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Antimicrobial and immunomodulatory activities of porcine cathelicidin Protegrin-1

Ali Javed [a,b], Trishana Oedairadjsingh [a], Irene S. Ludwig [a], Thomas M. Wood [c], Nathaniel I. Martin [c], Femke Broere [a], Markus H. Weingarth [b], Edwin J.A. Veldhuizen [a,*]

[a] Faculty of Veterinary Medicine, Department of Biomolecular Health Sciences, Division Infectious Diseases & Immunology, Section Immunology, Utrecht University, the Netherlands
[b] NMR Spectroscopy, Bijvoet Centre for Biomolecular Research, Department of Chemistry, Faculty of Science, Utrecht University, the Netherlands
[c] Biological Chemistry Group, Institute of Biology Leiden, Leiden University, the Netherlands

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ABSTRACT

Antimicrobial peptides (AMPs) are a promising alternative to antibiotics in the fight against multi-drug resistant and immune system-evading bacterial infections. Protegrins are porcine cathelicidins which have been identified in porcine leukocytes. Protegrin-1 is the best characterized family member and has broad antibacterial activity by interacting and permeabilizing bacterial membranes. Many host defense peptides (HDPs) like LL-37 or chicken cathelicidin 2 (CATH-2) have also been shown to have protective biological functions during infections. In this regard, it is interesting to study if Protegrin-1 has the immune modulating potential to suppress unnecessary immune activation by neutralizing endotoxins or by influencing the macrophage functionality in addition to its direct antimicrobial properties. This study showed that Protegrin-1 neutralized lipopolysaccharide- (LPS) and bacteria-induced activation of RAW macrophages by binding and preventing LPS from cell surface attachment. Furthermore, the peptide treatment not only inhibited bacterial phagocytosis by murine and porcine macrophages but also interfered with cell surface and intracellular bacterial survival. Lastly, Protegrin-1 pretreatment was shown to inhibit the amastigote survival in Leishmania infected macrophages. These experiments describe an extended potential of Protegrin-1's protective role during microbial infections and add to the research towards clinical application of cationic AMPs.

1. Introduction

Antimicrobial peptides (AMPs) are small charged molecules which play a crucial role in the innate immune system as a first line of defense against invading bacteria, fungi, parasites and viruses (Ganz, 2003; Kang et al., 2017). From disrupting membranes and targeting cytoplasmic processes to cell agglutination, they can have diverse mechanisms of activity against pathogens (Bolintineanu et al., 2010; Florin et al., 2017; Mani et al., 2006; Sinha et al., 2017). The long-term utilization of antibiotics have caused the emergence of drug resistance, which is a leading challenge in clinical infection treatment (Munita and Arias, 2016; Park et al., 2011). In this regard, AMPs, owing to their novel and diverse mechanisms of actions, possess potent activity against drug resistant bacteria (Chung and Khanum, 2017; Mwangi et al., 2019). Apart from direct antimicrobial activity, AMPs have been shown to have a large number of other biological functions such as regulation of the immune response, antitumor activity, wound healing and angiogenesis in multi-cellular organisms (Mookherjee et al., 2020; Pfalzgraff et al., 2018; Roudi et al., 2017). This broad range of activities has led to AMPs also being referred to as host defense peptides (HDPs) (e.g., cathelicidins, defensins) as it better captures the *in vivo* function of combating infection by direct antimicrobial as well as immunomodulatory activities (Coorens et al., 2017c; Scott et al., 2002; Wan et al., 2014)

Cathelicidins are one of the two main classes of HDPs endogenously expressed by epithelial and immune cells of various organisms and the expression has been shown to surge in case of infection and inflammation (Lee et al., 2005a; van Harten et al., 2018). Protegrins are a class of five cathelicidins produced by porcine neutrophils of which Protegrin-1 is the most abundant and best characterized member (Choi et al., 2014). It is a short cationic AMP that contains a β-hairpin structure stabilized by two disulfide bonds (Fig. 1 **A**) and has been shown to have potent

* Correspondence to: Yalelaan 1, Utrecht 3584 CL, the Netherlands
E-mail address: e.j.a.veldhuizen@uu.nl (E.J.A. Veldhuizen).

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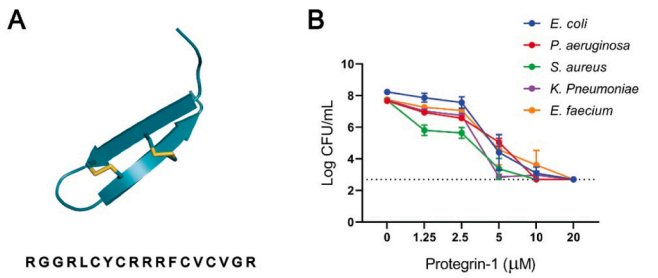

Fig. 1. (A) Structure of Protegrin-1 and its amino acid sequence are shown. The structure was created with PBD ID 1PG1 using Pymol. (B) Antibacterial activity of Protegrin-1 against 10^6 CFU/mL E. coli (blue), P. aeruginosa (red), S. aureus (green), K. Pneumoniae (purple) and E. faecium (orange) was tested using a colony count assay. Surviving bacterial colonies were detected after incubation with tested peptides for 3 h in MHB. The dotted line shows the detection limit of the assay. Shown are the mean ± SEM of three independent experiments (in duplicate).

activity against bacteria, including multi-drug resistant strains (Fahrner et al., 1996). Its antimicrobial potency *in vitro* and *in vivo*, and corresponding antibacterial mechanisms are described thoroughly (Capone et al., 2010; Cheung et al., 2008; Jang et al., 2011; Khandelia and Kaznessis, 2007; Lee et al., 2005b; Zeng et al., 2020), however the knowledge of its immunomodulatory functions is scarce. Some of its biological functions described include its protective effect in an murine intestinal inflammation model (Huynh et al., 2019) and its role in human mast cell mediated wound healing (Gupta et al., 2015). As far as its specificity for the porcine system is concerned, it has been shown to enhance proliferation and inhibit apoptosis of porcine granulosa cells (Pan et al., 2021).

In this study, the knowledge of Protegrin-1's indirect activity against microbial infections in terms of its anti-endotoxin capacity and modulation of immune cell-function in general was studied in a porcine perspective. A synthetic version of Protegrin-1 was used to assess its lipopolysaccharide (LPS) binding/ neutralizing activity as well as its capacity to influence macrophages' intrinsic functions like phagocytosis and microbial killing.

