agents. However, many CHDPs were further demonstrated to exert multiple potential immunomodulatory functions, including the modulation of cell death, raising questions about the nature of their primary physiologic roles and the possibility of developing novel therapeutics with both microbicidal and immunomodulatory activities.

(Received in original form July 9, 2009 and in final form December 21, 2009)

This work was funded by the Wellcome Trust, the Norman Salvesen Trust Emphysema Research Trust, and the Canadian Institutes for Health Research. D.J.D. is a Wellcome Trust Research Career Development Fellow (Fellowship 078265), P.E.B. holds a Medical Research Council/Asthma UK PhD studentship, C.C. was supported by the Canadian Cystic Fibrosis Foundation, and R.E.W.H. is a Canada Research Chair.

Correspondence and requests for reprints should be addressed to Donald J. Davidson M.B.Ch.B., Ph.D., Medical Research Council/University of Edinburgh Centre for Inflammation Research, Queen's Medical Research Institute, W2.05, 47 Little France Crescent, Edinburgh EH16 4TJ, Scotland, UK. E-mail: Donald.Davidson@ed.ac.uk

Am J Respir Cell Mol Biol Vol 43, pp 692–702, 2010

Originally Published in Press as DOI: 10.1165/rmb.2009-0250OC on January 22, 2010

Internet address: www.atsjournals.org

One of the CHDPs demonstrating the most significant immunomodulatory potential to date is LL-37. This cationic, amphipathic peptide is the predominant cleavage product of human cationic antimicrobial peptide (hCAP)-18, the sole human cathelicidin (reviewed by Zanetti) (1). LL-37 is stored at high concentrations in the specific granules of neutrophils, and is produced by epithelial cells and some leukocytes. It can be detected in airway surface liquid, plasma, sweat, and other body fluids, and is upregulated in response to infection and inflammation (1, 2). In addition to direct microbicidal capability, the modulatory potential of LL-37 is considerable, with *in vitro* and *in vivo* studies suggesting a broad range of activities that could modify innate inflammatory processes and adaptive immune responses (2). The physiological significance of LL-37 to human disease is demonstrated by the increased susceptibility to infection of individuals with morbus Kostmann (in which defective neutrophils are cathelicidin-deficient) (3), and is also suggested by the association between hCAP-18 expression and susceptibility to skin infections in psoriasis and atopic dermatitis (4). In addition, studies using a mouse model deficient in cathelin-related antimicrobial peptide (mCRAMP), the murine ortholog of LL-37, demonstrated increased susceptibility to infections of the skin, gastrointestinal system, urinary tract, and cornea (5–8). Despite this clear evidence of a critical role for cathelicidin expression in innate defense against infection, the relative roles of the microbicidal and immunomodulatory activities of this peptide remain unclear.

Gene therapy augmentation demonstrated that the expression of LL-37 in the murine lung can enhance the clearance of pulmonary *Pseudomonas aeruginosa* (9), an important opportunistic pulmonary pathogen of immunocompromised individuals and those with cystic fibrosis (10). However, the mechanisms underlying enhanced defense against infection in this model remain unclear, with the concentrations of LL-37 detected unlikely to be directly microbicidal under physiological conditions (9, 11). Multiple mechanisms are likely involved in the host defense against lung infection with *P. aeruginosa*, ranging from simple mucociliary clearance and innate microbicidal components of airway surface liquid, to the activity of professional phagocytes. In addition, the apoptosis and subsequent removal of infected epithelial cells were described as innate defense mechanisms at diverse epithelial surfaces (12–14), required for the clearance of invasive *P. aeruginosa* from the murine lung (12). Such a mechanism may be an important component of host defenses, removing bacteria that have evaded other defenses and invaded epithelial cells.

LL-37 was previously demonstrated to modulate cell death pathways (15–21). We previously demonstrated that high concentrations of LL-37 can induce apoptosis in airway epithelial cell lines and primary cells *in vitro*, and in murine airways *in vivo* (15, 17). Moreover, LL-37 was shown to induce mitochondrial depolarization in alveolar epithelial cells (18).

However, the roles of the Bcl2-family proteins, which can regulate mitochondrial membrane potential, and of the key apoptosis-inducing caspase proteins in LL-37, which can induce apoptosis of airway epithelial cells, remain uncertain. Further, it is unclear whether LL-37-induced apoptosis might be primarily detrimental, with overexpression of LL-37 damaging normal epithelial integrity, or whether at much lower, more physiological concentrations, LL-37 expression could enhance innate defenses by promoting targeted apoptosis to facilitate the clearance of pathogens. To address these issues, we studied the ability of LL-37 to induce apoptosis in airway epithelial cells infected with the invasive lung pathogen *P. aeruginosa*.

We demonstrate that LL-37 can induce Bax-dependent mitochondrial membrane depolarization in airway epithelial cells in a dose-dependent manner, with the release of cytochrome *c*, and that this is synergistically enhanced by infection with *P. aeruginosa*. However, at physiologically relevant concentrations of LL-37, the activation of caspase-9 and caspase-3, and DNA fragmentation, only occurred in the presence of both peptide and bacteria, but not with either stimulus alone. This synergistic induction of apoptosis was caspase-dependent and partly Bax-dependent, and required specific bacteria-epithelial cell interaction with whole, live bacteria capable of epithelial-cell invasion.

MATERIALS AND METHODS

Reagents

Dulbecco's modified Eagle's medium (DMEM), L-glutamine, non-essential amino acids (NEAAs), PBS, trypsin/EDTA, and FBS were all purchased from PAA Laboratories (Somerset, UK). Primary normal human bronchial epithelial (NHBE) media and growth supplements were purchased from Lonza (Wokingham, UK). Fibronectin, BSA, Tween-20, Luria Bertani broth, formalin, chemiluminescence peroxidase substrate, and 3-[(3-cholamidopropyl)dimethylammonio]-1-propanesulfonate hydrate were all supplied by Sigma-Aldrich (Dorset, UK). Mouse collagen type IV (Cultrex) was purchased from Stratech Scientific, Ltd. (Suffolk, UK). Ultrosor G was obtained from Pall Pharmaceuticals (Hampshire, UK). Precise protein polyacrylamide gels, M-PER protein extraction reagent, and protease/phosphatase inhibitor cocktails were supplied by Thermo Scientific (Loughborough, UK). Vectashield Hardset mounting medium with 4',6-diamidino-2-phenylindole (DAPI) was supplied by Vector Laboratories (Peterborough, UK). The Bax-inhibiting peptide V5 (BIP-V5) and Z-VAD-FMK were supplied by Merck Chemicals, Ltd. (Nottingham, UK). Rabbit anti-human cleaved caspase-3, caspase-8, and caspase-9 antibodies (catalogue numbers 9661, 9496, and 9505, respectively), anti-human X-linked inhibitor of apoptosis protein (XIAP) (catalogue number 2045), anti-human pan actin (catalogue number 4968), and horseradish peroxidase (HRP)-conjugated goat anti-rabbit IgG antibodies were purchased from Cell Signaling Technologies (Danvers, MA). We extracted *Pseudomonas aeruginosa* PAO1 lipopolysaccharide (LPS) using a 90% aqueous phenol solution at 65°C and ultracentrifugation, followed by quantification using a limulus amoebocyte lysate assay (Cambrex, Wokingham, UK). LL-37 (sequence LLGDFFRKSKEKIGKEFKRIVQRIKDFLRNLVPRTE; molecular weight 4,493.33) was synthesized by *N*-(9-fluorenyl) methoxycarbonyl chemistry at the Nucleic Acid/Protein Service Unit at the University of British Columbia (Vancouver, Canada), as described previously (22). Scrambled LL-37 control peptide (sequence RSLEGTDRLFVRLKNSRKLEFKDIKIGIKREQFVKIL) was purchased from CSS-Albachem, Ltd. (East Lothian, UK). Peptides were purified by reverse-phase high-performance liquid chromatography, and were at least 98% pure. LL-37, and scrambled LL-37 control peptide, were dissolved in endotoxin-free water (Sigma-Aldrich) and stored at -20°C until further use. The concentration of peptides in solution was determined by amino-acid analysis, and tested to ensure that they were free of endotoxin.

