Cathelicidin LL-37 Increases Lung Epithelial Cell Stiffness, Decreases Transepithelial Permeability, and Prevents Epithelial Invasion by Pseudomonas aeruginosa

Fitzroy J. Byfield, Marek Kowalski, Katrina Cruz, Katarzyna Leszczynska, Andrzej Namiot, Paul B. Savage, Robert Bucki and Paul A. Janmey

J Immunol 2011; 187:6402-6409; Prepublished online 16 November 2011;
doi: 10.4049/jimmunol.1102185
http://www.jimmunol.org/content/187/12/6402

References
This article cites 57 articles, 30 of which you can access for free at:
http://www.jimmunol.org/content/187/12/6402.full#ref-list-1

Subscription
Information about subscribing to The Journal of Immunology is online at:
http://jimmunol.org/subscription

Permissions
Submit copyright permission requests at:
http://www.aai.org/About/Publications/JI/copyright.html

Email Alerts
Receive free email-alerts when new articles cite this article. Sign up at:
http://jimmunol.org/alerts
Cathelicidin LL-37 Increases Lung Epithelial Cell Stiffness, Decreases Transepithelial Permeability, and Prevents Epithelial Invasion by *Pseudomonas aeruginosa*

Fitzroy J. Byfield,*† Marek Kowalski,*† Katrina Cruz,*† Katarzyna Leszczyńska,‡ Andrzej Namiot,§ Paul B. Savage,¶ Robert Bucki,*† and Paul A. Janmey*†

In addition to its antibacterial activity, the cathelicidin-derived LL-37 peptide induces multiple immunomodulatory effects on host cells. Atomic force microscopy, F-actin staining with phalloidin, passage of FITC-conjugated dextran through a monolayer of lung epithelial cells, and assessment of bacterial outgrowth from cells subjected to *Pseudomonas aeruginosa* infection were used to determine LL-37’s effect on epithelial cell mechanical properties, permeability, and bacteria uptake. A concentration-dependent increase in stiffness and F-actin content in the cortical region of A549 cells and primary human lung epithelial cells was observed after treatment with LL-37 (0.5-5 μM), sphingosine 1-phosphate (1 μM), or LPS (1 μg/ml) or infection with PAO1 bacteria. Other cationic peptides, such as RK-31, KR-20, or WLBU2, and the antibacterial cationic steroid CSA-13 did not reproduce the effect of LL-37. A549 cell pretreatment with WRW4, an antagonist of the transmembrane formyl peptide receptor-like 1 protein attenuated LL-37’s ability to increase cell stiffness. The LL-37-mediated increase in cell stiffness was accompanied by a decrease in permeability and *P. aeruginosa* uptake by a confluent monolayer of polarized normal human bronchial epithelial cells. These results suggested that the antibacterial effect of LL-37 involves an LL-37-dependent increase in cell stiffness that prevents epithelial invasion by bacteria. The Journal of Immunology, 2011, 187: 6402–6409.

*Catonic antibacterial peptides (CAPs) play an important role in innate host defense, particularly by preventing bacterial infections in the eye and at mucosal surfaces that form a shield between host and the external environment. In humans, this function is typically mediated by defensins and cathelicidins, which are produced primarily by neutrophils and epithelial cells. Both of these peptide agents have the capacity to kill various microorganisms (1–3). The only known cathelicidin peptide expressed in humans is LL-37, a 37-aa C-terminal domain of human (h)CAP-18 (4). The importance of cathelicidins as an effective defense against pathogenic microorganisms is illustrated by the high occurrence of periodontal disease in patients with severe congenital neutropenia accompanied by a deficiency of LL-37 in saliva (5), as well as an animal study showing severe bacterial skin infections in mice lacking murine cathelicidin-related CAPs (6). Overexpression of hCAP-18 after pulmonary gene transfer in mice results in increased *Pseudomonas aeruginosa* clearance in the course of pulmonary challenge (7). The spectrum of LL-37 activities includes many other immunomodulatory effects, such as chemotactic activation of monocytes, neutrophils, and T cells mediated through the G protein-coupled formyl peptide receptor-like 1 (8); airway epithelial cell IL-8 production, proliferation, and wound closure mediated by the epidermal growth factor receptor (9, 10); and possibly IL-1β processing and release via direct activation of P2X7 receptors (11). The cellular mechanisms and signaling pathways involved in the multiple functions of LL-37 are of interest because they might provide new strategies for antibacterial and immunomodulatory drug development (12, 13).