2. Materials and methods

2.1. Peptide synthesis

Protegrin-1 was synthesized by solid-phase peptide synthesis (SPPS) as follows: Rink Amide resin (150 mg, 0.684 mmol.g^{-1}) was loaded into the peptide synthesizer for a 0.1 mmol scale. Linear peptide encompassing AA-1 to AA-18 were assembled using microwave irradiation at 90 ℃ (resin bound AA:Fmoc-AA:DIC:Oxyma, 1:5:5:5 molar eq.). DMF was used as solvent and Fmoc deprotections were carried out with piperidine:DMF (1:4, v-v). Amino acid side chains were protected as follows: ^{t}Bu for Tyr, Trt for Cys, and Pbf for Arg. Following coupling and Fmoc deprotection of the final Fmoc-Arg(Pbf)-OH the linear peptide was removed from the CEM Liberty Blue, washed with DCM and directly treated with TFA:TIS:H_2O (95:2.5:2.5, 10 mL) for 90 minutes. The reaction mixture was added to cold MTBE:hexanes (1:1), spun down at 4500 RPM for 5 minutes and the resulting precipitate washed once more with MTBE:hexanes (1:1). The crude linear peptide with free Cys sidechains was dissolved in 1.0 mL DMF and then sonicated to ensure good dissolution followed by further addition of 180 mL of a mixture of 100 mM NH_4HCO_3 and MeCN (2:1). The mixture was stirred overnight while bubbling air through the solution followed by evaporation of MeCN and subsequent lyophilization. The crude peptide was purified by preparative HPLC using a Dr. Maisch Reprosil-Pur 120 C18-AQ column (250 ×25 mm, 10 μm) using a gradient of 10–100 % buffer B over 45 minutes (buffer A: 95 % H_2O, 5 % MeCN, 0.1 % TFA; buffer B: 5 % H_2O, 95 % MeCN, 0.1 % TFA) to afford 17 mg of the peptide as a white powder (8 % yield based on resin loading). The purity of the synthetic protegrin was determined to be >95 % by analytical HPLC and the disulfide pattern confirmed by co-injection of the synthetic peptide with an authentic sample (Tebu-bio, the Netherlands), and by Mass Spectrometry (Fig. S1).

2.2. Bacterial strains

Clinical bacterial isolates including multi-resistance species (Veldhuizen et al., 2013) were included in direct or indirect antibacterial assays. The strains tested were *Escherichia* (*E.*) *coli* 078, *Pseudomonas* (*P.*) *aeruginosa* VW178, *Staphylococcus* (*S.*) *aureus* S0385, *Klebsiella* (*K.*) *pneumoniae* NCTC-13443 and *Enterococcus* (*E.*) *faecium* E155 (University Medical Center Utrecht). For phagocytosis experiments, avian pathogenic *E. coli* (APEC) (Cuperus et al., 2016) transformed with green fluorescent protein (GFP) in PWM1007 plasmid as described previously (Peng et al., 2018) was used. All strains were grown on tryptic soy agar (TSA) plates (Oxoid Ltd, Basingstoke, Hampshire, UK). Liquid cultures were grown in Mueller-Hinton Broth (MHB, Becton, Dickinson and Company).

2.3. Parasite

Leishmania (L.) *infantum* was isolated out of a lymph node aspirate from an infected dog. Promastigotes were cultured in RPMI 1640 medium (Gibco, Thermo Fisher Scientific, Waltham, MA, USA) supplemented with 10 % fetal calf serum (FCS) (Bodinco B.V., Alkmaar, The Netherlands), 1x non-essential amino acids (NEAA) (Gibco), 100 units/mL Penicillin, 100 μg/mL Streptomycin, 10 μg/mL Hemin and 10 μM Xanthine (all Sigma) at 26 °C. Medium was refreshed twice a week.

2.4. Cell culture

RAW 264.7 cells (ATCC TIB-71) were cultured in DMEM (Gibco), while porcine alveolar macrophages 3D4/31 (ATCC CRL-2844) were cultured in RPMI-1640 (Gibco) supplemented with 10 % FCS (Bodinco B.V.), 1x non-essential amino acids (NEAA) (Gibco), 100 units/mL Penicillin, 100 μg/mL Streptomycin, 10 μg/mL Hemin and 10 μM Xanthine (all Sigma) at 37 °C, 5.0 % CO_2.

2.5. Lactate dehydrogenase (LDH) release assay

RAW 264.7 and 3D4/31 macrophages were seeded (5×10^4 cells/well) in a 96-well plate and left at 37 °C for overnight adherence. Cells were then stimulated with different concentrations of Protegrin-1 (0–20 μM). After 24 h, cytotoxicity was measured as fraction of lactate dehydrogenase (LDH) release in the supernatant compared to maximum LDH release of unstimulated cells treated with Triton X-100 detergent to completely lyse cells, using the Cyto Tox 96 nonradioactive cytotoxicity kit (Promega), according to the manufacturer's instructions.

2.6. WST-1 cell metabolic activity assay

RAW 264.7 and 3D4/31 macrophages were stimulated with Protegrin-1 (0–20 μM) as described above. After 24 h incubation, the medium was replaced by 100 μL of culture medium containing 10 % water-soluble tetrazolium 1 (WST-1) (Roche, Basel, Switzerland). Colorimetric changes were measured after 15–20 min of incubation, at 450 nm using iMark™ Microplate Absorbance Reader (Bio-Rad). Cell metabolic activity was calculated as percentage of the no peptide control set to 100 %.

2.7. Track dilution assay

Bacterial killing by HDPs was assessed using track dilution assays, as

described before [47]. In short, 10^6 colony forming units (CFU)/mL bacteria were incubated with different concentrations of Protegrin-1 for 3 h at 37 °C in a polypropylene U-bottom microtiter plate (Corning, New York, USA). After incubation, 10-fold dilutions were prepared using the corresponding medium and 10 μL of each dilution was plated on appropriate agar plates. Plates were incubated at 37 °C and colonies were counted after 24 h. Minimal bactericidal concentration (MBC) was defined as ≤500 CFU/mL.

