LL-37 Opsonizes and Inhibits Biofilm Formation of *Aggregatibacter actinomycetemcomitans* at Subbactericidal Concentrations

Asaf Sol,^a Ofir Ginesin,^a Stella Chaushu,^b Laila Karra,^c Shunit Copenhagen-Glazer,^a Isaac Ginsburg,^a Gilad Bachrach^a

Institute of Dental Sciences^a and Department of Orthodontics,^b The Hebrew University—Hadassah School of Dental Medicine, and Department of Pharmacology and Experimental Therapeutics, Institute for Drug Research, School of Pharmacy, Faculty of Medicine, The Hebrew University of Jerusalem,^c Jerusalem, Israel

Host defense peptides are immediate responders of the innate immunity that express antimicrobial, immunoregulatory, and wound-healing activities. Neutrophils are a major source for oral host defense peptides, and phagocytosis by neutrophils is a major mechanism for bacterial clearance in the gingival tissue. Dysfunction of or reduction in the numbers of neutrophils or deficiency in the LL-37 host defense peptide was each previously linked with proliferation of oral *Aggregatibacter actinomycetemcomitans* which resulted in an aggressive periodontal disease. Surprisingly, *A. actinomycetemcomitans* shows resistance to high concentrations of LL-37. In this study, we demonstrated that submicrocidal concentrations of LL-37 inhibit biofilm formation by *A. actinomycetemcomitans* and act as opsonins and agglutinins that greatly enhance its clearance by neutrophils and macrophages. Improved uptake of *A. actinomycetemcomitans* by neutrophils was mediated by their opsonization with LL-37. Enhanced phagocytosis and killing of *A. actinomycetemcomitans* by murine macrophage-like RAW 264.7 cells were dependent on their preagglutination by LL-37. Although *A. actinomycetemcomitans* is resistant to the bactericidal effect of LL-37, our results offer a rationale for the epidemiological association between LL-37 deficiency and the expansion of oral *A. actinomycetemcomitans* and indicate a possible therapeutic use of cationic peptides for host defense.

Antimicrobial peptides are positively charged amphipathic components of the innate immunity in insects, vertebrates, and humans that mediate a broad range of antimicrobial activity (1). Their production is induced by injury or microbial burden, and their microbial targets include the outer and inner membranes and cytoplasmic components. In mammals, apart from their direct microbicidal activity, they act as multifunctional effectors that elicit cellular processes to promote anti-infective and tissue repair responses (2). Since the recognition of their immunoregulatory functions, antimicrobial peptides have been referred to as alarmins (3) or host defense peptides (HDPs), and their protective immunomodulatory activities are being tested as a novel therapeutic approach (4).

Apart from protection against systemic and skin pathogens (5–7) and against lung infections (8), HDPs also maintain a balance in the oral microflora (1, 9, 10). The oral HDPs include α - and β -defensins, histatins, and the cathelicidin LL-37 (11–13).

Periodontitis, the primary cause of tooth loss after the age of 35 (14), is a common disease (15) that involves damage to the tooth-supporting tissue. Periodontal disease results largely from an inappropriate immune response to dysbiotic communities in bacterial biofilms at subgingival sites (16–19).

Dysfunction of neutrophils or reduction in neutrophil numbers was previously associated with the outgrowth of the periodontopathogenic *Aggregatibacter actinomycetemcomitans* and with the appearance of an aggressive, rapidly progressing periodontal disease (20, 21). Neutrophils are a major source for LL-37 (22). The importance of oral LL-37 has been shown in patients with Kostmann syndrome treated with granulocyte colony-stimulating factor (G-CSF) and in patients with Papillon-Lefevre syndrome. Patients with morbus Kostmann suffer from severe congenital neutropenia. Treatment with recombinant G-CSF restores their levels of neutrophils. However, despite treatment with G-CSF, these patients remain deficient in LL-37 and α -defensin HNP-1 and develop severe periodontal disease (9). Patients suf-

fering from Papillon-Lefevre syndrome lack LL-37 because of an inherited deficiency in serine proteinases that activate LL-37 by cleaving it from its hCAP-18 precursor, and similarly to individuals with Kostmann syndrome, they suffer from severe periodontal disease (23). The aggressive periodontal disease that develops in LL-37-deficient individuals with morbus Kostmann or Papillon-Lefevre syndrome is believed to be promoted by an overgrowth of *A. actinomycetemcomitans* (9, 23).

Surprisingly, although a lack of LL-37 was correlated with disease caused by *A. actinomycetemcomitans* overgrowth, this bacterium does not appear to be sensitive to LL-37 when tested *in vitro* (24). We therefore searched for additional mechanisms by which LL-37 might control the growth of *A. actinomycetemcomitans* in the oral cavity.

Phagocytosis by neutrophils is a major host defense mechanism for bacterial clearance in the space between the tooth and the surrounding gingival tissue (called gingival sulcus) (25–27). *A. actinomycetemcomitans*, a Gram-negative capnophilic microorganism (28, 29), can evade neutrophils by utilizing its 116-kDa pore-forming leukotoxin, which lyses human neutrophils and monocytes that express the β 2-integrin lymphocyte function-associated molecule 1 (LFA-1) on their surface (30–33). Leukotoxin

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Address correspondence to Gilad Bachrach, giladba@ekmd.huji.ac.il.

A.S. and O.G. contributed equally to this article.

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production varies among *A. actinomycetemcomitans* strains. The JP2 genotype produces large amounts of leukotoxin due to a 530-bp mutational deletion in the promoter region of the *lkt/ltx* gene, which encodes leukotoxin (34). Strains of this genotype were associated with aggressive periodontitis in subjects of African origin (35, 36).