Primary and Transformed Epithelial-Cell Culture

The 16HBE14o⁻ transformed human bronchial epithelial cells were a kind gift from Dieter Gruenert at the University of California, San Francisco. Cells were grown in standard submerged culture and maintained in DMEM supplemented with 1% L-glutamine (vol/vol), 1% NEAA (vol/vol), and 10% FBS (vol/vol). Culture flasks were coated with a basement layer of collagen IV (5 µg/ml), fibronectin (10 µg/ml), and BSA (100 µg/ml) before cell culture at 37°C, 5% CO₂. Normal human bronchial epithelial cells from a single donor were purchased from Lonza. Cells were grown in standard submerged culture and maintained in bronchial epithelial growth media (Lonza), supplemented with bronchial epithelial cell SingleQuots growth factors and supplements (Lonza) as a serum substitute, in strict accordance with the manufacturer's instructions.

All assays were conducted in Ultrosor G serum-substitute supplemented media, consisting of phenol red-free DMEM supplemented with 1% Ultrosor G (vol/vol), 1% L-glutamine (vol/vol), and 1% NEAA (vol/vol).

Bacterial Strains and Culture

In addition to *P. aeruginosa* PAO1, these studies used the following strains of *P. aeruginosa*: clinical isolate J1386 (a clonal isolate of J1385, originally from an individual with cystic fibrosis) (23), Δ *mexAB-oprM* mutant (a gift from Keith Poole) (24), PAO1*exsA::Ω* mutant (a gift from Dara Frank) (25), *P. aeruginosa pilA* mutant (a gift from Eva Lorenz) (26), and the isogenic PAO1 control strains for these mutants. Studies involving genetically modified bacteria were performed according to Scientific Advisory Committee on Genetic Modification Health and Safety Executive Certificate GM207/07.2.

All *P. aeruginosa* strains were grown in Luria Bertani (LB) broth at 37°C in an orbital shaker (250 rpm) overnight, to achieve a stationary-phase suspension. Before use, bacterial suspensions diluted 1:20 in fresh LB broth were incubated at 37°C for 90 minutes to reach log phase. Bacterial suspensions were standardized via dilution to an optical density of 0.1 at 595 nm, using spectrophotometry (WPA UV 1101, Biotech Photometer; Biochrom Ltd., Cambridge, UK), centrifuged at 1,500 × *g* for 15 minutes (keeping supernatant where required for use in place of live bacteria), and resuspended in PBS before immediate addition to epithelial cells. Where required, bacteria were heat-killed (60°C for 60 minutes in an orbital shaker) or ultraviolet light (UV)-killed (exposed to a constant UV source for 2 hours in a sealed glass Petri dish), with killing confirmed by overnight culture. To determine the direct microbicidal activity of LL-37, *P. aeruginosa* were resuspended in Ultrosor G serum-substitute supplemented media before the immediate addition of LL-37 at the concentrations stated. After incubation for 1 hour at 37°C, serial dilutions were performed, and 100-µl aliquots of these (and the original bacterial suspension) were spread onto LB agar plates in triplicate, and incubated overnight at 37°C before counting the number of colony-forming units (CFUs). For studies to determine if the function of the *ΔmexAB-oprM* mutant could be rescued by soluble factors released by PAO1, PAO1 was added to 16HBE14o⁻ cells at a multiplicity of infection (MOI) of 10:1 and incubated for 18 hours at 37°C with 5% CO₂. After incubation, the supernatant was collected and filtered through a 0.22-µm filter unit. The sterility of filtered supernatant was confirmed by culturing 50 µl on LB agar plates for 24 hours. Filtered supernatant (1:4 dilution in treatment medium) was simultaneously added together with LL-37 and *ΔmexAB-oprM* to 16HBE14o⁻ cells, and the cells were incubated for 1 hour and analyzed using the mitochondrial depolarization technique.

Mitochondrial Depolarization Assay

The 16HBE14o⁻ cells were seeded at 2.5 × 10⁴ cells per well in a 96-well plate and cultured at 37°C, 5% CO₂. Cells were exposed to LL-37 (or scrambled LL-37 control peptide) at the concentrations described in the presence and absence of (1) log-phase *P. aeruginosa* at an MOI of 10:1; (2) heat-killed or UV-killed bacteria (MOI 10:1), *P. aeruginosa* PAO1 LPS (1 µg/ml) or *P. aeruginosa* supernatant, all prepared as described above; or (3) log-phase *P. aeruginosa* PAO1 (MOI 10:1), separated from the epithelial cells by a Transwell semipermeable polyester membrane with 0.4-µm pore size (Corning Life Sciences,

Amsterdam, Netherlands), and incubated for 1 hour at 37°C, 5% CO₂. All treatments were conducted in Ultrosor G serum-substitute supplemented media. After treatments, cells were washed once with PBS prewarmed to 37°C, the supernatant was aspirated, and 50 µl of Mitocapture solution (Cambridge Bioscience, Cambridge, UK) at 37°C were added to each well, before incubation at 37°C for 30 minutes in the dark. Cells were then washed twice with PBS at 37°C, resuspended in 50 µl of Mitocapture buffer at 37°C, and imaged immediately using an Axiovert S100 inverted fluorescent microscope (Zeiss UK, Welwyn Garden City, UK). For each membrane, at least four random fields of view were counted with a minimum of 300 cells in total, and the number of apoptotic cells (displaying diffuse, green fluorescence) was expressed as a percentage of the number of healthy cells (displaying punctate red mitochondrial fluorescence). Data were corrected for a background level of approximately 10% positive cells observed in control untreated samples. For inhibition studies, the culture medium in each well was replaced with treatment medium containing 100 µM BIP-V5 for 1 hour before treatment.

Cytochrome c Assay

The 16HBE14o⁻ cells were seeded at 1×10^6 cells per well in a six-well plate and cultured at 37°C, 5% CO₂. Cells were exposed to LL-37 at the concentrations described in the presence and absence of log-phase *P. aeruginosa* PA01 (MOI 10:1), and incubated for 90 minutes at 37°C, 5% CO₂. Cells were washed once with PBS, and 0.5 ml of trypsin/EDTA was added to each well to detach cells. Ultrosor G serum-substitute supplemented media (0.5 ml) was added to each well and suspensions were centrifuged in microtubes at $850 \times g$ for 2 minutes. Mitochondrial and cytosolic fractions were then prepared using a Mitochondrial Isolation Kit (Thermo Scientific, Loughborough, UK) according to the manufacturer's instructions. Cytochrome c concentrations in each fraction were assessed using a Cytochrome c ELISA Kit (Merck Chemicals, Ltd.), according to the manufacturer's protocol.

Western Immunoblotting

The 16HBE14o⁻ cells were seeded at 1×10^6 cells per well in six-well plates and cultured at 37°C, 5% CO₂. Cells were exposed to LL-37 at the concentrations described, in Ultrosor-G serum-substitute supplemented media, in the presence and absence of log-phase *P. aeruginosa* PA01 (MOI 10:1) and incubated for 3 hours at 37°C, 5% CO₂. Cells were washed once with ice-cold PBS and lysed with 300 µL M-PER protein extraction reagent (Thermo Scientific) containing a cocktail of protease, phosphatase, and metalloprotease inhibitors. Protein concentrations were determined by bicinchoninic acid assay (Thermo Scientific). Equivalent total protein concentration lysates (15–40 µg) were resolved on either 10% or 12% precast Precise Protein polyacrylamide gels (Thermo Scientific), transferred to polyvinylidene fluoride membranes (Bio-Rad Laboratories, Ltd., Hemel Hempstead, UK), blocked for 1 hour with Tris-buffered saline and 0.1% Tween-20 (TBST) containing 5% skimmed milk powder (TBST/milk), and then incubated with antibodies specific for cleaved caspase-3 (1 in 5,000 dilution), cleaved caspase-9 (1 in 1,000 dilution), XIAP (1 in 1,000 dilution), or pan-actin (1 in 2,000 dilution) in TBST/milk overnight at 4°C. Membranes were washed for 15 minutes in TBST and then incubated with a 1 in 5,000 dilution of HRP-conjugated goat anti-rabbit antibody (in TBST/milk) or a 1 in 5,000 dilution of HRP-conjugated goat anti-mouse antibody (in TBST/milk) for 1 hour at room temperature. Membranes were washed for 30 minutes and developed with chemiluminescence peroxidase substrate (Sigma-Aldrich) according to the manufacturer's instructions, and imaged on CL-Xposure film (Thermo Scientific). Equal loading of protein was confirmed by examining the expression of actin as a loading control.

