12-22-2021

Bactericidal effects and stability of LL-37 and CAMA in the presence of human lung epithelial cells

Regina Geitani
Saint Joseph University

Carole Ayoub Moubareck
Saint Joseph University; Zayed University, carole.ayoubmoubareck@zu.ac.ae

Floriane Costes
Sorbonne University; Pasteur Institute

Léa Marti
Sorbonne University; Pasteur Institute

Gabrielle Dupuis
Sorbonne University; Pasteur Institute

See next page for additional authors

Microbes and Infection 24 (2022) 104928

Contents lists available at ScienceDirect

Microbes and Infection

journal homepage: www.elsevier.com/locate/micinf

Original article

Bactericidal effects and stability of LL-37 and CAMA in the presence of human lung epithelial cells

Regina Geitani [a, *], Carole Ayoub Moubareck [a, b], Floriane Costes [c, d], Léa Marti [c, d], Gabrielle Dupuis [c, d], Dolla Karam Sarkis [a, 1], Lhousseine Touqui [c, d, **, 1]

[a] Microbiology Laboratory, School of Pharmacy, Saint Joseph University, Beirut, Lebanon
[b] College of Natural and Health Sciences, Zayed University, Dubai, United Arab Emirates
[c] "Sorbonne Université", INSERM UMR_S 938, "Centre de Recherche Saint-Antoine" (CRSA), Paris, France
[d] "Mucoviscidose and Bronchopathies Chroniques", Department "Santé Globale", Pasteur Institute, Paris, France

ARTICLE INFO

Article history:
Received 25 January 2021
Accepted 16 December 2021
Available online 23 December 2021

Keywords:
Cationic antimicrobial peptides
Human lung epithelial cell lines
Pseudomonas aeruginosa
Bactericidal activity
Immunomodulatory activity

ABSTRACT

Cationic antimicrobial peptides (CAMPs) are important actors in host innate immunity and represent a promising alternative to combat antibiotic resistance. Here, the bactericidal activity of two CAMPs (LL-37 and CAMA) was evaluated against *Pseudomonas aeruginosa* (PA) in the presence of IB3-1 cells, a cell line derived from patients with cystic fibrosis. The two CAMPs exerted different effects on PA survival depending on the timing of their administration. We observed a greater bactericidal effect when IB3-1 cells were pretreated with sub-minimum bactericidal concentrations (Sub-MBCs) of the CAMPs prior to infection. These findings suggest that CAMPs induce the production of factors by IB3-1 cells that improve their bactericidal action. However, we observed no bactericidal effect when supra-minimum bactericidal concentrations (Supra-MBCs) of the CAMPs were added to IB3-1 cells at the same time or after infection. Western-blot analysis showed a large decrease in LL-37 levels in supernatants of infected IB3-1 cells and an increase in LL-37 binding to these cells after LL-37 administration. LL-37 induced a weak inflammatory response in the cells without being toxic. In conclusion, our findings suggest a potential prophylactic action of CAMPs. The bactericidal effects were low when the CAMPs were added after cell infection, likely due to degradation of CAMPs by bacterial or epithelial cell proteases and/or due to adherence of CAMPs to cells becoming less available for direct bacterial killing.

While the search for alternatives to antibiotics is becoming increasingly important [1,2], cationic antimicrobial peptides (CAMPs) are gaining attention as potential therapeutic agents [3]. CAMPs are an extremely diverse group of low molecular weight natural peptides that form part of the first line of the innate immune defense of most living organisms, ranging from insects to plants to animals, including humans [4]. As effectors of innate immunity, CAMPs exhibit rapid action and a broad spectrum of activity against both Gram-positive and Gram-negative bacteria, fungi, viruses, and parasites, independently from their resistance profiles [5,6]. These peptides act as effective modulators of inflammation, including angiogenesis, cytokine production, chemotactic functions, modulation of immune cell differentiation, and the initiation of adaptive immunity [7]. The CAMP LL-37 is a member of the cathelicidins obtained by extracellular cleavage with proteinase-3 of the C-terminal part of the only human cathelicidin identified to date called human cationic antimicrobial protein (hCAP18) [6,8]. LL-37 is produced by many human cell types, such myeloid cells, neutrophils, epithelial cells, mast cells, monocytes, and keratinocytes. Epithelial cells of the respiratory tracts represent a major source of this peptide [5,9]. The production of this 37 amino acid peptide is induced by inflammatory or infectious stimuli and has antimicrobial activity against both Gram-positive and Gram-negative bacteria. It has a net positive charge of +6 at a physiological pH and exerts its direct antibacterial activity by either disrupting bacterial membranes adopting a toroidal-pore model [10], or interfering with intracellular processes following to

* Corresponding author.
** Corresponding author. "Sorbonne Université", INSERM UMR_S 938, "Centre de Recherche Saint-Antoine" (CRSA), Paris, France.
E-mail addresses: regina.geitani@net.usj.edu.lb (R. Geitani), lhousseine.touqui@pasteur.fr (L. Touqui).
[1] authors with equal contribution.

https://doi.org/10.1016/j.micinf.2021.104928

Abbreviations

ATCC	American type culture collection
CAMPs	cationic antimicrobial peptides
CF	cystic fibrosis
CFTR	cystic fibrosis transmembrane conductance regulator
CFU	colony-forming unit
Cytotoxicity %	percentage cytotoxicity
DMEM	Dulbecco's modified Eagle's medium
ECCMID	European congress of clinical microbiology and infectious diseases
ELISA	enzyme-linked immunosorbent assay
FBS	fetal bovine serum
hCAP18	human cationic antimicrobial protein
HLEC	human lung epithelial cell line
IL	interleukin
LB	Luria Bertani
LDH	lactate dehydrogenase
LPS	lipopolysaccharide
MBC	minimal bactericidal concentration
MIC	minimal inhibitory concentration
MHA	Mueller Hinton agar
MOI	multiplicity of infection
OD	optical density
PA	*Pseudomonas aeruginosa*
PAK	wildtype PA strain
PAP	alkaline protease
PBS	phosphate-buffered saline
PDVF	polyvinylidene difluoride
PE	*Pseudomonas aeruginosa* elastase
PGE_2	prostaglandin E_2
PMSF	phenylmethylsulfonyl fluoride
RPMI	Roswell park memorial institute
SDS-PAGE	sodium dodecyl sulfate-polyacrylamide gel electrophoresis
SD	standard deviation
SPSS	statistical package software for social science
Sub-MBCs	sub-minimum bactericidal concentrations
Supra-MBCs	supra-minimum bactericidal concentrations
TNF-α	tumor necrosis factor-α
TBS-Tween	tris-buffered saline-Tween

translocation. Furthermore, LL-37 neutralizes the activity of bacterial lipopolysaccharide (LPS). Besides its direct antimicrobial activity, LL-37 can modulate inflammation; it is chemotactic for neutrophils, monocytes, mast cells and T cells, induces degranulation of mast cells, affects transcriptional responses in macrophages, and maintains a balance between pro- and anti-inflammatory mediators, helping in combating the infection and limiting the inflammatory response to prevent damage to the host [6,11]. Moreover, LL-37 promotes wound vascularization and angiogenesis, and they have also been implicated in anti-cancer activities [11].

The CAMP CAMA is a synthetized cecropin-melittin 15 amino acid hybrid peptide, composed of N-terminal alpha-helical segment of cecropin A(amino acid 1–7) and melittin A(2–9). It is a novel small peptide with a net positive charge of +8 at a physiological pH that exerts a potent antibacterial and synergistic activity by disrupting bacterial outer membrane through forming ion-permeable channels in toroidal-pore and carpet-like models [12]. Cecropins were originally isolated from the hemolymph of a North American silk moth, *Hyalophora cecropia* [13], while melittin was isolated from honey bee [6].