Several reports regarding the susceptibility of *A. actinomycetemcomitans* to neutrophils have been contradictory. Some reported efficient phagocytosis and killing (37), while others found complement-mediated phagocytosis of *A. actinomycetemcomitans* to be generally inefficient and uptake of antibody-opsonized bacteria to result in the rapid cell death of neutrophils (38). Oral *A. actinomycetemcomitans* strains were divided into seven serotypes, a, b, c, d, e, f, and g (39, 40). Increased resistance to phagocytic killing has been shown for serotype b strains. This increased resistance was reduced by mutations preventing the formation of the serotype b-specific polysaccharide antigen (41).

Cationic proteins, including the full-length hCAP-18 precursor of LL-37, were previously shown to be capable of acting as opsonins that enhance phagocytosis of systemic pathogens by macrophages and monocytes (42–45). LL-37 at low concentrations was shown to inhibit biofilm formation by *Pseudomonas aeruginosa* (46). Here we show that at subbactericidal concentrations, LL-37 eliminates *A. actinomycetemcomitans* by inhibition of biofilm formation and by opsonization.

MATERIALS AND METHODS

Peptides. LL-37 (LLGDFFRKSKEKIGKEFKRIVQRIKDFLRNLVPRTES), tetramethylrhodamine-labeled LL-37 (TMR-LL-37), scrambled LL-37 (sLL-37) (GLKLRFEFKSKIGEFKLTPEVRFDRDIKLKDNRSVQR), and 6-carboxyfluorescein (6-FAM)-labeled scrambled LL-37 were purchased from Genemed Synthesis Inc. (San Antonio, TX). The peptides were purified by high-pressure liquid chromatography (HPLC), and purity (greater than 90%) was determined by mass spectrometry.

Bacterial strains, cell lines, and growth conditions. *Aggregatibacter actinomycetemcomitans* ATCC 29523, JP2, and Y4 were cultured in 0.5% yeast extract, 1.5% Bacto tryptone, 0.75% D-glucose, 0.25% NaCl, 0.075% L-cysteine, 0.05% sodium thioglycolate, and 4% NaHCO₃. Strains VT726S, VT726S Ltx[−] (a kind gift of D. Galli), and ATCC 33384 (a kind gift of U. K. Gursoy) (38) were cultured in tryptic soy broth supplemented with 0.6% yeast extract (TSBYE medium). Kanamycin (100 µg/ml) was added for growth of the leukotoxin-deficient strain VT726S Ltx[−]. *A. actinomycetemcomitans* was grown at 37°C in 5% CO₂ or under anaerobic conditions, as specified. *Escherichia coli* ATCC 25922 was grown in LB (Difco, MD, USA) under aerobic conditions. Bacterial purity was determined by phase-contrast microscopy and by Gram staining. The RAW 264.7 murine macrophage cell line (kindly supplied by G. Nussbaum) was grown as described before (47) in Dulbecco modified Eagle medium (DMEM) supplemented with 10% fetal calf serum, 2 mM L-glutamine, and penicillin and streptomycin (both at 100 µg/ml). All cell growth reagents were purchased from Biological Industries (Kibbutz Beit Haemek, Israel).

Growth inhibition by LL-37. A microdilution assay was used. Portions (150 µl) containing approximately 1×10^5 (for strains JP2, ATCC 29523, and Y4) or 1×10^6 (for strains VT726S, VT726S Ltx[−], and ATCC 33384) bacteria from overnight cultures were distributed into wells of 96-well plates (Nunc, Denmark) containing 50 µl of LL-37 or scrambled LL-37 diluted in phosphate-buffered saline (PBS) (as specified). Plates were incubated for 16 h for *E. coli* and 24 h for *A. actinomycetemcomitans*. Bacterial growth was determined by measuring the absorbance at 595 nm using a GENios microplate reader (Tecan, Austria). Percent growth inhibition was calculated relative to growth of bacteria not treated with LL-37. The MIC was defined as the concentration that inhibited bacterial growth

by more than 95% compared to that of controls. Data presented are means and standard deviations from three independent experiments performed in triplicate.

LL-37 binding to *A. actinomycetemcomitans*. Overnight-cultured *A. actinomycetemcomitans* ($\sim 10^9$ CFU/ml, verified by plating on blood agar plates) was labeled with fluorescein isothiocyanate (FITC) (0.1 mg/ml in PBS; Sigma-Aldrich) for 30 min at room temperature and washed three times in PBS. Labeled bacteria (approximately 6×10^6 bacteria) were incubated for 10 min at room temperature with tetramethylrhodamine-labeled LL-37 (4.4 µM and 11.1 µM final concentrations). Bacteria were washed twice with PBS and resuspended in 50 µl PBS. From each sample, 5 µl was analyzed using fluorescence microscopy.

Binding of labeled LL-37 and scrambled LL-37 to *A. actinomycetemcomitans* strains was also quantified by flow cytometry. Overnight-cultured bacteria (20 µl) were incubated with tetramethylrhodamine-labeled LL-37 (0, 4.4, 6.6, 8.8, and 11.1 µM) or 6-FAM-labeled scrambled LL-37 (6.6, 8.8, and 11.1 µM) for 30 min at room temperature. The percentage of peptide-bound bacteria was measured by flow cytometry using an Eclipse fluorescence-activated cell sorter (FACS) analyzer (Icyte, USA). Analysis of raw data was performed using the FlowJo 7.6.5 software (Tree-Star, USA).

Inhibition of biofilm formation by LL-37. Overnight cultures of *A. actinomycetemcomitans* (20 µl, approximately 2×10^7 cells) were inoculated in 180 µl fresh appropriate broth in 96-well plates (Nunc, Denmark) containing LL-37 or sLL-37 diluted in PBS (0.4, 4, 6.6, 8.8, 11.1, and 22.2 µM final dilutions). Plates were incubated for 24 h. Biofilm quantification using crystal violet staining was performed as described previously (48). Wells were washed twice with PBS, stained with 0.1% crystal violet for 30 min, and rinsed twice. Two hundred microliters of 30% acetic acid was added to each well, and the plates were incubated for 30 min at room temperature with shaking. The crystal violet solution was collected, and the optical density (OD) at 595 nm was measured using the GENios microplate reader (TECAN, Austria). Percent inhibition of biofilm formation was calculated relative to biofilm formation by bacteria not treated with LL-37.

