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Activation of Neutrophils by the Two-Component Leukotoxin LukE/D from *Staphylococcus aureus*: Proteomic Analysis of the Secretions

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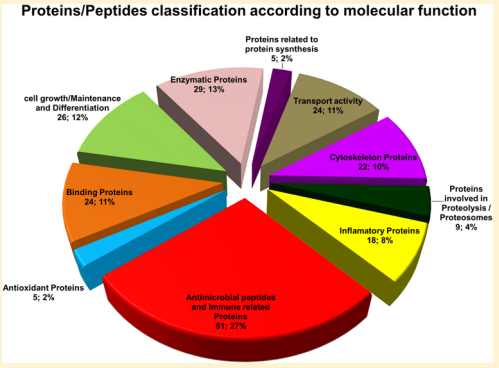
Supporting Information

ABSTRACT: *Staphylococcus aureus* is responsible for severe bacterial infections in hospitals and healthcare facilities. It produces single and bicomponent toxins (leukotoxins and hemolysins) that hinder innate immune function. Leukotoxin subunits bind to leukocyte cell membrane thus inducing transmembrane pores and subsequently, cell lysis. Leukotoxin LukE/D is a member of the bicomponent toxin family, but to date, no study concerning its involvement in host-pathogen interactions has been reported. In the present study, we performed the proteomic analysis of the secretions recovered after activation of human neutrophils by leukotoxin LukE/D. The neutrophil secretions were purified by RP-HPLC and different fractions were analyzed by Edman sequencing, LC-MS/MS, immunoblotted for chromogranin-derived peptides and further analyzed for antimicrobial properties. Proteomic analysis revealed that neutrophil secretions constitute a large number of proteins related with immune boosting mechanisms, proteolytic degradation, inflammatory process and antioxidant reactions.



KEYWORDS: *Staphylococcus aureus, Leukotoxin LukE/D, Neutrophils, Chromogranin A/B, Proteomic Analysis, Antimicrobial Peptides, Innate Immunity*

INTRODUCTION

Staphylococcus aureus (*S. aureus*), a commensal bacterial strain present in about 20−30% of the general population, is responsible for numerous nosocomial infections.[1] It causes a wide range of illnesses from minor skin infections to some of life-threatening diseases, such as sepsis, osteomyelitis, pneumonia, endocarditis, and meningitis.[2] Mostly, *S. aureus* infection starts with bacterial adhesion to host tissues and continues with colonization.[3,4] Colonization is associated with adhesion proteins that belong to the "microbial surface components recognizing adhesive matrix molecules" (MSCRAMMs) family. These proteins are important for the attachment of bacteria to fibronectin,[5] plasma clot, etc.[6] In addition, *S. aureus* produces several virulence factors, including toxins[7] such as staphylococcal enterotoxins (SEs); toxic shock syndrome toxin-1 (TSST-1); exfoliative toxins A and B (ETA and ETB); α-, β-, γ-, and δ-hemolysins; the bicomponent Panton-Valentine Leucocidin (PVL);[8] and the LukE/D[9] leukotoxin. LukE/D consists of two subunits: LukE and LukD. The toxin subunits bind to leukocyte cell membranes, inducing Ca^{2+} activated trans-membrane pores and subsequent cell lysis.[9−11] Expression of LukE/D is associated with several common diseases. As an example, it has been isolated with both epidermolysin A and B from 78% of retrospective and prospective cases of impetigo.[10] In addition, LukE/D has been isolated from 93.6% of patients with *S. aureus* associated postantibiotic diarrhea, in combination with the enterotoxins (80.9% of isolates).[12] However, no study regarding the involvement of LukE/D on the host-pathogen interactions has been reported so far.

Neutrophils (PMNs) are part of the first line of defense against pathogens, as they are involved in infection clearing and contribute to the development of adaptive immunity. Previous data indicate that in humans, chromogranins (CgA and CgB) are present in secretory granules of PMNs[13,14] and are overexpressed in plasma of the patients with systemic inflammatory response syndrome (SIRS) or sepsis.[15] Several endogenous CgA- and CgB-derived fragments have been described as antimicrobial peptides (AMPs).[16] In addition to their direct antimicrobial effect against pathogens, they activate PMNs,[17] inducing a calmodulin-mediated influx of calcium and therefore the secretion of innate immune components such as

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AMPs and cytokines.[18] A comprehensive understanding of the regulation of neutrophils responses could be obtained by proteomic analysis of the secretions from activated neutrophils.[19,20] Here, by using proteomics and computational analysis, we identified the secreted proteins from LukE/D-activated neutrophils in order to better define the effects of the dynamic regulatory network of neutrophils.

MATERIALS AND METHODS

Preparation of Leukotoxin LukE/D

LukE and LukD were purified from *S. aureus* Newman strain (NTCC 8178) according to previous studies.[9,21,22] Briefly, one colony was grown in 5 mL of YCP medium composed of 3% (w/v) yeast extract (Oxoid), 2% (w/v) bacto-casamino acids (Difco), 2% (w/v) sodium pyruvate (Merck), 0.25% (w/v) Na_2HPO4, 0.042% (w/v) KH_2PO_4, pH 7.0 at 37 °C for 18 h with vigorous shaking. Proteins were precipitated from the supernatant with the help of 80% (w/v) ammonium sulfate, resuspended and subjected to a first chromatography on a 50 mL XK26 SP Sepharose fast flow column onto an AktaPurifyer-10 (GE-Healthcare, Uppsala, Sweden).[21] The further purification was achieved by subjecting the eluted fractions separately to a two-step chromatography including a 8 mL Mono S cation exchange FPLC column (GE-Healthcare, Uppsala, Sweden) followed by a 8 mL ReSource-Iso hydrophobic FPLC column (GE-Healthcare, Uppsala, Sweden) conditioned into a HR-10 column (GE-Healthcare, Uppsala, Sweden).[21,22] Purified compounds were controlled by SDS-PAGE (12%) on PHAST System (GE-Healthcare, Uppsala, Sweden) and solubilized in 50 mM Na-phosphate buffer (pH 7.0) supplemented with 150 mM NaCl.[8,9] At $A_{280\ nm}$ of 1.0. LukE and LukD were stored at −80 °C until use. Purity and integrity of LukE and LukD were analyzed by Edman sequencing and MALDI-TOF.

Isolation of Polymorphonuclear Neutrophils

Human PMNs were prepared with a purity of 98% as previously described[23,24] from Buffy Coat from healthy sex nondiscriminated donors, obtained from the Centre de Transfusion Sanguine de Strasbourg (France). Briefly, 12 mL of lympho-Prep solution (EuroBio, Courtaboeuf Cedex B, France) was added to 30 mL of Buffy Coat from healthy donors and centrifuged at 200*g* for 20 min. After centrifugation the lower dark band was collected without disturbing the sediment and washed with neutrophil buffer (EGTA buffer) (140 mM NaCl, 5 mM KCl, 10 mM glucose, 0.1 mM EGTA (ethylene glycol-bis-(2-aminoethylether) N,N,Ǹ,Ǹ-tetraacetic acid), 10 mM HEPES, 3 mM Tris-base, pH 7.3). PMNs were further processed by dextran sedimentation[25] for 30 min and then centrifugated at 1600*g* for 6 min. The remaining impurities of red blood cells were lysed by hypotonic shock in distilled water (18 mL) for 45 s and immediately suspended in a 2 mL hypertonic NaCl (9% w/v) solution to a concentration of 0.9% (w/v).[26] Finally, cells were washed with EGTA buffer and suspended at a concentration of 5×10^6 cells/mL in the same buffer.

Flow Cytometry Measurements

For the flow cytometry analysis, PMNs were cultured at a concentration of 5×10^5 cells/mL in EGTA buffer. Flow cytometry measurements were carried out using a FacSort flow cytometer (Becton-Dickinson, Le Pont de Claix, France) equipped with a 1 mW argon laser tuned to 488 nm.[27] Activity of LukE/D on PMNs was evaluated using flow cytometry of cells previously incubated with ethidium bromide (25 nM)[28] for 10 min prior to addition of LukE and LukD. Ethidium fluorescence was measured using the Cell QuestProTM software (Becton Dickinson and Company, Franklin Lakes, NJ) by using (FL3: λ_{Em} = 650 nm).[27] Cells were supplemented with different concentrations of each leukotoxin component: 2, 3, 6, 10, 20, 40, and 60 nM. The first image was taken at the time of addition of the toxins. Subsequently, fluorescence was recorded from 5 to 30 min post-treatment. As a control, the fluorescence measuring for dead cells was considered as 100. Fluorescence measurements in the absence of toxin were subtracted from the data and the results are presented as percentiles.

Intracellular calcium kinetics were measured by using flow cytometry with cells previously loaded with 5 μM of Fluo-3 (Molecular Probes, New Brunswick, NJ) for 1 h at 37 °C, washed and resuspended in 0.1 mM EGTA buffer supplemented with 1.1 mM Ca^{2+}.[28] Fluo-3 measurement were carried out every 30 s during 10 min from the fluorescence light 1 (FL1: λ_{Em} = 530 nm) using the Cell Quest Pro software (Becton-Dickinson, Le Pont de Claix, France).[27] Data represent an average of at least three independent experiments.

