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Papareddy, Praveen; Kasetty, Gopinath; Kalle, Martina; Bhongir, Ravi; Mörgelin, Matthias; Schmidtchen, Artur; Malmsten, Martin

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PO Box 117
221 00 Lund
+46 46-222 00 00

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

3

4 Praveen Papareddy^{1,2*}, Gopinath Kasetty^{1,3}, Martina Kalle¹, Ravi KV Bhongir^{1,3},
5 Matthias Mörgelin², Artur Schmidtchen^{1,4} and Martin Malmsten⁵

6

7 ¹Division of Dermatology and Venereology, Lund University, Lund, Sweden

8 ²Division of Infection Medicine, Department of Clinical Sciences, Lund University,
9 Lund, Sweden

10 ³Respiratory Medicine and Allergology, Department of Clinical Sciences, Lund
11 University, Lund, Sweden

12 ⁴Dermatology, LKCMedicine, Nanyang Technological University, Singapore

13 ⁵Department of Pharmacy, Uppsala University, Uppsala, Sweden

14

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

16 **Keywords**

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

18

19 **Corresponding author:**

20 Praveen Papareddy, Division of Dermatology and Venereology, Department of Clinical
21 Sciences, Lund University, Biomedical Center, Tornavägen 10, SE-22184 Lund,
22 Sweden.

23 Email: praveen.papareddy@med.lu.se

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

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

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

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

49

50 **Introduction**

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

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

69

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

79

80 **Material and Methods**

81 **Peptides**

82 The peptides NLF20 (NLFRKLTHRLFRRNFGYTLLR) and omiganan
83 (ILRWPWWPWRK-NH₂) were synthesized by Biopeptide Co., San Diego, USA,
84 while LL-37 (LLGDFFRKSKEKIGKEFKRIVQRIKDFLRNLPRTES) was from
85 Innovagen AB, Lund, Sweden. The two latter peptides were included as biological effect
86 benchmark peptides. The purity (>95%) of these peptides was confirmed by mass
87 spectral analysis (MALDI-ToF Voyager).

88

89 **Microorganisms**

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

98

99 **Viable-count analysis**

100 *E. coli* ATCC 25922, *P. aeruginosa* ATCC 27853, or *S. aureus* ATCC 29213 bacteria
101 were grown to mid-logarithmic phase in Todd-Hewitt (TH) medium (Becton and
102 Dickinson, Maryland, USA). The microorganisms were then washed and diluted in 10
103 mM Tris, pH 7.4, containing 5 mM glucose. Following this, bacteria (50 μ L; 2 x
104 10^6 cfu/mL) were incubated, at 37°C for 2 h, with NLF20 or LL-37 (at 0.03, 0.06, 0.3,
105 0.6, 3, 6, 30, 60 μ M) in 10 mM Tris, 0.15 M NaCl, with or without 20% human citrate-
106 plasma. In the experiments using 50% whole blood, *S. aureus* ATCC 29213 and *P.*
107 *aeruginosa* ATCC 27853 bacteria (50 μ L; 2 x 10^8 cfu/mL) were incubated at 37°C for 1
108 h in the presence of peptide at 60 (for *P. aeruginosa*) and 120 μ M (*P. aeruginosa* and *S.*
109 *aureus*) (the high concentrations used to achieve high bactericidal effect). To quantify
110 bactericidal activity, serial dilutions of the incubation mixtures were plated on TH agar,
111 followed by incubation at 37°C overnight and determination of the number of colony-
112 forming units (cfu). Hundred % survival was defined as total survival of bacteria in the
113 same buffer and under the same condition in the absence of peptide. Significance was
114 determined using the statistical software SigmaStat (SPSS Inc., Chicago, IL, USA).

115

116 **Minimal inhibitory concentration (MIC) determination**

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

122 serial dilutions from a stock solution. Ten μL of each concentration was added to each
123 corresponding well of a 96-well microtiter plate (polypropylene, Costar Corp.) and 90
124 μL of bacteria ($1.1 \times 10^6/\text{mL}$) in MH medium added. The plate was incubated at 37°C for
125 16-18 h. MIC was taken as the lowest concentration where no visual growth of bacteria
126 was detected.

127

128 **Radial diffusion assay**

129 Essentially as described earlier,^{26, 27} bacteria were grown to mid-logarithmic phase in 10
130 mL of full-strength (3% w/v) trypticase soy broth (TSB) (Becton-Dickinson). The
131 microorganisms were then washed once with 10 mM Tris, pH 7.4. Subsequently, 4×10^6
132 cfu were added to 15 mL of the underlay agarose gel, consisting of 0.03% (w/v) TSB,
133 1% (w/v) low electroendosmosis type (EEO) agarose (Sigma-Aldrich), and 0.02% (v/v)
134 Tween 20 (Sigma-Aldrich). The underlay was poured into a \varnothing 144 mm Petri dish. After
135 agarose solidification, 4 mm-diameter wells were punched and 6 μL peptide solution of
136 required concentration added to each well. Plates were incubated at 37°C for 3 h to allow
137 peptide diffusion. The underlay gel was then covered with 15 mL of molten overlay (6%
138 TSB and 1% Low-EEO agarose in distilled H_2O). Antimicrobial activity of a peptide
139 was visualized as a clearing zone around each well after 18-24 h of incubation at 37°C .