To determine the adhered and intracellular bacterial load, RAW 264.7 and 3D4/31 macrophages were seeded in 96-well and 24-well plates respectively and were incubated with different concentrations of Protegrin-1 for 24 h. Subsequently, after removing supernatant and washing of the cells three times with PBS, *E. coli* was added to the culture at a multiplicity of infection (MOI) = 5 for RAW and MOI = 10 for 3D4/31 cells, and then incubated at 37 °C, 5 % CO_2 for an additional 1 h. Afterwards, cells were washed again and lysed with 0.1 % Triton X and then plated out in serial dilutions as described above. To study intracellular bacterial survival, cells were cultured in cell culture media supplemented with 100 μg/mL Gentamicin for 1 h and subsequently washed before lysis. To calculate the number of intracellular bacteria, CFU of gentamicin treated samples were subtracted from those of respective non-gentamicin controls.

2.8. Nitric oxide inhibition assay

RAW 264.7 cells were seeded in 96-well plate as described above. Then cells were stimulated with 20 ng/mL *E. coli* O111:B4 LPS (InvivoGen), 10 ng/mL *E. coli* K-12 LPS (InvivoGen), 50 ng/mL *P. aeruginosa* 10 LPS (Sigma) or 50 ng/mL Lipid A (MPLA or DPLA) from *E. coli* F583 (Sigma) with or without 0–20 μM Protegrin-1 in DMEM for 24 h. After incubation, the nitrite production in the supernatant was measured using the Griess assay [48]. Briefly, 50 μL cell culture supernatant was mixed with 50 μL 1 % Sulfanilamide (Sigma-Aldrich, Zwijndrecht, the Netherlands) and incubated at room temperature in the dark for 5 min. Then 50 μL 0.1 % N-(1-Naphthyl)ethylenediamine dihydrochloride (VWR International B.V., Amsterdam, the Netherlands) was added and incubated at room temperature in the dark for 5 min. Sodium nitrite (Sigma) was used as a standard to accurately determine the nitrite concentration in the cell supernatant. Samples were measured at 590 nm using iMark™ Microplate Absorbance Reader (Bio-Rad).

2.9. Enzyme-linked immuno-sorbent assay (ELISA)

RAW 264.7 cells were seeded as described above. *P. aeruginosa* (diluted to 1×10^6 CFU/mL in DMEM) was treated separately with heat (1 h at 90 °C), 1 mg/mL gentamicin (1 h at 37 °C), 20 μM Protegrin-1 or chicken cathelicidin 2 (CATH-2) (1 h at 37 °C) and DMEM only (untreated sample) (1 h at 4 °C). After treatment, the bacteria were added to the RAW264.7 cells for 2 h of incubation at 37 °C. After that, TNF-α production was determined in the supernatant by ELISA using ELISA Duoset kits (R&D systems, Minneapolis, MN) according to the manufacturer's protocol.

2.10. Isothermal titration calorimetry (ITC)

ITC was performed with a Low Volume NanoITC (TA Instruments-Waters LLC, New Castle, DE, USA). The 50 μL syringe was filled with 200 μM LPS in 1:3 H_2O:phosphate buffered saline (PBS) for titration into 164 μL of 12.5 μM Protegrin-1 (50 μM in case of K-12 LPS) in identical buffer. Titrations were incremental with 2 μL injections at 300 s intervals. Experiments were performed at 37 °C. For all calculations performed, an estimated molecular weight of 20 kDa for LPS was applied. The calorimetric data obtained were analyzed using NanoAnalyze Software Version 3.8.0 (TA instruments-Waters LLC), where the integrated heat signal is corrected for the heat of dilution of the titrant.

2.11. Flow cytometry

RAW 264.7 or 3D4/31 macrophages were seeded (2×10^5 cells/well) in a 24-well plate and left overnight for adherence. Subsequently, they were stimulated with or without different concentrations of Protegrin-1 for 24 h at standard cell culture conditions. Cells were then washed three times and incubated with GFP-labeled *E. coli* (MOI=10 for RAW 264.7 and MOI=50 for 3D4/31) or 50 μg/mL pHrodo *E. coli* bioparticles for 60 min. Then after washing twice with PBS, cells were harvested using Trypsin-EDTA (0.25 %) and transferred to a U-bottom 96-well plate. Subsequently cells were stained with 1/800 ViaKrome808 (Beckman Coulter, Indianapolis, IN, USA) in FACS Buffer (1x PBS supplemented with 2 % FCS, 0.005 % NaN_3). For LPS binding experiment, RAW 264.7 cells (seeded as described above) were incubated with 200 ng/mL biotin labeled *E. coli* O111:B4 LPS in the absence or presence of different concentrations of Protegrin-1 for 60 min. Then, after washing and harvesting cells as described above, they were stained with 1/800 ViaKrome808 (Beckman Coulter, Indianapolis, IN, USA) and 1/400 Streptavidin-PE (BD Pharmingen) in FACS Buffer.

After 30 min incubation at 4 °C in the dark, cells were washed and resuspended in 150 μL FACS buffer for measurement. Samples were measured on a Beckman Coulter Cytoflex LX (Faculty of Veterinary Medicine, Utrecht University). Acquired data were analyzed using FlowJo Software v.10.7 (FlowJo LLC, Ashland, OR, USA).

2.12. Amastigote burden assay

RAW 264.7 macrophages were seeded (5×10^4 cells/well) in a 8-well culture slide s (Lab-Tek™, BD Biosciences) and left overnight for adherence at 37 °C, 5.0 % CO_2. Then the macrophages were stimulated with or without different concentrations of Protegrin-1 for 24 h at standard cell culture conditions. Afterwards, cells were infected with *L. infantum* promastigotes (MOI = 20) and incubated for 24 h. Finally, cells were washed with PBS, fixed with ice-cold methanol for 5 min and stained with Giemsa stain. To determine the amastigote burden, the number of amastigotes per 200 infected macrophages was counted under a light microscope. The mean number of amastigotes per infected macrophage was determined by dividing the total number of amastigotes counted by the number of infected macrophages

2.13. Statistical analysis

Statistical analysis was performed using GraphPad Prism version 9.3.1 (471). All quantitative measurements were conducted as three independent measurements, which were made in duplicate. One-way ANOVA was used as an appropriate method to test for significant differences, followed by Sidak's multiple comparison test. A p value of < 0.05 was considered significant.