*P. aeruginosa* is a major opportunistic pathogen causing chronic pneumonia in immunocompromised patients during prolonged hospitalization, particularly in patients who are ventilated and those who are neutropenic, and is responsible for lethal pulmonary infection in most cystic fibrosis (CF) subjects. It is generally assumed that *P. aeruginosa*-induced lung inflammation involves binding to airway cells and subsequent direct and indirect cytotoxic effects (14, 15). One possible result of bacteria–lung epithelial cell interaction is bacterial internalization (16), although the mechanism of entry and the role of intracellular organisms in the epithelial life cycle remain unclear (17–21). It was proposed that bacterial uptake might result in desquamation of infected cells and bacteria clearance (14), intracellular survival of bacteria without killing the host cells, and host cell death through bacteria-mediated cytotoxicity (18). In contrast, the outcome of *P. aeruginosa* internalization might also depend on lung epithelial cell apoptosis likely induced via the CD95/CD95 ligand system (22), with involvement of the CF transmembrane conductance regulator (20). It was recently reported that apoptosis of infected epithelial cells targets *P. aeruginosa* into apoptotic bodies, which are subsequently internalized and digested by surrounding cells (20). This mechanism might be defective in CF, permitting *P. aeruginosa*...
internalization without epithelial cell apoptosis, resulting in intracellular bacteria survival, division, and escape from the host immune system (22). Internalization and activation of apoptosis might also depend on the growth phase of *P. aeruginosa* (only organisms in mid-growth phase interact with epithelial cells) (23) and the strain type (19, 24, 25). Generally, clinical isolates of *P. aeruginosa* exhibit both invasive and cytotoxic phenotypes. Invasion and cytotoxicity occur as independent events, but both involve host cell protein tyrosine kinase activity. Invasion requires both Src family tyrosine kinases and calcium-calmodulin activity (24, 26).

In this article, we report that LL-37 induces polymerization of cortical actin in lung epithelial cells and leads to an increase in cell stiffness and a decrease in epithelial permeability. These effects might contribute to an observed decrease in *P. aeruginosa* internalization.

**Materials and Methods**

**Materials**

LL-37 (LGDFFKRKSKIEKFKVRQIKDFLRNLVYPTES), RK-31 (RKKSKIEKFKVRQIKDFLRNLVYPTES), KR-20 (KRIVQIKDFLRNLVYPTES), and WLB2U (RWRVVRVRWVRYVVRVYVVR) peptides were purchased from Peprotech (Chantilly, VA). FITC-dextran (average molecular mass, 4 kDa), ATP, periodate-oxidized sodium salt (ox-ATP) (Aldrich), and *P. aeruginosa* LPS from serotype 10 (Escherichia coli LPS) were purchased from Sigma-Aldrich (St. Louis, MO). *Pseudomonas* isolation agar (PIA), Luria–Bertani broth, and trypsin. Trypsinized and seeded at a density of 2 × 10^5 cells/ml (Bronchial epithelial growth media without triiodothyronine supplemented with 0.2% Triton X-100 for 5 min at room temperature. After visualization of the actin, the fixed and permeabilized cells were stained with 2 µM rhodamine-labeled phallolidin (Invitrogen) in PBS for 30 min. For NHBEs, the polarized monolayer was stained with anti-actin antibody (Ab) (1:1000) (Abcam, Cambridge, MA). The assessment of hICAP-18/LL-37 expression in A549 cells was performed by immunostaining both nonpermeabilized and permeabilized samples (0.2% Triton X-100). A549 cells treated with exogenous LL-37 (2 µM) were used as a positive control. The presence of mucin on the surface of NHBEs was evaluated using staining with Texas Red-conjugated lectin (data not shown). Fluorescence was imaged using a LEICA DM IRBE microscope.