Preparation of neutrophils. Human peripheral blood was collected from healthy donors in accordance with a protocol that was approved by the institutional ethics committee. Neutrophils were isolated using Histopaque Percoll (Sigma) as described before (49) or by negative selection (50) and brought to a concentration of 1×10^7 cells/ml in Hanks buffer (Biological Industries, Kibbutz Beit Haemek, Israel). Neutrophil purity was determined by flow cytometry using antibodies to CD66b and CD16 (BioLegend, USA).

***A. actinomycetemcomitans* opsonization by LL-37.** *A. actinomycetemcomitans* ($\sim 10^9$ CFU/ml) was labeled with FITC as described above and added at a multiplicity of infection of 125 to neutrophils and a multiplicity of infection of 100 to RAW cells in the absence or presence of increasing concentrations of LL-37 (6.6 µM, 8.8 µM, and 11.1 µM), or scrambled LL-37 (11.1 µM). Samples were incubated at 37°C in 5% CO₂ for 1 h, and binding of labeled bacteria to the cells was measured by flow cytometry using the Accuri C6 FACS analyzer (BD, USA). Analysis of raw data was performed using the FlowJo 7.6.5 software (TreeStar, USA). In addition, 5-µl samples were fixed with 1% paraformaldehyde and analyzed by fluorescence microscopy.

Fluorescence microscopy of phagocytosis. RAW 264.7 cells were grown to confluence at 37°C in 5% CO₂ in Lab-Tek 8-well glass-bottom chamber slides (Nunc, USA). The wells were washed three times in ice-cold PBS. After the third wash, 200 µl of FITC-labeled *A. actinomycetemcomitans* cells ($\sim 2 \times 10^5$ CFU) preincubated with or without increasing concentrations of tetramethylrhodamine-labeled LL-37 (6.6 µM, 8.8 µM, and 11.1 µM) were added to the adherent cells in each chamber. Slides were incubated for 2 h at 37°C in 5% CO₂ and washed three times in ice-cold PBS. The slides were then fixed with 3.7% paraformaldehyde for 5 min and washed twice with ice-cold PBS, and 200 µl of 100% glycerol was added. Opsonization of *A. actinomycetemcomitans* by tetramethyl-

FIG 1 Resistance of *A. actinomycetemcomitans* to LL-37. (A) MICs of *A. actinomycetemcomitans* strains tested in this study. (B and C) Growth inhibition (see Materials and Methods) of *A. actinomycetemcomitans* strains and *E. coli* ATCC 25922 grown in the presence of increasing concentrations of LL-37 (B) or scrambled LL-37 (C) (22.2 μM). *, $P < 0.05$ compared to untreated control (no LL-37) using the Student t test.

rhodamine-labeled LL-37 and phagocytosis by murine RAW cells were analyzed by fluorescence microscopy.

Killing of *A. actinomycetemcomitans* by murine macrophages. Approximately 2×10^5 RAW 264.7 cells were added to 24-well plates (Nunc, Denmark), allowed to adhere for 2 to 4 h at 37°C in 5% CO₂, and washed three times in PBS. *A. actinomycetemcomitans* JP2 preincubated with or without increasing concentrations (6.6 μM, 8.8 μM, and 11.1 μM in DMEM) of LL-37 or sLL-37 was added at a 1:10 ratio of infection. Plates were incubated for 2 h at 37°C in 5% CO₂. The medium from each well, which contained extracellular bacteria, was collected, and the remaining adherent cells were lysed with double-distilled water (DDW) for 20 min at room temperature. The cell lysate containing intracellular and cell-bound bacteria was combined with the medium containing the extracellular bacteria, diluted, plated on blood agar, and incubated for 48 h at 37°C in a Bactron anaerobic (85% N₂, 10% H₂, and 5% CO₂) environmental chamber (Sheldon Manufacturing Inc., Cornelius, OR). The resulting colonies were counted, and the percentage of viable bacteria in each sample was then determined by comparing the number of CFU from a control tube without added macrophages (100% viability) to the number of CFU obtained for each bacterial strain incubated with macrophages.

Fluorescence microscopy and Image analysis. An Olympus Fluoview 300 (FV300) confocal microscope (Olympus, Japan) was used. Fluorescence intensity thresholds were set manually for red and green pixels. All analyses were done using FV-10 Fluoview software (Olympus, Japan) and the Image-Pro analyzer (Media Cybernetics, USA). Fold intensity of green and red pixels was measured relative to no LL-37 treatment. Colocalization was measured for green and red pixels, and value shown are Pearson's correlation calculated by Image-Pro software.

Statistical methods. Unless otherwise specified, all data presented are means and standard deviations from three independent experiments performed in triplicate. For statistical significance, Student t test analysis was

used. A statistical test was considered significant (*) when the P value was < 0.05 .

RESULTS AND DISCUSSION

***A. actinomycetemcomitans* is resistant to LL-37.** As previously observed (24), *A. actinomycetemcomitans* treated with LL-37 at physiological ionic strength maintained growth at all tested LL-37 concentrations (up to 100 μg/ml) (Fig. 1A). The antimicrobial potency of the LL-37 used was confirmed using *E. coli*, and as expected (51), it resulted in complete growth inhibition at concentrations equal to or greater than 4.4 μM (Fig. 1A and B). Of the six tested *A. actinomycetemcomitans* strains, growth of four was not affected by LL-37. Growth of strains ATCC 29523 and Y4 was partially (67% and 56%, respectively) inhibited at LL-37 concentrations of ≥ 8.8 μM (40 μg/ml) (Fig. 1B). *A. actinomycetemcomitans* Y4 is a serotype b strain. It therefore appears that in contrast to resistance to complement-mediated phagocytosis, where access of complement to the lipopolysaccharide is blocked (41), the serotype b-specific polysaccharide antigen does not provide increased resistance to LL-37.