140

141 **Fluorescence microscopy**

142 The impermeant probe FITC (Sigma-Aldrich, St. Louis, USA) was used for monitoring
143 bacterial membrane permeabilization. *E. coli* ATCC 25922 bacteria were grown to mid-
144 logarithmic phase in TSB medium. Bacteria were washed and re-suspended in buffer
145 (10 mM Tris, pH 7.4, 0.15 M NaCl, 5 mM glucose) to yield a suspension of 1×10^7
146 cfu/mL. Hundred μL of the bacterial suspension was incubated with 30 μM of the
147 respective peptides at 30°C for 30 min. Microorganisms were then immobilized on poly
148 (L-lysine)-coated glass slides by incubation for 45 min at 30°C , followed by addition
149 onto the slides of 200 μL of FITC (6 $\mu\text{g}/\text{mL}$) in buffer and a final incubation for 30 min
150 at 30°C . The slides were washed and bacteria fixed by incubation, first on ice for 15
151 min, then at room temperature for 45 min in 4% paraformaldehyde. The glass slides
152 were subsequently mounted on slides using Prolong Gold antifade reagent mounting
153 medium (Invitrogen, Eugene, USA). Bacteria were visualized using a Nikon Eclipse
154 TE300 (Nikon, Melville, USA) inverted fluorescence microscope equipped with a

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

158

159 **Electron Microscopy**

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

169

170 **Hemolysis assay**

171 EDTA-blood was centrifuged at 800 g for 10 min, whereafter plasma and buffy coat
172 were removed. The erythrocytes were washed three times and re-suspended in PBS, pH
173 7.4, to a 5% suspension. The cells were then incubated with end-over-end rotation for 1
174 h at 37°C in the presence of peptides (3-60 μM). 2% Triton X-100 (Sigma-Aldrich)
175 served as positive control. Following this, the samples were centrifuged at 800 g for 10
176 min and the supernatant was transferred to a 96 well microtiter plate. In the experiments
177 with blood infected by bacteria, citrate-blood was diluted (1:1) with PBS. The cells were
178 then incubated with end-over-end rotation for 1 h at 37°C in the presence of peptides
179 (30, 60 or 120 μM) and either *E. coli*, *P. aeruginosa*, *S. pyogenes* or *S. aureus* bacteria,
180 or the fungus *C. albicans* (2×10^8 cfu/mL). In another experiment, designed to evaluate
181 peptide-induced hemolysis over longer time, whole blood diluted in 50% RPMI or
182 erythrocytes diluted in 50% PBS were incubated with NLF20 for 18 h 37°C. In all cases,
183 the absorbance of hemoglobin release was measured at λ 540 nm and is expressed as %
184 of Triton X-100-induced hemolysis.

185

186 **Lactate dehydrogenase (LDH) assay**

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

199

200 **MTT assay**

201 Sterile filtered MTT (3-(4,5-dimethylthiazolyl)-2,5-diphenyl-tetrazolium bromide;
202 Sigma-Aldrich) solution (5 mg/mL in PBS) was stored protected from light at -20°C
203 until usage. HaCaT keratinocytes, 3000 cells/well, were seeded in 96 well plates and
204 grown in serum free keratinocyte-SFM/BPE-rEGF medium to confluency. Peptides
205 investigated were then added at 3-60 μ M. After incubation over night, 20 μ L of the
206 MTT solution was added to each well and the plates incubated for 1 h in CO₂ at 37°C.
207 The MTT-containing medium was then removed by aspiration. The blue formazan
208 product generated was dissolved by the addition of 100 μ L of 100% DMSO per well,
209 and the plates gently swirled for 10 min at room temperature to dissolve the precipitate.
210 The absorbance was monitored at 550 nm, and results given represent mean values from
211 triplicate measurements.

212

213 **LPS effects on macrophages *in vitro***

214 3.5×10^5 cells (RAW264.7 macrophages) were seeded in 96-well tissue culture plates
215 (Nunc, 167008) in phenol red-free DMEM (Gibco) supplemented with 10% FBS and
216 antibiotics. Following 6 h of incubation to permit adherence, cells were stimulated with
217 10 ng/mL *E. coli* LPS (0111:B4) or *P. aeruginosa* LPS (Sigma), with and without
218 peptide of various doses. The levels of NO in culture supernatants were determined after
219 24 hours from stimulation using the Griess reaction.²⁸ Phenol-red free DMEM with FBS

220 and antibiotics were used as a blank. A standard curve was prepared using 0-80 μ M
221 sodium nitrite solutions in ddH₂O.

222

223 **Cytokine assay**

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

229

230 **Clotting Assays**

231 Clotting times were analyzed using a coagulometer (Amelung, Lemgo, Germany). The
232 prothrombin time (PT) and the Thrombin clotting time (TCT) were measured as follows:
233 Hundred μ L of fresh human citrate plasma together with indicated concentrations of
234 NLF20 were pre-warmed for 1 min at 37°C before clot formation was initiated by
235 adding 100 μ L a clotting reagent (PT-thromboplastin reagent (Trinity Biotech), TCT:
236 Thrombin reagent (Technoclone)). To record the activated partial thromboplastin time
237 (aPTT), 100 μ L of a kaolin-containing solution (Technoclone) was added to the plasma-
238 peptide mix and incubated for 200 sec before clot formation was initiated by adding 100
239 μ L of 30 mM fresh CaCl₂ solution.

