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Published in:
Journal of Antimicrobial Chemotherapy

DOI:
10.1093/jac/dkv322

2016

NLF20 - an antimicrobial peptide with therapeutic potential against invasive *Pseudomonas aeruginosa* infection

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Running title: Potential of NLF20 against *Pseudomonas aeruginosa* infection

Keywords

Heparin cofactor II, peptide, antimicrobial, bacteria, coagulation, inflammation, sepsis

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Objectives: Increasing resistance to antibiotics makes antimicrobial peptides interesting as novel therapeutics. Here, we report on studies of the peptide NLF20 (NLFRKLTHRLFRRNFGYTLR), corresponding to an epitope of the D helix of heparin cofactor II (HCII), a plasma protein mediating bacterial clearance.

Methods: Peptide effects were evaluated by a combination of *in vitro* and *in vivo* methods, including antibacterial, anti-inflammatory, and cytotoxicity assays, fluorescence and electron microscopy, as well as experimental models of endotoxin shock and *Pseudomonas aeruginosa* sepsis.

Results: The results showed that NLF20 displayed potent antimicrobial effects against the Gram-negative bacteria *Escherichia coli* and *Pseudomonas aeruginosa*, the Gram-positive *Bacillus subtilis* and *Staphylococcus aureus*, as well as the fungi *Candida albicans* and *Candida parapsilosis*. Importantly, this antimicrobial effect was retained in human blood, particularly for *P. aeruginosa.* Fluorescence and electron microscopy studies showed that the peptide exerted membrane-breaking effects. In an animal model of *P. aeruginosa* sepsis, NLF20 reduced bacterial levels, resulting in improved survival. Reduced mortality was observed also in experimental animal models of endotoxin shock, which was paralleled with modulated IFN-■ IL-10, and coagulation responses.

Conclusions: Together, these results indicate that functional epitopes of HCII may have therapeutic potential against bacterial infection.

Introduction

The human pathogen *Pseudomonas aeruginosa* causes and/or aggravates a spectrum of diseases, including bacterial conjunctivitis and keratitis, otitis, postoperative and burn wound infections, chronic leg ulcers, pneumonia, and cystic fibrosis.[1-3] Considering also growing problems with resistance development against conventional antibiotics, new bactericidal agents against *P. aeruginosa* are needed, and there is significant current

interest in the potential use of antimicrobial peptides (AMP) as novel treatment modalities. [4] From a therapeutic perspective, AMPs should display high bactericidal potency, but low toxicity against (human) eukaryotic cells. Combinational library approaches,[5] use of stereoisomers composed of D-amino acids,[6] or cyclic D,L-α-peptides,[7] high-throughput based screening assays,[8, 9] quantitative structure-activity relationship (QSAR) approaches, [4, 8, 10, 11] and identification of endogenous peptides,[12-17] have all been used for identifying selective and therapeutically interesting AMPs. [4, 18] In addition to their direct antimicrobial effects, AMPs have recently been found to exhibit also multifaceted immunomodulatory activities, such as those observed for LL-37, [19, 20] and peptides derived from thrombin,[12, 21, 22] including functions within angiogenesis, chemotaxis, and wound-healing.[19] These biological properties indicate that AMPs may have a clinical potential also in disorders where targeting of inflammatory pathways is beneficial, such as in sepsis.[19, 23]

The serine proteinase inhibitor heparin cofactor II (HCII), found in plasma, was recently shown to mediate bacterial clearance upon proteolytic activation.[24] Mice deficient in HCII displayed increased susceptibility to infection by *P. aeruginosa.* Correspondingly, decreased levels of HCII were observed in wild-type animals challenged with bacteria. Since the antibacterial activities were mapped to the A and D helices of HCII, supplementation with these functional epitopes could be an attractive strategy to target bacterial infection. In this work, we therefore explored whether NLF20 (NLFRKLTHRLFRRNFGYTLR), an AMP derived from helix D of HCII, may have therapeutic potential against invasive *P. aeruginosa* infection.

Material and Methods

Peptides

The peptides NLF20 (NLFRKLTHRLFRRNFGYTLR) and omiganan (ILRWPWWPWRRK-NH_2) were synthesized by Biopeptide Co., San Diego, USA, while LL-37 (LLGDFFRKSKEKIGKEFKRIVQRIKDFLRNLVPRTES) was from Innovagen AB, Lund, Sweden. The two latter peptides were included as biological effect benchmark peptides. The purity (>95%) of these peptides was confirmed by mass spectral analysis (MALDI-ToF Voyager).

Microorganisms

Bacterial and fungal isolates, *Escherichia coli* ATCC 25922, *Pseudomonas aeruginosa* ATCC 27853, *Staphylococcus aureus* ATCC 29213, *Streptococcus pyogenes* AP1, *Bacillus subtilis* ATCC 6633, *Candida albicans* ATCC 90028, and *Candida parapsilosis* ATCC 90018, were all obtained from the Department of Bacteriology, Lund University Hospital. The *P. aeruginosa* strain PA01 was a generous gift from Dr. B. Iglewski (University of Rochester). The clinical isolate *P. aeruginosa* 15159 was originally derived from a patient with a chronic leg ulcer and obtained from the Department of Bacteriology, Lund University Hospital, Sweden.

Viable-count analysis

E. coli ATCC 25922, *P. aeruginosa* ATCC 27853, or *S. aureus* ATCC 29213 bacteria were grown to mid-logarithmic phase in Todd-Hewitt (TH) medium (Becton and Dickinson, Maryland, USA). The microorganisms were then washed and diluted in 10 mM Tris, pH 7.4, containing 5 mM glucose. Following this, bacteria (50 µL; 2 x 10^6 cfu/mL) were incubated, at 37°C for 2 h, with NLF20 or LL-37 (at 0.03, 0.06, 0.3, 0.6, 3, 6, 30, 60 µM) in 10 mM Tris, 0.15 M NaCl, with or without 20% human citrate-plasma. In the experiments using 50% whole blood, *S. aureus* ATCC 29213 and *P. aeruginosa* ATCC 27853 bacteria (50 µL; 2 x 10^8 cfu/mL) were incubated at 37°C for 1 h in the presence of peptide at 60 (for *P. aeruginosa*) and 120 µM (*P. aeruginosa* and *S. aureus*) (the high concentrations used to achieve high bactericidal effect). To quantify bactericidal activity, serial dilutions of the incubation mixtures were plated on TH agar, followed by incubation at 37°C overnight and determination of the number of colony-forming units (cfu). Hundred % survival was defined as total survival of bacteria in the same buffer and under the same condition in the absence of peptide. Significance was determined using the statistical software SigmaStat (SPSS Inc., Chicago, IL, USA).

Minimal inhibitory concentration (MIC) determination

MIC assay was carried out by a microtiter broth dilution method as previously described in the clinical and laboratory standards institute (CSLI) guidelines.[25] In brief, fresh overnight colonies were suspended to a turbidity of 0.5 McFarland units and further diluted in Mueller-Hinton broth (Becton Dickinson). For determination of MIC, peptides were dissolved in water at concentration 10 times higher than the required range by

serial dilutions from a stock solution. Ten μL of each concentration was added to each corresponding well of a 96-well microtiter plate (polypropylene, Costar Corp.) and 90 μL of bacteria ($1.1x10^6$/mL) in MH medium added. The plate was incubated at 37°C for 16-18 h. MIC was taken as the lowest concentration where no visual growth of bacteria was detected.