3. Results

3.1. Protegrin-1 effectively kills gram-positive and gram-negative bacteria

Antimicrobial activity of Protegrin-1 was tested against clinical isolated of two Gram-positive (*S. aureus* and *E. faecium*) and three Gram-negative (*E. coli*, *P. aeruginosa* and *K. Pneumoniae*) bacteria by colony count assays. Protegrin-1 was active against all these bacteria showing MBC values between 10 and 20 μM (Fig. 1**B**).

3.2. Protegrin-1 is not toxic against porcine and murine macrophages

Cytotoxicity of Protegrin-1 against porcine 3D4/31 and murine RAW 264.7 macrophage cells was determined by LDH release and WST-1 cell metabolic activity assays. Except for the highest concentration tested (20 μM) against 3D4/31 cells, no significant toxicity was observed in the LDH release assay (Fig. 2**A,C**). Metabolic activity as determined by WST-

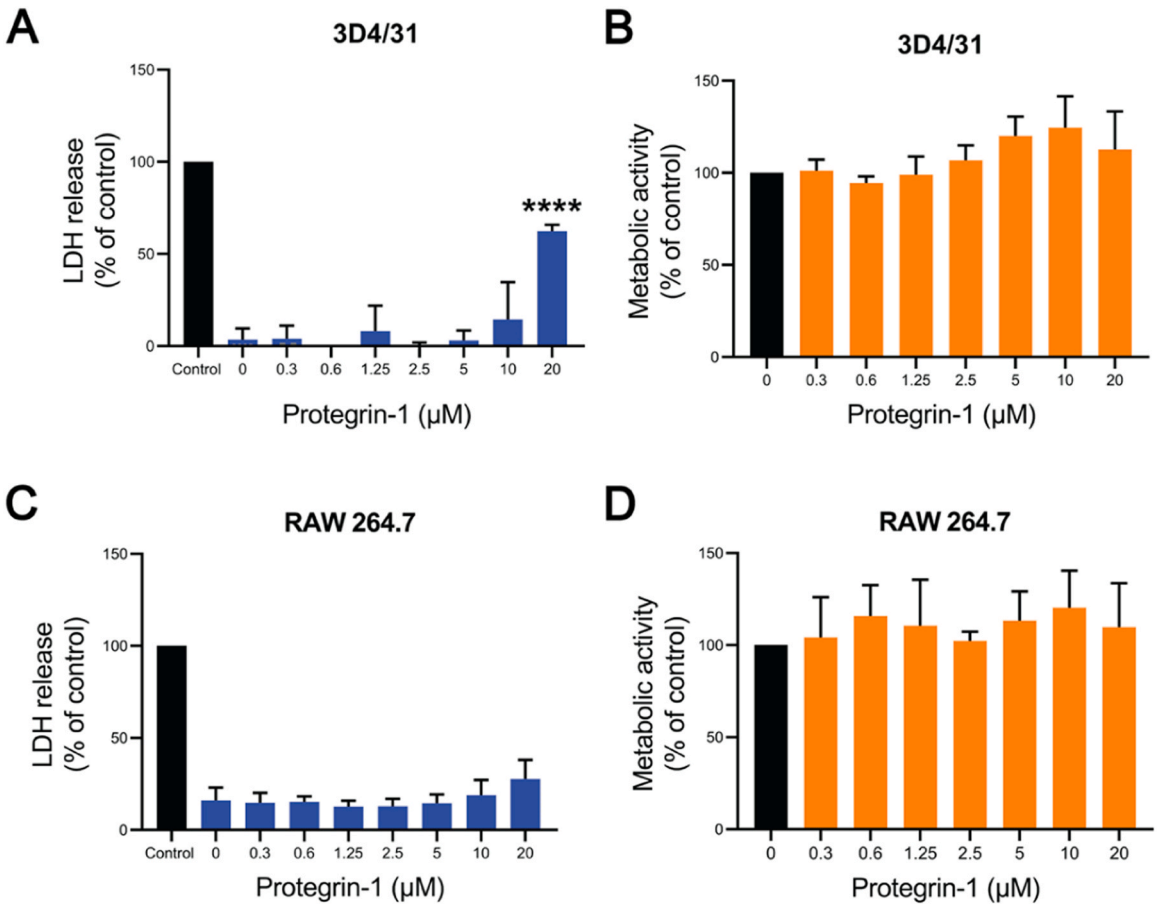

Fig. 2. Protegrin-1 is not toxic towards porcine and murine macrophages. Cytotoxicity against 3D4/31 (**A, B**) and RAW 246.7 cells (**C, D**) were tested using LDH release and WST-1 assays. Values were calculated as percentage of the Triton X-100 treated and untreated control in LDH release and WST-1 assay, respectively. Shown are the mean ± SEM of three independent experiments in duplicate. Data points that are significantly different from 0 μM control are denoted by * ($p < 0.05$); ** ($p < 0.005$); *** ($p < 0.001$); **** ($p < 0.0001$).