In Situ Cell Death Detection by Terminal Deoxynucleotidyl Transferase-Mediated Deoxyuridine Triphosphate Nick-End Labeling Assay

Transwell polyester-permeable supports (pore size, 0.4 µm; diameter, 6.5 mm; Corning Life Sciences) were equilibrated for 45 minutes in culture media (DMEM supplemented with 10% FBS [vol/vol], 1% L-glutamine [vol/vol], and 1% NEAA [vol/vol]) before the addition of 100 µl of medium containing 2.5×10^5 16HBE14o⁻ cells/ml into the

apical compartment, with 600 µl culture medium in the basal compartment, and cultured at 37°C, 5% CO₂. For primary bronchial epithelial cell experiments, Transwell supports were equilibrated with NHBE culture media for 45 minutes before the addition of 100 µl of NHBE media containing 2.5×10^5 NHBE cells/ml into the apical compartment, with 600 µL of NHBE culture medium in the basal compartment, and cultured at 37°C, 5% CO₂. Before treatments, culture media in both the apical and basal compartments were replaced with Ultrosor G serum-substitute supplemented media. Cells were exposed to LL-37 at the concentrations described in the presence and absence of log-phase *P. aeruginosa* PA01 (MOI 10:1), and incubated for 6 hours at 37°C, 5% CO₂. Cells were fixed in 10% neutral-buffered formalin (3.7% formaldehyde) for 10 minutes, washed once in PBS, permeabilized in ice-cold 0.1% Triton X-100/0.1% sodium citrate for 3 minutes, and washed twice with PBS. An *in situ* cell death detection kit (Roche Applied Science, West Sussex, UK) was used according to the manufacturer's instructions. The membranes with cells were mounted in 50 µL Vectashield Hardset (containing DAPI), and at least four random fields of view were counted (each containing more than 100 cells), using an Axiovert S100 fluorescent microscope, and analyzed using OpenLAB 3.0 software (Improvision/Perkin Elmer, Waltham, MA). The number of terminal deoxynucleotide transferase dUTP nick-end labeling (TUNEL)-positive cells was expressed as a percentage of the number of DAPI-positive nuclei. The total number of DAPI-positive nuclei counted for each condition was determined, to evaluate total cell number. For inhibition studies, cells were prepared as described, and culture medium in each well was replaced with treatment medium containing either 100 µM Bax inhibiting peptide V5 (Merck Chemicals, Ltd.) or 50 µM of the broad-spectrum caspase inhibitor, Z-VAD-FMK (Merck Chemicals, Ltd.), for 1 hour before treatment.

Gentamicin Exclusion Assay

The capacity of *P. aeruginosa* isolates and mutants to invade epithelial cells was assessed using a gentamicin exclusion assay. Briefly, 16HBE14o⁻ cells were exposed to strains of log-phase *P. aeruginosa* (MOI 10:1) for 60 minutes in Ultrosor G-serum-substitute supplemented media. The media were removed from all wells, and cells were incubated with fresh media for 60 minutes with or without gentamicin (50 µg/ml) to kill extracellular bacteria. The media were then aspirated from gentamicin-treated cells, and these cells were vigorously washed with PBS and lysed with PBS containing 0.1% Triton X-100, and then plated on LB agar to determine internalized bacterial numbers. Media and/or epithelial cell lysates from wells without gentamicin were also plated on LB agar, to determine the number of associated bacteria and total infectious load. The CFUs were quantified by culturing overnight on LB agar plates at 37°C.

Statistical Analysis

Statistical analyses were performed using Graphpad Prism version 5 for Windows (GraphPad Software Inc., La Jolla, CA). Statistical significance was assessed either using one-way ANOVA with Tukey's *post hoc* test, or two-way ANOVA with Bonferroni's *post hoc* test where appropriate. $P \leq 0.05$ was considered significant.

RESULTS

LL-37 and *P. aeruginosa* Synergistically Induce Epithelial Cell Death

To determine the capacity of LL-37 to induce cell death in infected airway epithelial cells, the human bronchial epithelial cell line 16HBE14o⁻ was infected with *P. aeruginosa* PA01, with or without concurrent exposure to LL-37. These cells were examined for nuclear DNA fragmentation by TUNEL assay at 6 hours (Figure 1A). *Pseudomonas aeruginosa* alone did not induce cell death, and LL-37 alone induced cell death only at higher concentrations. However, concurrent exposure to both stimuli synergistically induced significant levels of cell

death at greater than or equal to 20 $\mu\text{g/ml}$ of LL-37 ($P \leq 0.01$), and even at an LL-37 concentration of 20 $\mu\text{g/ml}$ that had no effect alone. A control scrambled LL-37 peptide had no effect. Total cell counts demonstrated no loss of cells during analysis (data not shown). To confirm these observation in nontransformed cells, primary human airway epithelial cells were used, and demonstrated the same response, with significant cell death induced in the presence of physiologically inflammatory levels of LL-37 only when infected with *P. aeruginosa* PAO1 (Figure 1B).

To determine whether the cell death observed was apoptosis, cleavage of the key executioner caspase, caspase-3, was determined by Western immunoblot in 16HBE14o⁻ cells at 3 to 6 hours after infection with *P. aeruginosa* PAO1, with or without concurrent exposure to LL-37. No activation was detected in response to LL-37 alone, or *P. aeruginosa* alone. However, concurrent exposure to both stimuli resulted in caspase-3 activation at 4 hours and thereafter (Figure 1C and data not shown). These data indicate that the cell death

induced synergistically by LL-37 and *P. aeruginosa*, but not by high concentrations of LL-37 alone, is caspase-dependent apoptosis. This finding is supported by the observation that preincubation with the polycaspase inhibitor Z-VAD-FMK significantly ($P \leq 0.001$) inhibited the synergistic induction of cell death by *P. aeruginosa* and LL-37 (Figure 1A), reducing it to approximately the level induced by LL-37 alone at that concentration.

In addition, cleavage of caspase-9 (a key cytochrome c-activated initiator caspase) was also observed in response to infection with *P. aeruginosa* PAO1, only in the presence of LL-37 (Figure 1D). Caspase-9 activation was not detected in response to LL-37 alone, or *P. aeruginosa* alone. In contrast, the activation of caspase-8 (a key death receptor-activated initiator caspase) was not evident (data not shown). These data demonstrate a synergistic induction of intrinsic apoptosis-inducing pathways.