Preparation of Secretions after PMNs Stimulation by Leukotoxin LukE/D

PMNs were cultured in EGTA buffer solution at 10^7 cells/mL. The two components of leukotoxin (LukE and LukD) were used each at 10 nM. Cells were stimulated with 1.1 mM $CaCl_2$ for 25 min at 37 °C and centrifuged at 800*g* for 10 min.[28] The supernatants were recovered and stored at −80 °C.

RP-HPLC of Neutrophils Secretions

Total protein concentration was measured using a Bradford Assay,[29] and 1 mg of total proteins was purified by RP-HPLC using a DIONEX Dual Gradient System (Dionex, Sunnyvale, CA) and a Nucleosil RP300−5C18 column (5 μm particle size, 300-Å porosity; Macherey-Nagel, Hoerdt, France) at 20 °C. Absorbance was monitored at 214 nm, at room temperature (25 °C). The solvent system consists of 0.1% (v/v) trifluoroacetic acid in water (solvent A) and 0.1% (v/v) trifluoroacetic acid in 70% (v/v) acetonitrile−water (solvent B) with a flow rate at 700 μL/min. A gradient with elution time reference is indicated on the chromatograms. The fractions were collected every 1 min interval and concentrated by speed-vac and stored at −20 °C.

Western Blot Analysis

Secretion samples were loaded on a 15% SDS-PAGE and transferred to PVDF membrane (Hybond-P, Amersham Bioscience, Little Chalfont, Bucks, UK). During migration a voltage of 50 V was applied for the first 30 min and then 110 V till end and 75 V during transfer for 70 min. For immunodetection Millipore Snap i.d. 2.0 protein detection system (Millipore, Billerica, MA) was used, using Chemi-Blocker as blocking agent. The antibodies used are as follows: mouse monoclonal anti-CgA (anti-CgA_{53-57}),[13,30] rabbit polyclonal anti-CgB (anti-$CgB_{547-560}$).[31] Immunolabeled bands were visualized by autoradiography after addition of ECL reagent (Amersham Bioscience, Buckinghamshire, UK).

Antibacterial Assay

Micrococcus luteus (A270) and *S. aureus* (ATCC 25923) strains were used to assess the antibacterial properties of the secreted material. Bacterial strains were precultured aerobically at 37 °C

in a Mueller Hinton Broth (MHB) medium (Merck KGaA, Darmstadt, Germany), pH 7.3. For each isolate, one colony was transferred to 10 mL of MHB medium and incubated at 37 °C for 18−20 h. Bacteria were resuspended in MHB medium, and antibacterial activity was assessed by measuring the inhibition of bacterial growth. We incubated 10−20 μL of PMNs secretion fractions purified by RP-HPLC with 90 μL of a midlogarithmic phase culture of bacteria, with a starting absorbance of 0.001 at 620 nm. In the initial inoculums, bacteria were quantified to 5 × 10^5 colony forming unit (CFU)/mL by agar plate spreading method.[32] Tetracycline (10 μg/mL) and Cefotaxime (0.1 μg/mL) were used as a positive control. Microbial growth was assessed by the increase of absorbance after 20 h incubation at 37 °C.[33,34] The $A_{620\ nm}$ values of control cultures growing in the absence of secretions and antibiotics were considered as 100% growth. Moreover, the $A_{620\ nm}$ value with the antibiotics (Tetracycline and Cefotaxime) was considered as 100% inhibition (absence of bacterial growth was confirmed by agar plate spreading). Each assay was performed at least three times.

Antifungal Activity

Neurospora crassa, *Aspergillus fumigatus*, *Candida albicans*, and *Candida tropicalis* strains were tested to evaluate the antifungal properties for the secreted material.

For the filamentous fungi, spores were resuspended at a concentration of 10^4 spores/mL, in Potato Dextrose Broth (PDB) medium (Difco, Becton Dickson Microbiology system, Sparks, MD) in half-strength, supplemented with Tetracycline (10 μg/mL) and Cefotaxime (0.1 μg/mL) to suppress bacterial growth.[35] Aliquots of PMN secretions purified by RP-HPLC (20 μL) were incubated with 80 μL of fungal spores. The suspension was incubated at 30 °C for 24 h without agitation.[34,35] Fungal growth was monitored microscopically after 24 h. Media alone was used as negative control and corresponds to 100% fungal growth and voriconazole (1 μg/mL) was used as a positive control and corresponds to 100% growth inhibition.

Concerning yeast, the protocol described above to test the antibacterial properties of the secretions was used, with two exceptions: the culture medium was Sabouraud (BioMerieux S.A., Marcy l'Etoile, France) and the incubation temperature was modified to 30 °C with agitation. Media alone was used as negative control and corresponds to 100% fungal growth and voriconazole (1 μg/mL) was used as a positive control and corresponds to 100% growth inhibition.

Proteomic Characterization by Edman Sequencing

The N-terminal sequencing was carried out by automatic Edman sequencing method, with a sequencer bi-cartridge (Procise 473A, Applied Biosystems, Foster City, CA).[13] For sequences identification a query was made in the SWISS-prot database http://web.expasy.org/blast/. The protein characteristic parameters were given by http://www.uniprot.org/.

Tryptic Digestion

After RP-HPLC, collected fractions were concentrated using a SpeedVac. Collected fractions were submitted to a reduction with 10 μL of 10 mM DTT in 25 mM NH_4HCO_3 (1 h at 57 °C) and alkylation with 10 μL of 55 mM iodoacetamide in 25 mM NH_4HCO_3 at room temperature. For tryptic digestion, the trypsin (12.5 ng/μL; Promega V5111, Madison, WI) was suspended in 2 mL of 25 mM NH_4HCO_3.The enzymatic digestion was performed at 37 °C during 4 h at an estimated ratio enzyme/substrate 1/50. Before nano LC-MS/MS analysis, 1 μL of H_2O/TFA 0.1% was added and fractions were concentrated using a SpeedVac.

Mass Spectrometry Analysis

NanoLC-MS/MS was performed using a nanoACQUITY ultra performance liquid chromatography (UPLC) system (Waters, Milford, MA) coupled to a maXis 4G Q-TOF mass spectrometer (BrukerDaltonics, Bremen, Germany). The system was fully controlled by HyStar 3.2 (Bruker Daltonics). The UPLC system was equipped with a Symmetry C18 precolumn (20 × 0.18 mm, 5 μm particle size, Waters, Milford, MA) and an ACQUITY UPLC BEH130 C18 separation column (75 μm × 200 mm, 1.7 μm particle size, Waters). The solvent system consisted of 0.1% formic acid in water (solvent A) and 0.1% formic acid in acetonitrile (solvent B). Three μL of each sample was injected. Peptides were trapped during 1 min at 15 μL/min with 99% A and 1% B. Elution was performed at 60 °C at a flow rate of 450 nL/min, using a linear gradient of 6−35% B over 28 min. The mass spectrometer was operating in positive mode, with the following settings: source temperature was set to 160 °C while dry gas flow was at 5 L per minute. The nanoelectrospray voltage was optimized to −5000 V. External mass calibration of the TOF was achieved before each set of analyses using Tuning Mix (Agilent Technologies, Paolo Alto, CA) in the mass range of 322−2722 m/z. Mass correction was achieved by recalibration of acquired spectra to the applied lock masses (methylstearate ([M+H]+ 299.2945 m/z) and hexakis(2,2,3,3,-tetrafluoropropoxy)phosphazine ([M+H]+ 922.0098 m/z)). For tandem MS experiments, the system was operated with automatic switching between MS and MS/MS modes in the range of 100−2500 m/z (MS acquisition time of 0.4 s, MS/MS acquisition time between 0.05 s (intensity >250 000) and 1.25 s (intensity <5000)). The six most abundant ions (absolute intensity threshold of 1500), were selected from each MS spectrum for further isolation and CID fragmentation using nitrogen as collision gas. Ions were excluded after acquisition of one MS/MS spectrum and the exclusion was released after 0.2 min. Peak lists in Mascot generic format (.mgf) were generated using Data Analysis (version 4.0; Bruker Daltonics, Bremen, Germany).

MS/MS Data Interpretation

The peak list has been searched against a NCBInr-derived combined target-decoy database (created 2010−03−23, containing 219120 target sequences plus the same number of reversed decoy sequences) using Mascot (version 2.4.1, Matrix science, London, England). During the database search, up to one missed cleavage by trypsin and two variable modifications (oxidation of Methionine (+16 Da), carbamidomethylation of Cysteine (+57 Da), were considered. The search window was set to 15 ppm for precursor ions and 0.05 Da for fragment ions. Mascot result files (.dat) were imported into the Scaffold 3 software (version 3.00.03; Proteome Software Inc., Portland, OR) used to validate MS/MS based peptide and protein identifications. Mascot identifications required at least ion minus identity scores greater than 0 and ion scores greater than 40. Protein identification was accepted if the protein contained at least 1 identified peptide. The false discovery rate (FDR) was calculated to be <1% based on the number of decoy hits.

