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Citrullination Alters Immunomodulatory Function of LL-37 Essential for Prevention of Endotoxin-Induced Sepsis

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Cathelicidin LL-37 plays an essential role in innate immunity by killing invading microorganisms and regulating the inflammatory response. These activities depend on the cationic character of the peptide, which is conferred by arginine and lysine residues. At inflammatory foci in vivo, LL-37 is exposed to peptidyl arginine deiminase (PAD), an enzyme released by inflammatory cells. Therefore, we hypothesized that PAD-mediated citrullination of the arginine residues within LL-37 will abrogate its immunomodulatory functions. We found that, when citrullinated, LL-37 was at least 40 times less efficient at neutralizing the proinflammatory activity of LPS due to a marked decrease in its affinity for endotoxin. Also, the ability of citrullinated LL-37 to quench macrophage responses to lipoteichoic acid and poly(I:C) signaling via TLR2 and TLR3, respectively, was significantly reduced. Furthermore, in stark contrast to native LL-37, the modified peptide completely lost the ability to prevent morbidity and mortality in a mouse model of β-galactosamine-sensitized endotoxin shock. In fact, administration of citrullinated LL-37 plus endotoxin actually exacerbated sepsis due to the inability of LL-37 to neutralize LPS and the subsequent enhancement of systemic inflammation due to increased serum levels of IL-6. Importantly, serum from septic mice showed increased PAD activity, which strongly correlated with the level of citrullination, indicating that PAD-driven protein modification occurs in vivo. Because LL-37 is a potential treatment for sepsis, its administration should be preceded by a careful analysis to ensure that the citrullinated peptide is not generated in treated patients. The Journal of Immunology, 2014, 192: 5363–5372.

Host defense peptides are evolutionarily ancient components of the innate immune system and act as effector molecules in host defense against pathogens (1). Various families of such peptides have been identified in different mammalian species, including ∼30 cathelicidins, although humans express only 1. Human cationic protein of 18 kDa (2) is expressed in epithelial tissues and myeloid cells (3, 4). Thirty-seven C-terminal amino acid residues of human cationic protein of 18 kDa comprise a cationic host defense peptide (LL-37), which has a broad spectrum of antimicrobial activity (5). In addition to its direct microbicidal role, LL-37 mediates other effects, including cytotoxicity, chemotaxis, epithelial cell activation, angiogenesis, and epithelial wound repair (4, 6–8). Moreover, LL-37 is a potent regulator of innate immunity because it strongly modulates the responses of myeloid cells to pathogen-associated molecular patterns (9). The positively charged amphipathic peptide, LL-37 (10, 11), interacts with the negatively charged LPS molecule, thereby inhibiting the binding of LPS to CD14+ cells. Thus, LPS-induced cytokine production is reduced (12). Inhibiting LPS is critical for abrogating the deleterious effects of certain infections, including intra-abdominal sepsis and endotoxemia (13). Because the biological activity of LL-37 is dictated by its charge (it is cationic), hydrophobicity, and amphipathicity (14, 15), it is anticipated that a change in these parameters will affect the physiological functions of the peptide. Such modifications can be caused by citrullination of the Arg residues within LL-37 by peptidylarginine deiminases (PAD), enzymes present alongside the peptide at infected/inflammatory sites.

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The online version of this article contains supplemental material.

Abbreviations used in this article: CRP, C-reactive protein; hMDM, human monocyte-derived macrophage; LTA, lipoteichoic acid; MALP, macrophage-activating lipopeptide; NET, neutrophil extracellular trap; OAS, overall assessment score; PAD, peptidyl arginine deiminase; PEST, penicillin-streptomyycin.

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Significantly, citrullinated chemokines were identified in inflamed tissues in vivo, indicating the involvement of PAD2 and/or PAD4, which are expressed in myeloid cells (23, 24).

LL-37 contains five Arg residues and is therefore easily citrullinated in vitro by PAD2 (25). Because LL-37 is released in large amounts in response to inflammatory stimuli, it is highly plausible that it is a good substrate for PADs, which are secreted into the inflammatory milieu. However, little is known about how citrullination affects those biological activities of LL-37 that are relevant to its role in regulating innate immune responses to TLR agonists. To address this question, we examined how citrullinated LL-37 affected the interaction between LPS and human monocyte-derived macrophages (hMDMs) or the murine macrophage cell line, RAW 264.7. We found that citrullinated LL-37 was unable to block the LPS-mediated activation of inflammatory macrophages. This was because citrullination led to a marked reduction in the affinity of LL-37 for LPS. This allowed LPS to bind CD14 on the macrophage cell surface and stimulate the expression of proinflammatory mediators. Citrullination of LL-37 also reduced its anti-inflammatory activity against other TLR agonists and host inflammatory mediators. Consistent with this, we found that citrullination of LL-37 abrogated its ability to prevent endotoxic shock in a mouse model.