Radial diffusion assay

Essentially as described earlier,[26, 27] bacteria were grown to mid-logarithmic phase in 10 mL of full-strength (3% w/v) trypticase soy broth (TSB) (Becton-Dickinson). The microorganisms were then washed once with 10 mM Tris, pH 7.4. Subsequently, $4x10^6$ cfu were added to 15 mL of the underlay agarose gel, consisting of 0.03% (w/v) TSB, 1% (w/v) low electroendosmosis type (EEO) agarose (Sigma-Aldrich), and 0.02% (v/v) Tween 20 (Sigma-Aldrich). The underlay was poured into a Ø 144 mm Petri dish. After agarose solidification, 4 mm-diameter wells were punched and 6 μl peptide solution of required concentration added to each well. Plates were incubated at 37ºC for 3 h to allow peptide diffusion. The underlay gel was then covered with 15 mL of molten overlay (6% TSB and 1% Low-EEO agarose in distilled H_2O). Antimicrobial activity of a peptide was visualized as a clearing zone around each well after 18-24 h of incubation at 37°C.

Fluorescence microscopy

The impermeant probe FITC (Sigma-Aldrich, St. Louis, USA) was used for monitoring bacterial membrane permeabilization. *E. coli* ATCC 25922 bacteria were grown to mid-logarithmic phase in TSB medium. Bacteria were washed and re-suspended in buffer (10 mM Tris, pH 7.4, 0.15 M NaCl, 5 mM glucose) to yield a suspension of $1x10^7$ cfu/mL. Hundred μl of the bacterial suspension was incubated with 30 μM of the respective peptides at 30°C for 30 min. Microorganisms were then immobilized on poly (L-lysine)-coated glass slides by incubation for 45 min at 30°C, followed by addition onto the slides of 200 μl of FITC (6 μg/mL) in buffer and a final incubation for 30 min at 30°C. The slides were washed and bacteria fixed by incubation, first on ice for 15 min, then at room temperature for 45 min in 4% paraformaldehyde. The glass slides were subsequently mounted on slides using Prolong Gold antifade reagent mounting medium (Invitrogen, Eugene, USA). Bacteria were visualized using a Nikon Eclipse TE300 (Nikon, Melville, USA) inverted fluorescence microscope equipped with a

Hamamatsu C4742-95 cooled CCD camera (Hamamatsu, Bridgewater, USA) and a Plan Apochromat ×100 objective (Olympus, Orangeburg, USA). Differential interference contrast (Nomarski) imaging was used for visualization of the microbes themselves.

Electron Microscopy

For transmission electron microscopy and visualization of peptide effects on bacteria, *P. aeruginosa* ATCC 27853 and *S. aureus* ATCC 29213 (1-2 x 10^6 cfu/sample) were incubated for 2 h at 37°C with the peptides (30 μM). Samples of *P. aeruginosa* and *S. aureus* suspensions were adsorbed onto carbon-coated copper grids for 2 min, washed briefly by two drops of water, and negatively stained by two drops of 0.75 % uranyl formate. The grids were rendered hydrophilic by glow discharge at low pressure in air. All samples were examined with a Jeol JEM 1230 electron microscope operated at 80 kV accelerating voltage. Images were recorded with a Gatan Multiscan 791 charge-coupled device camera.

Hemolysis assay

EDTA-blood was centrifuged at 800 g for 10 min, whereafter plasma and buffy coat were removed. The erythrocytes were washed three times and re-suspended in PBS, pH 7.4, to a 5% suspension. The cells were then incubated with end-over-end rotation for 1 h at 37°C in the presence of peptides (3-60 μM). 2% Triton X-100 (Sigma-Aldrich) served as positive control. Following this, the samples were centrifuged at 800 g for 10 min and the supernatant was transferred to a 96 well microtiter plate. In the experiments with blood infected by bacteria, citrate-blood was diluted (1:1) with PBS. The cells were then incubated with end-over-end rotation for 1 h at 37°C in the presence of peptides (30, 60 or 120 μM) and either *E. coli, P. aeruginosa*, *S. pyogenes* or *S. aureus* bacteria, or the fungus *C. albicans* (2 x 10^8 cfu/mL). In another experiment, designed to evaluate peptide-induced hemolysis over longer time, whole blood diluted in 50% RPMI or erythrocytes diluted in 50% PBS were incubated with NLF20 for 18 h 37°C. In all cases, the absorbance of hemoglobin release was measured at λ 540 nm and is expressed as % of Triton X-100-induced hemolysis.

Lactate dehydrogenase (LDH) assay

HaCaT keratinocytes were grown to confluency in 96 well plates (3000 cells/well) in serum-free keratinocyte medium (SFM) supplemented with bovine pituitary extract and recombinant EGF (BPE-rEGF) (Invitrogen, Eugene, USA). The medium was then removed, and 100 µL of the peptide added (at 3-60 µM, diluted in SFM/BPE-rEGF or in keratinocyte-SFM supplemented with 20% human serum). The lactate dehydrogenase (LDH)-based TOX-7 kit (Sigma-Aldrich, St. Louis, USA) was used for quantification of LDH release from the cells. Results represent mean values from triplicate measurements, and are given as fractional LDH release compared to the positive control, consisting of 1% Triton X-100 (yielding 100% LDH release). In another set of experiments, LDH release in mouse plasma was analysed according to manufacturer's instructions (Uscn Life Science Inc., Wuhan, China). The data are represented as LDH release in Units from three mice.

MTT assay

Sterile filtered MTT (3-(4,5-dimethylthiazolyl)-2,5-diphenyl-tetrazolium bromide; Sigma-Aldrich) solution (5 mg/mL in PBS) was stored protected from light at -20°C until usage. HaCaT keratinocytes, 3000 cells/well, were seeded in 96 well plates and grown in serum free keratinocyte-SFM/BPE-rEGF medium to confluency. Peptides investigated were then added at 3-60 µM. After incubation over night, 20 µL of the MTT solution was added to each well and the plates incubated for 1 h in CO_2 at 37°C. The MTT-containing medium was then removed by aspiration. The blue formazan product generated was dissolved by the addition of 100 µL of 100% DMSO per well, and the plates gently swirled for 10 min at room temperature to dissolve the precipitate. The absorbance was monitored at 550 nm, and results given represent mean values from triplicate measurements.

LPS effects on macrophages *in vitro*

3.5×10^5 cells (RAW264.7 macrophages) were seeded in 96-well tissue culture plates (Nunc, 167008) in phenol red-free DMEM (Gibco) supplemented with 10% FBS and antibiotics. Following 6 h of incubation to permit adherence, cells were stimulated with 10 ng/mL *E. coli* LPS (0111:B4) or *P. aeruginosa* LPS (Sigma), with and without peptide of various doses. The levels of NO in culture supernatants were determined after 24 hours from stimulation using the Griess reaction. [28] Phenol-red free DMEM with FBS

and antibiotics were used as a blank. A standard curve was prepared using 0-80 μM sodium nitrite solutions in ddH20.