Given the absence of caspase-3 activation in response to concentrations of LL-37 at which peptide alone induced cell

Figure 1. LL-37 and *P. aeruginosa* synergistically induce DNA fragmentation and caspase activation in airway epithelial cells. Human bronchial epithelial cell line 16HBE14o⁻ (A, C, D) or primary human bronchial epithelial cells (B) were incubated for 6 hours (A, B) or 5 hours (C, D) over a range of LL-37 concentrations (or scrambled LL-37 [sLL-37] at 50 $\mu\text{g/ml}$) in Ultrosor G serum-substitute supplemented media, in the presence and absence of log-phase *P. aeruginosa* PAO1 (MOI 10:1) added concurrently. (A, B) Cells were treated as described, with or without preincubation for 1 hour with the polycaspase inhibitor Z-VAD-FMK (50 μM), and were then fixed. Apoptosis was assessed by TUNEL assay. Four random fields of view, each containing more than 100 cells, were counted for each sample, and the number of TUNEL-positive cells was expressed as a percentage of the number of DAPI-positive nuclei. Data represent mean values $\pm$ SEM, for $n \geq 3$ independent experiments for each condition. Two-way ANOVA with Bonferroni *post hoc* test was used to compare LL-37/*P. aeruginosa*-treated samples with LL-37 only-treated samples at corresponding concentrations, or LL-37/*P. aeruginosa*/Z-VAD-FMK-treated samples with LL-37/*P. aeruginosa*-treated samples at corresponding concentrations. * $P \leq 0.05$, ** $P \leq 0.01$. (C, D) Whole-cell protein lysates were prepared and analyzed by SDS-PAGE and Western immunoblotting. Immunoblots were performed using antibodies specific for cleaved caspase-3, XIAP, cleaved caspase-9, or actin. Images shown are representative of $n \geq 3$ independent experiments.

death, the expression levels of XIAP (a potent caspase inhibitor) were examined, but no effect on expression levels was evident (Figure 1C).

***Pseudomonas aeruginosa* Infection of Airway Epithelial Cells Synergistically Enhances LL-37–Mediated Mitochondrial Depolarization and Cytochrome *c* Release**

To determine the role of mitochondria in LL-37–induced cell death, 16HBE14o[−] cells were infected with *P. aeruginosa* PAO1, with or without concurrent exposure to LL-37. After 1 hour, the mitochondrial membrane potential was determined as an early indicator of apoptosis (Figure 2A). The LL-37 alone induced a dose-dependent increase in mitochondrial depolarization at greater than or equal to 20 $\mu\text{g/ml}$. *Pseudomonas aeruginosa* alone had no effect, but synergized with LL-37 to induce significantly greater mitochondrial depolarization than LL-37 alone, even inducing increased depolarization at low (10 $\mu\text{g/ml}$) LL-37 concentrations that had no effect alone ($P \leq 0.05$). Scrambled LL-37 peptide had no effect (Figure 2A). To determine whether this synergistic effect required an initial interaction between LL-37 and either the epithelial cell or the bacteria, which could subsequently alter bacteria–epithelial cell interactions, 16HBE14o[−] cells were infected with *P. aeruginosa* PAO1 for 1 hour, and washed before incubation with LL-37 for 1 hour. Under these conditions, the synergistic induction of mitochondrial depolarization was still evident, and even amplified at lower LL-37 concentrations (Figure 2B). This result indicates that infection with *P. aeruginosa* promotes airway epithelial cell susceptibility to LL-37–induced apoptosis.

To evaluate the consequences of mitochondrial depolarization, the intracellular localization of cytochrome *c* was examined 90 minutes after 16HBE14o[−] cells were infected with *P. aeruginosa* PAO1, with or without concurrent exposure to LL-37 (Figure 2C). The LL-37 alone induced a dose-dependent relocalization of cytochrome *c* from the mitochondria to the cytoplasm, reflecting the mitochondrial depolarization and TUNEL positivity observed, and reaching significance at 50 $\mu\text{g/ml}$ LL-37 ($P \leq 0.01$). *Pseudomonas aeruginosa* alone had no effect, but synergized with LL-37 to induce a highly significant translocation of cytochrome *c* at all concentrations of LL-37 tested ($P \leq 0.001$). This latter effect was surprisingly pronounced, with very significant translocation observed even at 10 $\mu\text{g/ml}$ of LL-37, a concentration at which significant cell death was not evident. Effects as yet unexplained on the mitochondria under these conditions (but not in response to peptide alone or bacteria alone) may have led to further translocation of cytochrome *c* from the mitochondria during sample preparation, with a resultant amplification of the effect observed. Cytoplasmic cytochrome *c* was detected by Western immunoblot in response to 10–30 $\mu\text{g/ml}$ LL-37 only in infected cells (data not shown). Thus, the cytoplasmic translocation of cytochrome *c* was clearly evident under these conditions.

To determine whether the LL-37–mediated induction of apoptosis was dependent on the key proapoptotic Bcl-2 family protein Bax, the effects of exposure to LL-37 and *P. aeruginosa* on mitochondrial depolarization (Figure 3A) and DNA fragmentation (Figure 3B) were evaluated after preincubation with the Bax-inhibiting peptide V5 (BIP-V5). At high concentrations of LL-37, at which LL-37 alone induced substantial mitochondrial depolarization and apoptosis, the inhibition of Bax significantly ($P \leq 0.01$) and almost completely blocked these effects. In contrast, Bax inhibition only partly inhibited the combined effect of LL-37 and *P. aeruginosa*. These data demonstrate that caspase-independent induction of cell death by LL-37 alone is Bax-dependent. However, additional, and as yet unidentified, components are required for the synergistic enhancement of

mitochondrial depolarization and induction of caspase-dependent apoptosis by LL-37 in *P. aeruginosa*–infected cells.

Synergistic Induction of Apoptosis by LL-37 and *P. aeruginosa* Requires Specific Bacteria–Epithelial Cell Interactions with Live Bacteria

To exclude the possibility that LL-37 exerted directly microbicidal effects on *P. aeruginosa* PAO1, bacterial viability was determined after exposure to LL-37 over the range of concentrations and in the culture media used for these studies (Figure 4A). No significant, direct microbicidal activity was evident.

To examine whether the synergistic induction of apoptosis by LL-37 and *P. aeruginosa* required infection with live bacteria, and/or could result from secreted products, 16HBE14o[−] cells were exposed to a range of bacterial stimuli in the presence or absence of concurrent exposure to 30 $\mu\text{g/ml}$ LL-37, and assessed for mitochondrial depolarization (Figure 4B). The highly significant ($P \leq 0.001$), synergistic induction of mitochondrial depolarization observed after exposure to live *P. aeruginosa* and LL-37 was completely lost if the bacteria used were dead (heat-killed or UV-killed), or substituted with bacterial culture supernatant, or LPS prepared from PAO1 (1 $\mu\text{g/ml}$). Furthermore, physical separation of the epithelial cells from the bacteria by a semipermeable membrane also completely prevented this effect. These data indicate that the synergistic induction of apoptosis by LL-37 and *P. aeruginosa* requires a physical interaction between the epithelial cells and viable bacteria, and is not simply the result of pathogen-sensing by extracellular pattern recognition receptors.

Synergistic Induction of Apoptosis by LL-37 and *P. aeruginosa* Is Isolate-Specific, and Independent of Type III Secretion System and Pilus Expression

To exclude the possibility that the synergistic induction of apoptosis by LL-37 and *P. aeruginosa* was specific to PAO1, a clinical *P. aeruginosa* isolate J1386 was examined (Figure 5A). A synergistic induction of mitochondrial depolarization was also observed in response to this clinical isolate in the presence of LL-37. This finding was substantially enhanced in comparison to that observed using the laboratory strain PAO1, with significant effects observed in infected cells after incubation with concentrations of LL-37 greater than or equal to 1 $\mu\text{g/ml}$ ($P \leq 0.01$). No direct microbicidal effect of LL-37 was observed on *P. aeruginosa* isolate J1386 (data not shown).

To examine whether common virulence factors differentially expressed by divergent *P. aeruginosa* isolates were necessary for this effect, mutant strains of *P. aeruginosa* PAO1 were used (Figures 5B and 5C). No substantial difference was evident when comparing an ExsA mutant with a defective type III secretion system (PAO1exsA:: Ω ; Figure 5B) or a pilus mutant (*pilA* mutant; Figure 5C) with their corresponding isogenic strains. No direct microbicidal effect of LL-37 was evident in either mutant strain (data not shown). These data demonstrate that common determinants of virulence associated with epithelial-cell interactions (pilus) and bacterially induced epithelial cell death (type III secretion system) are not required for the synergistic induction of apoptosis in LL-37–treated infected epithelial cells.