Materials and Methods
Reagents
Gentamicin, endotoxin (LPS from Escherichia coli O26:B6), lipoteichoic acid (LTA), poly(I:C), and Griess reagents were from Sigma-Aldrich (St. Louis, MO). FBS, RPMI 1640, DMEM, calcium- and magnesium-free PBS (without Ca\(^{2+}\) and Mg\(^{2+}\)), penicillin-streptomycin (PEST), and lymphocyte separation medium were obtained from PAA. Flagellin, R848, and Pam3CSK4 were purchased from Enzo Life Science. Macrophage-activating lipopeptide (MALP)-2 was obtained from Imgenex (San Diego, CA). The purity of TLR agonists was estimated for 95% flagellin (SDS-PAGE), 80% LPS, 95% MALP-2 (HPLC), 99% poly(I:C) (TLC), and 97% LTA, according to the manufacturer’s statement. All agonists, except LPS, were endotoxin free. Recombinant human PAD2 and PAD4 were obtained from Modiquest.

Peptide synthesis and purification
LL-37 and citrullinated forms of LL-37, LL-37\(_{7,29,34}\) (1 Cit), LL-37\(_{7,29,34}\) (3 Cit), and LL-37\(_{all, Cit}\) (5 Cit), were assembled using the Fmoc solid-phase peptide synthesis approach using either model 433A (Applied Biosystems, Foster City, CA) or model Liberty (CEM, Matthews, NC) automated peptide synthesizers, followed by cleavage in trifluoroacetic acid/phenol/thioanisole/ethanedithiol/water (10 ml: 0.75 g/0.5 ml/0.25 ml/0.5 ml) mixture at 25°C for 90 min; see Barlow et al. (26) for details. The peptides were purified by reversed phase HPLC (>98% purity), and their masses were confirmed by MALDI time-of-flight mass spectrometry. Following lyophilization, the peptides were obtained in the form of their trifluoroacetate salts. Stock solutions (10 mg/ml) were prepared in PBS stored in aliquots at −20°C. To confirm the purity of synthetic peptides, the Limulus amebocyte lysate test purchased from Lonza was performed. The sequences of LL-37 peptides, which were used in this work, are shown in Table I.

Citrullination by human PAD2 and PAD4
Peptides were diluted to a concentration of 1 mg/ml in PAD assay buffer (100 mM Tris HCl, 10 mM CaCl\(_2\), 5 mM DTT [pH 7.6]) and incubated with recombinant human PAD2 or PAD4 (Modiquest) at a concentration up to 23.3 U/mg peptide, for 2 h at 37°C. Citrullination was terminated by sample dilution in RPMI 1640 with 10% HBSS. Control samples were treated similarly, apart from the addition of PAD.

Cell culture
PBMCs were isolated from human blood obtained from the Red Cross (Krakow, Poland). The Red Cross deidentified blood materials as appropriate for the confidentiality assurance of human subjects. Thus, this study adheres to appropriate exclusions from the approval of human subjects. Briefly, PBMCs were isolated from EDTA-treated blood using a lymphocyte separation medium (PAA) density gradient yielding the fraction highly enriched in monocytes (90% CD14 positive), as described previously (27). Cells were plated at 3 \times 10^5/well in 24-well plates (Sarstedt) in RPMI 1640 (PAA) supplemented with 2 mM L-glutamine, 50 μg/ml gentamicin (Sigma-Aldrich), and 10% autologous human serum. After 24 h, nonadherent PBMCs were removed by washing with complete medium, and adherent cells were differentiated to hMDMs in this medium for 7 d, with fresh medium changed every 2 d. The phenotype of each batch of hMDMs was routinely controlled, after nonenzymatic detachment of cells, by immunofluorescent staining of CD14 (clone TUK4; DakoCytomation Denmark A/S, Glostrup, Denmark), CD16 (clone DiJ130c; DakoCytomation), CD11b (clone ICRF44; BD Biosciences), and CD209 (clone DCN46; BD Biosciences) and subsequent flow cytometry analysis (Supplemental Fig. 1). The cultures selected for further experiments were positive in at least 90% for the first three markers and <1% for CD209. The adherent cells acquired typical macrophage morphology, and resting (nonstimulated) cells did not produce inflammatory cytokine TNF-α or IL-6.

FIGURE 1. Citrullination abolishes the ability of LL-37 to quench the proinflammatory activity of LPS. hMDMs (A) and mouse RAW 264.7 (B) macrophages were stimulated with 10 ng/ml LPS in the presence of native or citrullinated LL-37 at the indicated concentrations (0.1–10 μg/ml). Citrullinated LL-37 was obtained by treatment of the native peptide with human PAD2 or PAD4 at 23.3 U/mg peptide. The level of TNF-α (A) and NO (B) in the culture supernatants was determined using ELISA or the Griess assay at 6 or 20 h poststimulation, respectively. Because neither the LL-37 nor the PAD enzymes alone induced the release of NO or TNF-α, for the sake of clarity these controls are not shown in the figure. Data represent the mean ± SD of three independent experiments. ns, Not significant; *p < 0.05, **p < 0.01, ***p < 0.001.
at 37°C in a humidified 5% CO2 atmosphere. Cells were passaged every 2–4 d using cell scraper.