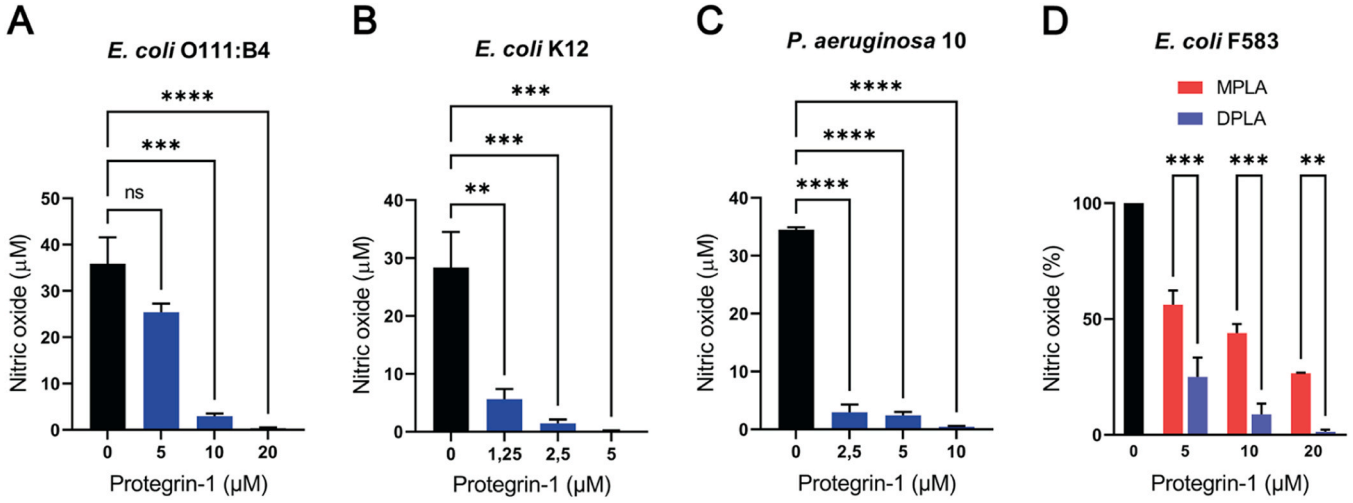

Fig. 3. Protegrin-1 inhibits LPS induced immune cell activation. LPS neutralization assay was performed by mixing different concentrations of Protegrin-1 with 20 ng/mL E. coli O111:B4 LPS (**A**), 10 ng/mL E. coli K-12 LPS (**B**), 50 ng/mL P. aeruginosa 10 LPS (**C**) or 50 ng/mL Lipid A (MPLA or DPLA) from E. coli F583 (**D**) for 5–10 min, and subsequently stimulating RAW 264.7 cells with this mixture. NO production was measured to determine macrophage activation. Shown are the mean ± SEM of three independent experiments (in duplicate). Data points that are significantly different from each other are denoted by * ($p < 0.05$); ** ($p < 0.005$); *** ($p < 0.001$); **** ($p < 0.0001$).

1 reaction was not affected by any concentration of Protegrin-1 for either cell type (Fig. 2**B,D**).

3.3. Protegrin-1 inhibits LPS and Lipid A induced immune cell activation

Cationic host defense peptides like LL-37 and CATH-2 are known to inhibit LPS induced immune cell activation, owing to their binding affinity for negatively charged LPS (Scheenstra et al., 2019). Similarly, Protegrin-1 inhibited LPS-induced activation of RAW macrophages, as measured by the nitric oxide (NO) production. Protegrin-1 effectively inhibited the NO produced in response to smooth LPS (Javed et al., 2023b) from *E. coli or P. aeruginosa* as well as rough LPS (Javed et al., 2023b) from *E. coli* K12. Interestingly, the highest activity of Protegrin-1 was measured against rough *E. coli* K12 where 1.25 μM already significantly ($p < 0.05$) reduced the NO production more than 5-fold, while 5 μM completely inhibited NO production. For the smooth LPS slightly higher concentrations were needed but 10 μM Protegrin-1 reduced the NO production to near-zero (Fig. 3**A-C**). Subsequently Protegrin-1 was tested to determine if it could also inhibit mono- and di-phosphorylated lipid A (MPLA and DPLA). As shown in Fig. 3**D**, DPLA was neutralized more efficiently than MPLA.

3.4. Protegrin-1 kills bacteria with limited immune cell activation

'Silent killing' experiments as described previously for CATH-2 (Coorens et al., 2017) were performed to determine if Protegrin-1 killed bacteria were still able to induce an inflammatory response of macrophages. RAW264.7 macrophages were stimulated with non-viable bacteria due to treatment with peptide (Proterin-1 or CATH-2), gentamicin, or heat, and viable (untreated) bacteria were used as control. After 2 h incubation of RAW cells with (treated) bacteria, TNF-α levels were measured by ELISA as a read out for macrophage activation. For all bacterial strains, bacteria killed by Protegrin-1 (or CATH-2) induced a significantly ($p < 0.05$) lower amount of TNF-α than live bacteria, while bacteria killed by gentamicin or heat resulted in similar activation of macrophages as live bacteria (Fig. 4 A-D). Only for *S. aureus* this effect was not observed, because heat-killed bacteria also resulted in significantly ($p < 0.05$) lower activation of RAW macrophages (Fig. 4**D).** These results show that Protegrin-1, similar to CATH-2 has dual activity simultaneously killing bacteria and subsequently preventing macrophage activation by non-viable bacteria.

3.5. Protegrin-1 binds LPS and prevents it from macrophage surface attachment

To study the ability of Protegrin-1 to bind LPS, which could explain the observed LPS neutralization (Fig. 3), ITC was performed to assess the binding kinetics of Protegrin-1 and LPS. These studies showed exothermic binding of Protegrin-1 to all three types of LPS tested, as indicated by the negative enthalpy (ΔH) values (Fig. 5). With Kd values in low μM range, no major differences were observed for Protegrin-1 binding to the different LPS's (Fig. 5**A-C**). The binding observed was enthalpy-driven with unfavorable entropy as indicated by the entropy factor (−TΔS) (Fig. 5**D,** Table 1), suggesting that mainly electrostatic interaction is mediating binding of Protegrin-1 with LPS.

Furthermore, the peptide's affinity for LPS was demonstrated by testing its capacity to bind LPS and thus prevent it from binding to the surface of RAW 264.7 cells. Biotin-labeled LPS was incubated with different concentrations of Protegrin-1 prior to its addition to RAW macrophages for 1 h. The PE signals obtained by flow cytometry after secondary staining of cell-surface bound LPS showed that Protegrin-1 incubation inhibited LPS binding to the cell surface (Fig. 5**E**)