Synergistic Induction of Apoptosis by LL-37 and *P. aeruginosa* Requires Epithelial-Cell Internalization of Bacteria

The internalization of *P. aeruginosa* by airway epithelial cells was proposed as a key component of the innate pulmonary host defense that is defective in cystic fibrosis (27). To determine the significance of bacterial internalization, a MexAB–OprM de-

letion mutant ($\Delta mexAB\text{-oprM}$), described as containing a defect in its ability to invade epithelial cells (28), was used. The 16HBE14o⁻ cells were infected with PAO1 $\Delta mexAB\text{-oprM}$, or the isogenic control, and a gentamicin-exclusion assay was performed to determine the extent of internalization into the epithelial cells (Figure 6A). Whereas internalization of the isogenic strain could be clearly demonstrated, negligible internalization of the PAO1 $\Delta mexAB\text{-oprM}$ bacteria occurred. Furthermore, the LL-37-mediated synergistic enhancement of apoptosis was significantly ($P \leq 0.001$) and completely lost when using PAO1 $\Delta mexAB\text{-oprM}$ (Figure 6B). The invasion defect of PAO1 $\Delta mexAB\text{-oprM}$ was previously shown to result from the absence of a bacterial secreted factor, and can be restored by the addition of supernatants from isogenic control bacteria exposed to epithelial cells (28). The LL-37-mediated synergistic enhancement of apoptosis was significantly restored ($P \leq 0.01$) when 16HBE14o⁻ cells were infected with PAO1 $\Delta mexAB\text{-oprM}$ in the presence of both LL-37 and supernatants from isogenic control bacteria exposed to epithelial

cells (Figure 6C). These data demonstrate that the epithelial-cell internalization of *P. aeruginosa* is required to facilitate the LL-37-mediated induction of apoptosis at physiologically inflammatory concentrations of peptide.

DISCUSSION

Cationic host-defense peptides, including LL-37, have been demonstrated to have multiple properties capable of modulating inflammation and immunity. The full extent of these properties remains to be determined, but understanding the physiological roles of CHDPs in health and disease, and their development as antimicrobial therapeutics, is clearly significant. Our results suggest a novel innate inflammomodulatory role for LL-37, preferentially inducing apoptosis in infected epithelial cells, with the potential to exert protective or detrimental effects.

The most critical mechanisms by which cathelicidins contribute to host defense against infections remain uncertain. In various models, LL-37 and mCRAMP exert antimicrobial effects *in vivo* (5–9), despite high minimum inhibitory concentration values that often exceed detectable physiologic levels. Recent studies implicated the vitamin D-dependent up-regulation of LL-37 in the intracellular killing of mycobacteria in mononuclear leukocytes (29, 30), perhaps in synergy with β -defensin 4 (31), and mCRAMP impairs the intracellular replication of *Salmonella* (32). Therefore, these peptides likely have direct antimicrobial roles where peptides are concentrated in favorable, controlled ionic conditions, and perhaps function synergistically with other agents. However, the function of LL-37 at epithelial surfaces, at the peptide concentrations reported, is less clear. In lungs, hCAP-18 was detected in bronchoalveolar lavage fluid from healthy infants at approximately 5 $\mu\text{g/ml}$, and was found at up to approximately 25 $\mu\text{g/ml}$ and at approximately 15 $\mu\text{g/ml}$ in infants with pulmonary infections and individuals with cystic fibrosis lung

Figure 3. LL-37-induced mitochondrial depolarization and DNA fragmentation involve Bax-dependent mechanisms. Human bronchial epithelial cells (16HBE14o⁻) were incubated for 1 hour (A) or 6 hours (B) over a range of LL-37 concentrations in Ultrosor G serum-substitute supplemented media, in the presence and absence of log-phase *P. aeruginosa* PA01 (MOI 10:1) added concurrently, with or without preincubation for 1 hour with Bax-inhibiting peptide V5 (BIP-V5; 100 µM). (A) Mitochondrial membrane depolarization was determined using Mitocapture dye, quantifying the percentage of apoptotic cells displaying diffuse green fluorescence (cells with depolarized mitochondria), compared with healthy cells displaying punctuate red fluorescence (cells with polarized mitochondrial membranes). Four random fields of view were counted for each sample (minimum of 300 cells per sample), and the number of apoptotic cells was expressed as a percentage of total number of cells. Data were corrected for a background level of approximately 10% positive cells in control untreated samples, and plotted as mean values $\pm$ SEM, for $n = 3$ independent experiments for each condition. A two-way ANOVA with Bonferroni *post hoc* test was used to compare LL-37-only treated samples with LL-37/BIP-V5-treated samples, or LL-37/*P. aeruginosa*-treated samples with LL-37/*P. aeruginosa*/BIP-V5-treated samples at corresponding concentrations. * $P \leq 0.05$, ** $P \leq 0.01$, *** $P \leq 0.001$. (B) Cells were fixed and apoptosis was assessed by TUNEL assay. Four random fields of view, each containing more than 100 cells, were counted for each sample, and the number of TUNEL-positive cells was expressed as a percentage of the number of DAPI-positive nuclei. Data represent mean values $\pm$ SEM, for $n = 3$ independent experiments for each condition. Two-way ANOVA with Bonferroni *post hoc* test was used to compare LL-37 only-treated samples with LL-37/BIP-V5-treated samples, or LL-37/*P. aeruginosa*-treated samples with LL-37/*P. aeruginosa*/BIP-V5-treated samples at corresponding concentrations ** $P \leq 0.01$, *** $P \leq 0.001$.

disease (in steady state), respectively (33, 34). At these sites, immunomodulatory roles may be of primary significance. Indeed, the potential significance of such activities was recently demonstrated in terms of the *in vivo* protection against infection in animal models using a synthetic CHDP derivative with no direct antimicrobial activity *in vitro* (35).

Figure 4. Synergistic induction of apoptosis by LL-37 and *P. aeruginosa* requires specific bacteria-epithelial cell interactions with whole, live bacteria. (A) *P. aeruginosa* PA01 was cultured to log-phase, then exposed to LL-37 over a range of concentrations for 1 hour at 37°C in Ultrosor G serum-substitute supplemented media. Serial dilutions were performed, incubated on LB agar plates in triplicate, and cultured for 16 hours before colony-forming units were counted. Data represent mean values $\pm$ SEM, for $n = 3$ independent experiments for each condition. (B) Human bronchial epithelial cells (16HBE14o⁻) were assessed for mitochondrial membrane depolarization using Mitocapture dye, as described in MATERIALS AND METHODS, after incubation for 1 hour with a range of concentrations of LL-37, in serum-substitute supplemented media, in the presence and absence of live log-phase *P. aeruginosa* PA01 (MOI 10:1), heat-killed or UV-killed PA01 (MOI 10:1), *P. aeruginosa* PA01 LPS (1 µg/ml), *P. aeruginosa* PA01 conditioned medium, or live *P. aeruginosa* PA01 (MOI 10:1) separated from the cells via a semipermeable polyester membrane with 0.4-µm pore size. Data represent mean values $\pm$ SEM, for $n = 3$ independent experiments for each condition. Two-way ANOVAs were performed to evaluate significance, with Bonferroni *post hoc* tests comparing LL-37 alone to LL-37/stimuli. *** $P \leq 0.001$.