Leukotoxin production was not found to affect the ability of *A. actinomycetemcomitans* to maintain growth in the presence of LL-37. As can be seen in Fig. 1B, the high-leukotoxin-producing JP2 strain, the intermediate-leukotoxin-producing VT726S strain, and the leukotoxin-deficient VT726Sltx⁻ strain were all similarly resistant to LL-37.

The ability of LL-37 to partially inhibit growth of *A. actinomycetemcomitans* ATCC 29523 and Y4 (as well as its ability to com-

FIG 2 LL-37 binds *A. actinomycetemcomitans* at submicrobicidal concentrations. (A) Fluorescence microscopy analysis of binding of 4.4 μM and 11.1 μM tetramethylrhodamine-labeled LL-37 (red) to FITC-stained *A. actinomycetemcomitans* JP2 (green). (B) Flow cytometry analysis of *A. actinomycetemcomitans* strains incubated with increasing concentrations of tetramethylrhodamine-labeled LL-37. Graphs indicate mean cell percentages ± standard deviations from three independent experiments.

pletely inhibit growth of *E. coli*) was sequence specific and was lost by sequence scrambling (Fig. 1C).

A previous report described growth inhibition of *A. actinomycetemcomitans* with a lower LL-37 concentration of 8.5 μM (38 μg/ml) (52). However, this inhibition was obtained in a non-physiological, low-ionic-strength environment (10 mM sodium phosphate buffer) (52).

Oral concentrations of LL-37 were shown to reach 0.1 μM (0.5 μg/ml) in saliva (53, 54) and 2.2 μM (10 μg/ml) in gingival crevicular fluid (GCF) of periodontitis patients (55), seemingly lower than that necessary to effectively inhibit *A. actinomycetemcomitans* growth. Saliva and GCF are constantly secreted, and therefore it is possible that newly secreted LL-37 might continuously accumulate on the pathogen's membrane to reach bactericidal concentrations (51). However, subinhibitory LL-37 concentrations might restrain the growth of *A. actinomycetemcomitans* by indirect mechanisms.

Binding of *A. actinomycetemcomitans* by subinhibitory concentrations of LL-37 is mediated by hydrophilic and hydrophobic interactions. Though growth of 4 of the tested *A. actinomycetemcomitans* strains was not inhibited at LL-37 concentrations as high as 22.2 μM (100 μg/ml) (Fig. 1), strong binding of fluorescently labeled LL-37 to all of the tested strains of *A. actinomycetemcomitans*

FIG 3 Binding of LL-37 to *A. actinomycetemcomitans* is sequence specific and sensitive to high ionic strength. (A) Flow cytometry analysis of *A. actinomycetemcomitans* JP2 incubated with increasing concentrations of tetramethylrhodamine-labeled LL-37 and 6-FAM-labeled scrambled LL-37. (B) *A. actinomycetemcomitans* incubated with tetramethylrhodamine-labeled LL-37 in the presence (+sLL-37) or absence (no sLL-37) of unlabeled 11.1 μM scrambled LL-37. (C) Flow cytometry histogram of binding of 11.1 μM tetramethylrhodamine-labeled LL-37 (TMR-LL-37) to *A. actinomycetemcomitans* JP2 at physiological ionic strength (PBS) (red) or high ionic strength (200 mM NaCl [black line] or 50 mM MgCl₂ [blue line]). The gray filled line represents JP2 without LL-37. Graphs and numbers indicate cell percentages ± standard deviations from two independent experiments.

temcomitans could be detected at concentrations as low as 4.4 μM (20 μg/ml) (Fig. 2). Similar to the partial growth inhibition of *A. actinomycetemcomitans* ATCC 29523 and Y4 by LL-37 (Fig. 1C), binding of LL-37 to *A. actinomycetemcomitans* JP2 was also sequence specific and was 90% diminished by scrambling (Fig. 3A, 6.6 μM). Binding of LL-37 to *A. actinomycetemcomitans* JP2 was not reduced by the addition of unlabeled sLL-37 (Fig. 3B), demonstrating that sLL-37 cannot compete with LL-37 for binding to *A. actinomycetemcomitans*. Scrambled LL-37 has the same charge as LL-37 but lacks the amphipathic structure and is disrupted in its hydrophobic domain. As both LL-37 and sLL-37 share the same charge, it appears that hydrophobic interactions, which are disrupted by scrambling the LL-37 sequence, are important for the ability of LL-37 to bind *A. actinomycetemcomitans*. However, binding of LL-37 to *A. actinomycetemcomitans* is also charge sen-

FIG 4 LL-37 inhibits biofilm formation of *A. actinomycetemcomitans*. Biofilm formation of *A. actinomycetemcomitans* strains on 96-well microtiter plates grown in the presence of increasing concentrations of LL-37 (A) or 22.2 μM scrambled LL-37 (B) for 24 h (see Materials and Methods) is shown.

sitive and was reduced by 85% and 50% in the presence of 50 mM MgCl₂ and 200 mM NaCl, respectively (Fig. 3C). These results demonstrate that both hydrophobic and hydrostatic interactions are involved in the binding of LL-37 to *A. actinomycetemcomitans*.

Based on our results so far, we concluded that at subinhibitory LL-37 concentrations, LL-37 binds to *A. actinomycetemcomitans*. Next, we tested whether LL-37 at low, sub-growth-inhibitory concentrations can exert nonbactericidal protective actions against the invading *A. actinomycetemcomitans*.