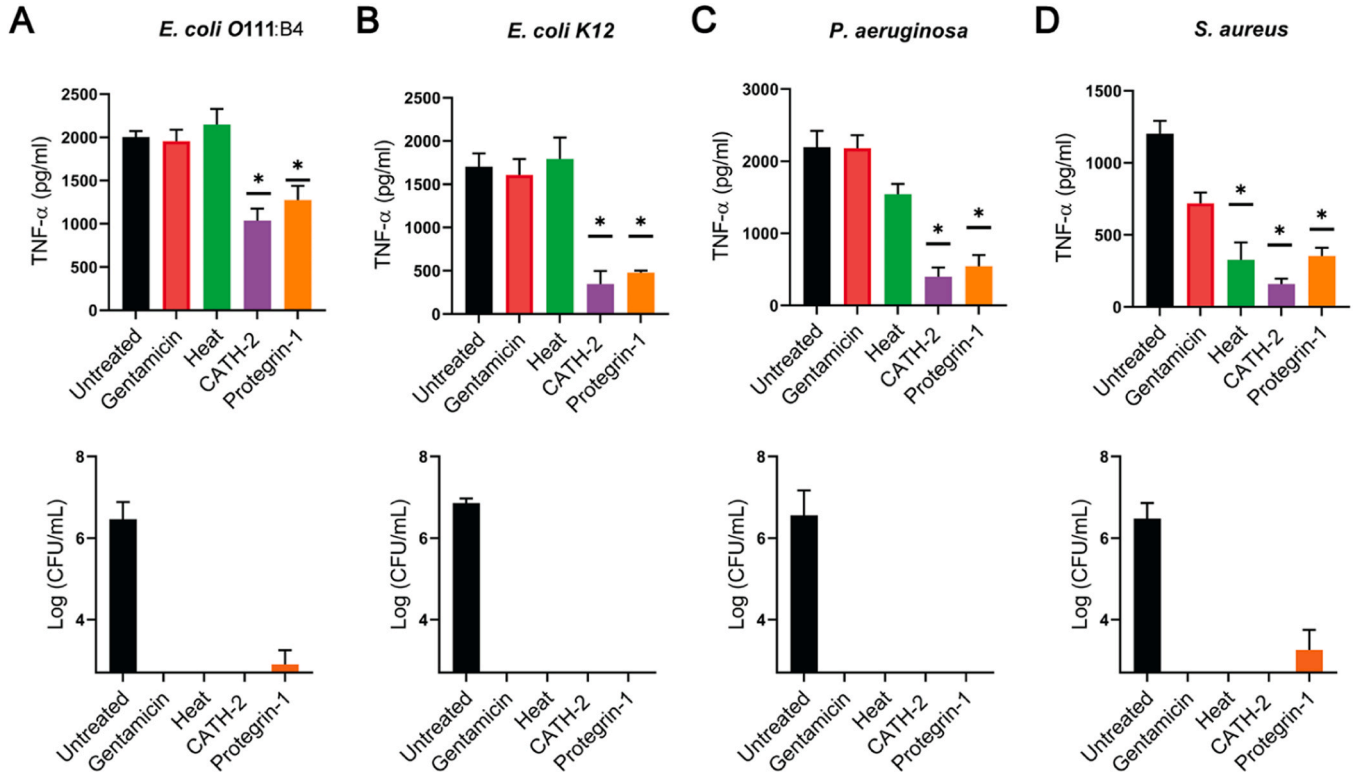

Fig. 4. Protegrin-1 kills bacteria with limited immune cell activation. Silent killing effect of Protegrin-1 was assessed by whole bacterial stimulation of macrophages. E. coli O111:B4 (**A**), E. coli K12 (**B**), P. aeruginosa (**C**) or S. aureus (**D**) cells were left untreated or were Protegrin-1/CATH-2 killed, heat killed, or gentamicin killed and used for stimulation of RAW 246.7 cells for 2 h (top) or colony count assays (bottom), after which TNF-α release was determined by ELISA. Shown are the mean ± SEM of three independent experiments (in duplicate). Data points that are significantly different from untreated control are denoted by * ($p < 0.05$).

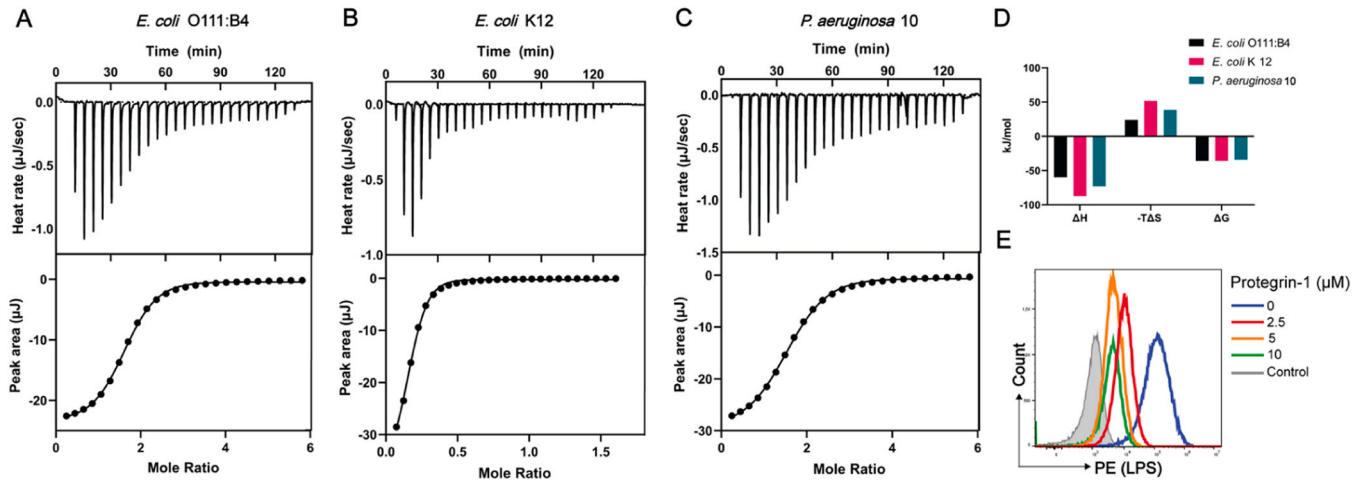

Fig. 5. Protegrin-1 binds LPS and prevents it from macrophage surface attachment. Thermodynamic binding of Protegrin-1 and E. coli O111:B4 (**A**), K12 (**B**) or P. aeruginosa 10 (**C**) LPS was measured with isothermal titration calorimetry (ITC). Every 300 seconds, 1.96 µl LPS solution was titrated into 164 µl peptide solution. Heat evolved was measured and normalized integrated heat was plotted against the molar ratio between LPS and the peptides. Independent model were used to fit the data and calculate Kd (µM), ΔG, ΔH and −TΔS (kJ mol^{-1}) (**D**). Effect of Protegrin-1 binding on LPS attachment to macrophages was determined by incubating Biotin labeled E. coli O111 LPS (200 ng/mL) and different Protegrin-1 concentrations for 10 min and then stimulating RAW 264.7 cells with this mixture for 1 h before washing. LPS was stained with Streptavidin-PE and its signal was measured by flow cytometry (**E**).

Table 1
Thermodynamic parameters derived from binding interactions of Protegrin-1 with LPS *E. coli* O111:B4, *E. coli* K12, *P. aeruginosa* 10 at 37 °C.