Cytokine assay

The cytokines IL-6, IL-10, MCP-1, IFN-γ, and TNF-α were measured in plasma from mice injected with LPS or *P. aeruginosa* (with or without peptide treatment), or alternatively NLF20 alone (1 mg/mouse), using the Cytometric bead array; Mouse Inflammation Kit (Becton Dickinson AB) according to the manufacturer's instructions. All plasma samples were stored at -20°C before the analysis.

Clotting Assays

Clotting times were analyzed using a coagulometer (Amelung, Lemgo, Germany). The prothrombin time (PT) and the Thrombin clotting time (TCT) were measured as follows: Hundred μL of fresh human citrate plasma together with indicated concentrations of NLF20 were pre-warmed for 1 min at 37°C before clot formation was initiated by adding 100 μL a clotting reagent (PT-thromboplastin reagent (Trinity Biotech), TCT: Thrombin reagent (Technoclone)). To record the activated partial thromboplastin time (aPTT), 100 μL of a kaolin-containing solution (Technoclone) was added to the plasma-peptide mix and incubated for 200 sec before clot formation was initiated by adding 100 μL of 30 mM fresh $CaCl_2$ solution.

LPS model *in vivo*

Male C57BL/6 mice (8-10 weeks, 22 +/- 5 g), were injected intraperitoneally with 200 μL of 18 mg *E. coli* 0111:B4 LPS (Sigma) per kg of body weight. Thirty minutes after LPS injection, 0.2 mg or 0.5 mg NLF20, or buffer alone, was injected intraperitoneally into the mice. Survival and status was followed during seven days. For blood collection, mice were sacrificed 20 h after LPS challenge, and lungs were removed and fixed. These experiments were conducted according to national guidelines and were approved by the Laboratory Animal Ethics Committee of Malmö/Lund (no. M228-10).

***P. aeruginosa* infection model**

The animal experiments were conducted according to national guidelines and were approved by the Laboratory Animal Ethics Committee of Malmö/Lund (no. M226-12 & M228-10). Animals were housed under standard conditions of light and temperature and

had free access to standard laboratory chow and water. *P. aeruginosa* 15159 bacteria were grown to logarithmic phase (OD_{620}~0.5), harvested, washed in PBS, diluted in the same buffer to either 2 x 10^8 cfu/mL (high levels) or 2 x 10^4 cfu/mL (low levels), and kept on ice until injection. Hundred µl of the bacterial suspension were injected intraperitoneally into C57BL/6 mice. Immediately or 60 min subsequent of bacterial injection, 0.5 mg NLF20 (in 10 mM Tris, pH 7.4) or buffer alone was injected i. p. or s. c. into the mice. (The purpose of injecting NLF20 peptide immediately after bacterial injection was to compare the NLF20 peptide activity with that of classical antibiotics.) The survival data were obtained by following the animals daily up to 7 days monitoring. Mice reaching the predefined endpoint-criteria were sacrificed and counted as non-survivors. In another experiment, mice were treated i. p. or s. c. with 0.5 mg of NLF20 or 300 mg/kg ceftazidime or 100 mg/kg levofloxacin injected 0 h or 1 h after bacterial infection. In order to study bacterial dissemination to target organs spleen, liver and kidney were harvested, placed on ice, homogenized, and colony-forming units determined. The P-value was determined using the Mann-Whitney U-test. Data from three independent experiments were pooled. In another experiment as previously described [29], *P. aeruginosa* Xen41 bacteria were grown to mid-exponential phase (A_{620} ~ 0.5), harvested, washed in PBS, diluted in the same buffer to 2-3 × 10^8 cfu/mL, and kept on ice until injection. Hundred µl of the bacterial suspension was injected intraperitoneally into male BALB/c mice. One and 4 h after bacterial injection, 0.5 mg of NLF20 peptide or buffer alone was administrated subcutaneously. Mice were anesthetized immediately, or 12 h after bacterial infection, followed by data acquisition and analysis using a Spectrum three-dimensional imaging system with Living Image®. For evaluation of animal survival, mice showing the defined and approved end point criteria (immobilization and shaking) were sacrificed by an overdose of isoflurane (Abbott) and counted as non-survivors.

Histochemistry

Lung biopsies were fixed in 10% formalin, rehydrated, and embedded in paraffin. Sections of 5 µm thickness were placed on polylysine-coated glass slides, deparaffinized in xylene, and rehydrated in graded alcohols and stained with hematoxylin and eosin by routine procedures.

Statistical analysis

Values are shown as mean with SEM. For statistical evaluation of two experimental groups, the Mann-Whitney U-test was used and for comparison of survival curves the log-rank test. To compare more than two groups, One-Way or Two-Way ANOVA with Bonferoni post-test were used. Viable count data are presented as mean with SD. All statistical evaluations were performed using the GraphPad Prism software 6.0. with *p-<0.005, **<0.001 and ***p<0.0001 and ns = not significant.

Results

To explore the antimicrobial spectrum of NLF20, we first investigated the effects in radial diffusion assays (RDA) against Gram-negative *Escherichia coli* and *Pseudomonas aeruginosa,* Gram-positive *Bacillus subtilis* and *Staphylococcus aureus*, as well as the fungi *Candida albicans* and *Candida parapsilosis* (Figure 1A). As can be seen, NLF20 activities well exceeded those observed for the human cathelicidin LL-37, a potent benchmark antimicrobial peptide. The antibacterial results were further substantiated by matrix-free viable count assay. Results from these dose-response experiments utilizing *E. coli, P. aeruginosa* and *S. aureus* confirmed that NLF20 displays significant antibacterial activity, also in presence of human citrated plasma (Figure 1B). In addition, kinetic studies demonstrated fast bacterial killing, indicating a direct bactericidal action compatible with many antimicrobial peptides (Figure 1C). MIC analyses according to CSLI against the above and other pathogens are presented in Supplementary Material, Table S1. Overall, NLF20 showed comparable activities to those observed for the two benchmarks omiganan, [30] and LL-37.[31] It was also noted that NLF20 displayed low MIC levels particularly against *P. aeruginosa*.

Next, studies employing the impermeant probe FITC showed that NLF20 permeabilized bacterial membranes of *E. coli* similarly to those seen after treatment with LL-37 (Figure 1D). Electron microscopy utilizing *P. aeruginosa* demonstrated extensive membrane damage, with cell envelopes devoid of their cytoplasmic contents, and intracellular material found extracellularly (Figure 1E). Again, similar findings were obtained with LL-37. These data indicate that NLF20 acts on bacterial membranes.