LL-37 inhibits *A. actinomycetemcomitans* biofilm formation at low concentrations. In order to withstand and avoid removal by the flow of the saliva or the gingival crevicular fluid, dental pathogens must attach to oral surfaces by constructing biofilms (16). Biofilms provide a protected mode of growth and are difficult to treat due to their high inherent resistance to antimicrobial agents (56). Biofilm formation or integration in an existing oral biofilm (also known as the dental plaque) is therefore essential for dental pathogens. LL-37 was previously shown to inhibit the formation of *Pseudomonas aeruginosa* biofilms at concentrations far below those which are required to kill or inhibit growth (46). As low concentrations of LL-37 were found to bind *A. actinomycetemcomitans*, we tested its ability to inhibit biofilm formation by *A. actinomycetemcomitans*. Biofilm formation of all tested *A. actinomycetemcomitans* strains was inhibited by LL-37 (though not in a dose-dependent manner in the tested range). LL-37 treatment of strains Y4 and ATCC 29523 (both of which are the most sensitive to growth inhibition by LL-37 [Fig. 1B]) led to a decrease of approximately 50% and 70% in biofilm mass (Fig. 4A). Biofilm for-

mation of both these strains was also most sensitive to sLL-37 (Fig. 4B), though to a lesser extent than to LL-37. Leukotoxin deletion in strain VT726S did not hamper biofilm formation of the mutant progeny VT726S Ltx⁻ (data not shown). For a reason yet unclear, leukotoxin deletion decreased the ability of the VT726S Ltx⁻ mutant to form biofilm in the presence of LL-37 (Fig. 4A) and sLL-37 (Fig. 4B).

LL-37 at low concentrations opsonizes *A. actinomycetemcomitans*, enhances its uptake by neutrophils, and increases its killing by macrophages. Phagocytosis by neutrophils is a major host defense mechanism for bacterial clearance in the gingival sulcus (25, 26). However, previous *in vitro* studies found *A. actinomycetemcomitans* to be relatively resistant to phagocytosis (38, 41, 52). The MCP-1 and MCP-2 host defense peptides (currently known as β-defensin A and B) were shown to act as opsonins and to enhance the ability of rabbit alveolar macrophages to ingest *Staphylococcus aureus*, *Klebsiella pneumoniae*, *Bordetella bronchiseptica*, and *Candida albicans* (45). The LL-37 precursor CAP18 was previously shown to opsonize *S. aureus* and to increase its phagocytosis by monocytes (but not granulocytes) (44). We therefore tested the ability of subbactericidal concentrations of LL-37 to opsonize *A. actinomycetemcomitans* and increase its uptake by human neutrophils and murine macrophages.

LL-37 added together with *A. actinomycetemcomitans* increased bacterial binding by neutrophils (as determined after 1 h of incubation). Opsonization of *A. actinomycetemcomitans* with 11.1 μM (50 μg/ml) LL-37 increased the binding of *A. actinomycetemcomitans* JP2 by neutrophils by approximately 10-fold (Fig. 5A) and increased the uptake of the four other tested strains (excluding strain ATCC 29523 where the increase in uptake was not statistically significant) by 2.8 to 9.5-fold (Fig. 5B). Leukotoxin inactivation in VT726S Ltx⁻ increased its LL-37-mediated uptake by neutrophils by 4-fold compared to that of its leukotoxin-producing VT726S parent strain. This is in agreement with the increased biofilm formation sensitivity to LL-37 generated by leukotoxin inactivation in the VT726S Ltx⁻ mutant.

Scrambled LL-37, which shares the same charge as LL-37 but lacks the amphipathic structure, produced a poor, nonstatistically significant improvement in binding of *A. actinomycetemcomitans* JP2 (Fig. 5A) and VT726S Ltx⁻ (Fig. 5C) by neutrophils. This is in agreement with the inability of sLL-37 to inhibit growth (Fig. 1) and to bind *A. actinomycetemcomitans* (Fig. 3). The weaker opsonization and growth inhibition activity by sLL-37 (compared with LL-37) is likely to result from its weaker affinity to *A. actinomycetemcomitans*, as demonstrated in Fig. 3.

In contrast to the case for neutrophils, adding LL-37 together with *A. actinomycetemcomitans* to nonactivated murine macrophage-like RAW 264.7 cells barely improved bacterial uptake as determined after 1 h (Fig. 6). However, incubating LL-37 with *A. actinomycetemcomitans* prior to its addition to the macrophage-like cells induced agglutination of the bacteria, and the bacterial aggregates were readily phagocytized by the RAW cells in an LL-37 dose-dependent manner (Fig. 7). A 3-fold increase in bacterial uptake was observed with an LL-37 concentration of 11.1 μM (Fig. 7, bottom panel). Three-dimensional reconstruction of the confocal imaging confirmed that the bacteria were indeed phagocytized and internalized by the RAW cells (see Movie S1 in the supplemental material). LL-37 agglutination improved killing of *A. actinomycetemcomitans* JP2 by the RAW cells in a statistically significant, dose-dependent manner, rising from 46% without ad-

FIG 5 LL-37 acts as an opsonin of *A. actinomycetemcomitans*. (A) FACS analysis (fold increase of mean fluorescence intensity [MFI]) of human neutrophils incubated with FITC-labeled *A. actinomycetemcomitans* JP2 in the absence (black lines) or presence (red lines) of 6.6 μ M, 8.8 μ M, and 11.1 μ M LL-37 and 11.1 μ M scrambled LL-37. Neutrophils without labeled bacteria and without LL-37 were used as baseline, which is represented by a filled blue histogram. (B) Fold increase of mean fluorescence intensity of *A. actinomycetemcomitans* (*A. a*) opsonization in the presence of increasing concentrations of LL-37. Neutrophils with *A. actinomycetemcomitans* only were used as a baseline. (C) Fold increase of mean fluorescence intensity of *A. actinomycetemcomitans* VT726S Ltx⁻ opsonization after incubation with LL-37 or scrambled LL-37. Neutrophils with labeled bacteria and no LL-37 were used as a baseline. (D) Fluorescence microscopy of human neutrophils (arrows) incubated with FITC-labeled *A. actinomycetemcomitans* JP2 (*A. a*) in the absence or presence of LL-37. *, $P < 0.05$ compared to baseline using the Student t test.