240

241 **LPS model *in vivo***

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

249

250 ***P. aeruginosa* infection model**

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

254 had free access to standard laboratory chow and water. *P. aeruginosa* 15159 bacteria
255 were grown to logarithmic phase ($OD_{620} \sim 0.5$), harvested, washed in PBS, diluted in the
256 same buffer to either 2×10^8 cfu/mL (high levels) or 2×10^4 cfu/mL (low levels), and
257 kept on ice until injection. Hundred μ l of the bacterial suspension were injected
258 intraperitoneally into C57BL/6 mice. Immediately or 60 min subsequent of bacterial
259 injection, 0.5 mg NLF20 (in 10 mM Tris, pH 7.4) or buffer alone was injected i. p. or s.
260 c. into the mice. (The purpose of injecting NLF20 peptide immediately after bacterial
261 injection was to compare the NLF20 peptide activity with that of classical antibiotics.)
262 The survival data were obtained by following the animals daily up to 7 days monitoring.
263 Mice reaching the predefined endpoint-criteria were sacrificed and counted as non-
264 survivors. In another experiment, mice were treated i. p. or s. c. with 0.5 mg of NLF20
265 or 300 mg/kg ceftazidime or 100 mg/kg levofloxacin injected 0 h or 1 h after bacterial
266 infection. In order to study bacterial dissemination to target organs spleen, liver and
267 kidney were harvested, placed on ice, homogenized, and colony-forming units
268 determined. The P-value was determined using the Mann-Whitney U-test. Data from
269 three independent experiments were pooled. In another experiment as previously
270 described²⁹, *P. aeruginosa* Xen41 bacteria were grown to mid-exponential phase ($A_{620} \sim$
271 0.5), harvested, washed in PBS, diluted in the same buffer to $2-3 \times 10^8$ cfu/mL, and kept
272 on ice until injection. Hundred μ l of the bacterial suspension was injected
273 intraperitoneally into male BALB/c mice. One and 4 h after bacterial injection, 0.5 mg
274 of NLF20 peptide or buffer alone was administrated subcutaneously. Mice were
275 anesthetized immediately, or 12 h after bacterial infection, followed by data acquisition
276 and analysis using a Spectrum three-dimensional imaging system with Living Image®.
277 For evaluation of animal survival, mice showing the defined and approved end point
278 criteria (immobilization and shaking) were sacrificed by an overdose of isoflurane
279 (Abbott) and counted as non-survivors.

280

281 **Histochemistry**

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

286

287 **Statistical analysis**

288 Values are shown as mean with SEM. For statistical evaluation of two experimental
289 groups, the Mann-Whitney U-test was used and for comparison of survival curves the
290 log-rank test. To compare more than two groups, One-Way or Two-Way ANOVA with
291 Bonferoni post-test were used. Viable count data are presented as mean with SD. All
292 statistical evaluations were performed using the GraphPad Prism software 6.0. with *p-
293 <0.005, **<0.001 and ***p<0.0001 and ns = not significant.

294

295 **Results**

296 To explore the antimicrobial spectrum of NLF20, we first investigated the effects in
297 radial diffusion assays (RDA) against Gram-negative *Escherichia coli* and *Pseudomonas*
298 *aeruginosa*, Gram-positive *Bacillus subtilis* and *Staphylococcus aureus*, as well as the
299 fungi *Candida albicans* and *Candida parapsilosis* (Figure 1A). As can be seen, NLF20
300 activities well exceeded those observed for the human cathelicidin LL-37, a potent
301 benchmark antimicrobial peptide. The antibacterial results were further substantiated by
302 matrix-free viable count assay. Results from these dose-response experiments utilizing
303 *E. coli*, *P. aeruginosa* and *S. aureus* confirmed that NLF20 displays significant
304 antibacterial activity, also in presence of human citrated plasma (Figure 1B). In addition,
305 kinetic studies demonstrated fast bacterial killing, indicating a direct bactericidal action
306 compatible with many antimicrobial peptides (Figure 1C). MIC analyses according to
307 CSLI against the above and other pathogens are presented in Supplementary Material,
308 Table S1. Overall, NLF20 showed comparable activities to those observed for the two
309 benchmarks omiganan,³⁰ and LL-37.³¹ It was also noted that NLF20 displayed low MIC
310 levels particularly against *P. aeruginosa*.

311

312 Next, studies employing the impermeant probe FITC showed that NLF20 permeabilized
313 bacterial membranes of *E. coli* similarly to those seen after treatment with LL-37 (Figure
314 1D). Electron microscopy utilizing *P. aeruginosa* demonstrated extensive membrane
315 damage, with cell envelopes devoid of their cytoplasmic contents, and intracellular
316 material found extracellularly (Figure 1E). Again, similar findings were obtained with
317 LL-37. These data indicate that NLF20 acts on bacterial membranes.

318

319 Frequently, AMPs efficient in killing bacteria also exhibit hemolytic and membrane
320 permeabilizing activities against eukaryotic cells, which risks translating into toxicity
321 issues. Importantly, however, NLF20 exerted only minor hemolytic activity at 30-60 μM
322 and standard incubation times, and displayed less permeabilization than LL-37 (Figure
323 2A). Similar findings were observed with respect to permeabilization of HaCaT cells
324 (Figure 2B), as well as effects on viability as monitored by MTT assay (Figure 2C).
325 Further demonstrating this selectivity between bacteria and human cell membranes
326 under physiological conditions, of importance for subsequent *in vivo* studies. Next,
327 NLF20 was added to human blood infected by various Gram-positive and Gram-
328 negative pathogens, as well as fungi. In such experiments, NLF20 caused almost
329 complete eradication of *P. aeruginosa*, *E. coli*, as well as *S. pyogenes*, with little (~ 2%
330 or less) accompanying hemolysis at a peptide dose of 30-60 μM (Figure 2D). (The lower
331 hemolysis in blood is due to anionic serum proteins competing with erythrocyte
332 membranes for NLF20 binding.³¹) The observed low simultaneous hemolysis mediated
333 by NLF20 under these conditions indicated a high selectivity of the peptide for bacteria
334 (Figure 2E). In contrast, NLF20 was not particularly active in human blood against the
335 Gram-positive *S. aureus*, as well as the fungus *C. albicans*, although the latter showing a
336 minor reduction at 120 μM NLF20. The reduced activity against some bacteria is due to
337 the presence of a high content of anionic serum proteins, able to bind cationic peptides
338 such as NLF20 and reduce their antimicrobial effect through ~~competitively~~-competition
339 for peptide binding with bacterial membranes. This effect is widely observed for
340 antimicrobial peptides,³² and is analogous to the reduced antimicrobial effects displayed
341 by most AMPs in the presence of growth media rich in anionic scavengers, such as in
342 standardized MIC determinations.³¹