A variety of CHDPs, including bovine cathelicidins and human α -defensins, were shown to affect eukaryotic cell death (18, 36). We previously showed that high (potentially supra-physiologic) concentrations of LL-37 induced apoptosis in pulmonary epithelial cells *in vitro* and *in vivo* (15, 17). However, the mechanisms involved remain undetermined. We demonstrate here that at these higher concentrations, LL-37 can induce mitochondrial depolarization and cytochrome *c* release

Figure 5. Synergistic induction of apoptosis by LL-37 and *P. aeruginosa* is isolate-specific and independent of type III secretion system and pilus expression. Human bronchial epithelial cells (16HBE14o⁻) were assessed for mitochondrial membrane depolarization using Mitocapture dye, as described in MATERIALS AND METHODS, after incubation for 1 hour with a range of concentrations of LL-37, in Ultrosor G serum-substitute supplemented media, in the presence and absence of (A) log-phase clinical *P. aeruginosa* isolate J1386 (MOI 10:1), (B) log-phase *P. aeruginosa* PA01 exsA::Ω or isogenic PA01 control strain (MOI 10:1), and (C) log-phase *pilA* *P. aeruginosa* mutant or isogenic PA01 control strain (MOI 10:1). Data represent mean values $\pm$ SEM, for $n = 3$ independent experiments for each condition. Two-way ANOVAs were performed to evaluate significance, with Bonferroni *post hoc* tests comparing (A) LL-37/*P. aeruginosa* to LL-37 alone, and (B) LL-37/*P. aeruginosa* mutant to LL-37/isogenic controls. * $P \leq 0.05$, *** $P \leq 0.001$.

in airway epithelial cells, confirming previous findings in alveolar epithelial cells (18). In addition, the LL-37-mediated induction of mitochondrial depolarization and the subsequent apoptosis of these cells can be completely blocked using the BIP-V5 peptide inhibitor of the proapoptotic Bcl-2 family

protein Bax. The BIP-V5 peptide mimics the Bax-binding domain of Ku70, preventing Bax translocation from cytosol to the mitochondria (37). This translocation is a central event in mitochondria-dependent apoptosis, with the subsequent activation and oligomerization of Bax and Bak resulting in either the nonspecific rupture of, or the formation of specific channels in, the outer mitochondrial membrane and release of cytochrome *c* (38). Interestingly, we demonstrate that the Bax-dependent LL-37-mediated release of cytochrome *c* did not cause an activation of caspase-3 or caspase-9 after exposure to LL-37 alone, yet resulted in a Bax-dependent DNA fragmentation. In addition, polycaspase inhibition resulted in only a partial inhibition of the apoptosis induced by high levels of LL-37 (15). These data suggest that the induction of apoptosis by high concentrations of LL-37 alone appears to be a Bax-dependent and predominantly caspase-independent process, and may implicate the liberation and activation of mitochondrial apoptosis-inducing factor (AIF) and/or endonuclease G. The mechanism by which LL-37 can interact with or activate Bax in airway epithelial cells is unclear. LL-37 could induce an opening of the mitochondrial permeability transition pore, as proposed for bovine myeloid antimicrobial peptide-28 (BMAP-28) (36). However, a study published during preparation of our manuscript described a calpain-dependent mechanism of LL-37-mediated Bax translocation to the mitochondria, responsible for the AIF-mediated apoptosis induced by very high concentrations (50–200 μg/ml) of LL-37 in Jurkat T leukemia cells. These findings are compatible with our data (21). Irrespective of this, we demonstrate that concentrations of LL-37 considered to be physiologically relevant during lung inflammation (10–30 μg/ml) induce minimal apoptosis in human airway epithelial cell lines and primary cells, in the absence of infection. This result suggests that under normal physiological conditions, LL-37 on epithelial surfaces would not be damaging.

In contrast to the effects of LL-37 alone, cells infected with *P. aeruginosa* demonstrated an enhanced susceptibility to the induction of apoptosis upon exposure to concentrations of LL-37 that had no effect alone, but not to control scrambled LL-37 peptide. This effect comprised a pronounced synergistic increase in mitochondrial depolarization, cytochrome *c* release, and DNA fragmentation, and was at least partly Bax-independent. In addition, the LL-37-mediated activation of caspase-3 and caspase-9 was evident only in infected cells, demonstrating activation of the intrinsic pathway of apoptosis. Although *P. aeruginosa* infection alone has been shown to induce extrinsic pathways of apoptosis via CD95/CD95L (12), we saw no activation of caspase-8 and no significant cell death in response to bacteria alone in our system. This finding may relate to the fairly low MOI used, and the timeframe examined in our studies, suggesting that the LL-37-mediated induction of apoptosis in infected epithelial cells is a much earlier (and mechanistically distinct) form of cell death compared with previously described, bacterially induced death receptor-mediated apoptosis. The intrinsic pathway of apoptosis is a mitochondrial-dependent mechanism of caspase activation involving cytochrome *c*-induced oligomerization of the cytosolic apoptotic protease activating factor-1 (Apaf-1), which recruits and activates procaspase-9, an upstream activator of effector caspases, such as caspase-3 (39). In addition, the mitochondrial release of Smac/DIABLO (second mitochondrial activator of caspases/direct IAP binding protein with low PI) (40) and Omi (also known as high temperature requirement factor A2 [HtrA2]) (41) leads to an inactivation of the inhibitor-of-apoptosis proteins (IAPs) that normally inhibit caspase activity. The increased apoptosis observed via TUNEL assay in infected cells exposed to LL-37 could be inhibited by the polycaspase in-

Figure 6. Synergistic induction of apoptosis by LL-37 and *P. aeruginosa* requires epithelial-cell internalization of bacteria. Human bronchial epithelial cells (16HBE14o⁻) were incubated for 60 minutes in Ultrosor G serum-substitute supplemented media, in the presence and absence of (MOI 10:1) log-phase *P. aeruginosa* strains PAO1, Δ mexAB-oprM mutant (A–C), isogenic PAO1 control strain (B), or Δ mexAB-oprM mutant added concurrently with sterile conditioned supernatant collected from 16HBE14o⁻ cells infected with PAO1 (C). (A) Invasion of epithelial cells by bacteria was determined by gentamicin exclusion, quantifying the number of viable CFUs surviving extracellular gentamicin treatment (50 μ g/ml). Data are plotted as mean values $\pm$ SEM, for $n = 3$ independent experiments plated in duplicate for each condition. (B, C) Infected epithelial cells were concurrently incubated with a range of concentrations of LL-37, and mitochondrial membrane depolarization was determined using Mitocapture dye, as described in MATERIALS AND METHODS. Data represent mean values $\pm$ SEM, for $n = 3$ independent experiments for each condition. Two-way ANOVAs were performed to evaluate significance, with Bonferroni *post hoc* tests comparing (B) LL-37/ Δ mexAB-oprM mutant to LL-37/isogenic controls, and (C) LL-37/ Δ mexAB-oprM mutant to LL-37/ Δ mexAB-oprM mutant in PAO1-conditioned supernatant. ** $P \leq 0.01$, *** $P \leq 0.001$.

inhibitor Z-VAD-FMK, reducing it to levels similar to those induced by LL-37 alone. Thus the synergistic effects are caspase-dependent, and occur in addition to predominantly caspase-independent pathways induced by higher concentrations of LL-37 alone. The caspase inhibition by IAPs may be reduced in infected cells, and although XIAP levels were unaffected, the roles of Smac/DIABLO and Omi HtrA2 in this system remain unknown. Therefore, a caspase-dependent pathway downstream of mitochondrial depolarization, induced by an alternate mechanism from that used by LL-37 alone, is responsible for the capacity of LL-37 to promote the apoptosis of cells infected with *P. aeruginosa*.