Frequently, AMPs efficient in killing bacteria also exhibit hemolytic and membrane permeabilizing activities against eukaryotic cells, which risks translating into toxicity issues. Importantly, however, NLF20 exerted only minor hemolytic activity at 30-60 μM and standard incubation times, and displayed less permeabilization than LL-37 (Figure 2A). Similar findings were observed with respect to permeabilization of HaCaT cells (Figure 2B), as well as effects on viability as monitored by MTT assay (Figure 2C). Further demonstrating this selectivity between bacteria and human cell membranes under physiological conditions, of importance for subsequent *in vivo* studies. Next, NLF20 was added to human blood infected by various Gram-positive and Gram-negative pathogens, as well as fungi. In such experiments, NLF20 caused almost complete eradication of *P. aeruginosa, E. coli,* as well as *S. pyogenes,* with little (~ 2% or less) accompanying hemolysis at a peptide dose of 30-60 μM (Figure 2D). (The lower hemolysis in blood is due to anionic serum proteins competing with erythrocyte membranes for NLF20 binding. [31]) The observed low simultaneous hemolysis mediated by NLF20 under these conditions indicated a high selectivity of the peptide for bacteria (Figure 2E). In contrast, NLF20 was not particularly active in human blood against the Gram-positive *S. aureus*, as well as the fungus *C. albicans*, although the latter showing a minor reduction at 120 μM NLF20. The reduced activity against some bacteria is due to the presence of a high content of anionic serum proteins, able to bind cationic peptides such as NLF20 and reduce their antimicrobial effect through ~~competitively~~ competition for peptide binding with bacterial membranes. This effect is widely observed for antimicrobial peptides,[32] and is analogous to the reduced antimicrobial effects displayed by most AMPs in the presence of growth media rich in anionic scavengers, such as in standardized MIC determinations.[31]

In another set of experiments, NLF20 toxicity was further evaluated following treatment of mice with NLF20. As shown in Figure S1, hemolysis after 18 hours is growing over time. At the highest peptide concentration used in the antimicrobial assays (313 μg/mL) however, it was only 5 % in 50% blood. Furthermore, LDH release after treatment with 1 mg/mL was 4800 U, which is low compared to that of LL-37.[33] Similarly, cytokine levels after peptide treatment at the same concentration are low,[34] and also histology on lung tissue indicates no visible toxic effects of peptide treatment.

Having demonstrated the antimicrobial effects of NLF20, as well as its relatively limited

toxicity, we next investigated possible anti-endotoxic effects of the peptide. As seen in Figure 3A, NLF20 inhibited LPS-induced NO-responses at 20-40 μM. In addition, measurements of the aPTT showed that NLF20 impaired the intrinsic pathway of coagulation in normal human plasma. Other parts of the coagulation system, as judged by the PT (monitoring the extrinsic pathway of coagulation) and the TCT (measuring thrombin induced fibrin network formation), were not significantly affected (Figure 3B).

Given the observed dual effects on cytokine responses and coagulation, the *in vivo* efficiency of NLF20 was next evaluated in a standardized mouse model of endotoxin-induced shock.[22] A dramatic improvement in survival rate of the animals was seen after treatment with the peptide (Figure 3C). NLF20-treated animals fully regained their weight after seven days (Figure 3D). Analyses of cytokines 8 h and 20 h after LPS injection showed no notable, or only minor, alterations of IL-6 and TNF-α, respectively, as well as of the pro-inflammatory cytokines IFN-γ and MCP-1, and a strong increase in the anti-inflammatory IL-10 (Figure 3E). Previous studies have shown that thrombocytopenia is as an important indicator for the severity of sepsis and disseminated intravascular coagulation.[35] Therefore, platelet numbers were measured in blood of LPS-injected mice treated with NLF20 at 8, 20 h, and 7 days after LPS injection. NLF20-treated animals showed significantly increased platelet numbers at 20 h, indicating that the peptide reduced platelet consumption at later stages in this endotoxin-model (Figure 3F). The levels were completely normalized in the survivors after seven days.

In order to explore whether NLF20 could be effective against invasive *P. aeruginosa* infection *in vivo*, the peptide was injected into mice infected with *P. aeruginosa.* Compared to the controls, immediate treatment with NLF20 yielded significantly lower bacterial numbers in the spleen, liver, and kidney of the animals infected with both low and high dose of bacteria (Figure 4A). Importantly, the antimicrobial effects, as evidenced by reductions in cfu, were present also after treatment was delayed and given subcutaneously 1 h after infection (Figure 4B). Similarly to the LPS model, platelet levels were increased in the peptide-treated animals, although recovery was not complete within the time-frame of the experiments (Figure 4C). It is also notable that treatment with only one dose of NLF20, particularly when given i. p., was sufficient to reduce mortality or prolong survival (Figure 4D). Finally, in a second set of experiments, the antimicrobial effects of NLF20 were compared to those of commonly

used antibiotics. The results showed that the peptide was able to reduce bacterial levels by 2-3 log, which was comparable to the bacterial reductions obtained with ceftazidime and levofloxacin (Figure 5). To further investigate the potential therapeutic effect of NLF20 in a clinically relevant bacterial sepsis model, we treated *P. aeruginosa* infected mice with NLF20 1 and 4 h after bacterial infection. The data showed that the two-dose treatment with NLF20 peptide prolonged mice survival (Figure 6A), paralleled by a reduced bacterial spread (Figures 6B and S2).

Discussion

Infections caused by Gram-negative bacteria are increasingly contributing to problems associated with antibiotic resistance, and remain a major cause of sepsis.[36] During sepsis, multiple proinflammatory cascades as well as tissue-factor related coagulative responses are activated.[36] Several clinical trials aimed at inhibiting these coagulative and pro-inflammatory responses have been conducted, including administration of several modulatory substances along with the traditional antibiotic regimen. Examples include antibodies against TNF-α,[37-39] or endotoxins, [40], antagonists against the interleukin-1 receptor,[41, 42] interleukin-6,[40] or platelet-activating factor (PAF) receptors, [43] or antithrombin III, [44-46] and other agents.[36, 47-49] Despite promising preclinical results, these drug candidates have all failed when tested in the clinic. While activated protein C (APC) has shown promise in preclinical as well as well as clinical studies, clinical trials were recently terminated due to lack of clinical efficiency (http://pi.lilly.com/us/xigris.pdf). Hence, the lack of dedicated therapeutics against sepsis remains, and current treatment is largely based on antibiotics in combination with supportive measures.

The capacity of proteolytically activated HCII to target bacteria and bind LPS, as well as its dramatic decrease in plasma during endotoxin shock and bacterial sepsis,[24] indicates a therapeutic potential of this protein. In an attempt to increase “drugability”, the present study aimed at characterizing the antimicrobial, immunomodulatory, and therapeutic potential of a HCII epitope. Thus, like the holoprotein, NLF20 was found to exert antimicrobial effects, particularly against *P. aeruginosa*, both in standard MIC assays and in physiological buffers including human plasma. The finding that NLF20 assumes

a helical conformation upon LPS binding, as well as interaction with LPS-coated or anionic liposomes,[50] further indicates a parallelism between NLF20 and other helical AMPs. In relation to its LPS-binding capacity, NLF20 showed anti-inflammatory effects *in vitro*, as well as *in vivo*, further illustrating the multifunctionality of AMPs.