343

344 In another set of experiments, NLF20 toxicity was further evaluated following treatment
345 of mice with NLF20. As shown in Figure S1, hemolysis after 18 hours is growing over
346 time. At the highest peptide concentration used in the antimicrobial assays (313 $\mu\text{g/mL}$)
347 however, it was only 5 % in 50% blood. Furthermore, LDH release after treatment with
348 1 mg/mL was 4800 U, which is low compared to that of LL-37.³³ Similarly, cytokine
349 levels after peptide treatment at the same concentration are low,³⁴ and also histology on
350 lung tissue indicates no visible toxic effects of peptide treatment.

351

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

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

359

360 Given the observed dual effects on cytokine responses and coagulation, the *in vivo*
361 efficiency of NLF20 was next evaluated in a standardized mouse model of endotoxin-
362 induced shock.²² A dramatic improvement in survival rate of the animals was seen after
363 treatment with the peptide (Figure 3C). NLF20-treated animals fully regained their
364 weight after seven days (Figure 3D). Analyses of cytokines 8 h and 20 h after LPS
365 injection showed no notable, or only minor, alterations of IL-6 and TNF- α , respectively,
366 as well as of the pro-inflammatory cytokines IFN- γ and MCP-1, and a strong increase in
367 the anti-inflammatory IL-10 (Figure 3E). Previous studies have shown that
368 thrombocytopenia is as an important indicator for the severity of sepsis and disseminated
369 intravascular coagulation.³⁵ Therefore, platelet numbers were measured in blood of LPS-
370 injected mice treated with NLF20 at 8, 20 h, and 7 days after LPS injection. NLF20-
371 treated animals showed significantly increased platelet numbers at 20 h, indicating that
372 the peptide reduced platelet consumption at later stages in this endotoxin-model (Figure
373 3F). The levels were completely normalized in the survivors after seven days.

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

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

393

394

395 **Discussion**

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

411

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

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

423

424 It is also interesting to compare the effects observed for NLF20
425 (NLF~~R~~KLTHRLFRRNFGYT~~L~~R) to those of the longer epitope KYE28
426 (KYEITTIHNLF~~R~~KLTHRLFRRNFGYT~~L~~R), containing the NLF20 sequence, as
427 well as those of the corresponding truncation in the other end of KYE28 (KYE21;
428 KYEITTIHNLF~~R~~KLTHRLFR). While both KYE21 and NLF20 display antimicrobial
429 and anti-inflammatory effects qualitatively similar to those displayed by KYE28, the
430 relative importance of the antimicrobial and the anti-inflammatory effects are shifted,
431 such that the main effect of KYE21 is anti-inflammatory, while NLF20 displays more
432 potent antimicrobial effects, along with partly retained anti-endotoxic effects as shown
433 here. The more potent antimicrobial activity of NLF20 was previously demonstrated to
434 be due to potent membrane destabilization of the highly charged NLF20, in turn
435 facilitated by higher electrostatically and hydrophobically driven adsorption to anionic
436 lipid membranes. In contrast, NLF20 displays somewhat poorer capacity than KYE28
437 and KYE21 to provide multiple scavenging alternatives to LPS-induced NF-~~κ~~B
438 activation, including peptide-induced scavenging of LPS at human cell surfaces
439 (mediated by lower adsorption and positive potential build-up at zwitterionic membranes
440 for NLF20 than for KYE21 and KYE28) and LPS micelle packing disruption (mediated
441 by a delicate balance of peptide binding to the lipid A and the polysaccharide regions of
442 LPS, and resulting effects on packing constraints in LPS micelles).⁵⁰

443

444 It must be stressed that the activities of NLF20 may not necessarily reflect all possible
445 activities mediated by the proteolytically activated HCII molecule. Thus, it is possible
446 that the bacterial binding and LPS-interactions mediated by this helix D peptide may be
447 complemented by other actions of distant structural motifs, potentially mediating other
448 cell-effects, phagocytosis, or distinct ligand interactions. Although some evidence
449 suggests that C-terminals of serpins, such as α_1 -antitrypsin and antithrombin III, may
450 interact with cell-receptors and mediate phagocytosis, little is known about such actions
451 for HCII, and clearly, this warrants further investigations. Nevertheless, the present data
452 indicate, with these limitations, that the current strategy of selecting one functional