The lung is the most exposed organ to microbes and lung infections are a major cause of death from infection worldwide. Opportunistic bacteria such as *Pseudomonas aeruginosa* (PA) are an important cause of lung infections in immunocompromised or critically ill patients [14]. PA is a ubiquitous organism that persists in both community and hospital settings due to its ability to survive on minimal nutritional requirements and tolerate a variety of physical conditions [15]. Patients with disrupted lung immunity or mucosal clearance, such as those with cystic fibrosis (CF), acquire bacterial infections that typically do not resolve, even with antibiotic treatment [14]. CF is a well-characterized, lethal, autosomal, recessive inherited disorder found predominantly in Caucasians due to mutations in the cystic fibrosis transmembrane conductance regulator (CFTR) gene, characterized by chronic lung bacterial infections. Such infections are the main cause of morbidity and mortality of CF patients. The major colonizing pathogen for people with CF is arguably PA [16,17]. PA lung infections resist the host response, as well as antibiotic treatment used to eradicate the microorganism from the lungs. The persistence of infection in CF airways is partially due to the ability of PA to develop resistance to antibiotics by mutating and the presence of abnormal amounts of bronchial mucus, which is a favorable niche for bacterial proliferation [18].

Traditionally, examination of the bactericidal effects of CAMPs has been performed in host cell-free media. Bactericidal activities of four CAMPs against clinical and laboratory strains of *Staphylococcus aureus*, *Streptococcus pneumoniae*, and PA, either antibiotic susceptible or multidrug-resistant has been published by our team previously [19]. Among the tested peptides, LL-37 and CAMA showed the most potent bactericidal activities against PA, therefore these two peptides were selected in the present work for further experimental studies in presence of human lung epithelial cell lines (HLECs) to reflect the natural conditions of host infections. We investigated the bactericidal activity of LL-37 and CAMA at three different timepoints (30 min pre-infection, at the same time as infection, and 30 min post-infection). Western-blot experiments were also performed to evaluate the stability of LL-37 when added to human cells infected with PA. The cytotoxicity and immunomodulatory effects of LL-37 and CAMA were assessed on two HLECs.

Part of this work was presented orally at the European congress of clinical microbiology and infectious diseases (ECCMID) 2019 in Amsterdam, Holland (April 13–16, presentation number O0424).

2. Materials and methods

2.1. CAMPs

LL-37 human cathelicidin (LLGDFFRKSKEKIGKEFKRIVQRIKD FLRNLVPRTES) at 96,7% purity and CAMA cecropin(1–7)-melittin A(2–9) (KWKLFKKIGAVLKVL) at > 95% were purchased from Bachem AG (Bubendorf, Switzerland). The synthetic peptides were dissolved in sterile water at a concentration of 2560 μg/mL and then aliquoted and stocked at −20 °C before use. Diluted solutions were prepared on the day of use.

2.2. Cell lines and culture conditions

HLECs used in this study consisted of the human bronchial epithelial IB3-1 and human adenocarcinoma alveolar epithelial

A549 cell lines. Both cell lines were purchased from the American type culture collection (ATCC). IB3-1 cells, derived from a CF patient with a ΔF508/W1282X mutant genotype and immortalized using adeno12/SV40, were grown in LHC-8 medium (Gibco) supplemented with 10% fetal bovine serum (FBS) (Sigma–Aldrich), 100 units/mL penicillin and 0.1 mg/mL streptomycin (Gibco), 1 mM HEPES buffer (Sigma–Aldrich), and 2 mM L-glutamine (Gibco) at 37 °C/5% CO_2. A549 cells were cultured in Dulbecco's modified Eagle's medium (DMEM) with Glutamax (Gibco) supplemented with 10% FBS, 100 units/mL penicillin and 0.1 mg/mL streptomycin at 37 °C/5% CO_2.

2.3. Bacteria and growth conditions

The wildtype PA strain (PAK) was purchased from the ATCC (strain ATCC 53308). A single bacterial colony from an overnight culture on Luria Bertani (LB) agar plates was grown overnight with shaking in 20 mL LB medium at 37 °C. The obtained bacterial suspension was further diluted in fresh LB and grown again for 4 h to achieve mid-log phase. After centrifugation at 3000×*g* at 4 °C for 15 min, the bacterial pellet was rinsed and suspended in an appropriate volume of phosphate-buffered saline (PBS). The bacterial concentrations were calculated according to the optical density (OD) value measured at 600 nm and further adjusted to the approximate desired concentration for in vitro bacterial infection experiments [20].

2.4. Infection of IB3-1 cultures with PAK and treatment with CAMPs

Infection experiments were performed to evaluate the bactericidal activity of the two peptides (LL-37 and CAMA) against PAK in the presence of IB3-1 cells. Two concentrations of each peptide were used, one lower and one higher than the minimal bactericidal concentration (MBC) of each peptide against PAK, designated sub-minimum bactericidal concentration (sub-MBC) and supra-minimum bactericidal concentration (supra-MBC), respectively. The MBC for each peptide was defined by first determining the minimal inhibitory concentration (MIC) values of LL-37 and CAMA against PAK by microbroth dilution, as described previously [21,22]. The MBC for each peptide was further defined by plating the contents of the first three wells showing no visible growth of bacteria onto Mueller Hinton Agar (MHA) plates, followed by incubation for 24 h [19,23]. The MIC is defined as the lowest concentration of an antimicrobial agent that prevents visible growth of a microorganism in an agar or broth dilution susceptibility test. Whereas the MBC is the lowest concentration of an antimicrobial agent that kills 99.9% of a particular organism [19]. Cell infections were performed in serum-free culture medium. IB3-1 cells were seeded in 12-well plates (TPP® tissue culture plates) and grown until confluence. Two hours before infection, culture medium was aspired from wells and a serum-free medium without antibiotics was gently added. Thirty minutes before infection, cells were counted using the trypan blue exclusion test of cell viability (Gibco) and stimulated with PAK at a multiplicity of infection (MOI) of 1. The CAMPs were added 30 min pre-infection, at the same time as infection, or 30 min post-infection. The plates were centrifuged at 80×*g* at 37 °C for 5 min and incubated at 37 °C/5% CO_2 to initiate infection and ensure similar contact between the mammalian cells and bacteria [20]. After 2 h of incubation, the wells were scraped and the cells homogenized gently and repeatedly with pipetting to guarantee obtaining all intra- and extra-cellular bacteria. Serial dilutions were plated onto LB agar plates and the plates incubated for 24 h at 37 °C. The bactericidal effect of LL-37 and CAMA was measured by counting bacterial colonies on the plates and expressing the value as colony-forming units (CFUs) relative to that of treatment in the absence of IB3-1 cells. A control well without drugs was used for each condition.

2.5. Assessment of LL-37 stability in IB3-1 cultures infected with PAK

IB3-1 cells were incubated with PAK for 30 min at a MOI of 1 and then supernatants were harvested, centrifuged to remove bacteria and non-adherent cells and stored at −20 °C until use. Later, after their thawing, these cell-free supernatants were incubated with exogenously added LL-37 at 1 μg/mL final concentration. This approach allows preventing any interaction between LL-37 and IB3-1 cells and/or PAK. In a second approach, LL-37 was directly added to the culture plates, allowing interaction with both IB3-1 cells and/or PAK. Cells extracts from IB3-1 cultures, infected or not with PAK at MOI 5 were analyzed. Furthermore, Western-blot analysis was also performed on cell extracts from IB3-1 cultures stimulated with vitamin D (Sigma–Aldrich) at a final concentration of 100 nM for 24 h. Vitamin D is known to up-regulate LL-37 expression by host cells. THP 1 cells were used as a positive control for LL-37 expression. THP 1 is a human monocytic cell line derived from an acute monocytic leukemia patient that were purchased from the ATCC and cultured in Roswell Park Memorial Institute (RPMI) 1640 medium (Bio-Rad). In certain experiments, cell-free supernatants from IB3-1, infected or not, were thawed and pre-treated with the serine protease inhibitor phenylmethylsulfonyl fluoride (PMSF, Sigma–Aldrich) at 2 mM final concentration for 30 min, before addition of LL-37.