RESULTS

Purification of LukE/D

LukE/D were isolated from the *S. aureus* Newman strain (NTCC 8178) as described in the Materials and Methods section. Once isolated, they were analyzed by RP-HPLC to determine their purity (Figure 1). HPLC peaks were collected and analyzed by SDS-PAGE after Coomassie Blue staining (Figure 1). Experimental masses obtained by SDS-PAGE and MALDI-TOF were in accordance with the theoretical molecular values reported in www.uniprot.org, (LukE: UniProt; A6QI09, LukD: UniProt; A6QI08). For each protein, the N-terminal sequence determined by Edman sequencing confirms the identification of LukE/D compared to UniProt sequences.

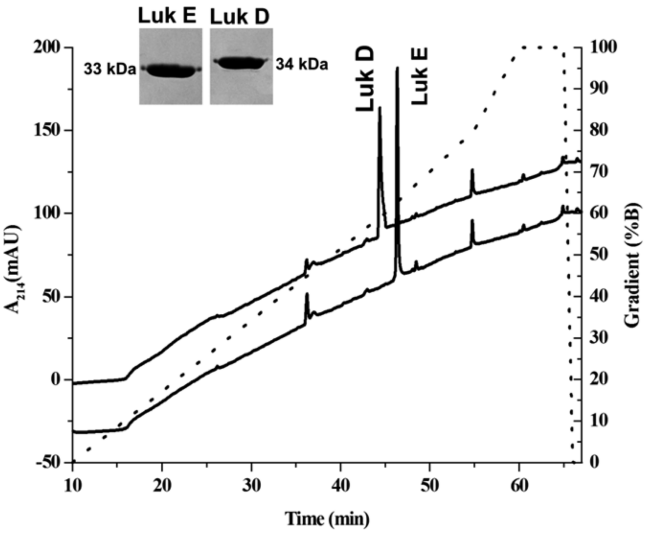

Figure 1. Reversed-phase HPLC of LukE and LukD and sequence analysis. LukE and LukD (10 μg) were separately loaded on a reverse phase Nucleosil RP300−5C18 column. Elution was performed at a flow rate of 700 μL/min with a linear gradient as indicated by dotted line on the right-hand scale. Fractions with one min time interval were collected for mass spectrometry. LukE and LukD were further loaded on a 12% SDS-PAGE and stained by Coomassie Blue.

LukE/D Stimulate PMNs

To determine the effects of LukE/D on PMNs activation, we carried out flow cytometry. Two parameters were evaluated: first, the kinetics of the ethidium bromide entry into the cells (Figure 2A) and second the kinetics of Ca^{2+} influx into the cells (Figure 2B). LukE and LukD were used at a concentration ranging from 2 to 60 nM (Figure 2A, B). Ethidium bromide uptake by PMNs is typically used as an indicator of pore formation.[23] We observed a rapid increase in ethidium bromide uptake, which continues during the first hour of the treatment (Figure 2A). These results are indicative of the toxicity of LukE/D toward PMNs. The presence of the toxins induced an increase of fluorescence and the time-course of ethidium bromide entry was dependent on the toxin concentration. Meanwhile an increase in calcium uptake by PMNs was also observed.

LukE/D at nanomolar concentrations stimulate PMNs by the entry of Ca^{2+} (Figure 2B), similarly to the effect observed for PVL,[17] which is mediated by the opening of the Ca^{2+} channels of the neutrophils plasma membrane.[24] With 10 nM of LukE/D, calcium uptake was rapid and later stabilized (Figure 2B). The observed increase in the intracellular calcium concentration is directly related to PMNs activation and production of cell secretions.[36] LukE/D leads to stimulation of PMNs, by triggering extracellular calcium influx and consequently production of secretory products. From the different concentrations tested, we selected 10 nM of LukE/D to induce cell secretion for further analyses, as 10 nM of LukE/D leads to a steady influx of calcium and abundant cell secretion.

Leukotoxin LukE/D Stimulates Production of AMPs and Other Immune Related Proteins

Secretions of PMNs were obtained after stimulation by 10 nM LukE/D, which leads to steady increase in ethidium positive cells (about 57% at 25 min incubation) (Supporting Information (SI) Figure 1A, B). Moreover, cell debris was evaluated 6% of total cells (SI Figure 1A, B). PMNs secretions were purified by RP-HPLC as described in the Materials and Methods section (Figure 3). One mg of PMN secretions was loaded on the HPLC column. Absorbance was monitored at 214 nm and elution was performed at a flow rate of 700 μL/min with a linear gradient, as indicated by the dotted line. Fractions were collected with 1 min time interval and marked

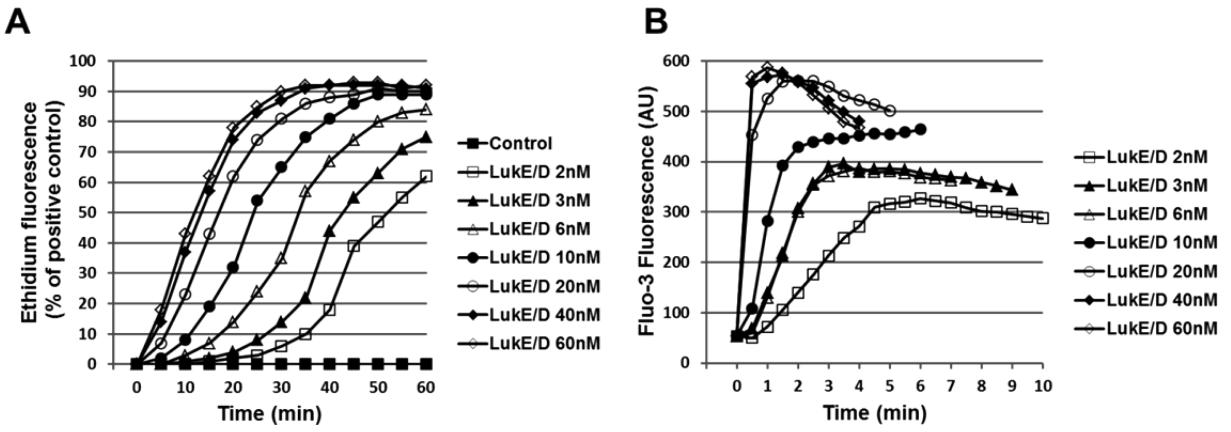

Figure 2. Time course of ethidium bromide and Ca2+ entry in human neutrophils. **A:** incubation with various concentrations (2, 3, 6, 10, 20, 40, and 60 nM) of LukE/D. **B:** Time course of Ca^{2+}entry in human neutrophils incubated with various concentrations of LukE/D (2, 3, 6, 10, 20, 40, and 60 nM).

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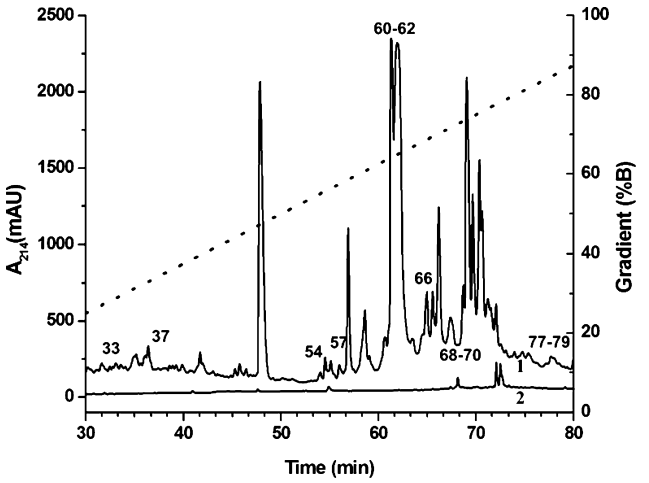

Figure 3. Reverse-phase HPLC of proteic material from LukE/D-induced PMNs secretions compared to the control. (1) After stimulation with 10 nM of LukE/D and (2) as a control, after treatment with PBS.

according to the collection time. Collected fractions were concentrated using a speed-vac. Compared to the control of nonstimulated PMNs (PBS) (Figure 3: Chromatogram 1), LukE/D stimulated neutrophils (Figure 3: Chromatogram 2) and induced a rapid secretion of a large number of immune related factors. The 223 secreted proteins identified by mass spectrometry are listed according to the RP-HPLC fraction numbers (SI Data 1), together with their number of unique peptides, sequence coverage, Mascot scores and the peptides used to identify them (SI Data 2).

Using gene ontology analysis software (GoTermMapper), all these factors were classified into different functional categories on the basis of their molecular functions, according to the Gene Ontology database analysis (Figure 4). Based on the theme of innate immunity, we decided to classify the proteins into the following functional categories: (1) AMPs and other immune related proteins, (2) binding proteins, (3) inflammatory proteins, (4) enzymatic proteins, (5) cell growth and differentiation proteins, (6) transport proteins, (7) cytoskeleton proteins, (8) antioxidant proteins, (9) proteins involved in proteolysis and (10) proteins related to protein synthesis (Figure 4).