It is also interesting to compare the effects observed for NLF20 (NLFRKLTHRLFRRNFGYTLR) to those of the longer epitope KYE28 (KYEITTIH**NLFRKLTHRLFRRNFGYTLR**), containing the NLF20 sequence, as well as those of the corresponding truncation in the other end of KYE28 (KYE21; KYEITTIHNLFRKLTHRLFR). While both KYE21 and NLF20 display antimicrobial and anti-inflammatory effects qualitatively similar to those displayed by KYE28, the relative importance of the antimicrobial and the anti-inflammatory effects are shifted, such that the main effect of KYE21 is anti-inflammatory, while NLF20 displays more potent antimicrobial effects, along with partly retained anti-endotoxic effects as shown here. The more potent antimicrobial activity of NLF20 was previously demonstrated to be due to potent membrane destabilization of the highly charged NLF20, in turn facilitated by higher electrostatically and hydrophobically driven adsorption to anionic lipid membranes. In contrast, NLF20 displays somewhat poorer capacity than KYE28 and KYE21 to provide multiple scavenging alternatives to LPS-induced NF-κB activation, including peptide-induced scavenging of LPS at human cell surfaces (mediated by lower adsorption and positive potential build-up at zwitterionic membranes for NLF20 than for KYE21 and KYE28) and LPS micelle packing disruption (mediated by a delicate balance of peptide binding to the lipid A and the polysaccharide regions of LPS, and resulting effects on packing constraints in LPS micelles).[50]

It must be stressed that the activities of NLF20 may not necessarily reflect all possible activities mediated by the proteolytically activated HCII molecule. Thus, it is possible that the bacterial binding and LPS-interactions mediated by this helix D peptide may be complemented by other actions of distant structural motifs, potentially mediating other cell-effects, phagocytosis, or distinct ligand interactions. Although some evidence suggests that C-terminals of serpins, such as a_1-antitrypsin and antithrombin III, may interact with cell-receptors and mediate phagocytosis, little is known about such actions for HCII, and clearly, this warrants further investigations. Nevertheless, the present data indicate, with these limitations, that the current strategy of selecting one functional

epitope of HCII may have therapeutic implications and also benefits due to a less complex mode of action, as well as easier production and enhanced stability of the peptide, while maintaining the endogenous character of the host response.

Acknowledgements

We wish to thank Ms. Lotta Wahlberg and Ms. Ann-Charlotte Strömdahl for expert technical assistance.

Funding

This work was supported by grants from the Swedish Research Council (projects 2012-1842 and 2012-1883), Knut and Alice Wallenberg Foundation, the Welander-Finsen, Thelma-Zoegas, Crafoord, Alfred Österlund, Lundgrens, and Kock Foundations, Ximmune AB, and The Swedish Government Funds for Clinical Research.

Transparency declarations

M. Malmsten and A. Schmidtchen are founders and own shares in XImmune AB, a company developing anti-inflammatory peptides for human therapy. All other authors: None to declare.

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Figure 1

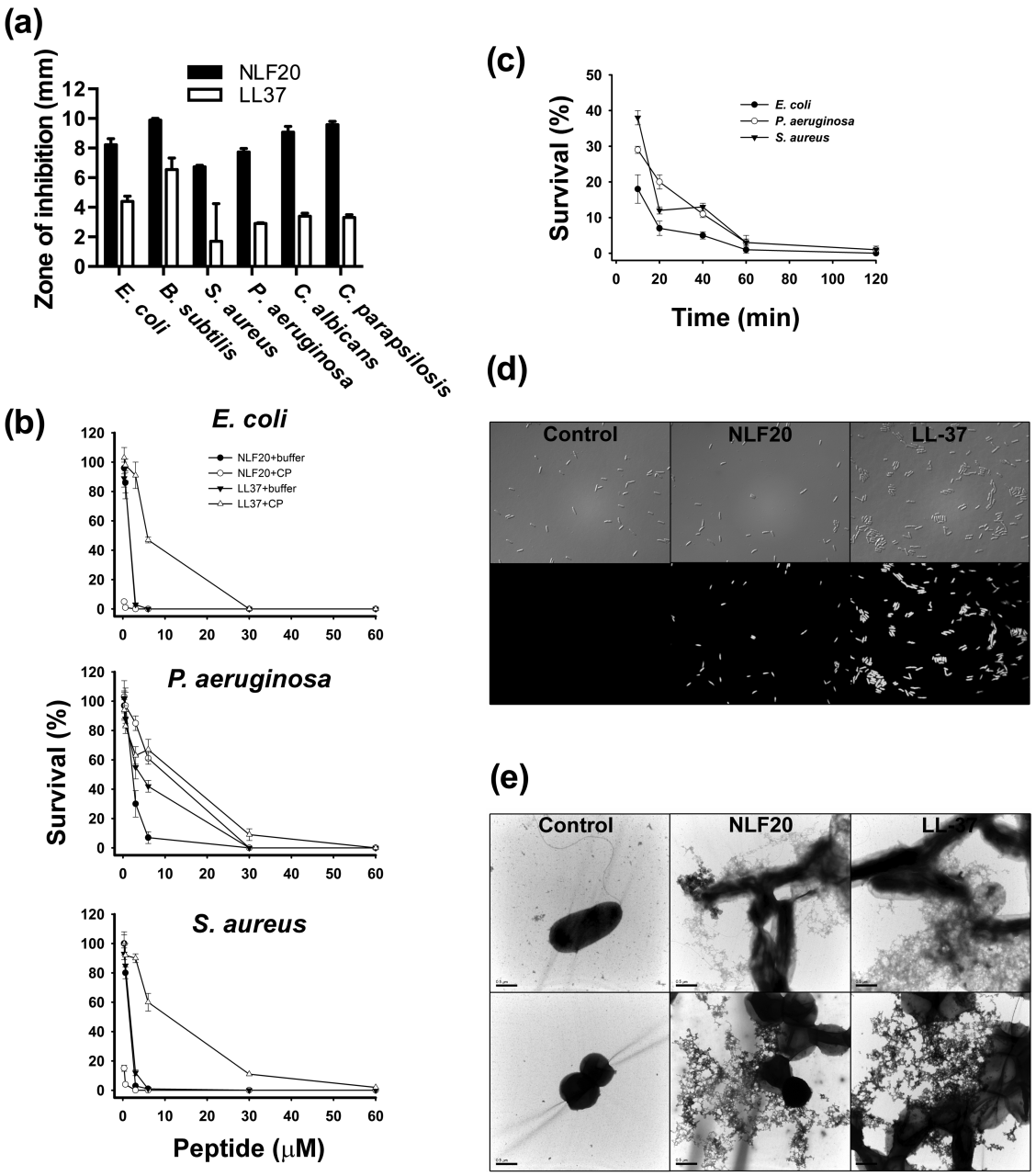

(a)
Zone of inhibition (mm)
NLF20
LL37
E. coli
B. subtilis
S. aureus
P. aeruginosa
C. albicans
C. parapsilosis
(b)
E. coli
P. aeruginosa
S. aureus
NLF20+buffer
NLF20+CP
LL37+buffer
LL37+CP
Survival (%)
Peptide (μM)
(c)
Survival (%)
E. coli
P. aeruginosa
S. aureus
Time (min)
(d)
Control
NLF20
LL-37
(e)
Control
NLF20
LL-37