FIG 6 LL-37 has only a moderate effect on binding of *A. actinomycetemcomitans* by murine macrophages. Macrophage-like RAW 264.7 cells were incubated with FITC-labeled *A. actinomycetemcomitans* JP2 in the absence (black lines) or presence (red lines) of increasing concentrations of LL-37 (6.6 μ M, 8.8 μ M, and 11.1 μ M). The fold increase in mean fluorescence intensity of *A. actinomycetemcomitans* JP2 binding to RAW cells in the presence of LL-37 is shown. RAW cells without labeled bacteria and with no LL-37 are represented by a filled blue line. RAW cells incubated with labeled bacteria without LL-37 were used as a baseline for fold increase calculations.

dition of LL-37 to 97% when cells were preopsonized with 11.1 μ M LL-37 (Fig. 8). Adding sLL-37 slightly increased the killing of *A. actinomycetemcomitans* JP2 by RAW cells but not in a statistically significant manner, in agreement with the sequence dependence of the antimicrobial action of LL-37 described above (Fig. 1, 3, and 5).

Conclusion. Dysfunction of neutrophils or reduction in their numbers (20, 21), as well as deficiency in LL-37, was previously linked with outgrowth of *A. actinomycetemcomitans* and the development of an aggressive periodontal disease (9, 23). Surprisingly, *A. actinomycetemcomitans* was found to be highly resistant to LL-37 (Fig. 1A and B). However, LL-37, even at low concentrations, bound *A. actinomycetemcomitans* (Fig. 2 and 3) and was found to inhibit its biofilm formation at low, submicrocidal concentrations (Fig. 4). Since cationic peptides (including LL-37) were shown to act as opsonins (42–45), we tested whether *A. actinomycetemcomitans* can be opsonized and cleared by neutrophils and macrophages at relatively low concentrations of LL-37. Our results demonstrate that at sub-growth-inhibitory concentrations, LL-37 efficiently opsonized *A. actinomycetemcomitans* and enhanced its removal by neutrophils (which are known to be abundantly present the gingival sulcus) (Fig. 5).

A. actinomycetemcomitans agglutination by LL-37 (rather than opsonization of planktonic bacteria) is suggested as a possible mechanism by which LL-37 improves phagocytosis and killing of *A. actinomycetemcomitans* by macrophages. The LL-37 concentra-

FIG 7 Preincubation of *A. actinomycetemcomitans* JP2 with LL-37 enhances its phagocytosis by murine macrophages. FITC-labeled *A. actinomycetemcomitans* (*A. a.*) was incubated in the absence of tetramethylrhodamine-labeled LL-37 or in the presence of increasing concentrations of tetramethylrhodamine-labeled LL-37 (6.6 μM , 8.8 μM , and 11.1 μM) for 10 min at room temperature, washed twice, incubated with macrophage-like RAW 264.7 cells for 2 h, washed twice, and analyzed using fluorescence microscopy. Colocalization analysis appears above the colocalization panels for each concentration. Mean fluorescence intensities from two independent experiments are shown in the bottom panel.

FIG 8 LL-37 enhances killing of *A. actinomycetemcomitans* by murine macrophages. *A. actinomycetemcomitans* JP2 was incubated with macrophage-like RAW 264.7 cells at a multiplicity of infection of 10 for 2 h in the presence of LL-37 or scrambled LL-37 at increasing concentrations. Murine macrophage killing of *A. actinomycetemcomitans* JP2 was determined after 2 h of incubation by plating the samples on blood agar. *A. actinomycetemcomitans* JP2 without macrophages was used as a reference of bacteria number and no killing (0%).

tions found in our study as necessary to improve the removal of *A. actinomycetemcomitans* by neutrophils and macrophages are still higher than those measured in saliva. Newly secreted LL-37 is likely to continuously accumulate on membranes of *A. actinomycetemcomitans*, reaching the concentrations required for mediating their clearance by local neutrophils and macrophages.

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REFERENCES

1. Zasloff M. 2002. Antimicrobial peptides of multicellular organisms. *Nature* 415:389–395.
2. Bernard JJ, Gallo RL. 2011. Protecting the boundary: the sentinel role of host defense peptides in the skin. *Cell. Mol. Life Sci.* 68:2189–2199.
3. Yang D, de la Rosa G, Tewary P, Oppenheim JJ. 2009. Alarmins link neutrophils and dendritic cells. *Trends Immunol.* 30:531–537.