Kinetic analyses at 37 °C were carried out for the two approaches. A 10 μL sample was removed at various timepoints (0.5, 1, 2, and 6 h) and stored at −20 °C until analysis. The stability of LL-37 in the supernatants was determined by assessing the level of exogenous LL-37 by western-blotting for the indicated time points under the different conditions (LHC-8, PAK, IB3-1, and IB3-1+PAK). In the second approach, LL-37 levels in the cell pellets were also determined to detect any interaction between LL-37 and IB3-1 cells at the indicated timepoints (0.5, 1, 2, and 6 h) and under same conditions (LHC-8, PAK, IB3-1, and IB3-1+PAK).

2.5.1. Western-blotting analyses

Cell lysates were prepared using 1X RIPA at pH 7.4 (Bio Basic, NY, USA). Proteins samples were diluted in LHC-8 medium with 4X Laemmli buffer (Bio-Rad) containing β-mercaptoethanol and loaded onto a 15% acrylamide gel and separated for 1–2 h at 130 V. The proteins were then transferred to a polyvinylidene difluoride (PDVF) membrane using an IBlot 2 type device (Invitrogen) and nonspecific binding sites were blocked with 5% milk in tris-buffered saline-Tween (TBS-Tween) buffer for 1 h. The membrane was then washed with TBS-Tween and subsequently incubated overnight with the primary anti-human antibody CAP18 (diluted 1:1000, Hycult Biotech) at 4 °C under shaking. The next day, the membrane was washed and incubated in the secondary anti-mouse IgG (antibody diluted 1:5000, Cell Signaling Technology) for 1 h at room temperature. The developer solution, containing equal volumes of the two reagents, was placed on the membrane for 5 min in the dark. All blots were imaged with ultraviolet rays using an LAS-3000 device.

2.6. Stimulation of HLECs with various CAMPs for cytotoxicity and immunological assays

HLECs were seeded in 12-well plates and cultured for one day to obtain a concentration of 1.6×10^6 cells/mL (1 mL per well). Before treatment, supernatants were removed, 1 mL serum-free medium was added to each well, and the cells allowed to rest for 1 h [24].

Subsequently, HLECs were stimulated with LL-37 (5, 10, 15, 25, 50, and 65 μg/mL) and CAMA (0.5, 5, 10, 15, 25, and 35 μg/mL) for 24 or 48 h. These concentrations were tested after showing potent bactericidal activity against PA [19].

Cell lysis due to treatment of the HLECs with various concentrations of CAMPs for 24 or 48 h was determined in vitro using the Lactate dehydrogenase (LDH)-based CytoTox 96®Non-Radioactive Cytotoxicity Assay according to the manufacturer's instructions (Promega). After the desired incubation time, supernatants were aliquoted, centrifuged at 400×*g* at 4 °C for 5 min to obtain cell-free samples, and the samples immediately analyzed for LDH. The dynamic range of the assay was determined using LDH released from non-treated cells as a negative control and LDH released from cells lysed with Triton X-100 as a control for maximum release. The average background values of the culture medium were subtracted from all values of the experimental wells. The corrected values were used in the following formula recommended by the manufacturer to compute the percentage cytotoxicity (cytotoxicity %):

Cytotoxicity % = 100*experimental LDH release (OD490)/ maximum LDH release (OD490)

The treatment was considered to not be cytotoxic for values < 10% [25].

The concentrations of interleukin (IL)-6, IL-8, and prostaglandin E_2 (PGE_2) in the supernatants were measured using a capture enzyme-linked immunosorbent assay (ELISA) following the manufacturer's suggestions (R&D Systems, Inc). After the desired time of incubation, the tissue culture supernatants were aliquoted, centrifuged at 3500×*g* at 4 °C for 5 min to obtain cell-free samples, and stored at −20 °C before assaying the cytokines.

2.7. Statistical analyses

All experiments were performed in at least three independent assays. The results are expressed as the mean ± standard deviation (SD) of the three independent assays and the data were analyzed using a general linear model procedure of *statistical package software for social science* (SPSS, version 20.00, SPSS Institute Inc., Chicago, IL, USA). The Shapiro Wilk test was used to evaluate the normality of the variables. Variance analyses followed by multiple comparisons were used to assess the efficacy of the CAMPs under all conditions. Kruskal–Wallis tests followed by Mann–Whitney tests were used to evaluate differences in cytotoxicity, and IL-6, IL-8, and PGE_2 levels relative to the control. *P* values ≤ 0.05 were considered statistically significant. Bands from the Western-blot experiments were quantified using Fiji software (Schindelin, J; Arganda-Carreras, I. & Frize, E. et al. (2012)). GraphPad Prism 7 software was used for ANOVA-type statistical tests. *P* values < 0.01 were considered statistically significant.

3. Results

3.1. Prophylactic effect of Sub-MBCs of LL-37 and CAMA against PAK

We first examined the efficacy of the CAMPs in the presence of IB3-1 cells. The killing potency of LL-37 and CAMA against PAK in the presence or absence of IB3-1 cells was determined at two different concentrations of each CAMP administrated at three different times. The MBC values for LL-37 and CAMA against PAK were 32 and 4 μg/mL, respectively [19]. Thus, the tested sub-MBCs (the concentration of an antimicrobial agent that is lower than its MBC) were 25 μg/mL for LL-37 and 2.5 μg/mL for CAMA and the Supra-MBCs (the concentration of an antimicrobial agent that is higher than its MBC) were 50 μg/mL for LL-37 and 5 μg/mL for CAMA. Overall, there was only marginal bactericidal activity of sub-MBCs of LL-37 and CAMA against PAK at all three timepoints (30 min pre-infection, same time as infection, and 30 min post-infection) in the absence of IB3-1 cells ($P > 0.05$ for all conditions, except for LL-37 30 min pre-infection) (Fig. 1A and B). However, in the presence of IB3-1 cells, the sub-MBCs of LL-37 and CAMA were able to significantly eliminate PAK organisms when added 30 min pre-infection ($P = 0.003$ for LL-37 and $P = 0.047$ for CAMA). Such a reduction in bacterial viability at sub-MBCs in the presence of IB3-1 cells was more significant than in their absence (Fig. 1A and B) ($P = 0$ for both LL-37 and CAMA). Indeed, in the presence of IB3-1 cells, sub-MBCs of LL-37 and CAMA were able to kill 83.5% and 99% of PAK, respectively. In their absence, these concentrations killed only 60.5% and 53%, respectively. However, the sub-MBCs of the tested peptides were ineffective if added at the same time as infection of the mammalian cells or 30 min post-infection ($P > 0.05$) (Fig. 1 A and B).

3.2. Decrease in the efficacy of LL-37 and CAMA when administrated at the same time as infection or 30 min post-infection of IB3-1 cells

In the absence of IB3-1 cells, LL-37 and CAMA exhibited total killing against PAK at the supra-MBC (50 μg/mL for LL-37 and 5 μg/mL for CAMA) at all three timepoints (30 min pre-infection, same time as infection, and 30 min post-infection) ($P < 0.05$) (Fig. 1A and B). However, in the presence of IB3-1 cells, supra-MBCs of LL-37 and CAMA were less effective if added at the same time as infection or post-infection, killing only 53.5% and 72.8% of PAK, respectively. Furthermore, addition of the supra-MBCs of these two peptides 30 min post-infection in the presence of IB3-1 cells appeared to be ineffective, showing no killing of PAK ($P > 0.05$) (Fig. 1A and B).