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

456

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466

467 **Transparency declarations**

468

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

472

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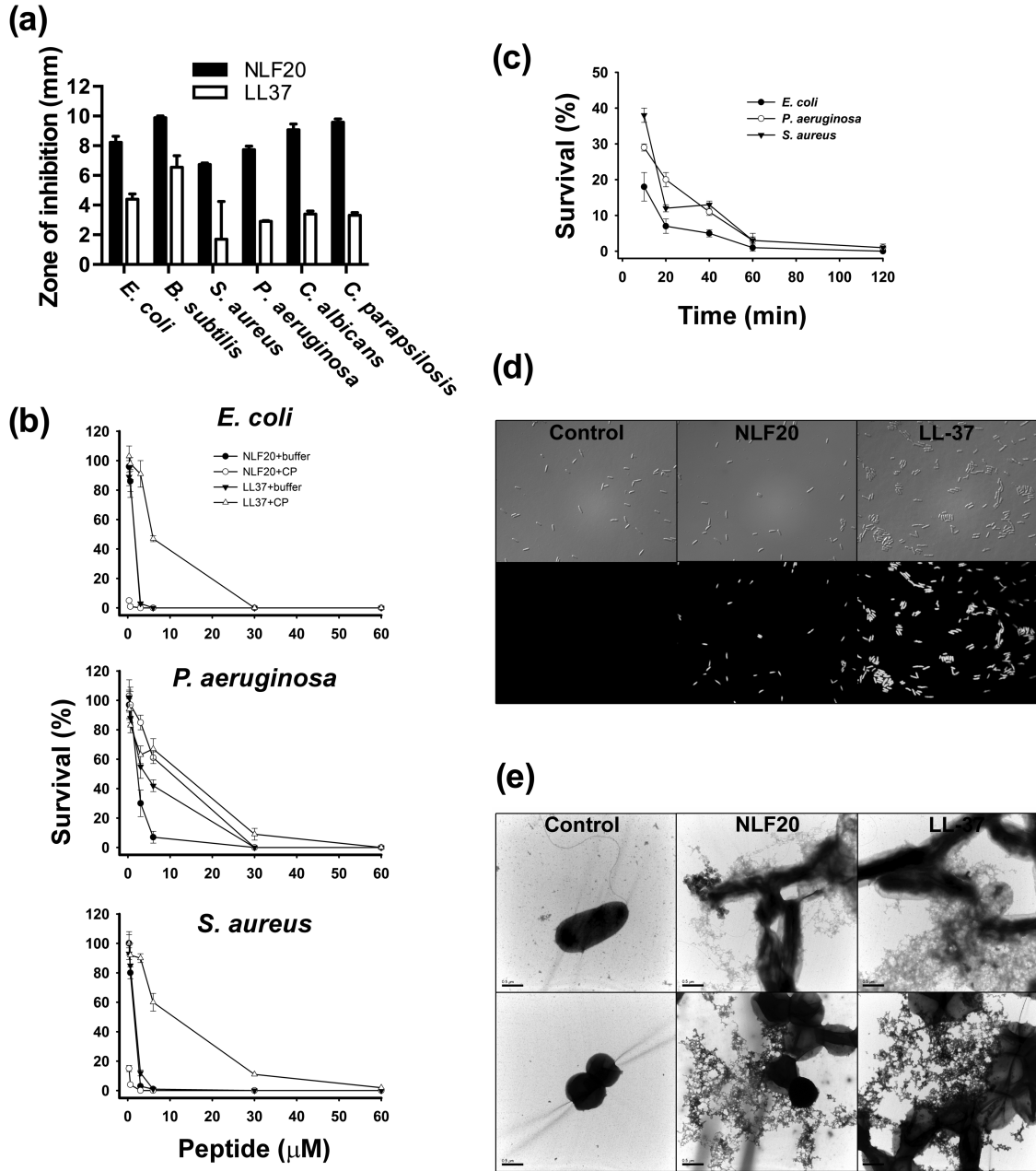
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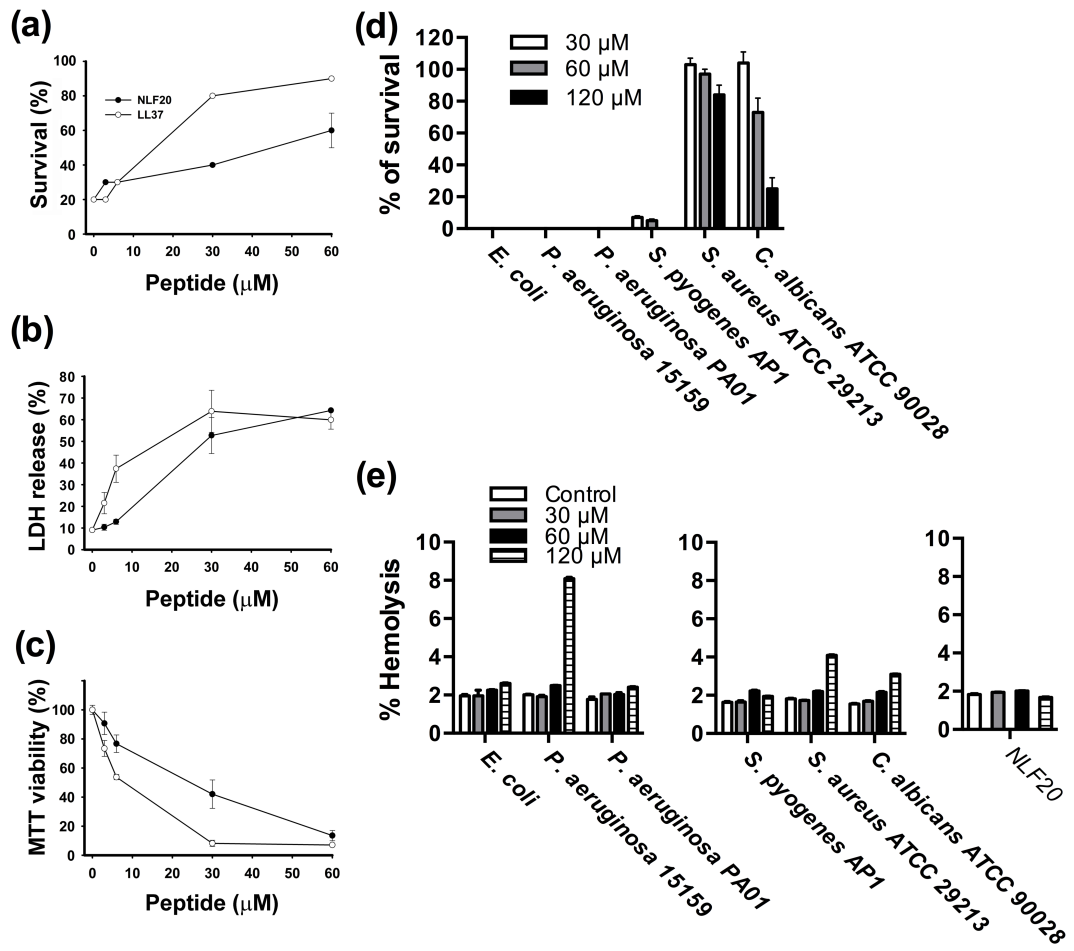
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Figure 1