4. Easton DM, Nijnik A, Mayer ML, Hancock RE. 2009. Potential of immunomodulatory host defense peptides as novel anti-infectives. *Trends Biotechnol.* 27:582–590.
5. Cole JN, Pence MA, von Kockritz-Blickwede M, Hollands A, Gallo RL, Walker MJ, Nizet V. 2010. M protein and hyaluronic acid capsule are essential for in vivo selection of covRS mutations characteristic of invasive serotype MIT1 group A *Streptococcus*. *mBio* 1(4):e00191–10. doi:10.1128/mBio.00191-10.
6. Nizet V, Ohtake T, Lauth X, Trowbridge J, Rudisill J, Dorschner RA, Pestonjamas V, Piraino J, Huttner K, Gallo RL. 2001. Innate antimicrobial peptide protects the skin from invasive bacterial infection. *Nature* 414:454–457.
7. Chromek M, Slamova Z, Bergman P, Kovacs L, Podracka L, Ehren I, Hokfelt T, Gudmundsson GH, Gallo RL, Agerberth B, Brauner A. 2006. The antimicrobial peptide cathelicidin protects the urinary tract against invasive bacterial infection. *Nat. Med.* 12:636–641.
8. Bergsson G, Reeves EP, McNally P, Chotirmall SH, Greene CM, Grealley P, Murphy P, O'Neill SJ, McElvaney NG. 2009. LL-37 complexation with glycosaminoglycans in cystic fibrosis lungs inhibits antimicrobial activity, which can be restored by hypertonic saline. *J. Immunol.* 183:543–551.
9. Putsep K, Carlsson G, Boman HG, Andersson M. 2002. Deficiency of antibacterial peptides in patients with morbus Kostmann: an observation study. *Lancet* 360:1144–1149.
10. Gupta S, Ghosh SK, Scott ME, Bainbridge B, Jiang B, Lamont RJ, McCormick TS, Weinberg A. 2010. *Fusobacterium nucleatum*-associated beta-defensin inducer (FAD-I): identification, isolation, and functional evaluation. *J. Biol. Chem.* 285:36523–36531.
11. Devine DA, Cosseau C. 2008. Host defense peptides in the oral cavity. *Adv. Appl. Microbiol.* 63:281–322.
12. Gorr SU. 2009. Antimicrobial peptides of the oral cavity. *Periodontol.* 2000 51:152–180.
13. Gursoy UK, Pollanen M, Kononen E, Uitto VJ. 2012. A novel organotypic dento-epithelial culture model: effect of *Fusobacterium nucleatum* biofilm on B-defensin-2, -3, and LL-37 expression. *J. Periodontol.* 83:242–247.
14. Loesche WJ, Grossman NS. 2001. Periodontal disease as a specific, albeit chronic, infection: diagnosis and treatment. *Clin. Microbiol. Rev.* 14:727–752.
15. Eke PI, Dye BA, Wei L, Thornton-Evans GO, Genco RJ. 2012. Prevalence of periodontitis in adults in the United States: 2009 and 2010. *J. Dent. Res.* 91:914–920.
16. Socransky SS, Haffajee AD, Cugini MA, Smith C, Kent RL, Jr. 1998. Microbial complexes in subgingival plaque. *J. Clin. Periodontol.* 25:134–144.
17. Hajishengallis G, Darveau RP, Curtis MA. 2012. The keystone-pathogen hypothesis. *Nat. Rev. Microbiol.* 10:717–725.
18. Hajishengallis G, Liang S, Payne MA, Hashim A, Jotwani R, Eskin MA, McIntosh ML, Alsam A, Kirkwood KL, Lambris JD, Darveau RP, Curtis MA. 2011. Low-abundance biofilm species orchestrates inflammatory periodontal disease through the commensal microbiota and complement. *Cell Host Microbe* 10:497–506.
19. Eskin MA, Jotwani R, Abe T, Chmela J, Lim JH, Liang S, Ciero PA, Krauss JL, Li F, Rauner M, Hofbauer LC, Choi EY, Chung KJ, Hashim A, Curtis MA, Chavakis T, Hajishengallis G. 2012. The leukocyte integrin antagonist Del-1 inhibits IL-17-mediated inflammatory bone loss. *Nat. Immunol.* 13:465–473.
20. Van Dyke TE, Levine MJ, Tabak LA, Genco RJ. 1981. Reduced chemotactic peptide binding in juvenile periodontitis: a model for neutrophil function. *Biochem. Biophys. Res. Commun.* 100:1278–1284.
21. Genco RJ, Christersson LA, Zambon JJ. 1986. Juvenile periodontitis. *Int. Dent. J.* 36:168–176.
22. Sorensen O, Arnljots K, Cowland JB, Bainton DF, Borregaard N. 1997. The human antibacterial cathelicidin, hCAP-18, is synthesized in myelocytes and metamyelocytes and localized to specific granules in neutrophils. *Blood* 90:2796–2803.
23. de Haar SF, Hiemstra PS, van Steenberg MT, Everts V, Beertsen W. 2006. Role of polymorphonuclear leukocyte-derived serine proteinases in defense against *Actinobacillus actinomycetemcomitans*. *Infect. Immun.* 74:5284–5291.
24. Altman H, Steinberg D, Porat Y, Mor A, Fridman D, Friedman M, Bachrach G. 2006. In vitro assessment of antimicrobial peptides as potential agents against several oral bacteria. *J. Antimicrob. Chemother.* 58:198–201.
25. Galicia JC, Benakanakere MR, Stathopoulou PG, Kinane DF. 2009. Neutrophils rescue gingival epithelial cells from bacterial-induced apoptosis. *J. Leukoc. Biol.* 86:181–186.
26. Fredman G, Oh SF, Ayilavarapu S, Hasturk H, Serhan CN, Van Dyke TE. 2011. Impaired phagocytosis in localized aggressive periodontitis: rescue by resolvins. *PLoS One* 6:e24422. doi:10.1371/journal.pone.0024422.
27. Nussbaum G, Shapira L. 2011. How has neutrophil research improved our understanding of periodontal pathogenesis? *J. Clin. Periodontol.* 38(Suppl 11):49–59.
28. Zambon JJ, Christersson LA, Slots J. 1983. *Actinobacillus actinomycetemcomitans* in human periodontal disease. Prevalence in patient groups and distribution of biotypes and serotypes within families. *J. Periodontol.* 54:707–711.
29. Shaddox LM, Huang H, Lin T, Hou W, Harrison PL, Aukhil I, Walker CB, Klepac-Ceraj V, Paster BJ. 2012. Microbiological characterization in children with aggressive periodontitis. *J. Dent. Res.* 91:927–933.
30. Lally ET, Kieba IR, Sato A, Green CL, Rosenbloom J, Korostoff J, Wang JF, Shenker BJ, Ortlepp S, Robinson MK, Billings PC. 1997. RTX toxins recognize a beta2 integrin on the surface of human target cells. *J. Biol. Chem.* 272:30463–30469.
31. Welch RA. 1991. Pore-forming cytotoxins of gram-negative bacteria. *Mol. Microbiol.* 5:521–528.
32. Johansson A, Sandstrom G, Claesson R, Hanstrom L, Kalfas S. 2000. Anaerobic neutrophil-dependent killing of *Actinobacillus actinomycetemcomitans* in relation to the bacterial leukotoxicity. *Eur. J. Oral Sci.* 108:136–146.
33. Kachlany SC, Fine DH, Figurski DH. 2000. Secretion of RTX leukotoxin by *Actinobacillus actinomycetemcomitans*. *Infect. Immun.* 68:6094–6100.
34. Brogan JM, Lally ET, Poulsen K, Kilian M, Demuth DR. 1994. Regulation of *Actinobacillus actinomycetemcomitans* leukotoxin expression: analysis of the promoter regions of leukotoxic and minimally leukotoxic strains. *Infect. Immun.* 62:501–508.
35. Haubek D, Poulsen K, Westergaard J, Dahlen G, Kilian M. 1996. Highly toxic clone of *Actinobacillus actinomycetemcomitans* in geographically widespread cases of juvenile periodontitis in adolescents of African origin. *J. Clin. Microbiol.* 34:1576–1578.
36. Yoshida A, Ennibi OK, Miyazaki H, Hoshino T, Hayashida H, Nishihara T, Awano S, Ansai T. 2012. Quantitative discrimination of *Aggregatibacter actinomycetemcomitans* highly leukotoxic JP2 clone from non-JP2 clones in diagnosis of aggressive periodontitis. *BMC Infect. Dis.* 12:253.
37. Wilson ME, Bronson PM, Hamilton RG. 1995. Immunoglobulin G2 antibodies promote neutrophil killing of *Actinobacillus actinomycetemcomitans*. *Infect. Immun.* 63:1070–1075.
38. Permpaich P, Kowolik MJ, Galli DM. 2006. Resistance of fluorescent-labelled *Actinobacillus actinomycetemcomitans* strains to phagocytosis and killing by human neutrophils. *Cell. Microbiol.* 8:72–84.
39. Gmur R, McNabb H, van Steenberg TJ, Baehni P, Mombelli A, Winkelhoff AJ, Guggenheim B. 1993. Seroclassification of hitherto non-typeable *Actinobacillus actinomycetemcomitans* strains: evidence for a new serotype e. *Oral Microbiol. Immunol.* 8:116–120.
40. Takada K, Saito M, Tsuzukibashi O, Kawashima Y, Ishida S, Hirasawa M. 2010. Characterization of a new serotype g isolate of *Aggregatibacter actinomycetemcomitans*. *Mol. Oral Microbiol.* 25:200–206.
41. Yamaguchi N, Kawasaki M, Yamashita Y, Nakashima K, Koga T. 1995. Role of the capsular polysaccharide-like serotype-specific antigen in resistance of *Actinobacillus actinomycetemcomitans* to phagocytosis by human polymorphonuclear leukocytes. *Infect. Immun.* 63:4589–4594.
42. Ginsburg I. 1987. Cationic polyelectrolytes: a new look at their possible roles as opsonins, as stimulators of respiratory burst in leukocytes, in bacteriolysis, and as modulators of immune-complex diseases. *Inflammation* 11:489–515.
43. Ginsburg I. 1989. Cationic polyelectrolytes: potent opsonic agents which activate the respiratory burst in leukocytes. *Free Radic. Res. Commun.* 8:11–26.
44. Heinzelmann M, Platz A, Flodgaard H, Miller FN. 1998. Heparin binding protein (CAP37) is an opsonin for *Staphylococcus aureus* and increases phagocytosis in monocytes. *Inflammation* 22:493–507.
45. Fleischmann J, Selsted ME, Lehrer RI. 1985. Opsonic activity of MCP-1 and MCP-2, cationic peptides from rabbit alveolar macrophages. *Diagn. Microbiol. Infect. Dis.* 3:233–242.
46. Overhage J, Campisano A, Bains M, Torfs EC, Rehm BH, Hancock RE.