3.3. Decrease in exogenous LL-37 levels in the supernatants of infected IB3-1 cells

Our results led us to postulate that LL-37 is likely degraded by proteases produced by IB3-1 cells upon their infection by PAK or that this peptide is sequestrated by IB3-1 cells and then becomes less available to kill the bacteria. The hypothesis stating that LL-37 is degraded by IB3-1 cells was tested by incubating the peptide with supernatants obtained from IB3-1 cells infected with PAK for 0.5, 1, 2, and 6 h under various conditions (LHC-8, PAK, IB3-1, and IB3-1+PAK), followed by Western-blot analysis of the supernatants. The bands observed in LHC-8 medium or in the presence of PAK alone were identical for all timepoints, showing that LL-37 was stable for up to 6 h under these conditions (Fig. 2A). However, the intensity of the LL-37 band in supernatants from uninfected IB3-1 cells decreased in a time-dependent manner, suggesting possible degradation. Degradation of LL-37 appeared to be even greater when it was incubated with supernatants from PAK infected IB3-1 cells. Indeed, LL-37 was no longer visible by Western-blotting after 6 h of incubation with supernatants of IB3-1 cells infected with PAK (Fig. 2A). Quantification of the Western-blots performed in triplicate from three independent samples under identical conditions showed a 28% decrease in LL-37 levels between 0.5 and 6 h when it was incubated with supernatants from uninfected IB3-1 cells, which reached 49% when it was incubated with supernatants from IB3-1 cells infected with PAK ($P < 0.01$) (Fig. 2 B).

When supernatants from IB3-1 cells were pretreated with the serine-protease inhibitor PMSF, no changes were observed in the magnitude of LL-37 degradation by these supernatants (Fig. 1 of the supplementary data).

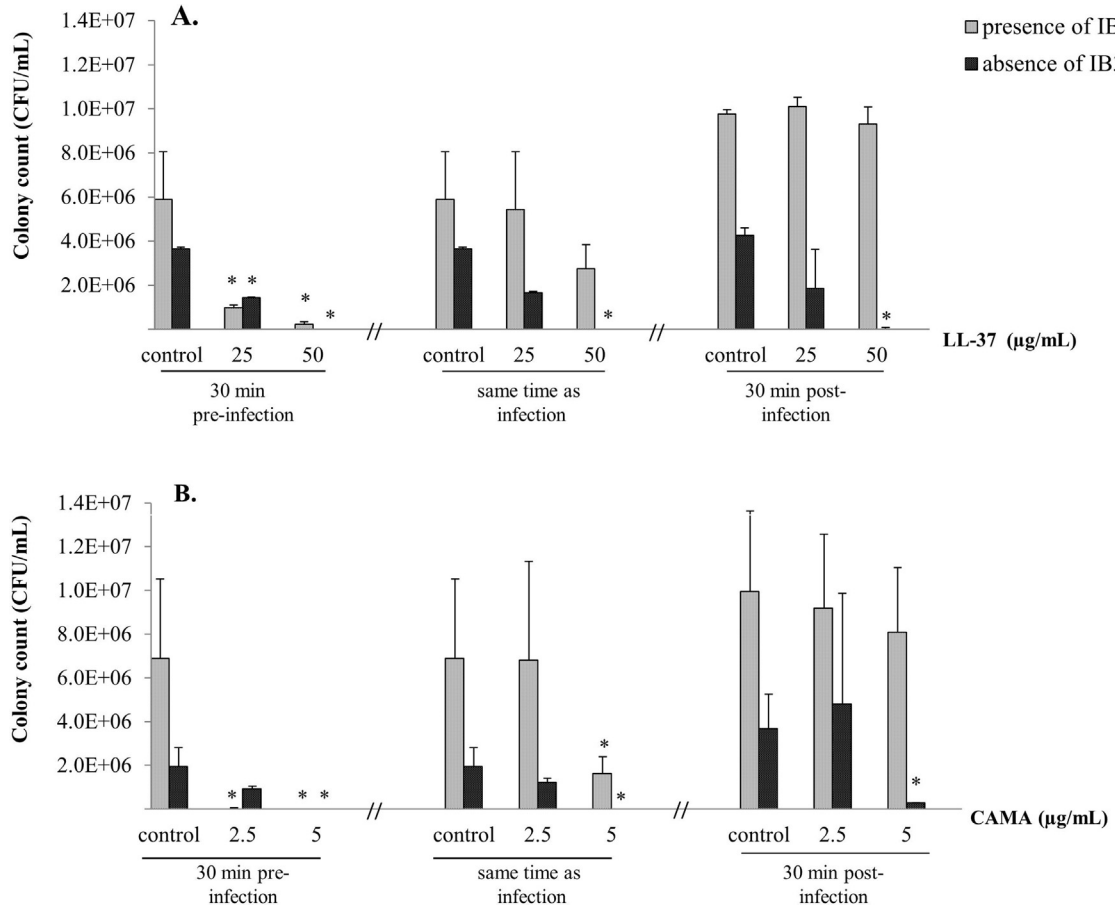

Fig. 1. Bactericidal effects of **A.** LL-37 and **B.** CAMA against PAK in the presence or absence of HLECs at three different timepoints of CAMP administration. Bar graphs show the means ± SD from three independent experiments of PAK survival expressed as colony forming units (CFU). *$P < 0.05$ for treated vs untreated controls.

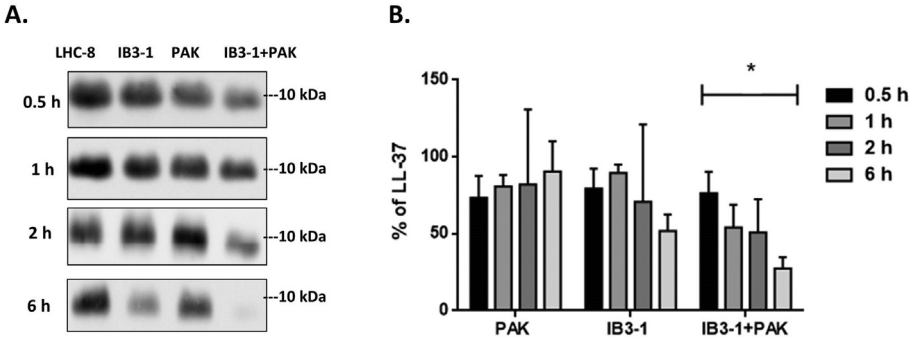

Fig. 2. Western-blot analysis of the stability of exogenously added LL-37 to IB3-1 supernatants. **A.** This panel shows the evolution of the levels of LL-37 added to IB3-1 supernatants in different conditions. **B.** Semi-quantification showing LL-37 levels (percentages). Data are expressed as the percentage of remaining LL-37 in the various conditions compared to those of LL-37 levels in LHC-8 culture medium; mean ± SEM; n = 3 separate experiments. *$P < 0.01$ for supernatants versus culture medium.

3.4. Increase of LL-37 quantity in IB3-1 cell pellets

We examined whether a portion of the exogenously added LL-37 was able to bind to IB3-1 cells by carrying out Western-blots on IB3-1 cell pellets under two culture conditions (IB3-1 and IB3-1+PAK) after incubations of 0.5, 1, 2, and 6 h. We observed a time-dependent increase in the intensity of the LL-37 bands for samples from both uninfected and infected IB3-1 cells (Fig. 3A). Quantification of the protein bands showed an increase of approximately 95% in the amount of cell-associated LL-37 between 0.5 and 6 h under the two conditions (Fig. 3B). Thus, LL-37 appears to bind to IB3-1 cells with high affinity (in both uninfected and infected IB3-1 cells).