**LPS effects on mouse macrophages in vitro**

RAW 264.7 cells (3.5 \(3\times10^5\)) were seeded in 96-well tissue culture plates in 100 \(\mu\)l phenol red-free DMEM/5% FBS/PEST. After ~6 h of incubation to permit adherence, medium was changed and cells were stimulated with 10 ng/ml LPS from *E. coli* (Sigma-Aldrich), in the presence of 0.01–0.1 \(\mu\)g/ml or absence of native LL-37, LL-37 treated with PAD2 or PAD4, or synthetic citrullinated forms of LL-37. Stimulation was performed in a total volume of 200 \(\mu\)l DMEM/10% FBS/antibiotics, in triplicates. The level of NO in culture supernatants was determined 20 h after stimulation using the Griess reaction. Nitrite (NO\(_2\)-), a stable product of NO degradation, was measured by mixing 50 \(\mu\)l culture supernatants with the same volume of Griess reagent (Sigma-Aldrich), and the absorbance at 540 nm was measured using spectrophotometer. Phenol red-free DMEM with FBS and antibiotics were used as a blank. Standard curve was prepared using 0–80 \(\mu\)M sodium nitrite solutions in dH2O.

Supernatants from stimulated RAW 264.7 cells were simultaneously analyzed for TNF-\(\alpha\) and IL-6 content by ELISA (BD Biosciences, San Jose, CA) assay, according to the manufacturer’s instructions.

**Binding of LPS to RAW 264.7 cells**

Effect of LL-37 on LPS binding to cells was determined according to the procedure described by Nagaoka et al. (12). To this end, RAW 264.7 cells were simultaneously analyzed for TNF-\(\alpha\) and IL-6 content by ELISA (BD Biosciences, San Jose, CA) assay, according to the manufacturer’s instructions.

**FIGURE 2.** The degree of impairment in the anti-inflammatory properties of LL-37 depends on the level of citrullination. The effect of citrullination on the ability of LL-37 to quench the proinflammatory effects of endotoxin was estimated by measuring NO release and IL-6 and TNF-\(\alpha\) secretion by RAW 264.7 cells and TNF-\(\alpha\) from hMDMs in the presence of absence of serum. Cells were stimulated with 10 ng/ml LPS in the presence of native LL-37 (10 \(\mu\)g/ml) or citrullinated synthetic forms of LL-37 (10 \(\mu\)g/ml; LL-37, LL-37,7,29,34, or LL-37all cit). A scrambled peptide (LL-37scrbl; comprising 37 aa residues found in LL-37 but randomly arranged) was used as a control. The NO level in the culture medium of RAW 264.7 cells at 20 h poststimulation was determined using the Griess assay (A). The amounts of IL-6 (B) and TNF-\(\alpha\) (C) secreted by RAW 264.7 cells and the amount of TNF-\(\alpha\) secreted by human macrophages (D–F) were measured by ELISA at 6 h postinduction. Because none of the LL-37 peptides alone induced the release of NO or TNF-\(\alpha\), for the sake of clarity these controls are not shown in the figure. Data represent the mean ± SD of three independent experiments. ns, Not significant; \(^*\)p < 0.05, \(^{**}\)p < 0.01, \(^{***}\)p < 0.001.

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**Table I.** The sequences of antimicrobial peptides that were used in this study

<table>
<thead>
<tr>
<th>LL-37 Peptide</th>
<th>Sequence</th>
</tr>
</thead>
<tbody>
<tr>
<td>LL-37</td>
<td>LLGDFRKSKEKIGKEFKRIVQRKDFNLVPRTES</td>
</tr>
<tr>
<td>LL-37(_{7,29,34})</td>
<td>LLGDFRKSKEKIGKEFKRIVQRKDFNLVPRTES</td>
</tr>
<tr>
<td>LL-37,7,29,34</td>
<td>LLGDFRKSKEKIGKEFKRIVQRKDFNLVPRTES</td>
</tr>
<tr>
<td>LL-37all cit</td>
<td>LLGDFRKSKEKIGKEFKRIVQRKDFNLVPRTES</td>
</tr>
<tr>
<td>LL-37scrbl</td>
<td>RLHSDTRFPFVRLANSKRLSFTEKDIKREQPQKIL</td>
</tr>
</tbody>
</table>

Citrulline residues (Cit) are bolded.
in DMEM supplemented with 5% FBS at 37°C for 15 min in the absence or presence of LL-37 or citrullinated LL-37 (LL-37

Cells were then washed twice with ice-cold PBS, and the LPS binding was analyzed by flow cytometry (FACSscan; BD Biosciences). The mean fluorescence intensity and percentage of cells labeled with Alexa488-conjugated LPS were measured in each group.