	E. coli O111:B4	*E. coli* K12	*P. aeruginosa* 10
K_a (μM^{-1})	1.018	1.033	0.629
ΔH (kJ mol^{-1})	−59.820 ± 1.3	−87,45 ± 2.1	−73,12 ± 1.7
TΔS (kJ mol^{-1})	24.150	51,74	38,8
ΔG (kJ mol^{-1})	−35.670	−35,71	−34,33
K_d (µM)	0.98 ± 0.14	0.96 ± 0.08	1.66 ± 0.22

3.6. Protegrin-1 inhibits bacterial phagocytosis by macrophages

In the next set of experiments it was investigated whether porcine and murine macrophages were able to take up GFP+ *E. coli*, and whether this uptake could be modulated by pre-incubation of the macrophages with Protegrin-1. In the absence of peptide, approximately 30 % of porcine (3D4/31) and RAW264.7 macrophages became GFP+ (Fig. 6**B, C**). At 10 µM, the highest concentration tested, Protegrin-1 could efficiently inhibit uptake of *E. coli* down to 20 % on average. A similar effect was observed when phagocytosis of pHrodo Green beads instead of

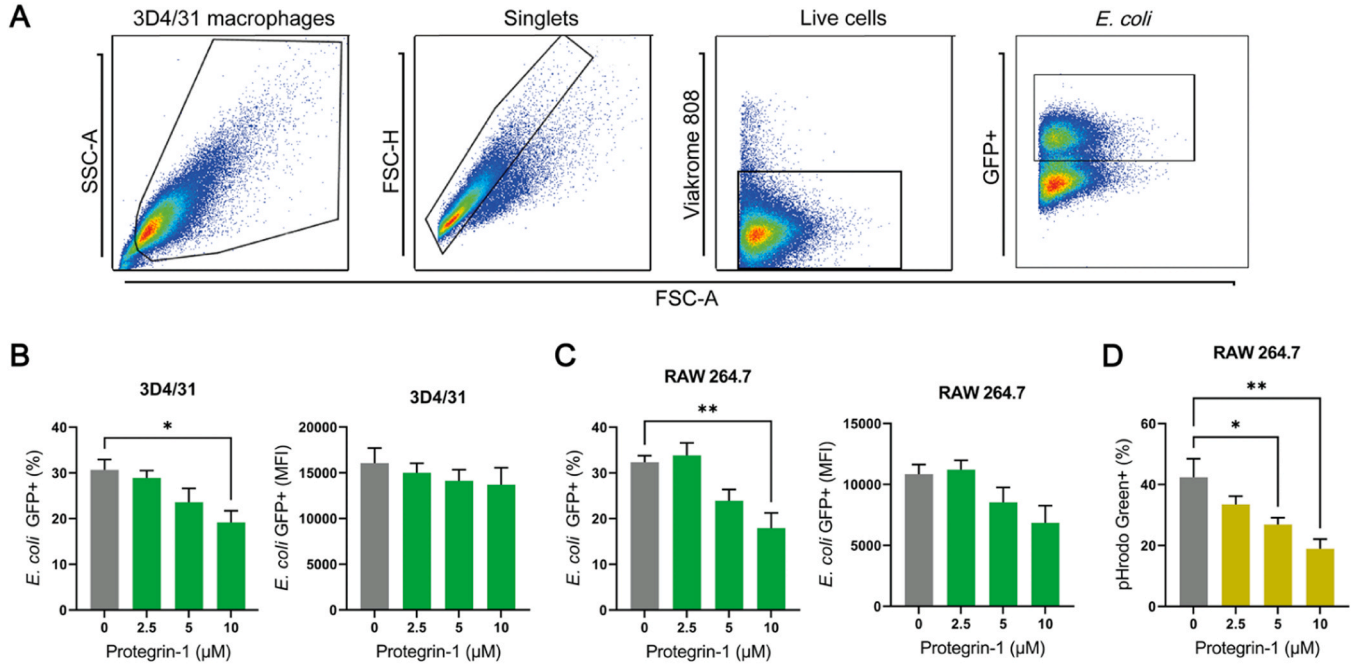

Fig. 6. Protegrin-1 inhibits bacterial phagocytosis by macrophages. 3D4/31 and RAW 264.7 cells were stimulated with Protegrin-1 (2.5–10 µM) for 24 h and then infected with GFP-labeled E. coli or treated with pHrodo E. coli for 60 min before washing, live dead staining and measuring the taken up bacteria or pHrodo signal by flow cytometry. (**A**) Gating strategy used to select the required populations. The percentage of macrophages that had taken up E. coli or pHrodo E. coli and geometric mean of GFP signal for 3D4/31 macrophages (**B**) and RAW 264.7 macrophages (**C,D**) was determined. Data is plotted as the mean ± SEM of three independent experiments (in duplicate). Data points that are significantly different from each other are denoted by * ($p < 0.05$); ** ($p < 0.005$).

GFP+ *E. coli* was measured in RAW264.7 cells pre-treated with Protegrin-1. Here a significant, concentration dependent decrease of beads uptake from 40 % to 20 % was observed (Fig. 6**D**).

3.7. Protegrin-1 modulates bacterial invasion and adherence to macrophages

Hiding and replication of intracellular bacterial pathogens during infection is one of the strategies to compromise host defense (Mitchell et al., 2016). We assessed the ability of Protegrin-1 to modulate *E. coli* adherence and invasion in porcine 3D4/31 and RAW264.7 macrophages. Protegrin-1 pre-treated macrophages were infected with *E. coli,* prior to treating them with or without gentamicin containing medium (to kill extracellular bacteria and determine the number of intracellular bacteria) and finally lysing the macrophages and plating out viable intracellular bacteria. Protegrin-1 significantly ($p < 0.05$) reduced the number of bacteria adhering to the cell surface and the intracellular bacterial survival in 3D4/31 macrophages (Fig. 7**A**). A similar tendency was observed in RAW macrophages but differences in the number of intracellular bacterial counts did not reach statistical significance in these experiments (Fig. 7**B**).

3.8. Protegrin-1 limits L. infantum amastigote survival

To evaluate the anti-amastigote activity of Protegrin-1, RAW264.7 cells were pretreated with Protegrin-1 for 24 h and subsequently infected with *L. infantum* amastigotes for 24 h. Amastigotes were then visualized under the light microscope and counted. The results showed that amastigote burdens in infected macrophages were almost 2-fold lower after 10 μM of Protegrin-1 pre-treatment (Fig. 8A,B).