**Extraction and gel electrophoresis analysis of F-actin**

A549 cells were grown to confluence in six-well plates, with each experimental condition performed in duplicate wells. After 48 h, cells were treated with various concentrations of LL-37 and WLB2U for 30 min. After two washes with ice-cold PBS, the cells were lysed by adding 300 µl/well of a 1% Triton X-100 solution containing 20 mM Tris-HCl (pH 7.5), 150 mM NaCl, 1 mM Na 2EDTA, 1 mM EGTA, 2.5 mM sodium pyrophosphate, 1 mM β-glycerophosphate, 1 mM Na 3VO 4, 1 µg/ml leupeptin, and 0.1 mM PMSF and incubated on ice for 20 min. The cell lyPhC from each group were pooled and centrifuged at 14,000 rpm for 5 min, and the supernatants (600 µl) were removed. The Triton-insoluble pellets were washed with cold PBS, centrifuged again, and resuspended in 40 µl sample buffer containing 60 mM Tris (pH 8), 5% mercaptoethanol, 2% SDS, 0.0025% bromphenol blue, and 10% glycerol. All samples were boiled for 10 min, and 15 µl each sample was subjected to SDS-PAGE (10% polyacrylamide gel). Actin was identified by its molecular mass (43 kDa) by comparison with purified actin from rabbit skeletal muscle as a positive control, as well as by Western blotting using mouse anti-β-actin Ab (1:10,000 TBST dilution; Sigma-Aldrich).

**Bacteria growth**

To obtain clinical strains of *P. aeruginosa*, a 10-µl aliquot of undiluted homogenized sputum (from subjects already identified as chronically infected with nonmucoid strains of *P. aeruginosa*) was inoculated onto a single plate of PIA. *P. aeruginosa* PAO1 and clinical isolates of *P. aeruginosa* from CF sputa were maintained in TSB with glycerol (25%) at −80°C. When required, bacteria from frozen stocks were streaked on PIA plates before the experiment. Bacteria were grown in TSB in all experiments.

**Bactericidal activity of LL-37 and gentamicin**

*P. aeruginosa* grown to mid-log phase at 37°C (controlled by the evaluation of OD at 600 nm) were resuspended in DMEM. LL-37 and gentamicin activities were evaluated using a bacterial-killing assay. After 15-, 30-, 60-, 120-, and 180-min incubations of PAO1 and 180-min incubations of clinical isolates (all bacterial suspensions in DMEM), with different concentrations of antibacterial agents at 37°C, samples were placed on ice and diluted 10–1000-fold. Ten-microliter aliquots of each dilution were spotted on PIA plates each dilution was counted the following morning. The CFU/ml of the individual samples were determined from the dilution factor.

**Bacteria-internalization assays**

The gentamicin-protection assay (20, 29, 30) was used to assess *P. aeruginosa* internalization. Before infection, cells were incubated with medium alone or with medium containing LL-37 (0.5–2 µM) for 30 min. Bacteria from each strain were grown to mid-logarithmic phase, washed, resuspended in DMEM, and added to the apical cell surface of cells grown to a pyramidal tip for indentation. The spring constant of the cantilever, calibrated by resonance measurements, was typically 0.06 N/m (DNPL, Veeco). To determine epithelial cell stiffness, atomic force microscopy measurements were made on single cells by indenting on three areas (1.6 µm) of the periphery of the cell body ~30 min after agonist addition. The average elastic modulus for each condition was calculated by averaging the measurements for each cell, following by averaging all obtained values.

**F-actin, acetylated α-tubulin, mucin, and hCAP-18/LL-37 staining**

A549 cells after treatment with various reagents or a non-treated confluent monolayer of human lung polarized epithelial cells were washed three times with PBS, fixed and incubated in 4% paraformaldehyde (Sigma-Aldrich) for 10 min at room temperature, and followed by a wash with PBS. Cells were then permeabilized with 0.2% Triton X-100 for 5 min at room temperature. To visualize F-actin, the fixed and permeabilized cells were stained with a 1:1000 solution of rhodamine-labeled phallolidin (Invitrogen) in PBS for 30 min. For NHBEs, the polarized monolayer was stained with anti-actin antibody (Ab) (1:1000) (Abcam, Cambridge, MA). The assessment of hICAP-18/LL-37 expression in A549 cells was performed by immunostaining both nonpermeabilized and permeabilized samples (0.2% Triton X-100). A549 cells treated with exogenous LL-37 (2 µM) were used as a positive control. The presence of mucin on the surface of NHBEs was evaluated using staining with Texas Red-conjugated lectin (data not shown). Fluorescence was imaged using a LEICA DM IRBE microscope.
LL-37 decreases P. aeruginosa cellular uptake