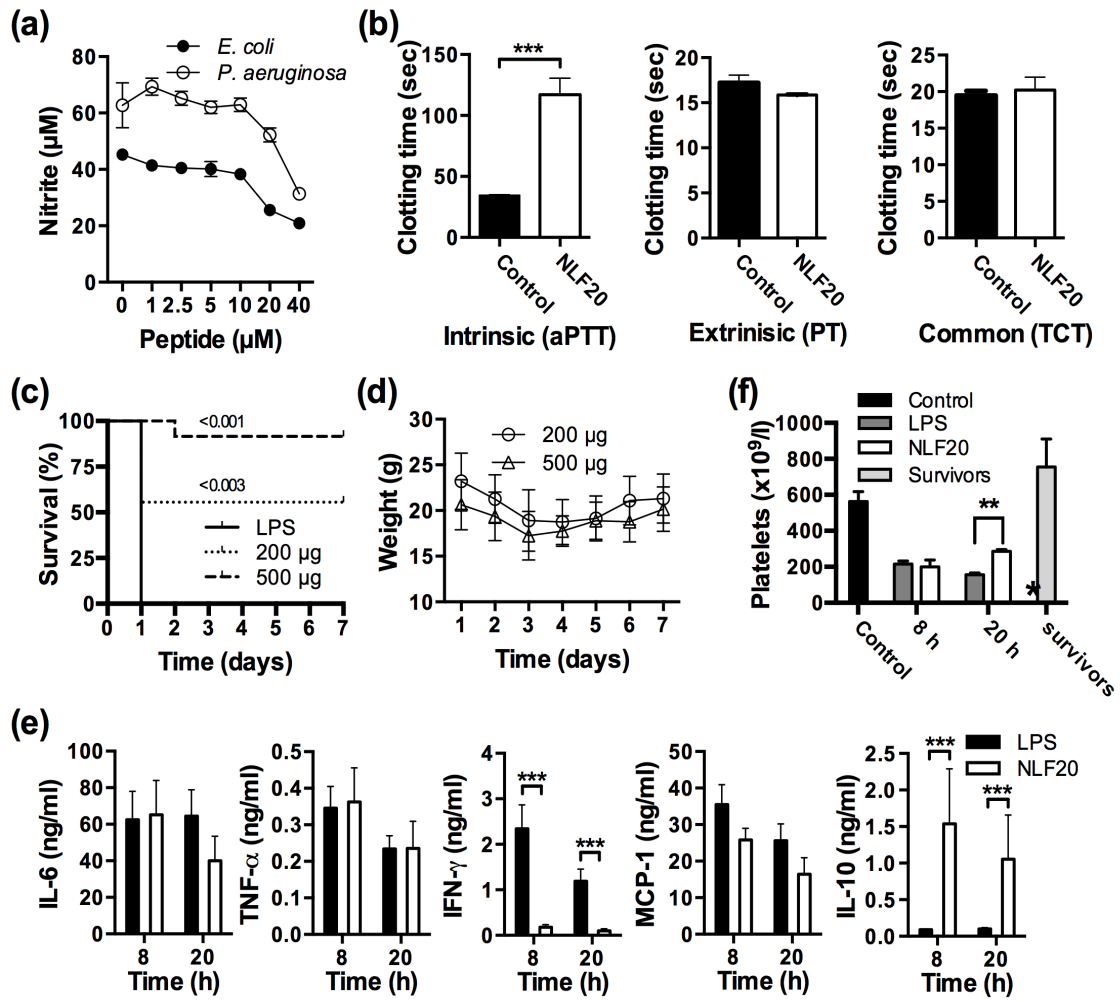
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Figure 2