The nature of the interaction between epithelial cells and bacteria required to make these cells susceptible to the apoptosis-inducing effects of LL-37 was investigated under a number of conditions and using mutants of *P. aeruginosa*. Neither dead bacteria nor soluble products produced by untreated or LL-37-treated bacteria could promote these synergistic effects. In the absence of physical contact between the epithelial cells and live bacteria, no effects were observed. In contrast, the effect of LL-37 was even more profound when a clinical strain of *P. aeruginosa* J1386 (isolated from an individual with cystic fibrosis) (23) was used, suggesting that this effect might be modified by isolate variation in virulence factors. PAO1 is classified as an “invasive” rather than “cytotoxic” strain of *P. aeruginosa* (although both can invade eukaryotic cells), and this invasiveness is proposed to require contact between bacteria and epithelial cells to stimulate the efflux of bacterial “invasive factors” (28). The Δ mexAB-oprM deletion mutant of *P. aeruginosa* PAO1 (24) is defective in terms of epithelial-cell invasion (despite normal adherence), and has diminished virulence *in vivo* as a consequence of the loss of the MexAB-OprM efflux system, proposed to be responsible for the efflux of these putative “invasive factors” (28). A synergistic induction of apoptosis was not evident in LL-37-treated epithelial cells infected with this mutant strain, but could be replicated by the addition of these unknown “invasive factors” from the isogenic wild-type PAO1 strain, demonstrating a requirement for invasiveness. In contrast, the PAO1exsA:: Ω mutant (25), in which the ExsA mutation impairs the ExsA-regulated type III secretion system, behaved identically to its isogenic wild-type PAO1 strain. Although a functional ExsA allele is required for *P. aeruginosa*-induced cytotoxicity, epithelial-cell invasiveness is independent of ExsA expression (42). Similarly, a *P. aeruginosa* pilA mutant (26) was largely able to synergize with LL-37 to induce apoptosis as effectively as its isogenic PAO1 wild-type strain. In this strain, *pilA* mutation results in an absence of pilus, proposed to be an important adhesin involved early in epithelial-cell interactions with *P. aeruginosa* (43). Interestingly, differences were observed in the sensitivity to the LL-37-induced mitochondrial depolarization of cells infected with our original PAO1 isolate, compared with isogenic controls for some of the mutants used. Additional investigations using these isolates may help in further defining the key events involved in this interaction. Nevertheless, the data suggest that the bacterial invasion of airway epithelial cells, but not ExsA-regulated type III secretion or pili expression, is critical in inducing enhanced susceptibility to LL-37-mediated apoptosis.

Our results describe a novel innate inflammomodulatory role for LL-37, preferentially inducing the apoptosis of infected epithelial cells. However, the extent to which this might contribute to innate epithelial defenses, or be manifest in pathologic damage to epithelial-barrier integrity, is unknown, and a fine balance could exist. Although LL-37 clearly has important roles in innate host defense against infection, chronically increased hCAP-18/LL-37 concentrations in cystic fibrosis

lung disease are correlated with increased lung damage (34), and elevated hCAP-18/LL-37 concentrations are associated with bronchiolitis obliterans syndrome (44) and the pathogenesis of psoriasis (45). Pulmonary epithelial-cell apoptosis plays a significant role in *P. aeruginosa* clearance from the murine lung (12). In addition, bladder epithelial-cell exfoliation after bacterial attachment plays a role in innate defense against invasive *Escherichia coli* (14), preventing the establishment of a safe niche and intracellular biofilm-like growth (46). Furthermore, the susceptibility of individuals with cystic fibrosis to pulmonary *P. aeruginosa* infection is proposed to relate, in part, to the failure of airway epithelial cells to internalize this bacterium, and thus an inability to clear *P. aeruginosa* by desquamation of infected cells (27). Thus, we propose that in the healthy host, LL-37, up-regulated during infection and inflammation, may promote the apoptosis and consequent clearance of *P. aeruginosa*-infected airway epithelial cells, as a component of the innate host defense against this pathogen. However, under pathologic conditions of excessive, chronic LL-37 exposure, or a failure of epithelial-cell internalization of *P. aeruginosa* (such as in cystic fibrosis), the epithelial-cell death induced by high concentrations of LL-37 alone may be detrimental to the host and contribute to chronic lung damage. The extent to which this effect might be common to other invasive bacteria, or else specific to *P. aeruginosa*, remains to be determined, but has clear significance for the possible use of LL-37 and related CHDPs as antimicrobial therapeutics.

Author Disclosure: P.G.B. has received sponsored grants from Asthma UK (\$1,001–\$5,000). J.R.W.G. has received compensation from the Transave Corp. for consultancies (\$5,001–\$10,000), has served on the board of Bayer (up to \$1,000), and has received industry-sponsored grants from Transave Corp (more than \$100,001) and from the Cystic Fibrosis Trust (more than \$100,001). A.J.S. received lecture fees from GlaxoSmithKline (up to \$1,000). None of the other authors has a financial relationship with a commercial entity that has an interest in the subject of this manuscript.

Acknowledgments: The authors thank Dieter Gruenert and the University of California, San Francisco, Department of Laboratory Medicine, for 16HBE14o⁺ cells; Keith Poole, Dara Frank, and Eva Lorenz for *P. aeruginosa* strains; and Robert Morgan, Manjeet Bains, Ivan Villanueva, Cathy Doherty, Alan Brown, Hsin-Ni Li, Adriano Rossi, Ian Dransfield, Simon Brown, Kev Dhaliwal, Olga Lucia Moncayo Nieto, Andy Conway Morris, Mark Marsden, Fiona Rossi, Sharon Hannah, and Sarah Fox for advice and assistance.