We recently observed an increase in cell stiffness after treating bovine aortic endothelial cells and mouse aortic endothelium with LL-37 (31). Similarly, human A549 and primary lung epithelial cells responded to LL-37 treatment with a concentration-dependent increase in their stiffness (Fig. 1). The elastic modulus of resting A549 cells was very close to that of primary lung epithelial cells (Fig. 1A, 1B). LL-37 increased A549 cell stiffness ∼30% more compared with the effects of truncated LL-37 moieties, such as RK-30 and KR-20, and ∼85% higher compared with the longer peptide WLB2, which in several experimental conditions showed stronger bactericidal activity than LL-37 (32, 33) (Fig. 1C). S1P, a bioactive lipid involved in regulation of epithelial permeability was shown in previous studies to increase cellular stiffness (31, 34). In this study, treatment of A549 cells with S1P for 30 min at 1 μM concentration resulted in an ∼1.7-fold increase in elastic modulus (data not shown), similar to the magnitude of stiffening induced by 5 μM LL-37 (Fig. 1A). CSA-13, a membrane-active, antibacterial cationic steroid had no effect on A549 cell stiffness at concentrations up to 2 μM. Twenty minutes after addition of P. aeruginosa PAO1 bacteria (10^7 CFU/ml) to A549 cells, we observed a statistically significant increase in epithelial cell stiffness, similar to the effect of purified P. aeruginosa LPS at a concentration of 1 μg/ml (Fig. 1D). Stiffening of A549 cells was also observed when LL-37 was added together with PAO1 bacteria (Fig. 1D). In contrast, purified LPS (0.1–1 μg/ml) decreased LL-37’s ability (2 μM) to induce A549 cell stiffening (Fig. 1E). This finding is in agreement with several studies showing inactivation of the antibacterial function of LL-37 by LPS (35–39). It is interesting to note that the elastic modulus of A549 cells treated with a premixed solution of LPS and LL-37 is significantly lower than the elastic modulus of cells treated with either agonist separately. This observation indicates a mutual inactivation of cell-stiffening capabilities of LPS and LL-37 upon their interaction.

The ability of LL-37 to induce endothelial cell stiffening in the presence of serum at a concentration that effectively prevents LL-37 bacterial killing (37) suggests that LL-37’s effects might be mediated by activation of plasma membrane receptors (31), rather than less specific interactions that mediate bacteria killing. A role for specific transmembrane proteins in the effect of LL-37 on epithelial cells is also supported by experiments showing inhibition of the stiffness increase in A549 cells subjected to LL-37 activation after treatment with the formyl peptide receptor-like 1 receptor antagonist WRW4 or the P2X7 receptor antagonist oxATP (Fig. 1F). The effect of the FMLP1 antagonist was much stronger than the limited effect of ox-ATP and the lack of inhibition by suramin, a weak antagonist of the P2X7 receptor.

LL-37 expression and F-actin organization in A549 cells

Immunofluorescence studies did not reveal a strong expression of hCAP-18/LL-37 in A549 cells (Fig. 2A) or polarized HBECs (data not shown). Positive staining with anti-LL-37 Ab was observed in A549 cells pretreated with 2 μM LL-37 for 30 min. This staining was not present when A549 cells were subjected to permeabilization before Ab addition. This result suggests that most of the LL-37 distributes to plasma membrane compartments, and its direct association with intracellular structures is unlikely. However, we observed an increase in cortical F-actin content in single and confluent A549 cells after the addition of LL-37. This observation provides a mechanism that can explain, at least in part, the LL-37-induced increase in cell stiffness. The intensity of F-actin staining in LL-37-stimulated cells (Fig. 2B) was similar to that observed after N-formylated peptide (formyl-methionyl-leucyl-phenylalanine) activation (data not shown). In ∼10% of a population of A549 cells (Fig. 2B), as well as in primary lung epithelial cells (data not shown), LL-37 induced plasma membrane bleb formation. Remodeling of cytosolic F-actin after LL-37 addition was accompanied by an increase in total F-actin concentration (Fig. 2C). Quantitative densitometry revealed that the phalloidin-stainable F-actin concentration in A549 cells increased ∼50% after the addition of 1–2 μM LL-37.