Response of macrophages to stimulation with TLR agonists

hMDMs or RAW 264.7 cells were incubated in RPMI 1640 or DMEM supplemented with 5% of serum (human serum or FBS) for various lengths of time (6 h for hMDMs, 20 h for RAW 264.7 cells) with the following TLR agonists: LPS (10 ng/ml), LTA (10 μg/ml), R848 (10 μM), Pam,C3K2 (1 μg/ml), MALP-2 (10 ng/ml), poly(I:C) (10 μg/ml), or flagellin (100 ng/ml) with or without native LL-37 or full citrullinated LL-37 (LL-37_all cit). As cell stimulation readout, we have determined the level of NO (the Griess reaction) and TNF-α (ELISA) released into media at 20 and 6 h poststimulation by RAW 264.7 and hMDM cells, respectively.

D-galactosamine–sensitized endotoxin shock model

A D-galactosamine–sensitized mouse model (28), which is highly susceptible to LPS, was used to investigate the ability of LL-37 and LL-37_all cit to suppress inflammatory reaction in vivo. Male BALB/c mice (8–10 wk, 22–25 g obtained from Jackson ImmunoResearch Laboratories) were i.p. injected with 100 g obtained from Jackson ImmunoResearch Laboratories) were i.p. injected with 100 μg D-galactosamine (1.2 mg/g, dissolved in saline) or D-galactosamine plus LPS (0.1 μg/g) with or without native or modified LL-37 (10 μg/ml). Survival rates and overall assessment score (OAS) ranging from 0 (normal) to 8 (death) were monitored every 1–2 h for first 24 h postinjection and then at 48 and 72 h later. Reduced motor activity, lethargy, shivering, and piloerection were recorded as symptoms of sepsis. Each one of these conditions was scored as 0 (no observable symptom), 1 (a noticeable symptom), and 2 (a severe symptom).

For the cytokine assay, C-reactive protein (CRP) quantification, and analysis of peritoneal inflammation, mice were sacrificed 6 h post-LPS challenge. Blood and peritoneal cells were collected by cardiac puncture and lavage, respectively. The cytokines IL-6, IL-10, MCP-1, IFN-γ, TNF-α, and IL-12p70 were measured in plasma using the cytoketric bead array mouse inflammation kit (BD Biosciences), according to the manufacturer’s instructions.

Analysis of the content of citrulline proteins and PAD activity

The citrulline presence (both free and incorporated in polypeptide chains) and PAD activity in blood serum were determined using the modified Boyde and Rahmatullah (29) method, based on the chemical modification of the citrulline side chain and colorimetric detection of the derivative. Briefly, for the activity measurement, 1 μl serum was mixed with 9 μl PBS in a 96-well microtiter plate, followed by addition of 40 μl 10 mM benzoyl-Arg-ethyl ester (Sigma-Aldrich) in 0.1 M Tris, 10 mM CaCl2, PBS in a 96-well microtiter plate, followed by addition of 40 μl 10 mM benzoyl-Arg-ethyl ester (Sigma-Aldrich) in 0.1 M Tris, 10 mM CaCl2, and 5 mM DTT. A plate was incubated for 1 h at 55°C. After incubation, the enzymatic reaction was stopped by the addition of 10 μl 5 M HClO4. The color was developed by adding 150 μl freshly prepared 1:2 mixture of solution A (0.5% diacetyl monoxime, 0.01% thiosemicarbazide in water) and B (0.25 mg/ml FeCl3, 2.4% H2SO4, 17% H3PO4 in water) to the enzymatic reaction. The OD535 nm was measured using SpectraMax microplate reader (Molecular Devices) against a sample blank. Sample blanks were incubated in parallel and contain serum and all reagents, except the substrate (benzoyl-Arg-ethyl ester). In the same time, the readout of the sample blanks against a reagent blank (samples containing all reagents, but without the substrate and serum) reflects the basal level of citrullination (free and peptidyl citrulline) in each serum sample. Free l-citrulline in the concentration range of 1–100 nmol/well was used to prepare a calibration curve. The enzymatic activity was defined as production of 1 μmol citrulline within 1-h incubation at 55°C.

Ethics statement

All studies were performed in accordance with European Union regulations for the handling and use of laboratory animals. The protocols were approved by the institutional Animal Care and Use Committees (Jagiellonian University, Krakow, Poland; Permit 11/2013).

Statistical analyses

Statistical comparisons were performed with Prism 4.0 software (GraphPad), using two-tailed Student t test for comparisons of two data sets and ANOVA for multiple comparisons. The survival curve was analyzed using the Mantel–Cox test. A p value <0.05 was considered to be significant.