Figure 2

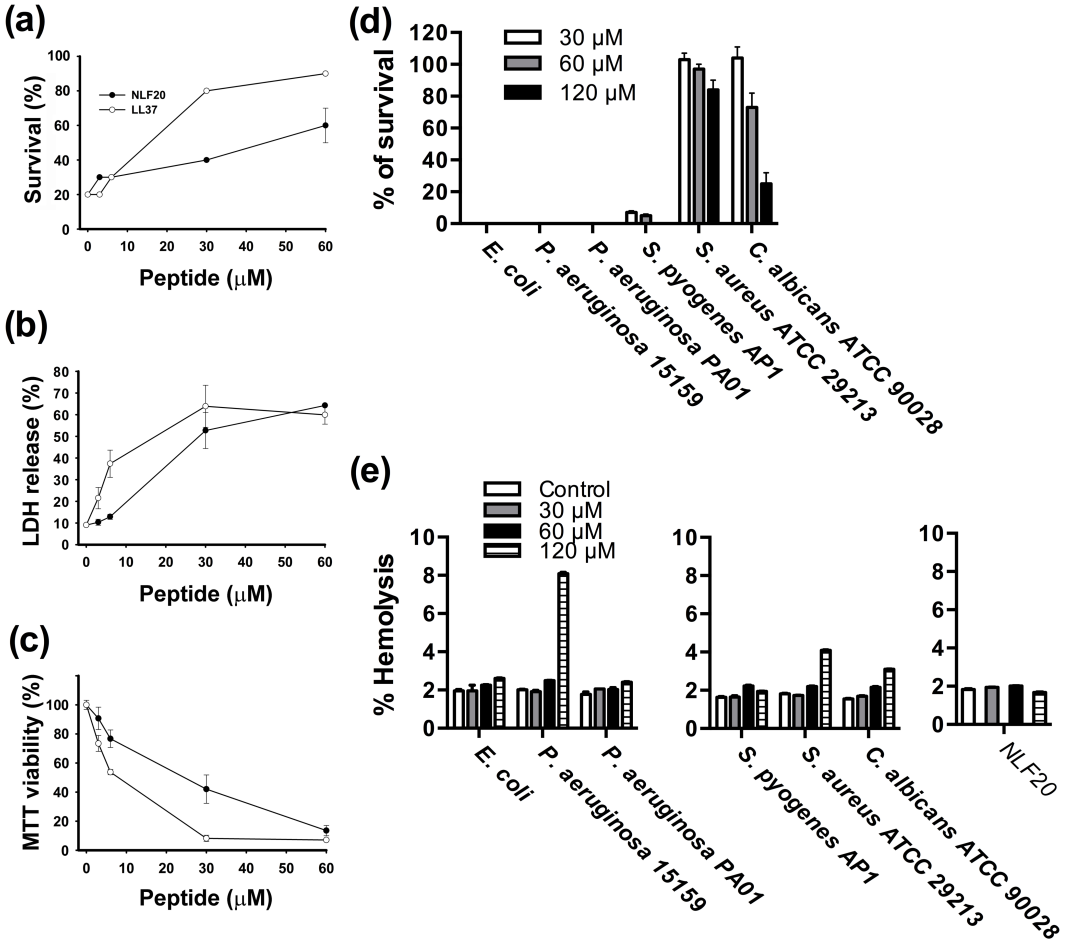
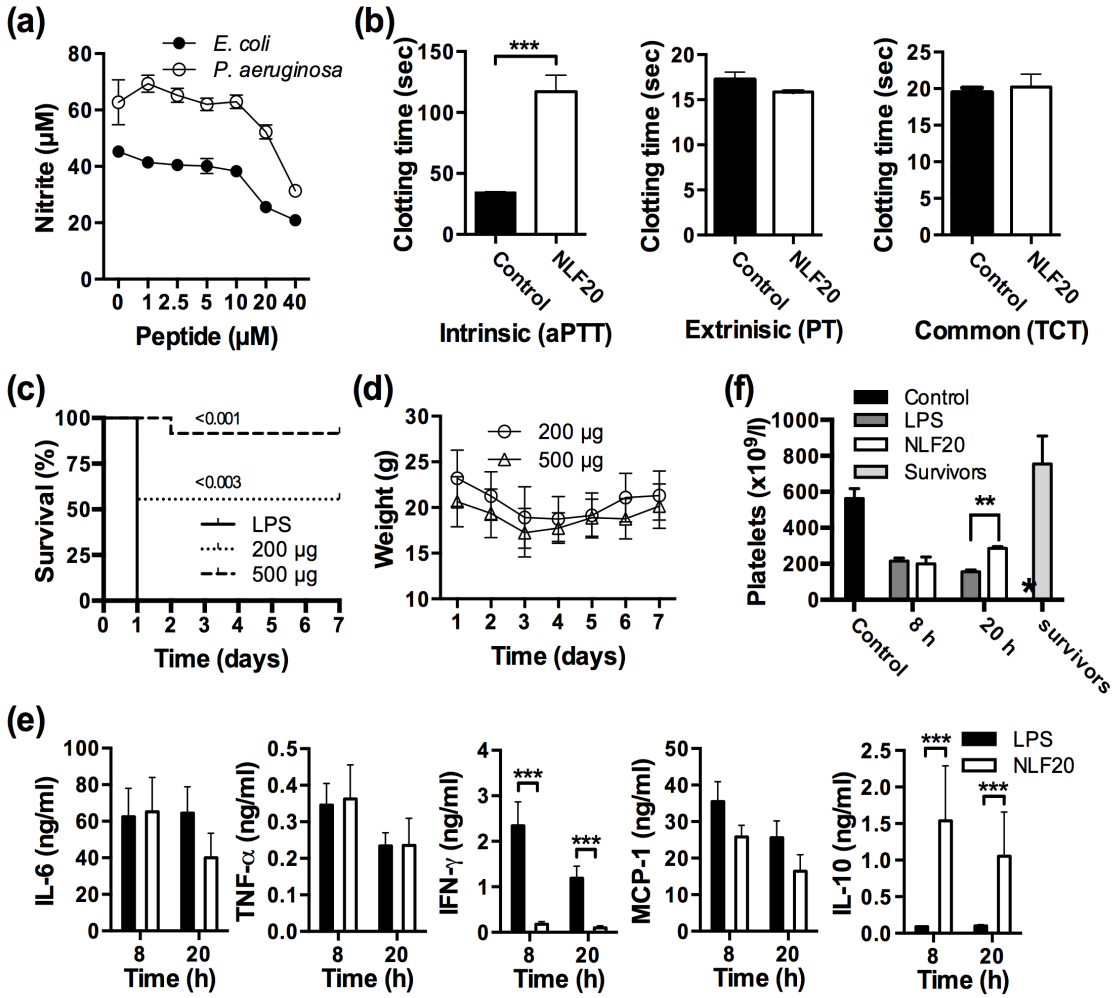