LL-37 decreases HBEC monolayer permeability

Polarized cell cultures of HBECs express cilia, as indicated by imaging of fluorescence after staining with an anti-acetylated α-tubulin Ab (Fig. 3A). In agreement with previous studies showing that endothelial cell stiffness can be linked to permeability changes after treatment with barrier-protective agonists (40), we observed a decrease in monolayer permeability after treatment with LL-37 (Fig. 3B) that coincided with an increase in lung epithelial cell stiffness (Fig. 1B). The effect on permeability occurred hours after addition of LL-37, reaching statistical significance within 6 h and increasing at 18 h. In contrast, addition of PAO1 had little or no effect on permeability until 18 h, at which time permeability was increased. There was an ∼10%, but not statistically significant, decrease in HBEC permeability following a 3-h infection with PAO1 bacteria. This observation could be explained by the initial localization of bacteria predominantly in the valleys formed at cell–cell junctions (data not shown), which could inhibit the passage of FITC-dextran. However, this early event of PAO1 bacterial infection is transitional. This strain pro-
duces several virulence factors, such as rhamnolipids, exotoxin A, LasA, B proteases, and ExoY, which are able to disrupt intercellular tight junctions (41), causing an increase in permeability in the later phase. Indeed, at 18 h we observed a significant increase in permeability of NHBE monolayers subjected to PAO1 infection compared with a noninfected monolayer. At the 18-h data point, we also observed a significantly lower fluorescence of FITC-dextran in the lower chamber for LL-37–treated wells, indicating that treatment with LL-37 results in persistent inhibition of polarized NHBE permeability (Fig. 3B).

LL-37 prevents uptake of PAO1 bacteria by A549 and NHBEs

With the use of the gentamicin-protection assay, we determined the number of internalized bacteria following infection of A549 cells with *P. aeruginosa* PAO1 and three nonmucoid *Pseudomonas* clinical isolates obtained from CF sputum. Because reduced internalization of PAO1 by A549 in the presence of LL-37 might be due to bactericidal effects of LL-37 prior to bacterial entry into the A549 cell, internalization of PAO1 was quantified in an experimental setting in which cells were treated with LL-37 for 30 min and then washed before bacteria addition, as well as when LL-37 was kept in the medium during the 3-h period of infection. The data in Fig. 4 show that LL-37, at concentrations up to 10 M, had no killing activity in DMEM against PAO1 bacteria or three clinical isolates of *P. aeruginosa* (data not shown) diluted in DMEM. Therefore, reduced PAO1 outgrowth after A549 cell lysis in this assay reflected reduced prior entry of the bacteria into the A549 cells.

At 3 h postinfection, PAO1 internalization by A549 cells was reduced upon pretreatment with LL-37 compared with infected cells without LL-37 treatment (Fig. 5A). Fig. 5B shows that 1 M LL-37 significantly decreased the uptake of these clinical isolates.
by A549 cells and primary lung epithelial cells, but had no effect on bacteria outgrowth in medium alone (Fig. 4).

There was a quantitative difference in susceptibility to *Pseudomonas* invasion between A549 cells and primary lung epithelial cells, likely resulting from differences in experimental settings. To observe invasion of polarized primary lung epithelial cells, protected by a mucin layer covering their apical surface and likely containing natural antibacterial agents expressed by fully differentiated NHBEs, the number of administered bacteria required was higher (∼10⁷ CFU) than the concentration needed to infect nonpolarized A549 cultures.

We also examined the effect of bacterial internalization on host cell viability by measurement of LDH concentration in the tissue culture medium. This analysis revealed no change between the LDH levels in medium collected from infected and noninfected cells 3 h postinfection (data not shown). This result is in agreement with a previous report showing a lack of PAO1 toxicity following infection of CFBE41o cells up to 24 h postinfection (20).