Results

The in vitro citrullination of LL-37 by PAD2 and PAD4 abrogates its anti-inflammatory properties

The cationic character of LL-37 facilitates electrostatic interactions with negatively charged molecules, such as the anionic lipid A domain of endotoxin (12). LL-37 binds to LPS and prevents it from binding to receptors expressed by monocytes and macrophages, a process that initiates inflammatory responses. Therefore, we tested whether the conversion of cationic Arg residues to neutral peptidyl citrulline residues, a reaction catalyzed by PADS, influences the anti-inflammatory properties of LL-37. hMDMs and RAW 264.7 cells were stimulated for 6 and 20 h with LPS (10 ng/ml) in the presence of either native LL-37 or the PAD2- or PAD4-treated peptide. As shown in Fig. 1, preincubating LL-37 with PAD2 or PAD4 removed its ability to neutralize LPS and quench the release of TNF-α (Fig. 1A) and NO (Fig. 1B) by macrophages. Neither of the PADS alone, nor LL-37 alone, had any effect on TNF-α and NO production at the concentrations used in this work.

The degree to which the ability of LL-37 to quench the proinflammatory activity of LPS is impaired depends on the level of citrullination

Depending on the incubation time and enzyme concentration, deimination of the Arg residues within LL-37 generates different levels of citrullination as shown by amino acid sequence analysis of the peptide incubated with PAD2, PAD4, and rabbit PAD (Supplemental Fig. 2). In concordance with observation by Kilsgaard...
et al. (25), Arg residues in position 7 were the most susceptible to citrullination, followed by Arg7,29 and Arg34. Because the level of citrullination may variably affect the ability of the peptide to neutralize LPS, we verified whether abrogating of the anti-inflammatory activity of LL-37 depends on the degree of citrullination. To this end, we stimulated RAW 264.7 cells with LPS alone (10 ng/ml) or with LPS in the presence of native LL-37 or peptides harboring different numbers of citrullinated Arg residues, as follows: Arg7Cit (LL-37Cit), Arg7,29,34Cit (LL-377,29,34Cit), or Arg7,19,23,29,34Cit (LL-377,29,34Cit) (Table I). We found that both LL-37 and LL-37Cit completely quenched NO release (Fig. 2A) and inhibited TNF-α and IL-6 secretion (Fig. 2B, 2C) by LPS-stimulated murine macrophages. By contrast, LL-377,29,34Cit and LL-37allCit only partially blocked the proinflammatory activity of LPS. The strongest ablation of LPS-neutralizing activity was observed with the fully citrullinated peptide (Fig. 2A). A scrambled peptide (LL-37scr), which was used as a control, showed no effect on LPS-mediated secretion of proinflammatory factors. Complete citrullination of LL-37 also attenuated its ability to interfere with the LPS-stimulated secretion of TNF-α by hMDMs (Fig. 2D). The effect was more significant in the presence of autologous serum (Fig. 2E) and was also observed in the presence of serum obtained from human AB blood (Fig. 2F). This suggests that posttranslational modification by PADs can ablate the anti-inflammatory activity of LL-37 against human cells. To further investigate effect of citrullination on LL-37 functions, we used the fully citrullinated form of the peptide (LL-377,29,34Cit).

Citrullination affects the ability of LL-37 to inhibit endotoxin-mediated cell stimulation

To examine the different abilities of native and modified LL-37 to quench the proinflammatory activity of LPS, we measured the inhibition (IC50) of LPS-induced macrophage stimulation. hMDMs or RAW 264.7 cells were stimulated with either LPS alone or with LPS plus different concentrations of native LL-37 or LL-37allCit. LL-37 strongly inhibited endotoxin-mediated TNF-α or NO secretion in a dose-dependent manner (IC50 = 5.21 μg for hMDMs, and IC50 = 3.4 μg for RAW 264.7 cells) (Fig. 3). By contrast, the citrullinated peptide was far less effective (IC50 = 25.4 μg for hMDMs, and IC50 = 143 μg) (Fig. 3). Even at very high nonphysiological concentrations of LL-37allCit (200 μg/ml = 44.4 μM), the level of NO release by control cells was still 36%. Taken together, these data indicate that, compared with the native peptide, citrullinated LL-37 is at least 5 (in case of hMDMs) or 40 (in case of RAW 264.7) times less potent at inhibiting LPS-mediated macrophage stimulation.