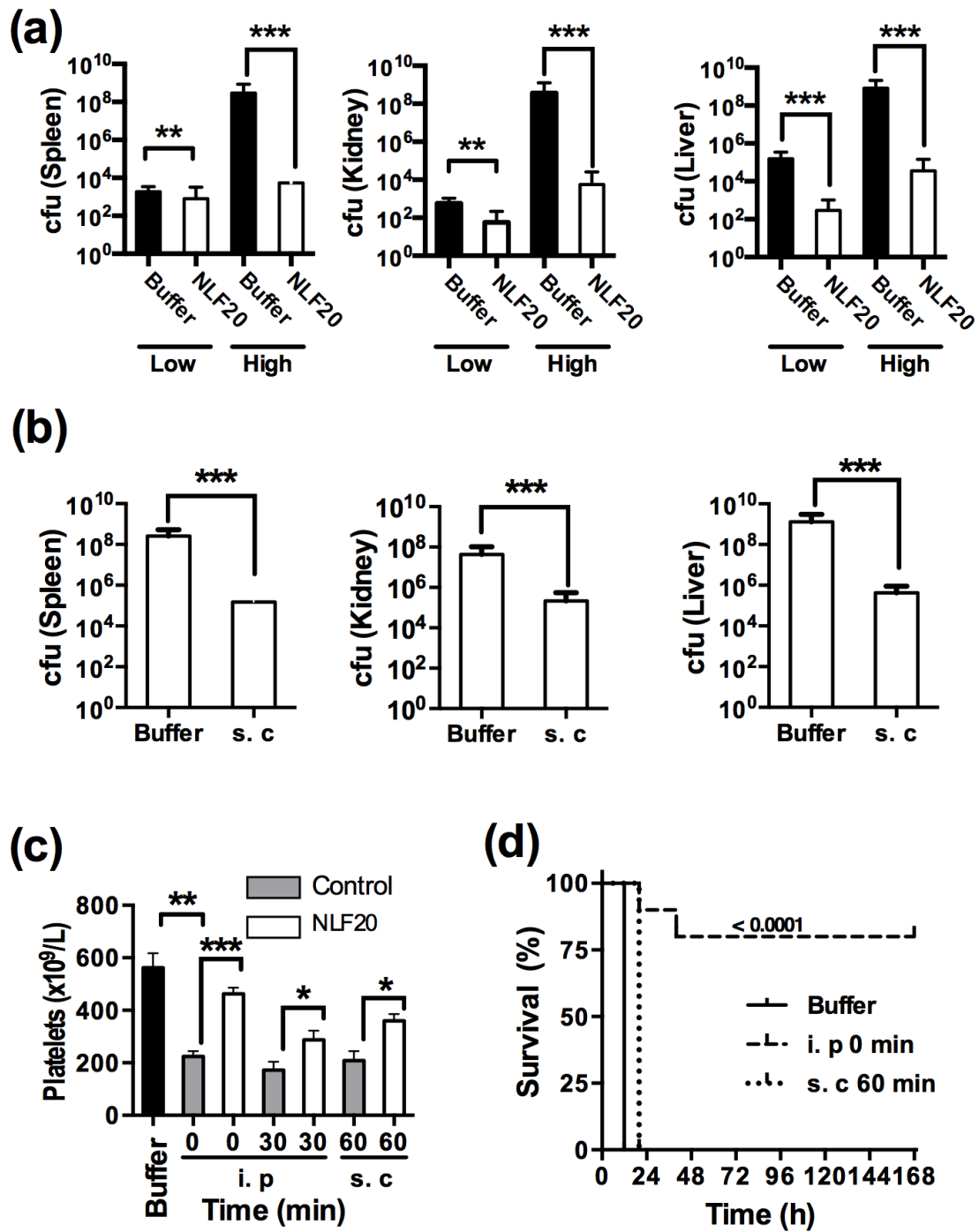
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Figure 3



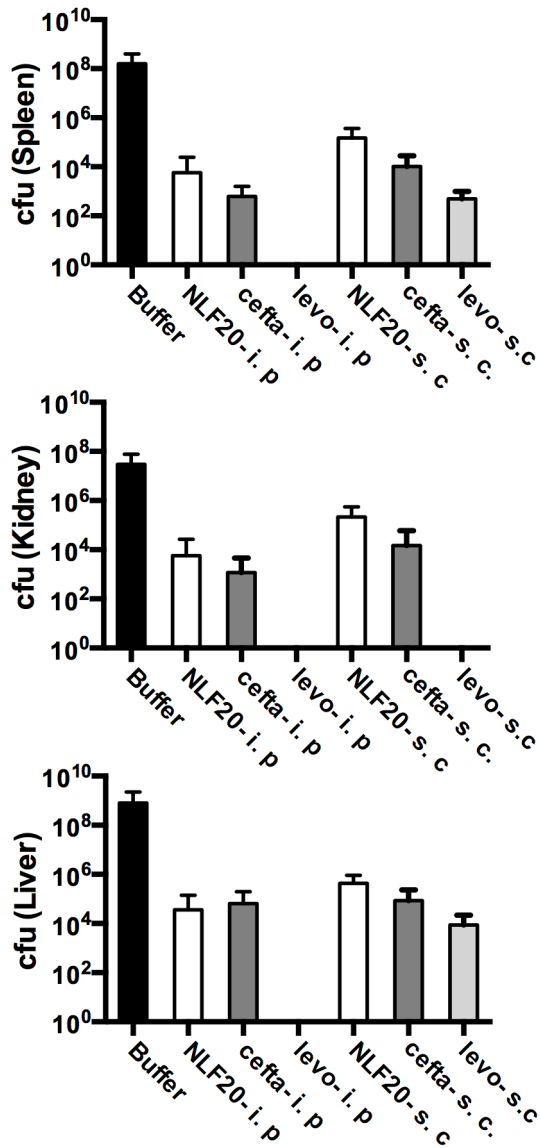
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Figure 4



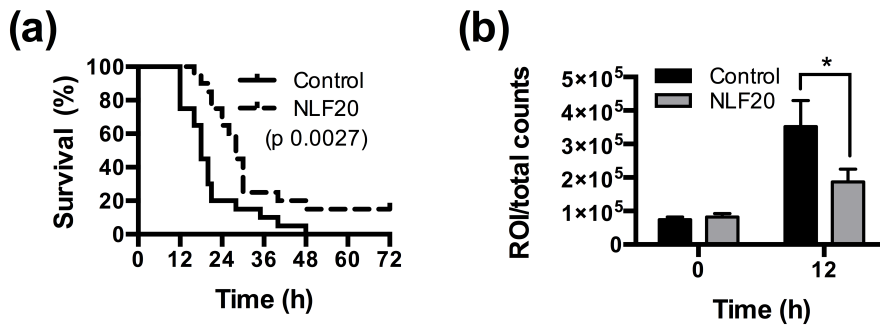
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Figure 5