2008. Human host defense peptide LL-37 prevents bacterial biofilm formation. *Infect. Immun.* 76:4176–4182.
47. Bachrach G, Ianculovici C, Naor R, Weiss EI. 2005. Fluorescence based measurements of *Fusobacterium nucleatum* coaggregation and of fusobacterial attachment to mammalian cells. *FEMS Microbiol. Lett.* 248:235–240.
 48. Kolodkin-Gal I, Cao S, Chai L, Bottcher T, Kolter R, Clardy J, Losick R. 2012. A self-produced trigger for biofilm disassembly that targets exopolysaccharide. *Cell* 149:684–692.
 49. Brinkmann V, Laube B, Abu Abed U, Goosmann C, Zychlinsky A. 24 February 2010. Neutrophil extracellular traps: how to generate and visualize them. *J. Vis. Exp.* doi:10.3791/1724.
 50. Elishmereni M, Alenius HT, Bradding P, Mizrahi S, Shikotra A, Minai-Fleminger Y, Mankuta D, Eliashar R, Zabucchi G, Levi-Schaffer F. 2011. Physical interactions between mast cells and eosinophils: a novel mechanism enhancing eosinophil survival in vitro. *Allergy* 66:376–385.
 51. Rosen G, Sela MN, Bachrach G. 2012. The antibacterial activity of LL-37 against *Treponema denticola* is dentilisin protease independent and facilitated by the major outer sheath protein virulence factor. *Infect. Immun.* 80:1107–1114.
 52. Ji S, Hyun J, Park E, Lee BL, Kim KK, Choi Y. 2007. Susceptibility of various oral bacteria to antimicrobial peptides and to phagocytosis by neutrophils. *J. Periodontol. Res.* 42:410–419.
 53. Bachrach G, Chaushu G, Zigmond M, Yefenof E, Stabholz A, Shapira J, Merrick J, Chaushu S. 2006. Salivary LL-37 secretion in individuals with Down syndrome is normal. *J. Dent. Res.* 85:933–936.
 54. Takeuchi Y, Nagasawa T, Katagiri S, Kitagawara S, Kobayashi H, Koyanagi T, Izumi Y. 2012. Salivary levels of antibacterial peptide (LL-37/hCAP-18) and cotinine in periodontitis patients. *J. Periodontol.* 83:766–772.
 55. Turkoglu O, Emingil G, Kutukculer N, Atilla G. 2009. Gingival crevicular fluid levels of cathelicidin LL-37 and interleukin-18 in patients with chronic periodontitis. *J. Periodontol.* 80:969–976.
 56. Stewart PS, Costerton JW. 2001. Antibiotic resistance of bacteria in biofilms. *Lancet* 358:135–138.