The pie chart representing the identified proteins revealed a major secretion of AMPs and other immune related proteins (27% of total secreted products) (Figure 4; Table 1). HPLC fraction numbers containing the identified proteins were listed together with their NCBI accession numbers and their identification probability. In addition, the type of granule containing the proteins is also listed next to their position. Other abundant secreted proteins belong to the binding proteins (11%), inflammatory proteins (8%), enzymatic proteins (13%), cell growth and differentiation proteins (12%), transport proteins (11%), cytoskeleton proteins (10%), antioxidant proteins (2%), proteins involved in proteolysis (4%), and proteins related to protein synthesis (2%) (Figure 4). All these secreted factors well illustrate acute activation of the neutrophil cell machinery.

Antimicrobial Activities of the PMNs Secretions

Antimicrobial assays were performed on RP-HPLC fractions against different bacterial, fungal, and yeast strains. Two Gram-positive bacteria (*M. luteus* and *S. aureus*) were tested for their antibacterial activity and *N. crassa*, *A. fumigatus*, *C. albicans*, and *C. tropicalis* were challenged for their antifungal activity. Several fractions were active against the pathogenic strains used. Fractions expressing 100% bacteria and fungi growth inhibition are presented in Tables 2 and 3, respectively.

Chromogranin A and B-Derived Peptides Are Secreted by PMNs in Response to LukE/D Stimulus

In LukE/D-induced PMNs secretions, CgA and CgB were identified (Figure 5A, B) by using two types of antibodies: a monoclonal anti-CgA (CgA_{53-57}) and a polyclonal anti-CgB

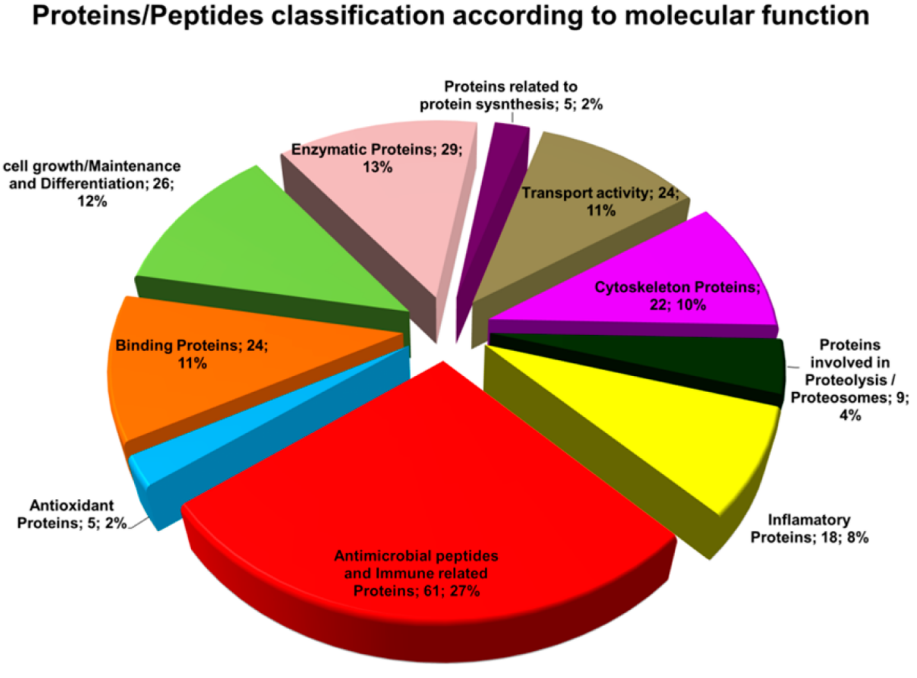

Figure 4. Pie chart representing the protein distribution according to their molecular function (GoTermMapper).

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Table 1. Identification of Proteins Involved in Innate Immunity by LC-MS/MS Analysis of RP-HPLC Fractions Corresponding to LukE/D-Induced PMN Secretions[a]

HPLC fraction number	protein name	protein accession numbers	protein molecular weight (Da)	total number of unique peptide	percentage sequence coverage (%)	granule type	reference
28−31, 74−75, 80	alpha-1 antitrypsin variant	gil 110350939	46 607.8	4	12	AZ	37
9	alpha-1-antichymotrypsin	gil112874	47 653.0	1	3	AZ	37
39−42, 51−59, 68−69, 75−79	alpha-enolase	gil119339	47 170.2	12	41	GE	14
28−31	anti-SARS-CoV S protein immunoglobulin kappa light chain	gil 110626504	23 566.5	3	24	GE	14
74	antithrombin-III	gil113936	52 604.1	1	2	SP, GE	14,37
62, 63	calreticulin	gil117501	48 142.9	1	7	GE, SP, AZ	14
56−57, 68	cathelicidin antimicrobial peptide	gil 119585258	19 591.6	5	30	GE, SP, AZ	14,38
63	cathepsin C	gil 119579767	35 640.0	2	11	GE, SP, AZ	14,38
37, 57−63	cathepsin G	gil115725	28 837.5	10	46	GE, SP, AZ	14,38
37, 60−63	cationic antimicrobial protein CAP37	gil227250	24 051.0	7	58	GE, AZ	14,38
59	CD93 antigen	gil 119630576	68 549.1	1	2	AZ	39
68	chitinase 3-like 1	gil23512215	42 527.9	8	29	SP	14,38
47−48	chitotriosidase-1	gil37999493	51 682.1	8	25	GE, SP, AZ	14
75	chromogranin A	gil180529	50 929.5	1	1	SP	14
37, 61−62	cofilin-1	gil116848	18 503.2	8	57	GE	14
61	complement factor properdin	gil 119579721	49 664.4	1	3	GE, SP, SV	14,38
59, 76	dermcidin isoform 2	gil 148271063	12 414.5	1	9	GE, SP	14
53−59	Eosinophil cationic protein	gil 147744558	18 385.3	5	37	GE, SP, AZ	14
78−80	neuroleukin	gil17380385	63 148.5	16	43	GE	14
28, 31, 34	hCG2029987	gil 119602340	43 900.2	7	28	GE	14
44	histocompatibility (minor) HA-1	gil 119589958	122 920.8	1	1	GE, SP	14,38
28	Ig alpha-1 chain C region	gil113584	37 653.8	3	20	GE, SP, AZ	14
28	Ig gamma 2 H chain	gil243169	38 721.8	3	14	GE	14
56−60	IgG receptor IIIB	gil11344591	26 189.0	3	15	GE, SP	14
57−61	IgG receptor precursor	gil183037	26 299.0	3	14	GE, SP	14
28, 31	immunoglobulin heavy chain constant region	gil10799664	35 919.3	4	19	GE, SP, AZ	14
37−38, 57−70, 72−79	lactoferrin	gil34412	78 338.5	42	69	GE, SP, AZ	14,38
61−63	lactoferrin precursor	gil12083188	78 382.5	42	69	GE, SP, AZ	14,38
77−79	leukotriene A-4 hydrolase	gil126353	69 287.1	15	36	SP	14
38−40, 42	lymphocyte-specific protein 1; WP34	gil462553	37 191.1	1	8	GE, SP	14
37−38, 60−63	lysozyme	gil1335210	14 700.5	6	59	GE, SP, AZ	14,38
44, 47, 70−72	matrix metallopeptidase 8 (neutrophil collagenase)	gil 119587431	53 426.3	10	31	GE, SP	14
69−79	myeloperoxidase	gil 119614877	88 763.7	17	28	GE, SP, AZ	14
40, 47, 50, 70−73, 79	neutrophil collagenase	gil116862	53 414.2	10	31	SP	14
54−55	neutrophil defensin 4	gil399352	10 504.1	1	18	SP, GE	14,38
71, 73−79	neutrophil elastase	gil119292	28 517.4	3	27	SP, AZ	14,38
63−68	neutrophil gelatinase-associated lipocalin	gil1171700	22 588.9	7	48	GE, SP	14,38
46−53, 60−63	neutrophil granule peptide HP1	gil228797	3448.1	4	83		
57	nonspecific cross reacting antigen	gil189102	38 139.8	2	10		