To address whether IB3-1 cells produce LL-37 during infection by PAK and whether this endogenous LL-37 interfere with the quantification of the exogenously added LL-37, Western-blot B analysis was performed. The results showed that LL-37 is not upregulated by this bacterium (Fig. 2A of the supplementary data). Furthermore, Western-blot analysis performed on cell extracts from IB3-1 cultures stimulated with vitamin D showed that these cells do not produce LL-37. Thus, IB3-1 cells are not able to synthesize LL-37 and the variations observed in the levels of LL-37

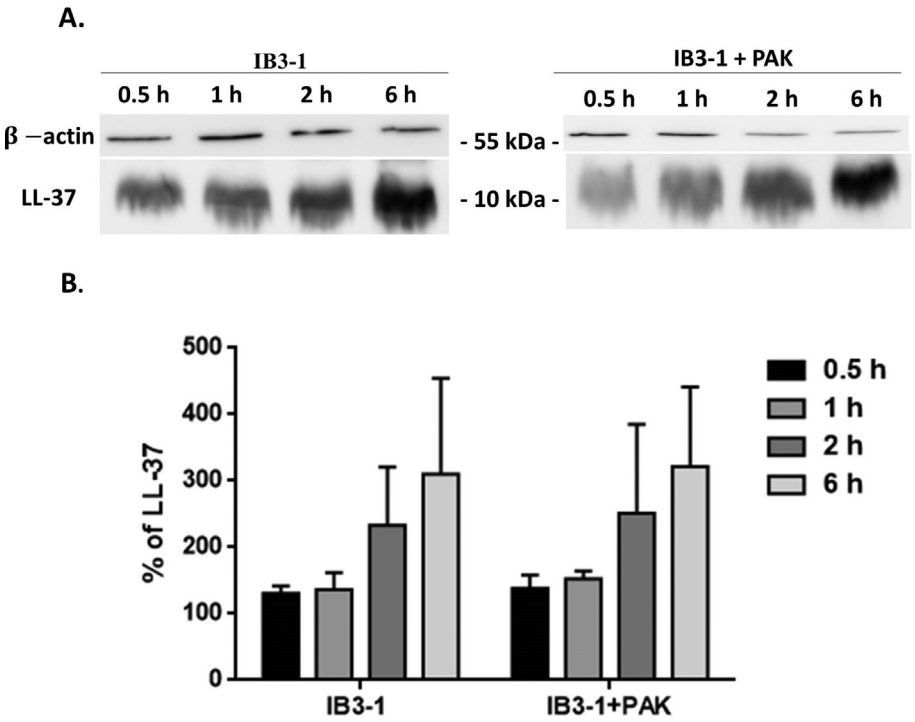

Fig. 3. Western-blot analysis of the binding of exogenously added LL-37 to IB3-1 pellets. **A.** Representation of LL-37 and β-actin in absence and presence of PAK. **B.** Semi-quantification, showing LL-37 levels present in the pellets normalized to that of β-actin. Data are expressed as percentages; mean ± SEM; n = 3 separate experiments.

associated to IB3-1 cell pellets are not due to an interference with endogenous LL-37 (Fig. 2B of the supplementary data).

3.5. No cytotoxic effect of CAMPs on HLECs after up to 48 h of treatment

We screened the two CAMPs (LL-37 and CAMA) for their cytotoxicity based on LDH release from the cytosol of two HLECs cell lines, IB3-1 and A549, 24 and 48 h post-treatment. LDH release was <5% for all tested conditions for both CAMPs and both cell lines (Fig. 4A–C). Thus, treatment with LL-37 and CAMA up to 50 and 35 μg/mL, respectively, had no cytotoxic effect on either the IB3-1 or A549 cell lines 24 and 48 h post-treatment.

3.6. LL-37 and CAMA moderately stimulate IL-6 and IL-8 release from IB3-1 cells

We examined the effect of the two CAMPs on cytokine/chemokine secretion by exposing IB3-1 and A549 cells to a range of LL-37 and CAMA concentrations of up to 50 μg/mL for LL-37 and ≤35 μg/mL for CAMA for 24 and 48 h, as these concentrations showed no cytotoxic effect on HLECs.

LL-37 and CAMA induced modest dose- and time-dependent IL-6 and IL-8 release from IB3-1 cells (Fig. 5A). We observed significantly greater IL-6 release following treatment with 25–50 μg/mL LL-37 ($P < 0.05$) and 5–35 μg/mL CAMA after 24 and 48 h ($P < 0.05$) than for untreated controls. We also observed significantly greater IL-8 release following treatment with 50 μg/mL LL-37 after 24 and 48 h ($P < 0.05$), 35 μg/mL CAMA after 24 h ($P < 0.05$), and 5–35 μg/mL CAMA after 48 h ($P < 0.05$) than for untreated controls (Fig. 5A). However, IL-6 and IL-8 levels in the supernatants were insignificant relative to positive controls stimulated with Tumor necrosis factor-α (TNF-α) or LPS. We observed no significant increase in PGE_2 levels following treatment of IB3-1 cells with either LL-37 or CAMA.

We obtained different results with the A549 cell line. A549 cells released neither IL-6 nor IL-8 following treatment with either LL-37 or CAMA (Fig. 5B). However, both peptides induced PGE_2 release from A549 cells in a time and dose-dependent manner, but it was not statistically significant ($P > 0.05$).

4. Discussion

The role of CAMPs in the mechanism of killing various bacteria has been extensively explored [26], but mostly in the absence of host cells [12,27]. However, host cells can potentially influence the bactericidal actions of CAMPs, thus introducing an important bias in the interpretation of the actual role of these peptides. Going beyond such studies, we investigated the bactericidal activity of LL-37 and CAMA against PAK, a laboratory strain of PA, in the presence of the bronchial epithelial cell line, IB3-1, derived from CF patients. Bronchial epithelial cells are the first target of bacteria, such as PA, that colonize patient airways [17]. Our results show that sub-MBCs of LL-37 and CAMA kill PAK more effectively when added to IB3-1 cells 30 min pre-infection than when added at the same time or 30 min post-infection. These findings suggest a possible interaction between CAMPs and IB3-1 cells, resulting in an enhanced bactericidal effect. Such improved bactericidal activity occurred at levels that are well within the range of the sub-MBCs. Thus, the effect of these two peptides against PAK appears to be indirect (host cell-mediated) via a mechanism that is yet to be precisely identified. It is well known that numerous CAMPs are able to bind to cellular receptors, inducing a variety of receptor-mediated functions [28], such as the stimulation of angiogenesis, cutaneous wound healing, and chemoattraction of inflammatory and immune cells [29]. Thus, it is conceivable that CAMPs can stimulate IB3-1 cells to produce factors that enhance the bactericidal effects of these peptides. However, LL-37 and CAMA showed much less bactericidal activity when added to IB3-1 cells at the same time of bacterial