Citrullination impairs the ability of LL-37 to prevent LPS binding to the cell surface

The reduced ability of LL-37 to block the proinflammatory activity of LPS appears to be related to a reduction in the affinity of the citrullinated peptide for endotoxin; thus, endotoxin can still interact with cell surface receptors (12). We next examined the effects of LL-37allCit on the binding of FITC-conjugated LPS to CD14+ RAW 264.7 cells by flow cytometry. When used alone, FITC-LPS clearly bound to the cell surface. This interaction was not affected by low concentrations of LL-37 (0.4 μg/ml), but was inhibited by 2 μg/ml LL-37 and was almost completely blocked by 10 μg/ml LL-37 (Fig. 4A). Conversely, the binding of FITC-LPS was only slightly inhibited by the citrullinated peptide (Fig. 4A). Even at the highest concentration tested (10 μg/ml LL-37allCit), 75% of cells were FITC-LPS+. By contrast, when cells were incubated with FITC-LPS in the presence of native LL-37, only 7.5% cells were positive (Fig. 4B). Taken together, these results reveal the mechanism underlying the observed reduction in the anti-inflammatory activity of citrullinated LL-37.

Citrullination abolishes the ability of LL-37 to prevent the mortality and morbidity associated with septic shock

Neutralization of LPS by LL-37 prevents the activation of CD14/TLR-4–expressing cells and protects mice from endotoxic shock. We used the well-established d-galactosamine–sensitized mouse
model (28) to examine the ability of citrullinated LL-37 to prevent the lethal effects of LPS. Intraperitoneal injection of β-galactosamine sensitized mice to the lethal effects of LPS; indeed, 90% of the sensitized mice died within 17 h postinjection of LPS (0.1 μg/g) (Fig. 5A). However, the administration of LL-37 (10 μg/g) completely prevented LPS-induced mortality (100% of animals survived). By stark contrast, LL-37all cit provided no protection at all, and all animals were dead within 16 h p.i. (Fig. 5A). An analysis of the OAS at 6 and 10 h postinfection revealed a significantly greater deterioration in the health of animals injected with LPS plus LL-37all cit than with LPS alone (Fig. 5B). There were no significant differences between these two groups of mice at later time points. Remarkably, animals cojected with native peptide (10 μg/g) showed no signs of morbidity (according to the OAS). The OAS for mice injected with LPS and native LL-37 was very low and not significantly different from that for control animals (mice injected with native LL-37 alone or with vehicle [β-galactosamine] alone) over the 16-h study period (Fig. 5B).

Examination of the peritoneal cavity revealed a significant increase in inflammatory cell infiltration into the peritoneum of animals treated with LPS plus LL-37 compared with that in animals injected with LPS plus LL-37all cit. In the latter group, the number of

![Figure 5. Citrullinated LL-37 increases the susceptibility of mice to endotoxic shock. β-galactosamine (1.2 mg/g)-sensitized BALB/c mice were injected i.p. with LPS (0.1 μg/g) without or with LL-37 or LL-37all cit (10 μg/g). Mice injected with β-galactosamine alone or with each LL-37 peptide alone were used as controls. The survival rate and OAS were estimated at 6–17 h postinjection. Data are expressed as the mean value for each group (n = 8–10/mice group). (A) Mortality is expressed as a percentage. The mortality in the control groups was zero. The survival of mice injected with LPS plus LL-37 or with LPS plus LL-37all cit was compared. Survival statistics were calculated using the Mantel–Cox test. **p < 0.01 (n = 10 mice per tested group). (B) OAS expressed as the mean ± SEM (n = 10 mice per tested group). In a separate experiment, mice were sacrificed 6 h after i.p. injection of LPS and/or the LL-37 peptides or buffer. Peritoneal lavage was then performed, and blood samples were collected (n = 8 per group). (C) Influx of inflammatory cells into the peritoneum. Data are expressed as the mean ± SEM; ***p < 0.001 (n = 10 per group). (D) The levels of CRP in mouse serum. Data are expressed as the mean ± SEM. *p < 0.05 (n = 10 per group). (E) Serum levels of TNF-α, IL-6, IL-10, MCP-1, IL-12p70, and IFN-γ measured using a cytokine bead array system. Values were compared between animals injected with LPS, LPS/LL-37, or LPS/LL-37all cit. The levels of each individual cytokine in the control mice are illustrated by the dotted gray lines. Data are expressed as the mean ± SEM. ns, Not significant; *p < 0.05, **p < 0.01, ***p < 0.001 (n = 10 per group).]
inflammatory cells was similar to that in nontreated controls and mice injected with LPS alone (Fig. 5C).

Serum analyses revealed that animals injected with LPS showed a significant increase in the levels of CRP (Fig. 5D) and five different cytokines (Fig. 5E) compared with control mice (the mean level of cytokines in control animals is marked by dotted gray lines). The exception was IL-12p70, which showed no difference in blood concentration between the groups. Importantly, the injection of LPS plus native LL-37 led to a significant reduction in CPR and cytokine levels. This was not observed upon injection of LPS plus the citrullinated peptide, clearly indicating the lack of any ability to prevent LPS-induced increases in CRP, IL-6, IFN-γ, and IL-10 (Fig. 5D, 5E). The results clearly demonstrate that citrullination of LL-37 abrogates its antiendotoxic effects in vivo, which are maintained both locally and systemically by the native peptide (13).