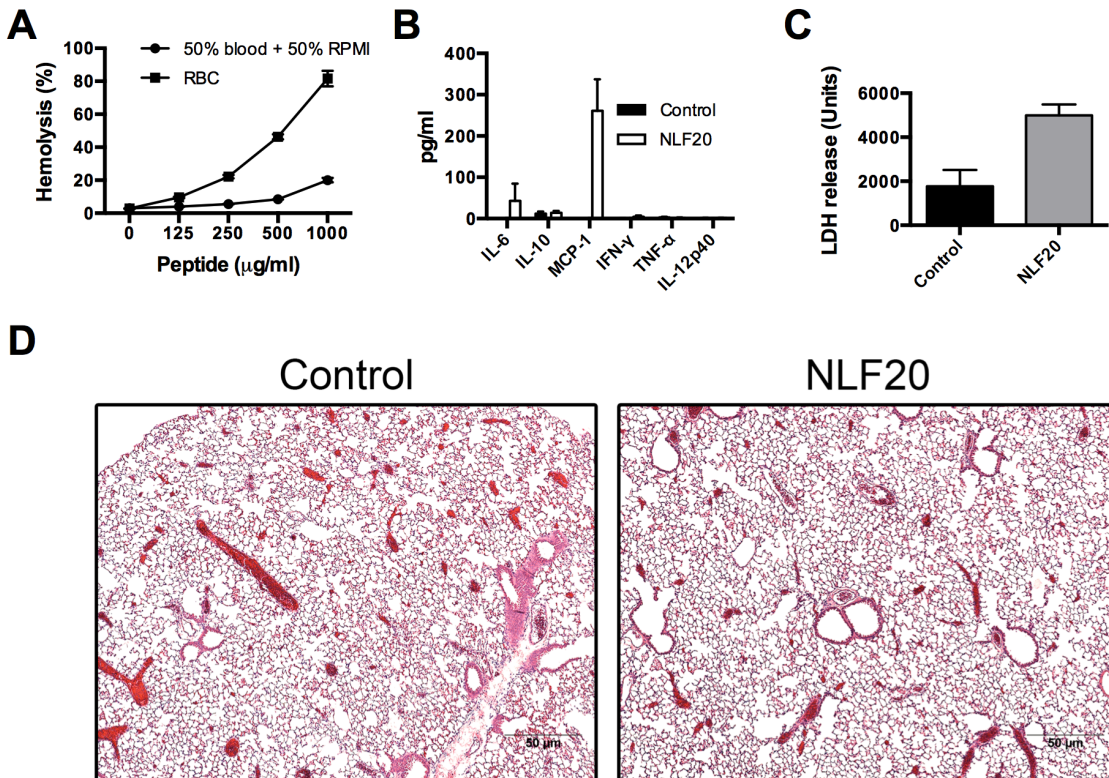
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Figure 6



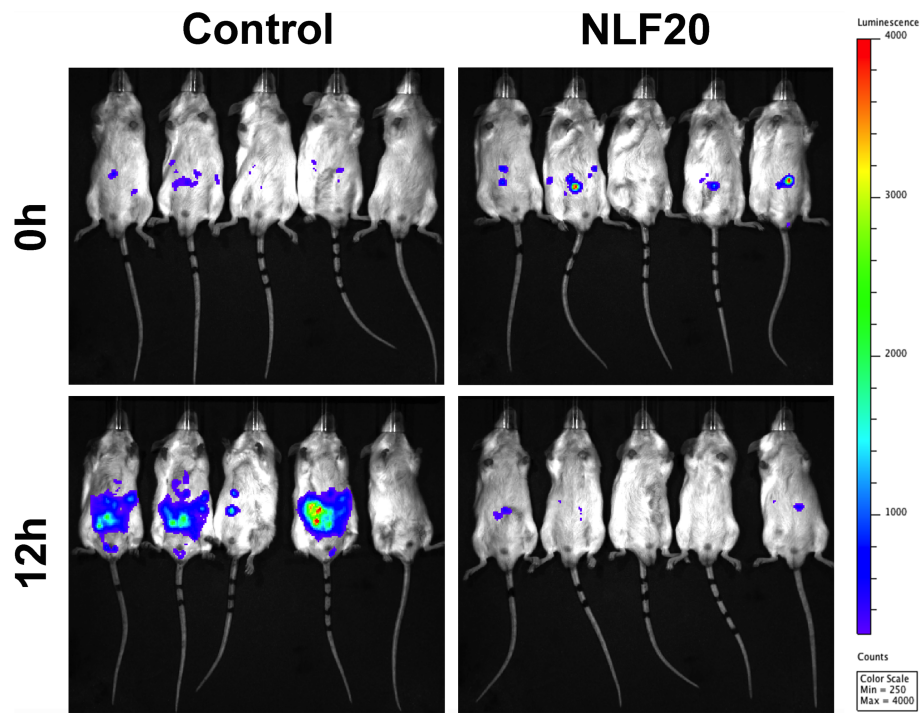
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Figure S1



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Figure S2



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651 **Table S1**

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Bacterial strains		MIC (μM)		
		NLF20	LL-37	Omiganan
<i>E. coli</i>	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
<i>P. aeruginosa</i>	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
<i>S. aureus</i>	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
<i>S. pyogenes</i>	AP1	2.5	1.2	5
<i>S. pneumoniae</i>	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