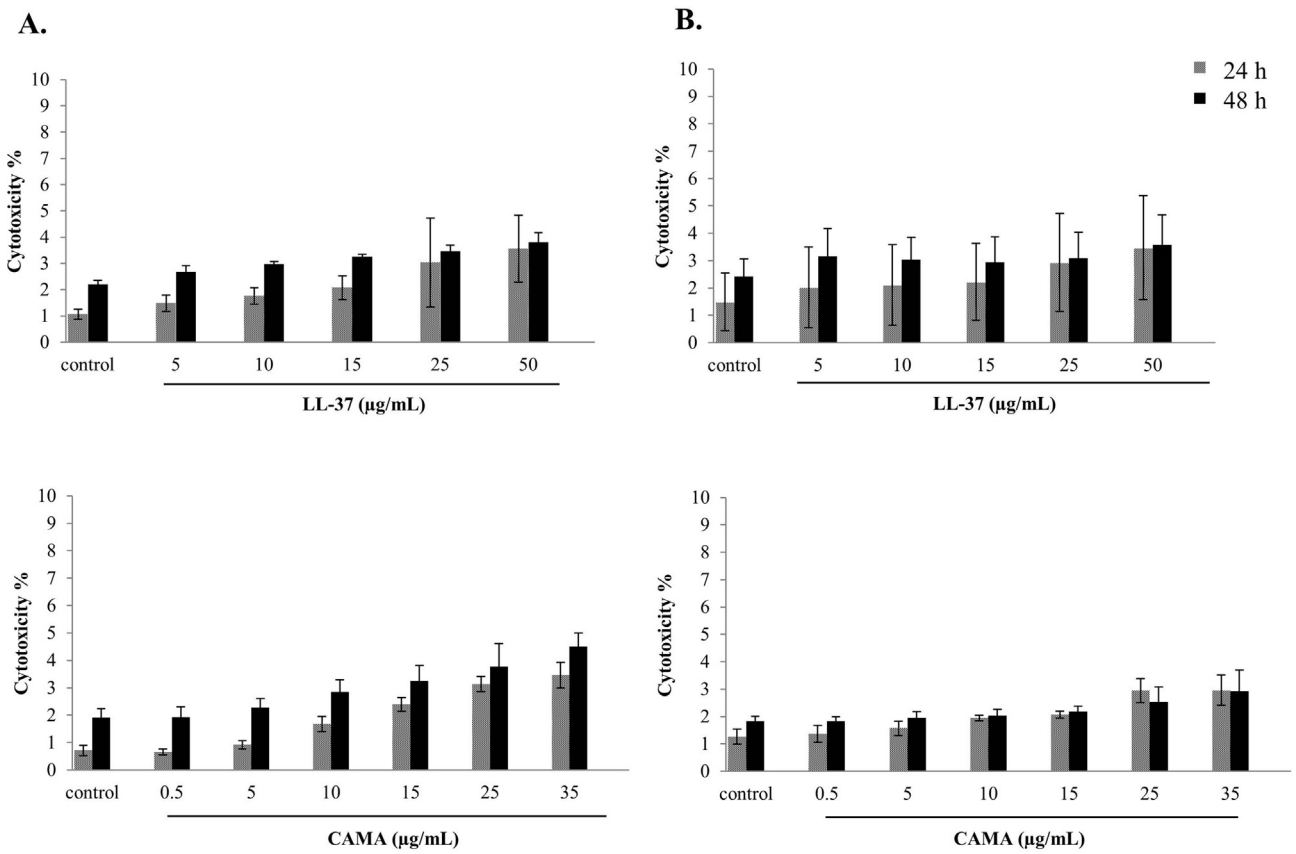

Fig. 4. LDH-based cytotoxicity (percentages) of various concentrations of LL-37 and CAMA on: **A.** IB3-1 and **B.** A549 cell lines at 24 and 48 h incubation with LL-37. Results are representative of the mean ± SD of at least three independent experiments.

administration or 30 min after than in the absence of host cells. This suggests probable inactivation of these peptides via a number of potential mechanisms, including degradation of the CAMPs by proteases released by IB3-1 cells or by PA and/or inhibition of their bactericidal effects by molecules that bind to CAMPs. Indeed, PA is known to produce extracellular enzymes (*P. aeruginosa* elastase (PAE), and alkaline protease (PAP)) in the early stages of infection, which may contribute to inactive CAMPs and ensure their survival in the host. Besides, PA can invade and survive within eukaryotic cells [30,31], which may allow this bacterium to escape the bactericidal actions of CAMPs. Bacterial internalization has been clearly documented for both epithelial [32] and phagocytic cells [33] in vitro, as well as in infected mice [34].

We performed Western-blotting experiments to determine whether degradation of LL-37 is the principal mechanism underlying the inhibition of its antibacterial action when administrated 30 min post-infection to IB3-1 cells. These experiments showed a decrease in the amount of LL-37 in the supernatants from uninfected IB3-1 cells, which was further decreased in the supernatants of infected IB3-1 cells. Interaction of IB3-1 cells with the PAK strain appeared to induce a greater decrease in the peptide, possibly due to the release of proteases by the IB3-1 cells or PAK. Our results also showed that when supernatants from IB3-1 cells were pretreated with the serine-protease inhibitor PMSF before addition of LL-37, no changes were observed in the magnitude of LL-37 degradation by these supernatants. This suggested that the apparent cleavage of LL-37 by supernatants from PAK infected IB3-1 cells is probably not due to the release by these cells of serine-proteases.

In addition, studies have shown that proteases (such as cathepsins) produced by human alveolar macrophages and neutrophils can be found in the sputum of CF patients during infection with PAK. Among them, cathepsins K and S have been shown to cleave LL-37 [35]. These proteases belong to the family of cysteine-proteases which are insensitive to PMSF, a finding in agreement with our results showing that LL-37 degradation by supernatants from infected IB3-1 cells was not prevented by pretreatment of these supernatants by PMSF. This is also in agreement with previous reports showing that bronchial epithelial cells from CF patients produce higher levels of cathepsins compared to non CF cells [36].

As mentioned above, it is possible that PA produces proteases, such as PAE, capable of degrading LL-37 [37]. Based on our results, there was no degradation of LL-37 in the presence of PAK alone but only in the presence of IB3-1 cells infected with PAK. Thus, it is likely that the interaction of bronchial epithelial cells with PA induces the production of proteases by the bacterial strain and/or IB3-1 cells that may cleave LL-37.

It is also possible that LL-37 can bind to IB3-1, which may then sequester this peptide, making it less available to direct bacterial killing. In support of our hypothesis, we found that the amount of LL-37 bound to the IB3-1 cells increased in a time-dependent manner, whether these cells were infected or not with PAK. As LL-37 is a strongly cationic peptide, it is possible that it binds to the membranes of bronchial epithelial cells with high affinity.

It could be argued that IB3-1 cells produce LL-37 during infection by PAK and that this endogenous LL-37 may interfere with the quantification of the exogenously added LL-37. We demonstrated that LL-37 is not upregulated by IB3-1 cells even when stimulated by vitamin D. Moreover, LL-37 mRNA expression by IB3-1 cells infected with PAK and its mutant ΔExoS (known to be less virulent