(a)
Survival (%)
NLF20
LL37
Peptide (μM)
(b)
LDH release (%)
Peptide (μM)
(c)
MTT viability (%)
Peptide (μM)
(d)
% of survival
30 μM
60 μM
120 μM
E. coli
P. aeruginosa 15159
P. aeruginosa PA01
S. pyogenes AP1
S. aureus ATCC 29213
C. albicans ATCC 90028
(e)
% Hemolysis
Control
30 μM
60 μM
120 μM
E. coli
P. aeruginosa 15159
P. aeruginosa PA01
S. pyogenes AP1
S. aureus ATCC 29213
C. albicans ATCC 90028
NLF20

Figure 3



630
631

Figure 4

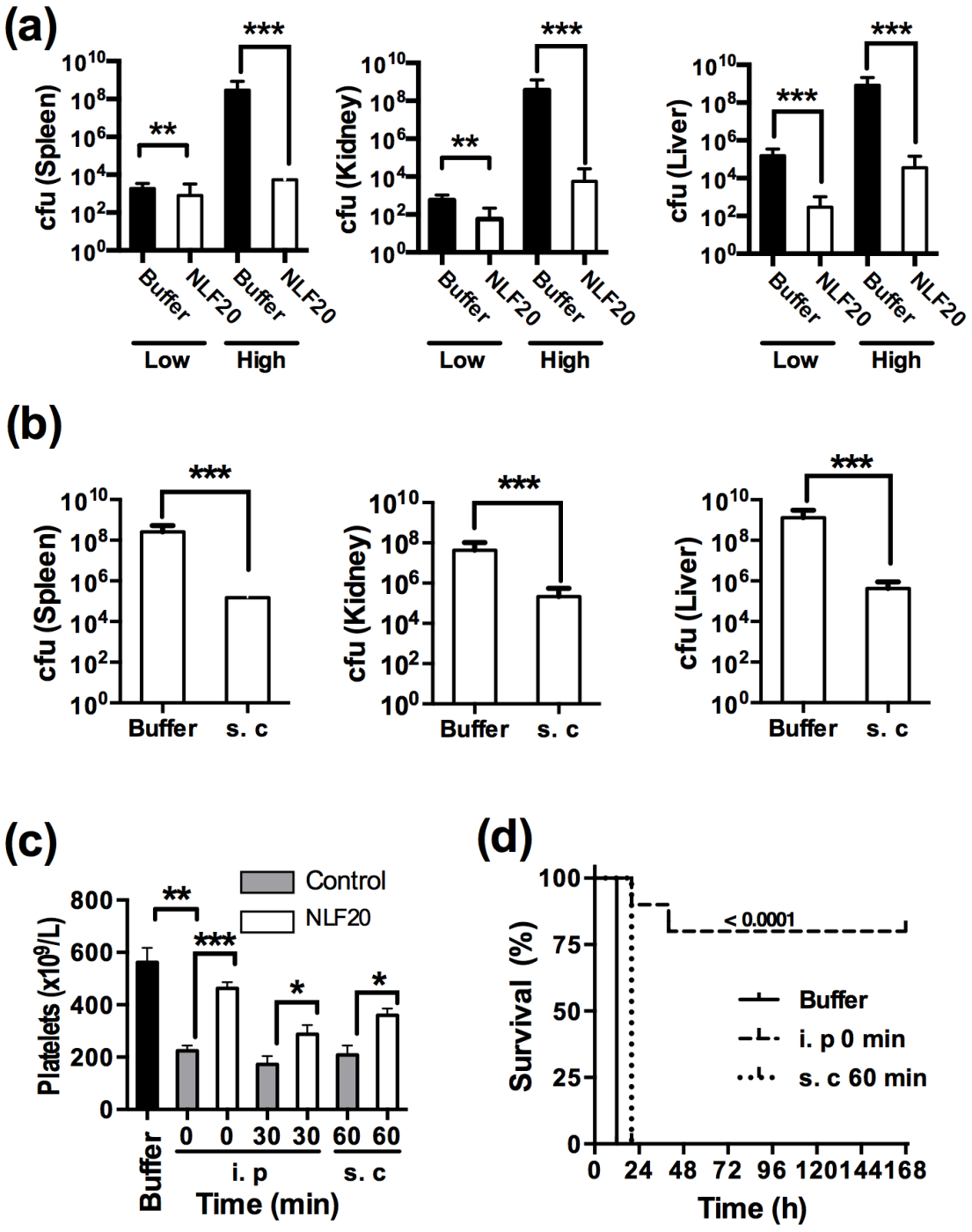

(a)
cfu (Spleen)
cfu (Kidney)
cfu (Liver)
Buffer
NLF20
Low
High
(b)
s. c
(c)
Control
NLF20
Platelets (x10⁹/L)
Time (min)
i. p
s. c
(d)
Survival (%)
Time (h)
≤ 0.0001
Buffer
i. p 0 min
s. c 60 min