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Table 1. continued

HPLC fraction number	protein name	protein accession numbers	protein molecular weight (Da)	total number of unique peptide	percentage sequence coverage (%)	granule type	reference
37, 60−64	peptidoglycan recognition protein 1	gil18202143	21 730.6	5	49	GE, SP	14
63−66, 74, 77−79	plastin-2	gil1346733	70 292.1	25	49	GE, SP, AZ	14
34, 54−58	polyubiquitin	gil11024714	68 492.8	5	8	GE, SP, AZ	14
78	PRO2783 (mitogen-activated protein kinase kinase 1-interacting protein 1)	gil11493534	13 589.0	1	13	SP	40
53, 57−63, 67, 76−77	profilin-1	gil130979	15 054.3	7	62	GE, SP, AZ	14
48	proteasome activator complex subunit 1	gil1170519	28 723.9	1	4		
79	proteasome activator subunit 2 (PA28 beta)	gil 119586505	21 752.7	1	12		
75	proteasome subunit alpha type-6	gil46397659	27 399.5	2	10		
76	proteasome subunit, alpha type, 3	gil 119601121	24 217.1	3	15		
79	proteasome subunit, alpha type 4	gil 119619571	25 047.6	3	18		
74	proteasome subunit, beta type 2	gil 119627813	20 208.6	1	9		
46, 67−74	protein S100-A11; calgizzarin	gil1710818	11 741.1	3	34	SP	14
69−72, 77−78	protein S100-A12;calgranulin-C	gil2507565	10 575.4	4	65	SP	14
73	protein S100-A4; metastasin	gil115601	11 729.0	1	8	GE, SP, AZ	14
74, 76−77	protein S100-A6; Calcyclin	gil116509	10 180.4	4	48	GE, SP AZ	14
34, 63, 66−80	protein S100-A8; calgranulin-A	gil115442	10 835.0	9	76	GE, SP, AZ	14
34, 36, 66−80	protein S100-A9; calgranulin-B	gil115444	13 242.3	8	80	GE, SP AZ	14
76−79	protein S100−P	gil134142	10 400.4	4	72	GE, SP, AZ	14
28	serum amyloid A1	gil 119588814	13 562.6	1	12	GE, SP AZ	14,37
60−61	SGP28 protein	gil1587691	27 640.1	4	28	SP	41
47−49, 66−67	vimentin	gil55977767	53 652.7	14	34	GE, SP, AZ	14

[a]GE: gelatinous granules, SP: specific granules, AZ: azurophilic granules.

Table 2. Antibacterial Assay on the RP-HPLC Fractions (n = 96), Each Containing 1 mg of LukE/D-Induced PMNs Secretions (Figure 3)[a]

antimicrobial HPLC Fraction no.	name of protein identified	active (100% inhibition) against	
		Micrococcus luteus	*Staphylococcus aureus*
54	neutrophil defensin IV, eosinophil cationic protein, polyubiquitin	X	
57	cathepsin G, cathelicidin, eosinophil cationic protein, Polyubiquitin, lactoferrin	X	X
60	azurocidin, eosinophil cationic protein, lysozyme	X	
61	cathepsin G, lactoferrin, lysozyme, azurocidin	X	
62	cathepsin G, lactoferrin, lysozyme, azurocidin	X	
66	lysozyme C, lactotransferrin, calgranulin A, calgranulin B	X	
68	calgranulin A, calgranulin B, calgizzarin, cathelicidin antimicrobial peptide, neutrophil gelatinase associated lipocalin, cathepsin G, lactoferrin	X	X
69	calgranulin A, calgranulin B, clgranulin C, calgizzarin,	X	X
70	calgranulin A, calgranulin B, calgranulin C, calgizzarin,	X	
77	calcyclin, lactoferrin, calgranulin A, calgranulin B, calgranulin C, calgizzarin, protein S100-P,	X	X
79	lactoferrin, calgranulin A, calgranulin B, protein S100-P,	X	

[a]Fractions with 100% of bacterial growth inhibition are indicated for each bacterial strain (*Micrococcus luteus* and *S. aureus*).

($CgB_{547-560}$). The control used in both cases is the granular proteic material of chromaffin cells (PSG), which is highly rich in Cgs and their derived peptides.[42] In the present experiment, we identified several fragments of both CgA and CgB: a major

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Table 3. Antifungal Assay on the Reverse Phase HPLC Fractions (n = 96), Each Containing 1 mg of LukE/D-Induced PMNs Secretions (Figure 3)[a]

antifungal HPLC fraction no.	protein identified	active (100% inhibition) against			
		Neurospora crassa	*Aspergillus fumugatus*	*Candida albicans*	*Candida tropicalis*
33	calgranulin A, calgranulin B, polyubiquitin	X			
37	cathepsin G, lactoferrin, cationic antimicrobial peptide, lysozyme	X	X	X	X
54	neutrophil defensin IV, eosinophil cationic protein, polyubiquitin	X		X	
57	cathepsin G, cathelicidin, eosinophil cationic protein, polyubiquitin, lactoferrin	X	X	X	X
60	azurocidin, eosinophil cationic protein, lysozyme	X	X		X
61	cathepsin G, lactoferrin, lysozyme, azurocidin	X			
62	cathepsin G, lactoferrin, lysozyme, azurocidin	X			
66	lysozyme C, lactotransferrin, calgranulin A, calgranulin B	X	X	X	
68	calgranulin A, calgranulin B, calgizzarin, cathelicidin antimicrobial peptide, Neutrophil gelatinase associated lipocalin, cathepsin G, lactoferrin	X	X	X	X
69	calgranulin A, calgranulin B, calgranulin C, calgizzarin,	X	X	X	X
70	calgranulin A, calgranulin B, calgranulin C, calgizzarin,	X	X	X	
77	calcyclin, lactoferrin, calgranulin A, calgranulin B, calgranulin C, calgizzarin, protein S100−P,	X	X		
78	calgranulin A, calgranulin B, calgranulin C, lactoferrin, protein S100−P,	X		X	X
79	lactoferrin, calgranulin A, calgranulin B, protein S100-P,	X	X		

[a]For each microorganism (*Neurospora crassa*, *Aspergillus fumigatus*, *Candida albicans*, and *Candida tropicalis*) fractions displaying 100% of fungal growth inhibition are indicated.

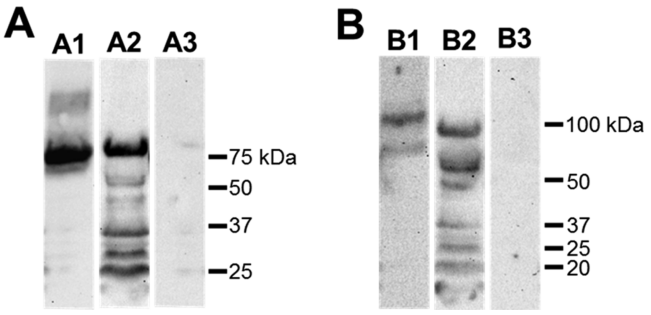

Figure 5. Immunodetection of CgA and CgB in the secretions of LukE/D-treated PMNs. **A:** Western blot analysis (12%, SDS-PAGE) with monoclonal antibody (anti- CgA_{47-68}). **A1,** Soluble material from chromaffin granules used as a positive control. **A2,** PMNs secretions obtained after stimulation with LukE/D. **A3,** Extra-cellular medium after PBS treatment. **B:** Western blot analysis (12%, SDS-PAGE) with polyclonal antibody anti-$CgB_{547-560}$. **B1,** Soluble material from chromaffin granules used as a positive control. **B2,** PMNs secretions obtained after stimulation with leukotoxin LukE/D. **B3,** Extra-cellular medium after PBS treatment.

band of 75 kDa (apparent molecular weight) was recovered and corresponds to full-length CgA (CgA_{1-439}) (Figure 5A). In addition, according to previous studies, several fragments were also recovered including CgA_{1-291} (51 kDa), CgA_{1-249} (34 kDa), CgA_{1-209} (28 kDa) and CgA_{1-115} (24 kDa)[43,44] (Figure 5A). Similarly, regarding CgB, a band of 100 kDa (apparent molecular weight), which corresponds to full-length CgB (CgB_{1-657}) (Figure 5B) as well as several shorter fragments were identified: $CgB_{156-657}$ (70 kDa), $CgB_{439-657}$ (37 kDa), $CgB_{498-657}$ (30 kDa), $CgB_{520-657}$ (25 kDa), and $CgB_{568-657}$ (20 kDa)[45] (Figure 5B).