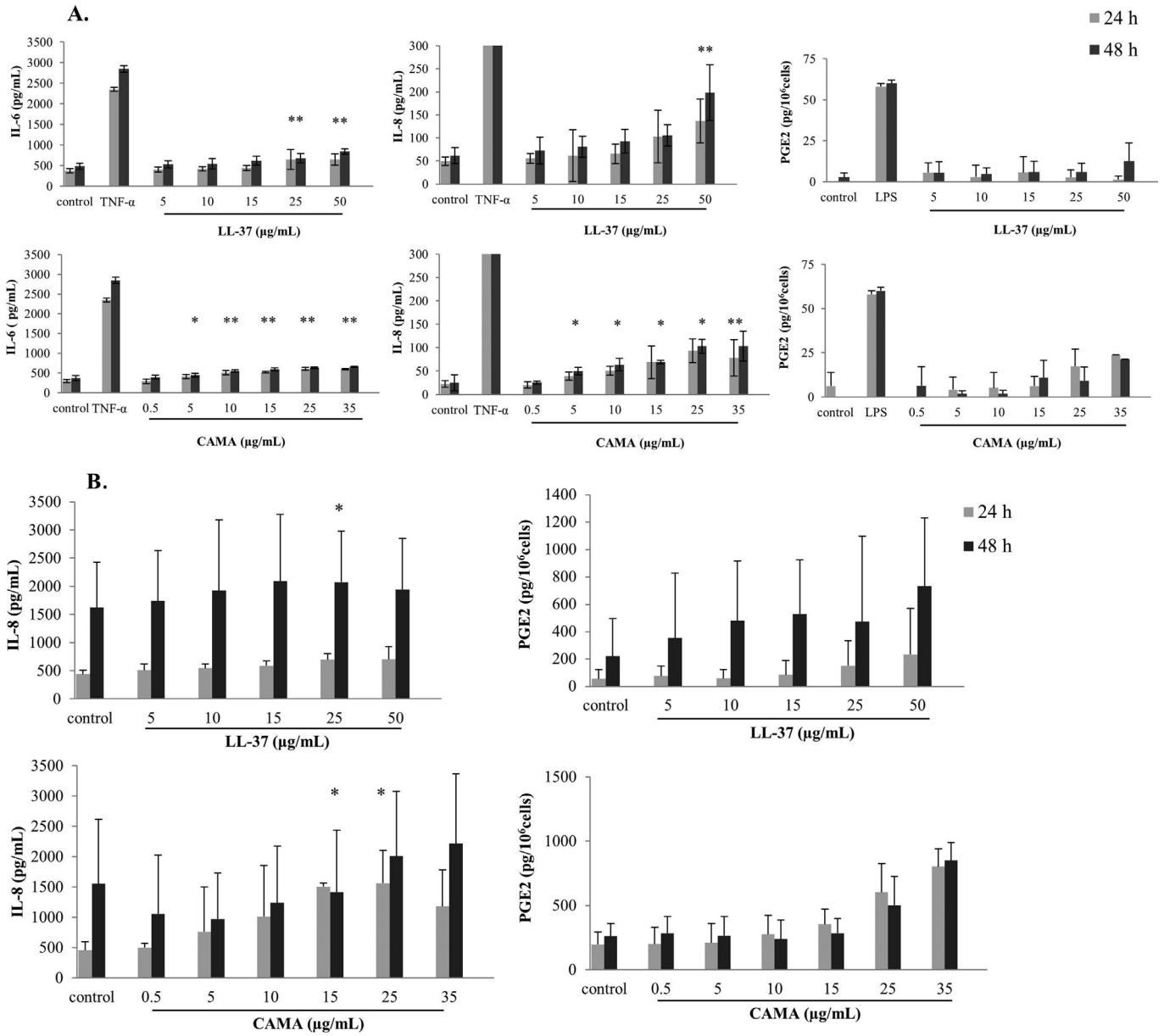

Fig. 5. Assessment of cytokine and chemokine release upon stimulation of HLECs with various concentrations of LL-37 or CAMA for up to 48 h. Bar graphs show the amount of IL-6, IL-8, and PGE_2 secreted into the culture supernatants of: **A**. IB3-1 and **B.** A549 cell lines. The values represent the means ± SD of three independent experiments. *$P < 0.05$ for stimulated vs unstimulated control cells. IB3-1 cells stimulated with LPS and TNF-α served as positive controls. LPS: lipopolysaccharide, TNF-α: tumor necrosis factor-α.

than the parental PAK) at MOI 1 and 5 were assessed by reverse transcription polymerase chain reaction (RT-PCR). Our results showed no LL-37 mRNA expression by IB3-1 cells infected or not with PAK (data not shown).

Plus, we revealed that the doses of the two CAMPs used is this study (≤50 μg/mL for LL-37 and ≤35 μg/mL for CAMA) were non-toxic for the two tested host cell lines for up to 48 h of treatment. Our results confirm the previously reported non-cytotoxicity of LL-37 after 24 h of treatment [38] and demonstrate its non-cytotoxicity on two types of host cells after 48 h of treatment [39]. Furthermore, we showed the hybrid peptide CAMA to also be non-toxic for two HLECs.

In addition, we showed that LL-37 and CAMA weakly stimulate IL-6 cytokine and IL-8 chemokine release from IB3-1 cells. PGE_2 release was not significant for either cell type. In the lung, IL-6 can act as a pro-inflammatory or anti-inflammatory agent, depending on the stimulus and inflammatory context [24]. We observed significantly enhanced IL-6 release upon treatment with LL-37 and CAMA. However, IL-6 release was very weak relative to that induced by the potent pro-inflammatory stimuli TNF-α and LPS. These results suggest a role of LL-37-mediated IL-6 release in the maintenance of the delicate inflammatory balance in the lungs, as reported elsewhere [24,40], and, for the first time, a similar role for CAMA-mediated IL-6 release. IL-8 is best known for its leukocyte chemotactic properties [25]. We observed a significant increase in the release of this chemokine upon LL-37 and CAMA treatment ($P < 0.05$) relative to non-treated controls. However, IL-8 release was very weak relative to that induced by the potent pro-inflammatory stimuli TNF-α and LPS, showing a non-pro-inflammatory effect of the tested concentrations of these peptides. PGE_2, synthesized by many mammalian cells and tissues throughout the body, has long been considered to be the principal

prostaglandin in acute inflammation. We observed no release of PGE_2 by LL-37-, and CAMA-treated HLECs, revealing no pro-inflammatory effect of the tested CAMPs. Overall, these results suggest that these CAMPs could be potential drug candidates to cure bacterial infection in clinical practice, as they show no excessive pro-inflammatory effects on host cells but rather induce the minimal inflammation crucial in the process of defense against bacterial infections. Finally, the slight differences in terms of interleukin and chemokine release by the two cell types tested in this study suggest cell-type specificity [40].

Our results support the potential prophylactic use of LL-37 and CAMA in the control of PA, an opportunistic bacterium known to infect immune-deficient or chronically ill patients. However, previously infected host cells may attenuate or even suppress the bactericidal effects of CAMPs. The protection of CAMPs from such inactivation is a perquisite step before proposing these compounds for potential therapeutic use.

Declaration of competing interest

The authors have no conflicts of interest to disclose.

Acknowledgements

The authors thank “Fondation Air Liquide” (Grant: S-CM19006) and Association «Les Motards du Viaduc de Millau » for funding the project. The authors thank Mrs Nora Touqui, a professional specialized in editing scientific papers.

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.micinf.2021.104928.