Both PAD activity and the level of citrullination are higher in mouse serum under septic conditions

The data we have gathered to date strongly argue that citrullination of LL-37 alters the immunomodulatory function of the peptide, which is essential for prevention of endotoxin-induced sepsis. To further support this theory, we next determined the levels of PAD activity and the content of citrulline (both free and encompassed in proteins/peptides) in mouse serum. We found that serum from mice exposed to endotoxin showed significantly ($p < 0.01$) higher PAD activity than that from healthy control animals (Fig. 6A). Consistent with this, the level of citrullination in serum collected from septic mice was markedly higher than that in control mice (Fig. 6B), and the levels of both showed a positive reciprocal correlation (Fig. 6C). Collectively, these data clearly show that the increases in PAD activity and in the amount of citrullinated proteins/peptides in the blood are associated with sepsis.

Citrullination abolishes the ability of LL-37 to downregulate cellular responses to TLR ligands and host inflammatory mediators

In addition to the effects of LPS, LL-37 also suppresses the proinflammatory effects of other bacterial TLR ligands, such as LTA, and those of endogenous inflammatory mediators, including IFN-γ (30). Therefore, we next investigated the effects of LL-37 citrullination on cellular responses to TLR agonists and IFN-γ. Human macrophages, which are sensitive to all types of TLR agonist (31), were treated with seven different TLR agonists in the presence of native and citrullinated LL-37, and the secretion of TNF-α was measured. We found that citrullination inhibited the anti-inflammatory effects of LL-37 against LTA and poly(I:C) (Fig. 7A) but had no effect on signaling elicited by other agonists. Moreover, compared with the native peptide, the ability of LL-37$_{ul,p70}$ to inhibit the production of TNF-α by hMDMs and NO by RAW 264.7 macrophages stimulated with IFN-γ alone or with IFN-γ plus LPS was markedly inhibited (Fig. 7B, 7C). Taken together, these findings suggest that the citrullination of LL-37 not only eliminates its antiendotoxic activity but also prevents it from neutralizing other inflammatory mediators.

Discussion

The large numbers of neutrophils that infiltrate a site of infection constitute the main source of cathelicidin LL-37, an important component of the host defense system. Apart from its direct bactericidal activity, LL-37 released from neutrophils (along with that locally produced by activated epithelial cells) effectively blocks the inflammatory response induced upon exposure to microbial Ags and viable pathogens (6, 13, 32). However, both the bactericidal and immunomodulatory effects of LL-37 are absent from a synthetic LL-37 peptide in which the Arg residues are replaced by citrulline residues (25). In this study, we showed that citrullination of LL-37 by human PAD2 and PAD4, which are coexpressed at sites of inflammation, completely inhibited the ability of LL-37 to neutralize the proinflammatory activity of this ubiquitous pathogen-associated molecular pattern. These findings were confirmed using synthetic citrullinated LL-37 peptides. In contrast to the study by Kilsgård et al. (25), we found that partially citrullinated LL-37 showed a significant decrease in immunomodulatory activity. This may be of physiological significance because it is plausible that several citrullinated forms of LL-37 (harboring different levels of citrullination) are generated in vivo.

In contrast to the native peptide, citrullinated LL-37 was unable to block the LPS-stimulated release of NO and TNF-α by macrophages (Fig. 2). Quantitative analysis revealed that modified
LL-37 was at least 40 times less potent at inhibiting LPS-mediated macrophage activation (Fig. 3). One may speculate that this could be compensated for by increasing the concentration of the modified peptide; however, this is unlikely, because citrullinated LL-37 was significantly less effective at inhibiting LPS than the native peptide even when used at a concentration of 200 μg/ml. Furthermore, such a high concentration of LL-37 is toxic to some cells (e.g., neutrophils) (11) and is not expected to occur in vivo. To summarize, our data indicate that citrullinated LL-37 (at physiological concentrations) is unable to prevent the LPS-induced activation of macrophages.

The mechanism underlying the reduced ability of citrullinated LL-37 to neutralize LPS was examined using the CD14⁺ murine macrophage cell line RAW264.7. Flow cytometry analysis revealed that, in contrast to the native peptide, citrullinated LL-37 was significantly less efficient at blocking the binding of FITC-conjugated LPS to RAW264.7 cells (Fig. 4). This appears to be due to its markedly reduced affinity for LPS. Such a mechanism agrees with the results of the in vitro experiments; however, we must bear in mind that, in contrast to direct chemotactic activity of LL-37, which is clearly compromised by citrullination (Fig. 5C), Taken together, the data presented in this study show that the ability of the citrullinated LL-37 to control inflammatory reaction is significantly altered (Fig. 5C–E) and may even contribute to severity of experimental sepsis (Fig. 5A, 5B).