DISCUSSION

LukE/D, a pore-forming leukotoxin produced by *S. aureus* strains infecting human, is specific against host defense cells and T-lymphocytes.[46−48] Several recent studies have underlined the crucial role of LukE/D in *S. aureus* virulence. LukE/D gene expression was significantly higher in community-associated methicillin-resistant *S. aureus* (*CA-MRSA*) compared with methicillin sensitive *S. aureus* (*MSSA*), suggesting that they contribute to bacterial virulence.[49] Analysis of the most prevalent genes associated with *MRSA* strains with type III *SCCmec* points out LukE/D in 72.9% of the isolates.[50] Furthermore, it was reported that in addition to PVL, a high prevalence of LukE/D was recorded in HIV (+) furuncles patients.[51] The role of LukE/D to trigger inflammation by acting synergistically with PVL to amplify IL-1b release in human macrophages has been demonstrated.[52] A biological mechanism explaining the toxic activity of LukE/D was proposed in a recent study.[48] Indeed, the authors demonstrate that LukE/D is specifically cytotoxic on human T-cell line expressing CCR5. Moreover, LukE, but not LukD binds to CCR5 in a time-dependent manner.[48]

In the present study, we characterized the proteins secreted by PMNs upon *S. aureus* LukE/D activation. PMNs are potent actors of innate immunity and serve as an important interface between innate and adaptive immunity by producing reactive oxygen species, antimicrobial peptides, chemokines and cytokines.[53,54]

The 223 proteins identified in the neutrophil secretions after proteomic analysis were classified according to their molecular function by using go.princeton.edu/cgi-bh/GoTermMapper (Figure 4). Major proteins correspond to AMPs and immune related proteins (27%). PMNs display a potent microbicidal activity including oxygen-dependent and oxygen-independent strategies to destroy invading pathogens.[54] The production of ROS derived from NADPH oxidase is critical for host defense mechanisms against invading microorganisms.[55] In relation with oxidative mechanisms, the proteomic analysis indicates that 2% of antioxidant proteins and oxygen-independent microbicidal agents include AMPs[38] such as defensins, cathelicidins, azurocidin, cathepsins, lactoferrin, chromogranin A, and lysozyme (Table 1). Enzymatic proteins, such as cathepsin, lysozyme, pyruvate kinase, neutrophil collagenase, eosinophil peroxidase, and matrix metallo peptidase correspond to 13% and are important for proteolysis of AMPs and to

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manage the neutrophil turnover. Proteins for cell growth and development, such as epidermal growth factor (EGF), elongation factors, olfactomedin 4 (OLFM4) and neuroleukin represent 12% of the total released proteins and they participate to the rapid turnover of PMNs (Figure 4). All these proteins are crucial for the dynamics of host-pathogen interactions with the multistep progression into mature PMNs.[56] Binding proteins correspond to 11% of the total proteins and include DNA binding proteins (histones, high mobility group protein B2), calcium binding proteins (calmodulin and its isoforms), adenylate cyclase and fatty acid binding proteins. The proteomic analysis also includes proteins involved in transport activity (hemoglobin, lactoferrin, and neutrophil gelatinase associated lipocalin), which play an important role in boosting innate defense at the site of infection. PMNs are the most efficient vertebrate motile defense cells. Their directional movement is governed by the binding of chemoattractants and chemokines to G-proteins coupled receptors. These motility responses start with the polymerization of F-actin and the rearrangement of cytoskeleton and cell crawling. The stimulation of neutrophils by LukE/D induces the release of cytoskeleton proteins (10%) such as actin, actinin, gelsolin, profilin, and vimentin. The proteins involved in the modulation of inflammation represent 8% of the total proteins and play a key role in the early pro-inflammatory signals, as they release proinflammatory molecules to promote efficient pathogen recognition and removal.[54] Numerous secreted proteins are pro-inflammatory molecules such as cathepsin, chitotriosidase, cofilin, complement C3, cysteine rich secretory protein, eosinophil cationic protein, eosinophil peroxidase, neutrophil elastase, defensin-4, proteasome proteins, S100 A12, calgranulin, vimentin. In contrast, others such as alpha-1 antitrypsin, serpin, antithrombin III, neuroleukin, lactoferrin, lysozyme, MMP8, peptidoglycan recognition protein 1 and polyubiquitin are anti-inflammatory. Collectively, these observations suggest that LukE/D can enhance rather than hinder the host innate immune response to *S. aureus* infection. Nevertheless, the ability of LukE/D to enhance neutrophils bactericidal activity *in vivo* needs further investigation. For the first time Brinkmann et al. reported that activated PMNs release their DNA that is laden with antimicrobial molecules and form PMNs extracellular traps (NETs).[57] Later, in 2007 with a very detailed series of imaging experiments it was demonstrated that the formation of NETs in response to *S. aureus* is the last step in a process of active PMN death.[58] It was also reported that over the period of 3 h, the PMNs underwent important morphological changes. The granular membranes disappeared and the nuclear, cytoplasmic and granular components were mixed together, allowing the extrusion of the NETs and therefore the antimicrobial role of PMNs even after cell death. PVL was described to be the major NET inducer, after inhibition of NET production by treatment with antiserum against PVL.[59] Our proteomic analysis of the PMNs secretions induced by LukE/D shows the presence of 6.25% cytoskeleton proteins (Figure 4) also suggesting the involvement of severe morphological changes.

Calcium is a universal secondary messenger involved in many cellular signal transduction pathways, regulating crucial functions such as secretion, cell motility, proliferation and cell death. Increase in intracellular Ca^{2+} derives mainly from two sources: internal stores releasing Ca^{2+} into the cytosol and Ca^{2+} channels in the plasma membrane that open to allow external Ca^{2+} to flow into the cell. In PMNs, calcium signaling has been reported to be involved in oxidase activation, cell degranulation and priming response to a wide range of proinflammatory molecules.[60] In the present study, we demonstrated that calcium binding proteins represent ~11% of the PMNs secreted proteins highlighting the importance of calcium in the stimulation of PMNs by LukE/D. For instance, vesicular amine transport (VAT-I) plays a crucial role in calcium regulated cell activation.[61]

Our previous studies have established the presence of CgA in neutrophils and its role as an immune activator.[17] Immunodetection of CgA (Figure 5) showed that LukE/D induces the predominant release of full-length CgA (75 kDa) but also of minor fragments with apparent molecular masses of 50, 35, 30, and 25 kDa that might correspond to fragments 1-298 1-210, 1-182, and 1-115. Moreover, immunodetection of CgB (Figure 5) shows the release of two major fragments corresponding to full-length CgB (100 kDa) and the 143-657 fragment (70 kDa). In addition, several minor fragments 259-657 (50 kDa), 306-657 (37 kDa), 555-657 (20 kDa), and 564-657 (10 kDa) were identified.

CONCLUSION

We previously described that an increase in intracellular free Ca^{2+} can induce secretion in PMNs.[62] These secretions can result from cell activation by the two LukE and LukD components of the LukE/D toxin of *S. aureus*. A similar effect was observed previously by using PVL of *S. aureus*.[23] Complement C3 factor (CD35) was recovered upon PVL treatment, which indicates neutrophil activation.[63] Here, we demonstrated that in addition to toxicity, LukE/D can also stimulate PMNs. Identification of trademark proteins from different PMNs granules underlines the ability of this leukotoxin to degranulate all type of compartments. PMNs secrete various proteins belonging to different functional categories, such as immune related proteins, antioxidant proteins, proteases, and inflammatory components. Beyond these functional categories, AMPs and immune activation proteins represent a significant proportion of the secretions in this specific inflammatory stimulus. The release of all these factors is crucial for innate immune functions. As an example, we recovered Cgs-derived fragments, which are active participants of the immune system. These findings underlie the importance to further study the role of leukotoxin LukE/D as immune activating components.

ASSOCIATED CONTENT

Supporting Information

Extended information of all the proteins/peptides identified by LC-MS/MS in PMNs secretions induced by *S. aureus* leukotoxin LukE/D, are listed in the Supporting Information. Data are presented according to RP-HPLC fraction numbers, which correspond to collection time (data 1). Identified proteins are provided with their NCBI database accession numbers, molecular weight, percent sequence coverage, and corresponding peptide sequences (data 1). Proteomic analysis was supplemented with Mascot Ion score, Mascot identity score and ion score-identity score (data 2). Proteins/peptides identified by only one unique peptide and with Ion score less than 40 are presented with MS/MS spectra (data 3). This material is available free of charge via the Internet at http://pubs.acs.org.

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Author Contributions

The manuscript was written through contributions of all authors. RA. has performed the experiments and prepared the manuscript and figures. B.-J.L. and D.K. have purified and characterized LukE/D. G.P. supervised the study and with C.M. participated in preparation of the manuscript. J.-M.S. and A.v.D. brought their expertise in proteomic studies; Y.H. and C.T. participated to interesting discussions and M.-H. M.-B. supervised the experiments and the preparation of the manuscript. All authors have given approval to the final version of the manuscript.

Notes

The authors declare no competing financial interest.

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REFERENCES

(1) Heyman, D. *Control of Communicable Diseases Manual*, 18th ed.; American Public Health Association: Washington DC, 2004.

(2) DeLeo, F. R.; Otto, M.; Kreiswirth, B. N.; Chambers, H. F. Community-associated meticillin-resistant *Staphylococcus aureus*. *Lancet* **2010**, *375* (9725), 1557−68.

(3) Foster, T. J.; Hook, M. Surface protein adhesins of *Staphylococcus aureus*. *Trends Microbiol.* **1998**, *6* (12), 484−8.

(4) Gordon, R. J.; Lowy, F. D. Pathogenesis of methicillin-resistant *Staphylococcus aureus* infection. *Clin Infect Dis.* **2008**, *46* (Suppl 5), S350−9.

(5) Switalski, L. M.; Patti, J. M.; Butcher, W.; Gristina, A. G.; Speziale, P.; Hook, M. A collagen receptor on *Staphylococcus aureus* strains isolated from patients with septic arthritis mediates adhesion to cartilage. *Mol. Microbiol.* **1993**, *7* (1), 99−107.