References

- Zanetti M. Cathelicidins, multifunctional peptides of the innate immunity. *J Leukoc Biol* 2004;75:39–48.
- Bowdish DM, Davidson DJ, Hancock REW. Immunomodulatory properties of defensins and cathelicidins. *Curr Top Microbiol Immunol* 2006;306:27–66.
- Putsep K, Carlsson G, Boman HG, Andersson M. Deficiency of antibacterial peptides in patients with morbus Kostmann: an observation study. *Lancet* 2002;360:1144–1149.
- Schauber J, Gallo RL. Antimicrobial peptides and the skin immune defense system. *J Allergy Clin Immunol* 2008;122:261–266.
- Nizet V, Ohtake T, Lauth X, Trowbridge J, Rudisill J, Dorschner RA, Pestonjama V, Piraino J, Huttner K, Gallo RL. Innate antimicrobial peptide protects the skin from invasive bacterial infection. *Nature* 2001;414:454–457.
- Iimura M, Gallo RL, Hase K, Miyamoto Y, Eckmann L, Kagnoff MF. Cathelicidin mediates innate intestinal defense against colonization with epithelial adherent bacterial pathogens. *J Immunol* 2005;174:4901–4907.
- Chromek M, Slamova Z, Bergman P, Kovacs L, Podracka L, Ehren I, Hofkelt T, Gudmundsson GH, Gallo RL, Agerberth B, et al. The antimicrobial peptide cathelicidin protects the urinary tract against invasive bacterial infection. *Nat Med* 2006;12:636–641.
- Huang LC, Reins RY, Gallo RL, McDermott AM. Cathelicidin-deficient (*cnlp* ^{-/-}) mice show increased susceptibility to *Pseudomonas aeruginosa* keratitis. *Invest Ophthalmol Vis Sci* 2007;48:4498–4508.
- Bals R, Weiner DJ, Mosconi AD, Meegalla RL, Wilson JM. Augmentation of innate host defense by expression of a cathelicidin antimicrobial peptide. *Infect Immun* 1999;67:6084–6089.
- Davidson DJ, Currie AJ, Speert DP. *Pseudomonas aeruginosa* infections in individuals with cystic fibrosis: North American perspective. In: Hauser A, Rello J, editors. Severe infections caused by *Pseudomonas aeruginosa*. Norwell: Kluwer Academic Publishers; 2003. pp. 71–89.
- Bowdish DM, Davidson DJ, Lau YE, Lee K, Scott MG, Hancock REW. Impact of LL-37 on anti-infective immunity. *J Leukoc Biol* 2005;77:451–459.
- Grassme H, Kirschnek S, Riethmueller J, Riehle A, von Kurthy G, Lang F, Weller M, Gulbins E. CD95/CD95 ligand interactions on epithelial cells in host defense to *Pseudomonas aeruginosa*. *Science* 2000;290:527–530.
- Cannon CL, Kowalski MP, Stopak KS, Pier GB. *Pseudomonas aeruginosa*-induced apoptosis is defective in respiratory epithelial cells expressing mutant cystic fibrosis transmembrane conductance regulator. *Am J Respir Cell Mol Biol* 2003;29:188–197.
- Mulvey MA, Lopez-Boado YS, Wilson CL, Roth R, Parks WC, Heuser J, Hultgren SJ. Induction and evasion of host defenses by type 1-piliated uropathogenic *Escherichia coli*. *Science* 1998;282:1494–1497.
- Barlow PG, Li Y, Wilkinson TS, Bowdish DM, Lau YE, Cosseau C, Haslett C, Simpson AJ, Hancock REW, Davidson DJ. The human cationic host defense peptide LL-37 mediates contrasting effects on apoptotic pathways in different primary cells of the innate immune system. *J Leukoc Biol* 2006;80:509–520.
- Nagaoka I, Tamura H, Hirata M. An antimicrobial cathelicidin peptide, human CAP18/LL-37, suppresses neutrophil apoptosis via the activation of formyl-peptide receptor-like 1 and P2X₇. *J Immunol* 2006;176:3044–3052.
- Lau YE, Bowdish DM, Cosseau C, Hancock REW, Davidson DJ. Apoptosis of airway epithelial cells: human serum sensitive induction by the cathelicidin LL-37. *Am J Respir Cell Mol Biol* 2006;34:399–409.
- Aarbiou J, Tjabringa GS, Verhoosel RM, Ninaber DK, White SR, Peltenburg LT, Rabe KF, Hiemstra PS. Mechanisms of cell death induced by the neutrophil antimicrobial peptides alpha-defensins and LL-37. *Inflamm Res* 2006;55:119–127.
- Zhang Z, Cherryholmes G, Shively JE. Neutrophil secondary necrosis is induced by LL-37 derived from cathelicidin. *J Leukoc Biol* 2008;84:780–788.
- Björstad Å, Askarieh G, Brown KL, Christenson K, Forsman H, Onnheim K, Li HN, Teneberg S, Maier O, Hoekstra D, et al. The host defence peptide LL-37 selectively permeabilises apoptotic leukocytes. *Antimicrob Agents Chemother* 2009;53:1027–1038.
- Mader JS, Mookherjee N, Hancock REW, Bleackley RC. The human host defense peptide LL-37 induces apoptosis in a calpain- and apoptosis-inducing factor-dependent manner involving Bax activity. *Mol Cancer Res* 2009;7:689–702.
- Gough M, Hancock REW, Kelly NM. Antiendotoxin activity of cationic peptide antimicrobial agents. *Infect Immun* 1996;64:4922–4927.
- Govan JR, Nelson JW. Microbiology of lung infection in cystic fibrosis. *Br Med Bull* 1992;48:912–930.
- Li XZ, Zhang L, Srikumar R, Poole K. Beta-lactamase inhibitors are substrates for the multidrug efflux pumps of *Pseudomonas aeruginosa*. *Antimicrob Agents Chemother* 1998;42:399–403.
- Frank DW, Nair G, Schweizer HP. Construction and characterization of chromosomal insertional mutations of the *Pseudomonas aeruginosa* exoenzyme S trans-regulatory locus. *Infect Immun* 1994;62:554–563.
- Lorenz E, Chemotti DC, Vandal K, Tessier PA. Toll-like receptor 2 represses nonpilus adhesion-induced signaling in acute infections with the *Pseudomonas aeruginosa* pila mutant. *Infect Immun* 2004;72:4561–4569.
- Pier GB, Grout M, Zaidi TS. Cystic fibrosis transmembrane conductance regulator is an epithelial cell receptor for clearance of *Pseudomonas aeruginosa* from the lung. *Proc Natl Acad Sci USA* 1997;94:12088–12093.
- Hirakata Y, Srikumar R, Poole K, Gotoh N, Suematsu T, Kohno S, Kamihira S, Hancock REW, Speert DP. Multidrug efflux systems play an important role in the invasiveness of *Pseudomonas aeruginosa*. *J Exp Med* 2002;196:109–118.
- Liu PT, Stenger S, Li H, Wenzel L, Tan BH, Krutzik SR, Ochoa MT, Schaubert J, Wu K, Meinken C, et al. Toll-like receptor triggering of a vitamin D-mediated human antimicrobial response. *Science* 2006;311:1770–1773.
- Martineau AR, Wilkinson KA, Newton SM, Floto RA, Norman AW, Skolimowska K, Davidson RN, Sorensen OE, Kampmann B, Griffiths CJ, et al. IFN- γ and TNF-independent vitamin D-inducible

- human suppression of mycobacteria: the role of cathelicidin LL-37. *J Immunol* 2007;178:7190–7198.
31. Liu PT, Schenk M, Walker VP, Dempsey PW, Kanchanapoomi M, Wheelwright M, Vazirnia A, Zhang X, Steinmeyer A, Zugel U, *et al.* Convergence of IL-1 β and VDR activation pathways in human TLR2/1-induced antimicrobial responses. *PLoS One* 2009; 4:e5810.
32. Rosenberger CM, Gallo RL, Finlay BB. Interplay between anti-bacterial effectors: a macrophage antimicrobial peptide impairs intracellular salmonella replication. *Proc Natl Acad Sci USA* 2004;101: 2422–2427.
33. Schaller-Bals S, Schulze A, Bals R. Increased levels of antimicrobial peptides in tracheal aspirates of newborn infants during infection. *Am J Respir Crit Care Med* 2002;165:992–995.
34. Chen CI, Schaller-Bals S, Paul KP, Wahn U, Bals R. Beta-defensins and LL-37 in bronchoalveolar lavage fluid of patients with cystic fibrosis. *J Cyst Fibros* 2004;3:45–50.
35. Scott MG, Dullaghan E, Mookherjee N, Glavas N, Waldbrook M, Thompson A, Wang A, Lee K, Doria S, Hamill P, *et al.* An anti-infective peptide that selectively modulates the innate immune response. *Nat Biotechnol* 2007;25:465–472.
36. Risso A, Braidot E, Sordano MC, Vianello A, Macri F, Skerlavaj B, Zanetti M, Gennaro R, Bernardi P. BMAP-28, an antibiotic peptide of innate immunity, induces cell death through opening of the mitochondrial permeability transition pore. *Mol Cell Biol* 2002;22: 1926–1935.
37. Sawada M, Hayes P, Matsuyama S. Cytoprotective membrane-permeable peptides designed from the BAX-binding domain of KU70. *Nat Cell Biol* 2003;5:352–357.
38. Martinou JC, Desagher S, Antonsson B. Cytochrome c release from mitochondria: all or nothing. *Nat Cell Biol* 2000;2:E41–E43.
39. Riedl SJ, Salvesen GS. The apoptosome: signalling platform of cell death. *Natl Rev* 2007;8:405–413.
40. Verhagen AM, Ekert PG, Pakusch M, Silke J, Connolly LM, Reid GE, Moritz RL, Simpson RJ, Vaux DL. Identification of DIABLO, a mammalian protein that promotes apoptosis by binding to and antagonizing IAP proteins. *Cell* 2000;102:43–53.
41. Suzuki Y, Imai Y, Nakayama H, Takahashi K, Takio K, Takahashi R. A serine protease, HTRA2, is released from the mitochondria and interacts with XIAP, inducing cell death. *Mol Cell* 2001;8: 613–621.
42. Fleiszig SM, Wiener-Kronish JP, Miyazaki H, Vallas V, Mostov KE, Kanada D, Sawa T, Yen TS, Frank DW. *Pseudomonas aeruginosa*-mediated cytotoxicity and invasion correlate with distinct genotypes at the loci encoding exoenzyme S. *Infect Immun* 1997;65: 579–586.
43. Hahn HP. The type-4 pilus is the major virulence-associated adhesin of *Pseudomonas aeruginosa*—a review. *Gene* 1997;192:99–108.
44. Anderson RL, Hiemstra PS, Ward C, Forrest IA, Murphy D, Proud D, Lordan J, Corris PA, Fisher AJ. Antimicrobial peptides in lung transplant recipients with bronchiolitis obliterans syndrome. *Eur Respir J* 2008;32:670–677.
45. Lande R, Gregorio J, Facchinetti V, Chatterjee B, Wang YH, Homey B, Cao W, Wang YH, Su B, Nestle FO, *et al.* Plasmacytoid dendritic cells sense self-DNA coupled with antimicrobial peptide. *Nature* 2007;449: 564–569.
46. Anderson GG, Palermo JJ, Schilling JD, Roth R, Heuser J, Hultgren SJ. Intracellular bacterial biofilm-like pods in urinary tract infections. *Science* 2003;301:105–107.