**Discussion**

Numerous studies concluded that human cathelicidin LL-37 peptide functions as an important mediator of innate immunity.
In addition to its antibacterial activity, LL-37 possesses several functions that are related to host defense, such as chemotactic, endotoxin-neutralizing, angiogenic, and wound-healing activities (3, 42, 43). This study showed that LL-37’s ability to induce an increase in lung epithelial F-actin content and cellular stiffness provides a possible explanation for its role in preventing epithelial invasion by pathogens and adding new insight into its pleiotropic nature. Together with its potential to preferentially promote apoptosis in the infected airway epithelium (44), LL-37’s ability to increase epithelial stiffness and decrease permeability in response to bacterial infection may serve as a first line of defense to prevent bacterial invasion. The effect of LL-37 on epithelial cells, together with its ability to neutralize LPS, might also provide an explanation for the finding that lung injury in guinea pigs subjected to i.v. endotoxin was significantly attenuated by treatment with a 32-aa C-terminal fragment of rabbit CAP18 (45).

Previous studies showed that P. aeruginosa strains, including PAO1, can survive in the cytoplasm of epithelial cells and suggest different mechanisms leading to bacterial uptake (20, 30, 46). At least one pathway of bacterial internalization is an actin-dependent endocytic process, as suggested by its inhibition by cytochalasin D (20). However, it is not clear how the different mechanisms resulting in Pseudomonas internalization are switched on, how such a decision is established at the level of bacteria and host cells, and which structures govern this process. It is plausible that modulation of cellular actin dynamics, either by an increase or decrease in F-actin content, can inhibit internalization of P. aeruginosa by the epithelium. Our findings support a model in which P. aeruginosa internalization can infect epithelial cells through more than one pathway (47), including targeting molecules associated with lipid rafts (48, 49), such as Lyn (50) and caveolin-2 (51). LL-37’s ability to interact with lipid rafts is suggested by its high surface activity (52), the caveolae-independent membrane raft-dependent endocytosis of an LL-37/DNA plasmid complex (53), and LL-37-mediated/raft-dependent LPS internalization (54). Because LL-37 activates multiple plasma receptors, and the cellular mechanical response to agonists depends on the receptor engaged (55), it is also possible that the LL-37-mediated increase in F-actin concentration results in the formation of distinct cytoskeletal networks with mechanical properties that affect bacterial invasion.

LL-37 appears to have a general, but integral, effect on the strengthening of epithelial barriers to invading pathogens in various compartments of the human body that is separate from, but complementary to, its bactericidal activity. It could be inferred from our results that during an acute or chronic bacterial infection of the respiratory tract, LL-37 functions to increase the stiffness of the epithelium, which correlates with and might be required to decrease epithelial permeability and reduce bacterial entry. A recent study by Wagh et al. (56) showed a complex distribution of cell stiffness at the site of an epithelial wound, with a decreased stiffness at the very edge and a significant increase observed 10–20 μm away from the leading edge compared with cells >2.75 mm away from the wound. It is tempting to hypothesize that LL-37-induced changes in actin assembly and cell stiffness play a role in its reported ability to accelerate epithelial wound healing (10, 57) at the site of infections.

Exposure of lung epithelial cells to PAO1 bacteria and LPS also resulted in a significant increase in their elastic modulus. This effect could indicate that the natural response of lung epithelium is to increase its stiffness in an attempt to prevent bacterial entry during infection, and the increased presence of LL-37 is able to preemptively induce and bolster this stiffening response. Alternately, LL-37 is able to bind to LPS, which could also result in a reduction in proinflammatory stimuli (39). However, this interaction might also result in a gradual functional sequestration of LL-37 during the course of the infection, depending on the relative amounts of LPS and LL-37. A better understanding of the complex interactions among LL-37, lung epithelial cells, and pathogen bacteria, such as P. aeruginosa, and its products, such as LPS, might enable more effective therapeutic strategies during respiratory tract infections.
Acknowledgments
We thank Melissa Mendez, Qi Wen, and Marianne Ferrin for assistance and patients of the Adult Cystic Fibrosis Center of the University of Pennsylvania for providing sputum samples.

Disclosures
The authors have no financial conflicts of interest.

References