(6) Hauck, C. R.; Ohlsen, K. Sticky connections: Extracellular matrix protein recognition and integrin-mediated cellular invasion by *Staphylococcus aureus*. *Curr Opin Microbiol.* **2006**, *9* (1), 5−11.

(7) Sauer, P.; Sila, J.; Stosova, T.; Vecerova, R.; Hejnar, P.; Vagnerova, I.; Kolar, M.; Raclavsky, V.; Petrzelova, J.; Loveckova, Y.; Koukalova, D. Prevalence of genes encoding extracellular virulence factors among meticillin-resistant *Staphylococcus aureus* isolates from the University Hospital, Olomouc, Czech Republic. *J. Med. Microbiol.* **2008**, *57* (Pt 4), 403−10.

(8) Prévost, G.; Cribier, B.; Couppie, P.; Petiau, P.; Supersac, G.; Finck-Barbançon, V.; Monteil, H.; Piémont, Y. Panton-Valentine leucocidin and gamma-hemolysin from *Staphylococcus aureus* ATCC 49775 are encoded by distinct genetic loci and have different biological activities. *Infect. Immun.* **1995**, *63* (10), 4121−9.

(9) Gravet, A.; Colin, D. A.; Keller, D.; Girardot, R.; Monteil, H.; Prévost, G. Characterization of a novel structural member, LukE-LukD, of the bi-component staphylococcal leucotoxins family. *FEBS Lett.* **1998**, *436* (2), 202−8.

(10) Gravet, A.; Couppie, P.; Meunier, O.; Clyti, E.; Moreau, B.; Pradinaud, R.; Monteil, H.; Prévost, G. *Staphylococcus aureus* isolated in cases of impetigo produces both epidermolysin A or B and LukE-LukD in 78% of 131 retrospective and prospective cases. *J. Clin. Microbiol.* **2001**, *39* (12), 4349−56.

(11) Joubert, O.; Viero, G.; Keller, D.; Martinez, E.; Colin, D. A.; Monteil, H.; Mourey, L.; Dalla Serra, M.; Prévost, G. Engineered covalent leucotoxin heterodimers form functional pores: Insights into S-F interactions. *Biochem. J.* **2006**, *396* (2), 381−9.

(12) Gravet, A.; Rondeau, M.; Harf-Monteil, C.; Grunenberger, F.; Monteil, H.; Scheftel, J. M.; Prévost, G. Predominant *Staphylococcus aureus* isolated from antibiotic-associated diarrhea is clinically relevant and produces enterotoxin A and the bicomponent toxin LukE-lukD. *J. Clin. Microbiol.* **1999**, *37* (12), 4012−9.

(13) Lugardon, K.; Raffner, R.; Goumon, Y.; Corti, A.; Delmas, A.; Bulet, P.; Aunis, D.; Metz-Boutigue, M. H. Antibacterial and antifungal activities of vasostatin-1, the N-terminal fragment of chromogranin A. *J. Biol. Chem.* **2000**, *275* (15), 10745−53.

(14) Lominadze, G.; Powell, D. W.; Luerman, G. C.; Link, A. J.; Ward, R. A.; McLeish, K. R. Proteomic analysis of human neutrophil granules. *Mol. Cell Proteomics* **2005**, *4* (10), 1503−21.

(15) Zhang, D.; Lavaux, T.; Voegeli, A. C.; Lavigne, T.; Castelain, V.; Meyer, N.; Sapin, R.; Aunis, D.; Metz-Boutigue, M. H.; Schneider, F. Prognostic value of chromogranin A at admission in critically ill patients: A cohort study in a medical intensive care unit. *Clin Chem.* **2008**, *54* (9), 1497−503.

(16) Metz-Boutigue, M. H.; Kieffer, A. E.; Goumon, Y.; Aunis, D. Innate immunity: Involvement of new neuropeptides. *Trends Microbiol.* **2003**, *11* (12), 585−92.

(17) Zhang, D.; Shooshtarizadeh, P.; Laventie, B. J.; Colin, D. A.; Chich, J. F.; Vidic, J.; de Barry, J.; Chasserot-Golaz, S.; Delalande, F.; Van Dorsselaer, A.; Schneider, F.; Helle, K.; Aunis, D.; Prévost, G.; Metz-Boutigue, M. H. Two chromogranin a-derived peptides induce calcium entry in human neutrophils by calmodulin-regulated calcium independent phospholipase A2. *PLoS One* **2009**, *4* (2), e4501.

(18) Ma, X.; Chang, W.; Zhang, C.; Zhou, X.; Yu, F. Staphylococcal panton-valentine leukocidin induces pro-inflammatory cytokine production and nuclear factor-kappa B activation in neutrophils. *PLoS One* **2012**, *7* (4), e34970.

(19) Borregaard, N.; Lollike, K.; Kjeldsen, L.; Sengelov, H.; Bastholm, L.; Nielsen, M. H.; Bainton, D. F. Human neutrophil granules and secretory vesicles. *Eur. J. Haematol.* **1993**, *51* (4), 187−98.

(20) Luerman, G. C.; Uriarte, S. M.; Rane, M. J.; McLeish, K. R. Application of proteomics to neutrophil biology. *J. Proteomics* **2009**, *73* (3), 552−61.

(21) Finck-Barbançon, V.; Prévost, G.; Piémont, Y. Improved purification of leukocidin from *Staphylococcus aureus* and toxin distribution among hospital strains. *Res. Microbiol.* **1991**, *142* (1), 75−85.

(22) Morinaga, N.; Kaihou, Y.; Noda, M. Purification, cloning and characterization of variant LukE-LukD with strong leukocidal activity of staphylococcal bi-component leukotoxin family. *Microbiol. Immunol.* **2003**, *47* (1), 81−90.

(23) Finck-Barbançon, V.; Duportail, G.; Meunier, O.; Colin, D. A. Pore formation by a two-component leukocidin from *Staphylococcus aureus* within the membrane of human polymorphonuclear leukocytes. *Biochim. Biophys. Acta* **1993**, *1182* (3), 275−82.

(24) Staali, L.; Monteil, H.; Colin, D. A. The staphylococcal pore-forming leukotoxins open Ca2+ channels in the membrane of human polymorphonuclear neutrophils. *J. Membr. Biol.* **1998**, *162* (3), 209−16.

(25) Colin, D. A.; Mazurier, I.; Sire, S.; Finck-Barbancon, V. Interaction of the two components of leukocidin from *Staphylococcus aureus* with human polymorphonuclear leukocyte membranes: Sequential binding and subsequent activation. *Infect. Immun.* **1994**, *62* (8), 3184−8.