Figure 5

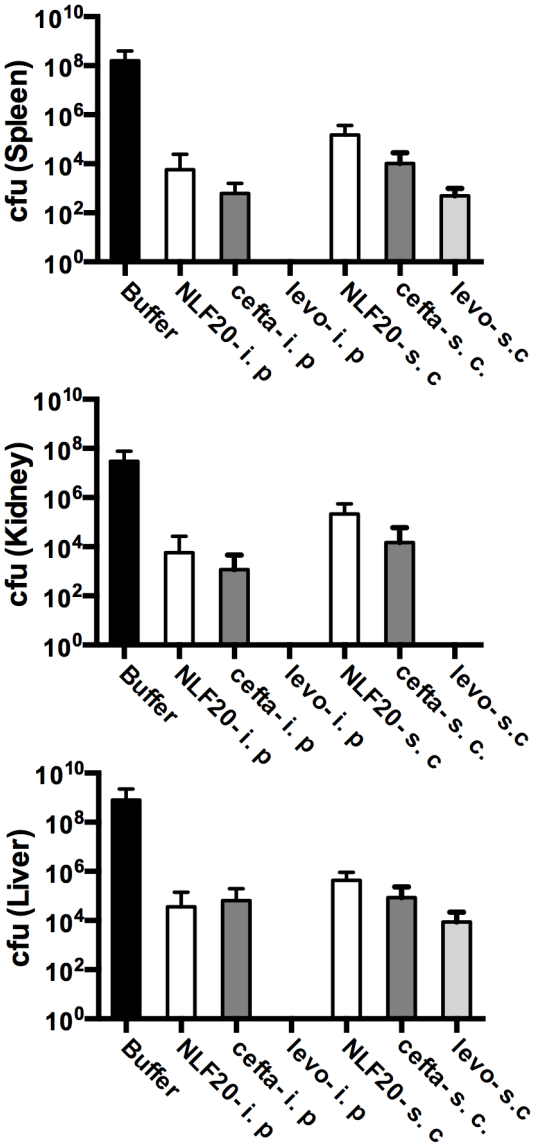


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Figure 6

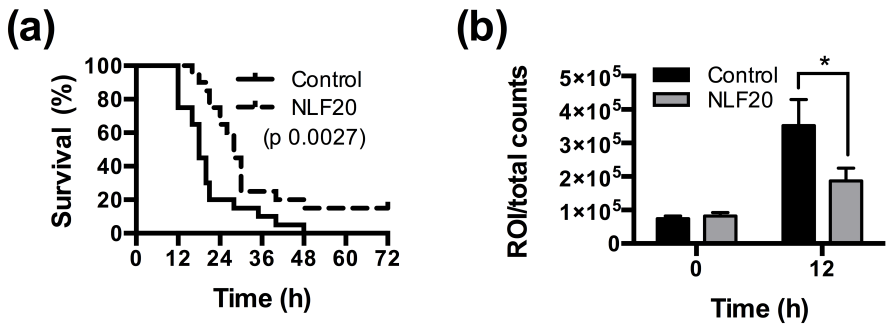

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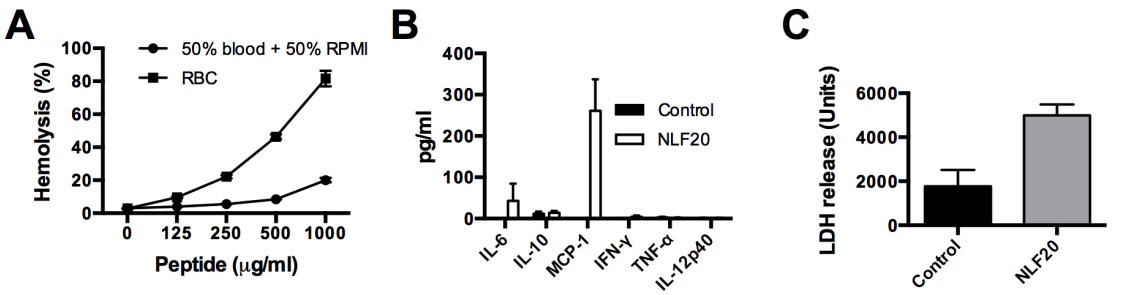
Figure S1



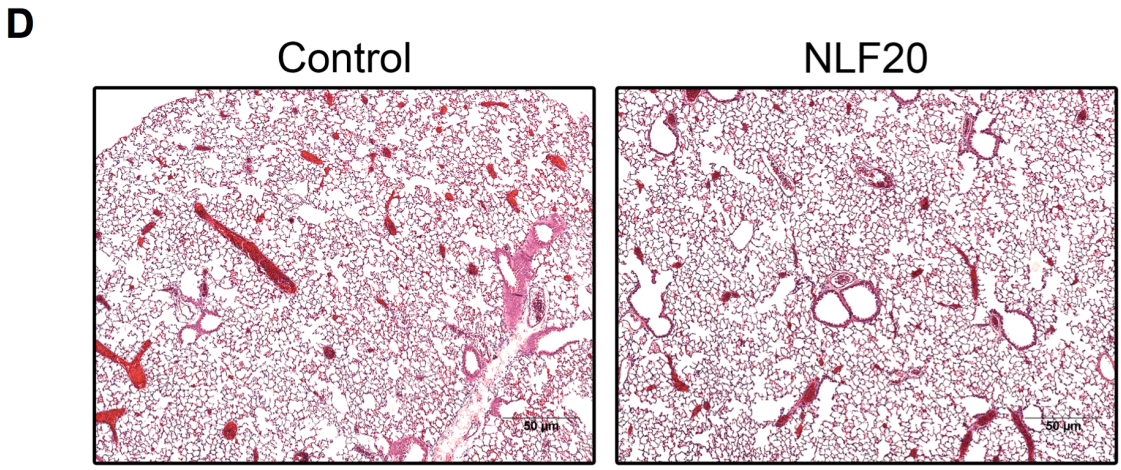


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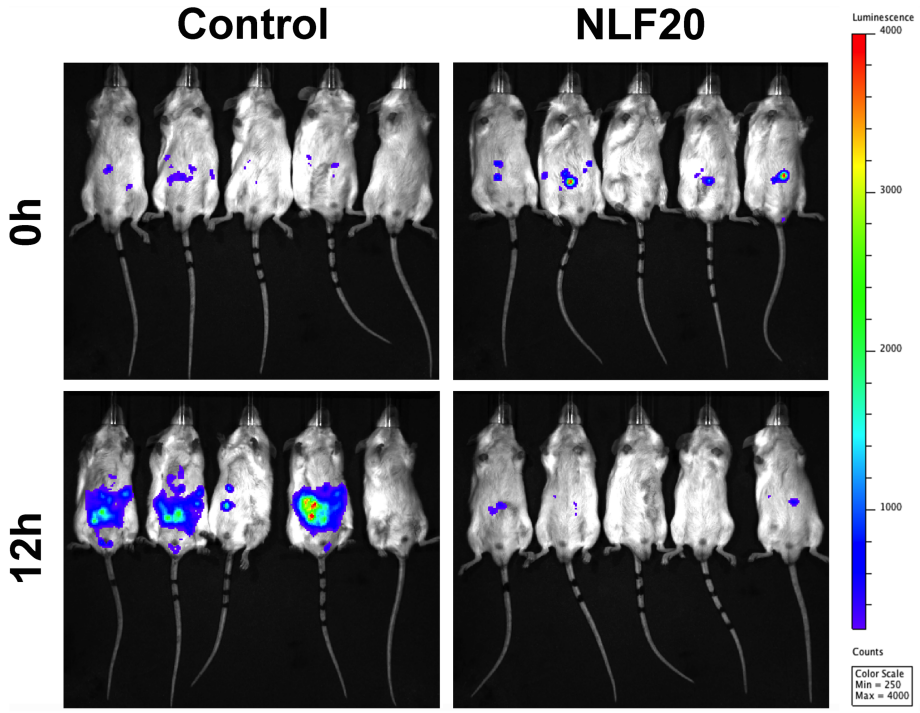
Figure S2


Control
NLF20
0h
12h
Luminescence
4000
3000
2000
1000
Counts
Color Scale
Min = 250
Max = 4000

651 **Table S1**

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653

Bacterial strains		MIC (μM)		
		NLF20	**LL-37**	**Omiganan**
E. coli	ATCC 25922	10	20	20
	Clinical isolate 37.4	20	5	20
	Clinical isolate 47.1	40	5	20
	Clinical isolate 49.1	40	10	10
P. aeruginosa	ATCC 27853	10-20	10	160
	Clinical isolate 15159	5	20	20
	Clinical isolate 10.5	20	10	40
	Clinical isolate 51.1	20	40	80
	Clinical isolate 62.1	10	20	20
	Clinical isolate 18488	2.5	20	20
S. aureus	ATCC 29213	10-20	40	10
	Clinical isolate 16065	20	10	5
	Clinical isolate 13430	20	20	10
	Clinical isolate 14312	20	10	20
	Clinical isolate 18800	20	5	2.5
	Clinical isolate 18319	10	10	20
S. pyogenes	AP1	2.5	1.2	5
S. pneumoniae	TIGR4	5	10	2.5
	D39	160		5
	Clinical isolate PJ1354	80	5	10
	Clinical isolate I-104	20	20	160
	Clinical isolate I-95	80	5	1.25