References

[1] Nigam A, Gupta D, Sharma A. Treatment of infectious disease: beyond antibiotics. Microbiol Res 2014;169:643–51.
[2] Aloush V, Navon-venezia S, Seigman-igra Y, Cabili S, Carmeli Y. Multidrug-resistant Pseudomonas aeruginosa : risk factors and clinical impact. Antimicrob Agents Chemother 2006;50:43–8.
[3] da Costa JP, Cova M, Ferreira R, Vitorino R. Antimicrobial peptides: an alternative for innovative medicines? Appl Microbiol Biotechnol 2015;99:2023–40.
[4] Mahlapuu M, Håkansson J, Ringstad L, Björn C. Antimicrobial peptides : an emerging category of therapeutic agents. Front Cell Infect Microbiol 2016;6.
[5] Guaní-Guerra E, Santos-Mendoza T, Lugo-Reyes SO, Terán LM. Antimicrobial peptides: general overview and clinical implications in human health and disease. Clin Immunol 2009;135:1–11. https://doi.org/10.1016/j.clim.2009.12.004.
[6] Geitani R, Moubareck CA, Xu Z, Sarkis DK. Expression and roles of antimicrobial peptides in innate defense of airway mucosa : potential implication in cystic fibrosis. Front Immunol 2020;11 June:1–11.
[7] Otvos Jr L. Immunomodulatory effects of anti-microbial peptides. Acta Microbiol Immunol Hung 2016;63:257–77. https://doi.org/10.1556/030.63.2016.005.
[8] De Smet K, Contreras R. Human antimicrobial peptides: defensins, cathelicidins and histatins. Biotechnol Lett 2005;27:1337–47.
[9] Hiemstra PS, Amatngalim GD, Does AM Van Der, Taube C. Antimicrobial peptides and innate lung defenses role in infectious and noninfectious lung diseases and therapeutic. Chest 2019;149:545–51. https://doi.org/10.1378/chest.15-1353.
[10] Pasupuleti M, Schmidtchen A, Malmsten M. Antimicrobial peptides : key components of the innate immune system. Crit Rev Biotechnol 2012;32:143–71.
[11] Xhindoli D, Pacor S, Benincasa M, Scocchi M, Gennaro R, Tossi A. The human cathelicidin LL-37 — a pore-forming antibacterial peptide and host-cell modulator. Biochim Biophys Acta 2016;1858:546–66. https://doi.org/10.1016/j.bbamem.2015.11.003.
[12] Mataraci E, Dosler S. In vitro activities of antibiotics and antimicrobial cationic peptides alone and in combination against methicillin-resistant Staphylococcus aureus biofilms. Antimicrob Agents Chemother 2012;56:6366–71.
[13] Steiner H, Hultmark D, Engstrom A, Bennich H, Boman H. Sequence and specifity of two antibacterial proteins involved in insect immunity. Nature 1981;292:246–8.
[14] Pragman AA, Berger JP, Williams BJ. Understanding persistent bacterial lung infections: clinical implications informed by the biology of the microbiota and biofilms. Clin Pulm Med 2016;23:57–66.
[15] Lister PD, Wolter DJ, Hanson ND. Antibacterial-resistant Pseudomonas aeruginosa: clinical impact and complex regulation of chromosomally encoded resistance mechanisms. Clin Microbiol Rev 2009;22:582–610.
[16] Lyczak JB, Cannon CL, Pier GB. Lung infections associated with cystic fibrosis. Antimicrob Agents Chemother 2002;15:194–222.
[17] Zhang L, Parente J, Harris SM, Woods DE, Hancock REW, Falla TJ. Antimicrobial peptide therapeutics for cystic fibrosis. Antimicrob Agents Chemother 2005;49:2921–7. https://doi.org/10.1128/AAC.49.7.2921-2927.2005.
[18] Moser C, Gennip MVAN, Bjarnsholt T, Jensen PØ, Lee B, Hougen HP, et al. Novel experimental Pseudomonas aeruginosa lung infection model mimicking long-term host – pathogen interactions in cystic fibrosis. APMIS 2009;2:95–107.
[19] Geitani R, Moubareck CA, Touqui L, Sarkis DK. Cationic antimicrobial peptides : alternatives and/or adjuvants to antibiotics active against methicillin-resistant Staphylococcus aureus and multidrug-resistant Pseudomonas aeruginosa. BMC Microbiol 2019;19:1–12.
[20] Pernet E, Guillemot L, Burgel P-R, Martin C, Lambeau G, Sermet-Gaudelus I, et al. Pseudomonas aeruginosa eradicates Staphylococcus aureus by manipulating the host immunity. Nat Commun 2014;5:5105. https://doi.org/10.1038/ncomms6105.
[21] National Committee for Clinical Laboratory standards. Approved Standards M7-A3. Methods for dilution susceptibility tests for bacteria that grow aerobically. 1993.
[22] Giacometti A, Cirioni O, Barchiesi F, Del Prete MS, Fortuna M, Caselli F, et al. In vitro susceptibility tests for cationic peptides: comparison of broth microdilution methods for bacteria that grow aerobically. Antimicrob Agents Chemother 2000;44:1694–6.
[23] Field D, Connor RO, Cotter PD, Ross RP, Hill C. In vitro activities of nisin and nisin derivatives alone and in combination with antibiotics against Staphylococcus biofilms. Front Microbiol 2016;7 April:1–11.
[24] Pistolic J, Li Y, Yu J, Filewod CJ. Host defence peptide LL-37 induces IL-6 expression in human bronchial epithelial cells by activation of the NF- B signaling pathway. J Innate Immun 2009;1:254–67.
[25] Mohamed F Ben, Garcia-verdugo I, Medina M, Balloy V. A crucial role of flagellin in the induction of airway mucus production by Pseudomonas aeruginosa. PLoS One 2012;7.
[26] Giacometti A, Cirioni O, Barchiesi F, Simona M, Prete D, Scalise G. Antimicrobial activity of polycationic peptides. Peptides 1999;20:1265–73.
[27] Dosler S, Mataraci E. In vitro pharmacokinetics of antimicrobial cationic peptides alone and in combination with antibiotics against methicillin resistant Staphylococcus aureus biofilms. Peptides 2013;49:53–8. https://doi.org/10.1016/j.peptides.2013.08.008.
[28] Lai Y, Gallo RL. AMPed up immunity : how antimicrobial peptides have multiple roles in immune defense. Trends Immunol 2009;30(February):131–41.
[29] Shaykhiev R, Beißwenger C, Ka K, Senske J, Damm T, Bals R, et al. Human endogenous antibiotic LL-37 stimulates airway epithelial cell proliferation and wound closure. Am J Physiol Lung Cell Mol Physiol 2015;289:842–8.
[30] Bernut A, Belon C, Soscia C, Bleves S. Intracellular phase for an extracellular bacterial pathogen : MgtC shows the way. Microb cell 2015;2:353–5.
[31] Buyck JM, Tulkens PM, Bambeke F Van. Pharmacodynamic evaluation of the intracellular activity of antibiotics towards Pseudomonas aeruginosa PAO1 in a model of THP-1 human monocytes. Antimicrob Agents Chemother 2013;57:2310–8.
[32] Garcia-medina R, Dunne WM, Singh PK, Brody SL. Pseudomonas aeruginosa acquires biofilm-like properties within airway epithelial cells. Infect Imm 2005;73:8298–305.
[33] Porto P Del, Cifani N, Guarnieri S, Gino E, Domenico D, Mariggio MA, et al. Dysfunctional CFTR alters the bactericidal activity of human macrophages against Pseudomonas aeruginosa. PLoS One 2011;6.
[34] Schmiedl A, Kerber-momot T. Bacterial distribution in lung parenchyma early after pulmonary infection with Pseudomonas aeruginosa. Cell Tissue Res 2010;342:67–73.
[35] Andrault P-M, Samsonov S, Weber G, Coquet L, Nazmi K, Bolsher J, et al. Antimicrobial peptide LL-37 is both a substrate of cathepsins S and K and a selective inhibitor of cathepsin L. Biochemistry 2015;54:2785–98.
[36] Mcnally P, Mcauley DF, Oglesby IK, Wohlford-lenane CL, Bartlett JA, Scott CJ, et al. miR-31 dysregulation in cystic fibrosis airways contributes to increased pulmonary cathepsin S production. Am J Respir Crit Care Med Vol 2014;190:165–74.
[37] Wretlind B, Pavlovskis OR. Pseudomonas aeruginosa elastase and its role in Pseudomonas infections. Clin. Infect. Dis. 5, S998–S1004 (1983. Clin Infect Dis 1983;5:S998–1004.
[38] Lau YE, Rozek A, Scott MG, Goosney DL, Davidson DJ, Hancock REW, et al. Interaction and cellular localization of the human host defense peptide LL-37 with lung epithelial cells. Infect Immun 2005;73:583–91.

[39] Johansson J, Gudmundsson GH, Rottenberg E, Berndt KD, Agerberth B. Conformation-dependent antibacterial activity of the naturally occurring human peptide LL-37 *. J Biol Chem 1998;273:3718–24.
[40] Chotjumlong P, Bolscher JG, Nazmi K, Reutrakul V, Supanchart C, Krisanaprakornkit WBS. Immunity involvement of the P2X 7 purinergic receptor and c-jun N-terminal and extracellular signal-regulated kinases in cyclooxygenase-2 and prostaglandin E 2 induction by LL-37. J Immun Innate Res 2013;5:72–83.