(26) Barrio, M. B.; Rainard, P.; Prévost, G. LukM/LukF'-PV is the most active *Staphylococcus aureus* leukotoxin on bovine neutrophils. *Microbes Infect.* **2006**, *8* (8), 2068−74.
(27) Meunier, O.; Falkenrodt, A.; Monteil, H.; Colin, D. A. Application of flow cytometry in toxinology: Pathophysiology of human polymorphonuclear leukocytes damaged by a pore-forming toxin from *Staphylococcus aureus*. *Cytometry* **1995**, *21* (3), 241−7.
(28) Moussa, L. B.; Werner, S.; Coraiola, M.; Colin, D. A.; Keller, D.; Sanni, A.; Serra, M. D.; Monteil, H.; Prévost, G. Site-directed mutagenesis to assess the binding capacity of class s protein of *Staphylococcus aureus* leucotoxins to the surface of polymorphonuclear cells. *J. Biomed. Biotechnol.* **2006**, *2006* (2), 80101.
(29) Bradford, M. M. A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. *Anal. Biochem.* **1976**, *72*, 248−54.
(30) Corti, A.; Sanchez, L. P.; Gasparri, A.; Curnis, F.; Longhi, R.; Brandazza, A.; Siccardi, A. G.; Sidoli, A. Production and structure characterisation of recombinant chromogranin A N-terminal fragments (vasostatins) – evidence of dimer-monomer equilibria. *Eur. J. Biochem.* **1997**, *248* (3), 692−9.
(31) Gasnier, C.; Lugardon, K.; Ruh, O.; Strub, J. M.; Aunis, D.; Metz-Boutigue, M. H. Characterization and location of post-translational modifications on chromogranin B from bovine adrenal medullary chromaffin granules. *Proteomics* **2004**, *4* (6), 1789−801.
(32) NCCLS. *Methods for Dilution Antimicrobial Susceptibility Tests for Bacteria That Grow Aerobically*, Approved Standard M07-A8, 8th ed.; National Committee for Clinical Laboratory Standards: Wayne, PA, 2003.
(33) Bulet, P.; Dimarcq, J. L.; Hetru, C.; Lagueux, M.; Charlet, M.; Hegy, G.; Van Dorsselaer, A.; Hoffmann, J. A. A novel inducible antibacterial peptide of *Drosophila* carries an O-glycosylated substitution. *J. Biol. Chem.* **1993**, *268* (20), 14893−7.
(34) Wu, M.; Hancock, R. E. Interaction of the cyclic antimicrobial cationic peptide bactenecin with the outer and cytoplasmic membrane. *J. Biol. Chem.* **1999**, *274* (1), 29−35.
(35) Broekaert, W. F.; Terras, F. R. G.; Cammue, B. P. A.; Vanderleyden, J. An automated quantitative assay for fungal growth inhibition. *FEMS Microbiol. Lett.* **1990**, *69* (1−2), 55−59.
(36) Granfeldt, D.; Harbecke, O.; Bjorstad, A.; Karlsson, A.; Dahlgren, C. Neutrophil secretion induced by an intracellular Ca^{2+} rise and followed by whole-cell patch-clamp recordings occurs without any selective mobilization of different granule populations. *J. Biomed. Biotechnol.* **2006**, *2006* (2), 97803.
(37) Rosales, J. L.; Ernst, J. D. Calcium-dependent neutrophil secretion: Characterization and regulation by annexins. *J. Immunol.* **1997**, *159* (12), 6195−202.
(38) Faurschou, M.; Borregaard, N. Neutrophil granules and secretory vesicles in inflammation. *Microbes Infect.* **2003**, *5* (14), 1317−1327.
(39) Burlak, C.; Whitney, A. R.; Mead, D. J.; Hackstadt, T.; Deleo, F. R. Maturation of human neutrophil phagosomes includes incorporation of molecular chaperones and endoplasmic reticulum quality control machinery. *Mol. Cell Proteomics* **2006**, *5* (4), 620−34.
(40) Mocsai, A.; Banfi, B.; Kapus, A.; Farkas, G.; Geiszt, M.; Buday, L.; Farago, A.; Ligeti, E. Differential effects of tyrosine kinase inhibitors and an inhibitor of the mitogen-activated protein kinase cascade on degranulation and superoxide production of human neutrophil granulocytes. *Biochem. Pharmacol.* **1997**, *54* (7), 781−9.
(41) Kjeldsen, L.; Cowland, J. B.; Johnsen, A. H.; Borregaard, N. SGP28, a novel matrix glycoprotein in specific granules of human neutrophils with similarity to a human testis-specific gene product and a rodent sperm-coating glycoprotein. *FEBS Lett.* **1996**, *380* (3), 246−50.
(42) Smith, A. D.; Winkler, H. Purification and properties of an acidic protein from chromaffin granules of bovine adrenal medulla. *Biochem. J.* **1967**, *103* (2), 483−92.
(43) Metz-Boutigue, M. H.; Garcia-Sablone, P.; Hogue-Angeletti, R.; Aunis, D. Intracellular and extracellular processing of chromogranin A. Determination of cleavage sites. *Eur. J. Biochem.* **1993**, *217* (1), 247−57.
(44) Gadroy, P.; Stridsberg, M.; Capon, C.; Michalski, J. C.; Strub, J. M.; Van Dorsselaer, A.; Aunis, D.; Metz-Boutigue, M. H. Phosphorylation and O-glycosylation sites of human chromogranin A (CGA79−439) from urine of patients with carcinoid tumors. *J. Biol. Chem.* **1998**, *273* (51), 34087−97.
(45) Fournier, I.; Gaucher, D.; Chich, J. F.; Bach, C.; Shooshtarizadeh, P.; Picaud, S.; Bourcier, T.; Speeg-Schatz, C.; Strub, J. M.; Van Dorsselaer, A.; Corti, A.; Aunis, D.; Metz-Boutigue, M. H. Processing of chromogranins/secretogranin in patients with diabetic retinopathy. *Regul. Pept.* **2011**, *167* (1), 118−24.
(46) Foster, T. J. The *Staphylococcus aureus* "superbug". *J. Clin. Invest.* **2004**, *114* (12), 1693−6.
(47) Foster, T. J. Immune evasion by staphylococci. *Nat. Rev. Microbiol.* **2005**, *3* (12), 948−58.
(48) Alonzo, F., 3rd; Kozhaya, L.; Rawlings, S. A.; Reyes-Robles, T.; DuMont, A. L.; Myszka, D. G.; Landau, N. R.; Unutmaz, D.; Torres, V. J. CCR5 is a receptor for *Staphylococcus aureus* leukotoxin ED. *Nature* **2013**, *493* (7430), 51−5.
(49) Shukla, S. K.; Karow, M. E.; Brady, J. M.; Stemper, M. E.; Kislow, J.; Moore, N.; Wroblewski, K.; Chyou, P. H.; Warshauer, D. M.; Reed, K. D.; Lynfield, R.; Schwan, W. R. Virulence genes and genotypic associations in nasal carriage, community-associated methicillin-susceptible and methicillin-resistant USA400 *Staphylococcus aureus* isolates. *J. Clin. Microbiol.* **2010**, *48* (10), 3582−92.
(50) Emaneini, M.; Jabalameli, L.; Iman-Eini, H.; Aligholi, M.; Ghasemi, A.; Nakhjavani, F. A.; Taherikalani, M.; Khoramian, B.; Asadollahi, P.; Jabalameli, F. Multiple-locus variable number of tandem repeats fingerprinting (MLVF) and virulence factor analysis of methicillin resistant *Staphylococcus aureus SCCmec* type III. *Pol. J. Microbiol.* **2011**, *60* (4), 303−7.
(51) Baba-Moussa, L.; Sina, H.; Scheftel, J. M.; Moreau, B.; Sainte-Marie, D.; Kotchoni, S. O.; Prévost, G.; Couppie, P. Staphylococcal Panton-Valentine leucocidin as a major virulence factor associated to furuncles. *PLoS One* **2011**, *6* (10), e25716.
(52) Perret, M.; Badiou, C.; Lina, G.; Burbaud, S.; Benito, Y.; Bes, M.; Cottin, V.; Couzon, F.; Juruj, C.; Dauwalder, O.; Goutagny, N.; Diep, B. A.; Vandenesch, F.; Henry, T. Cross-talk between *Staphylococcus aureus* leukocidins-intoxicated macrophages and lung epithelial cells triggers chemokine secretion in an inflammasome-dependent manner. *Cell Microbiol.* **2012**, *14* (7), 1019−36.
(53) Amulic, B.; Cazalet, C.; Hayes, G. L.; Metzler, K. D.; Zychlinsky, A. Neutrophil function: From mechanisms to disease. *Annu. Rev. Immunol.* **2012**, *30*, 459−89.
(54) Rigby, K. M.; DeLeo, F. R. Neutrophils in innate host defense against *Staphylococcus aureus* infections. *Semin Immunopathol.* **2012**, *34* (2), 237−59.
(55) DeCoursey, T. E.; Cherny, V. V.; Zhou, W.; Thomas, L. L. Simultaneous activation of NADPH oxidase-related proton and electron currents in human neutrophils. *Proc. Natl. Acad. Sci. U. S. A.* **2000**, *97* (12), 6885−9.
(56) Glasser, L.; Fiederlein, R. L. Functional differentiation of normal human neutrophils. *Blood* **1987**, *69* (3), 937−44.
(57) Brinkmann, V.; Reichard, U.; Goosmann, C.; Fauler, B.; Uhlemann, Y.; Weiss, D. S.; Weinrauch, Y.; Zychlinsky, A. Neutrophil extracellular traps kill bacteria. *Science* **2004**, *303* (5663), 1532−5.
(58) Fuchs, T. A.; Abed, U.; Goosmann, C.; Hurwitz, R.; Schulze, I.; Wahn, V.; Weinrauch, Y.; Brinkmann, V.; Zychlinsky, A. Novel cell death program leads to neutrophil extracellular traps. *J. Cell Biol.* **2007**, *176* (2), 231−41.
(59) Pilsczek, F. H.; Salina, D.; Poon, K. K.; Fahey, C.; Yipp, B. G.; Sibley, C. D.; Robbins, S. M.; Green, F. H.; Surette, M. G.; Sugai, M.; Bowden, M. G.; Hussain, M.; Zhang, K.; Kubes, P. A novel mechanism of rapid nuclear neutrophil extracellular trap formation in response to *Staphylococcus aureus*. *J. Immunol.* **2010**, *185* (12), 7413−25.
(60) Parekh, A. B.; Putney, J. W., Jr. Store-operated calcium channels. *Physiol Rev.* **2005**, *85* (2), 757−810.

(61) Koch, J.; Foekens, J.; Timmermans, M.; Fink, W.; Wirzbach, A.; Kramer, M. D.; Schaefer, B. M. Human VAT-1: A calcium-regulated activation marker of human epithelial cells. *Arch Dermatol Res.* **2003**, *295* (5), 203−10.
(62) Cockcroft, S.; Bennett, J. P.; Gomperts, B. D. The dependence on Ca^{2+} of phosphatidylinositol breakdown and enzyme secretion in rabbit neutrophils stimulated by formylmethionyl-leucylphenylalanine or ionomycin. *Biochem. J.* **1981**, *200* (3), 501−8.
(63) Mann, B. S.; Chung, K. F. Blood neutrophil activation markers in severe asthma: Lack of inhibition by prednisolone therapy. *Respir. Res.* **2006**, *7*, 59.