Citrullinated LL-37 is most likely generated within the inflammatory milieu, which is rich in neutrophil extracellular traps (NETs) (33). In this study, the peptide is exposed to neutrophil-derived PAD4, which catalyzes citrullination of histones essential for NET formation (34). Colocalization of histones and LL-37 on DNA strings (35) argues that NETs can be a source of citrullinated LL-37 at least in endotoxemia and sepsis in mice, conditions associated with abundant NETosis within the liver sinusoids (36). We anticipated that such extensive NETosis as well as cells dying of necrosis and apoptosis might serve as both a local and systematic source of PADs, which can also citrullinate proteins in the inflammatory milieu outside NETs. Indeed, we found increased levels of PAD activity and citrulline in the serum of septic mice compared with control animals. Importantly, the PAD activity showed a strong correlation with the level of citrullination in the serum (Fig. 6). Taken together, these data argue that extracellular PAD activity in septic mice citrullinates not only proteins/peptides associated with the NETs structure but also other proteins in the circulation, thereby affecting their biological activity (23, 24).

The results showing that injection of LL-37 in contrast to the native peptide cannot protect against the endotoxin-induced sepsis in mice may explain a puzzling clinical observation, as follows: rather than protecting patients from sepsis-induced death, increased levels of AMPs exacerbate the condition (37, 38). It is likely that, in such patients, LL-37 is citrullinated by PADs released from neutrophils. To verify this hypothesis, the levels of citrullinated LL-37 need to be measured in septic patients; unfortunately, there is no reliable method of quantifying (or even
detecting) citrullinated LL-37. Significantly, in stark contrast to other neutrophil-derived peptides/protiens, including α-defensins, lactoferrin, and heparin-binding protein (all of which are elevated in sepsis patients), the level of LL-37 (as detected by ELISA) remains unchanged (39). In this context, it is tempting to speculate that a significant amount of LL-37 is citrullinated and is, therefore, not recognized by the mAbs used in the ELISA. This idea is supported by our observation that citrullination of LL-37 prevents detection by mAbs in Western blotting and dot-blot assays. Taken together, the data suggest that it is very likely that the blood concentrations of native LL-37 versus citrullinated LL-37 may be a potential diagnostic biomarker for human sepsis.

The immunomodulatory role of LL-37 is not limited to its interaction with LPS. The peptide also exerts a strong influence on cellular signaling pathways triggered by other TLR ligands and host inflammatory mediators such as TNF-α and IFN-γ. LL-37 inhibits the release of proinflammatory cytokines by human monocycte cells stimulated with TLR2 and TLR4 agonists, modulates the activation of dendritic cells by TLR ligands (32), and affects cytokine expression mediated by TLR-2, -3, -4 and -7 signaling in human gingival fibroblasts (40). Most of these activities will likely be lost after citrullination. In this study, we showed that citrullination reduced the ability of LL-37 to quench the signaling pathways triggered by LTA, poly(I:C), and IFN-γ. LL-37 will likely be lost after citrullination. In this study, we showed that citrullination reduced the ability of LL-37 to quench the signaling pathways triggered by LTA, poly(I:C), and IFN-γ (Fig. 7). These data clearly indicate that the citrullination of LL-37 affects cellular responses to a broad range of microbial structural components and host-derived inflammatory mediators. Therefore, citrullination of LL-37 may also be relevant to the pathogenicity of sepsis caused by Gram-positive bacteria and fungi.

Disclosures

The authors have no financial conflicts of interest.

References


Fig. S1. *Analysis of surface markers typical for macrophages using flow cytometry.* Immunofluorescent staining of hMDMs using CD14 (clone: TUK4, DakoCytomation Denmark A/S, Glostrup, Denmark), CD16 (clone: DJ130c, DakoCytomation), CD11b (clone: ICRF44, Becton Dickinson and Co., Franklin Lakes, USA), and CD209 (clone: DCN46, Becton Dickinson) and subsequent flow cytometry analysis. The cultures selected for further experiments were positive in at least 90% for the first three markers and less than 1% for CD209. Courtesy of Dr. Krzysztof Guzik (Jagiellonian University).
Fig. S2. The degree of citrullination for individual Arg residues in LL-37 treated with PAD2, PAD4 and rabbit PAD. Citrullination was quantitated by amino acid sequence analysis using automated Edman degradation. The figure illustrates detection of citrulline and arginine in LL-37 incubated with PAD4 in cycle 7, 19, 23, 29 and 34. In addition an overlay of cycles is shown. The same analysis was performed for LL-37 treated with PAD2 and rabbit PAD (rPAD). Integration of Cit and Arg peaks allowed for calculation of the percentage of modification of individual Arg residues in PPD-treated LL-37 (Table).