4. Discussion

In this era of prevalent and increasing antibiotic resistance, the development of new antimicrobials and alternative therapies is crucial. To effectively reduce the use of antibiotics in not only human but also in veterinary medicine, novel strategies e.g. use of AMPs, are being tested. In this study, the antimicrobial and immunomodulatory activities of the porcine cathelicidin Protegrin-1 was explored.

AMPs can generally show some cytotoxicity towards mammalian cells, which is critical for their therapeutic applications. The cytotoxic effect of Protegrin-1 towards macrophages was assessed by LDH release and WST-1 proliferation assays. Both of the assays showed that the peptide was not cytotoxic towards these cells except for the LDH release in case of highest concentration tested in 3D4/31 (porcine macrophage) cells (Fig. 2**A**). This rules out the possibility of the peptide's toxicity to influence its observed effects on the macrophages at least for the concentrations used in this study. Protegrin-1's killing mechanism involves membrane pore formation similar to multiple other AMPs (Mani et al., 2006). In this study, bactericidal capacity of the synthetic Protegrin-1 was confirmed by MBC determination which showed that the peptide was active against clinical isolates of Gram-positive and Gram-negative bacteria (Fig. 1**B**). The antimicrobial spectrum of Protegrin-1 was broadened in this study with its observed activity against Leishmania infection of macrophages. Leishmaniasis is a parasitic zoonosis which is transmitted to humans by the bite of phlebotomine sand flies. Leishmania parasites exist as intracellular spherical amastigotes in mammalian hosts (Sunter and Gull, 2017). Several groups of AMPs have been shown to have significant action against diverse *Leishmania* species (El-Dirany et al., 2021). Amastigote burden assay in this study showed that 10 μM Protegrin-1 significantly ($p < 0.05$) lowered intracellular amastigote number (Fig. 8**A**). Peptides like BMAP-28 and 19–2.5 have been shown to have similar effect on *Leishmania* amastigotes (João et al., 2022; Lynn et al., 2011) but in a post-infection treatment rather than pre-treatment setting as employed in this study which indicates a cumulative protective activity of the Protegrin-1 against the infection.

In earlier studies, Protegrin-1 has been shown to inhibit lipo-oligosaccharide mediated TNF-α and nitric oxide release in human and murine macrophages (Zughaier et al., 2005), but whether LPS- binding by Protegrin-1 was essential for this, and which structural characteristics of LPS are required for the peptide's neutralizing activity have never been determined. In this study, the basis for this neutralization of LPS was systematically tested by using different LPSs (rough- vs smooth-type) and two lipid A variants. Results showed that Protegrin-1 was most active against rough-type LPS among the strains tested (Fig. 3**A-C**). Furthermore, the peptide showed more affinity for DPLA than MPLA in the similar readout (Fig. 3**D**). These experiments indicate the role of Lipid A structure in Protegrin-1's activity to bind and neutralize LPS. The presence of LPS modification could affect the mechanisms of membrane targeting peptides e.g., Colistin resistance in *E. coli* conferred by MCR-1 mediated ethanolamine addition at Lipid A phosphate groups (Simpson and Trent, 2019). Also, we have previously shown that some HDPs neutralized DPLA with more affinity than MPLA while the O-antigen variability didn't observably affect LPS neutralizing or bactericidal activity of these peptides (Javed et al., 2023a). This is a very importance point in studying the role of outer membrane (OM) remodeling in AMPs' mechanisms of action and in developing better LPS targeting peptides. Direct binding of Protegrin-1 to LPS was further confirmed by ITC. The peptide showed binding to all three types of LPS tested with almost similar affinity. The observed entropy factor ($-T\Delta S$) and binding enthalpy (ΔH) values (Fig. 5**A-D**), indicate that this binding was enthalpy driven. Cysteine-deleted variants of Protegrin-1 have been previously shown by ITC to interact with LPS exothermically, among which Arg-deficient analogs showed less affinity (Mohanram and Bhattacharjya, 2014). This, and a docking model suggested the critical role of cationic residues in the electrostatic interaction with LPS. Furthermore, porcine PMAP-36 and PR-39 have been shown to have biphasic binding pattern with simultaneous and sequential exothermic and endothermic binding (Javed et al., 2023a). Additionally the binding of Protegrin-1 with rough LPS was as effective as with the smooth LPS phenotype, indicating only a minimal involvement of the O-antigen in its interaction and possibly in its OM disruption activity. However, the LPS binding as shown by ITC does not necessarily directly explain the neutralization activity as the state of soluble LPS in culture medium is different from the micellar LPS in ITC experiments. This could be the reason why for example Murepavidin, a Protegrin-1 peptidomimetic was shown to be able to bind LPS in ITC studies but not neutralize LPS-induced macrophage activation (Javed et al., 2023b). Finally using flow cytometry it was again shown that indeed binding of Protegrin-1

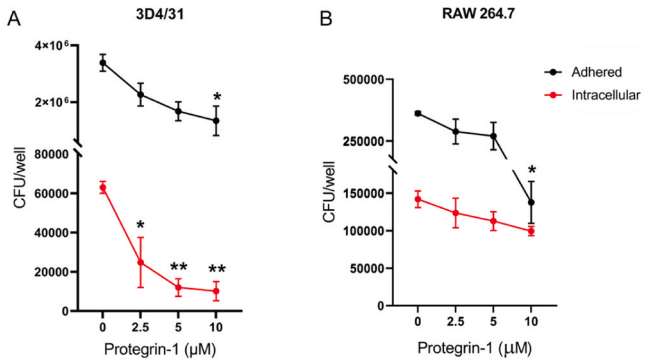

Fig. 7. Protegrin-1 modulates bacterial invasion and adherence to macrophages. Bacterial adhesion and invasion of RAW 264.7 (**A**) and 3D4/31 (**B**) macrophages was determined by colony count assay. Cells were stimulated with Protegrin-1 (2.5–10 μM) for 24 h and then infected with E. coli for 60 min before washing, treating with 100 ug/mL Gentamicin (for invasion), lysing the cells and plating out. Data is plotted as the mean ± SEM of three independent experiments (in duplicate). Data points that are significantly different from each other are denoted by * ($p < 0.05$); ** ($p < 0.005$).

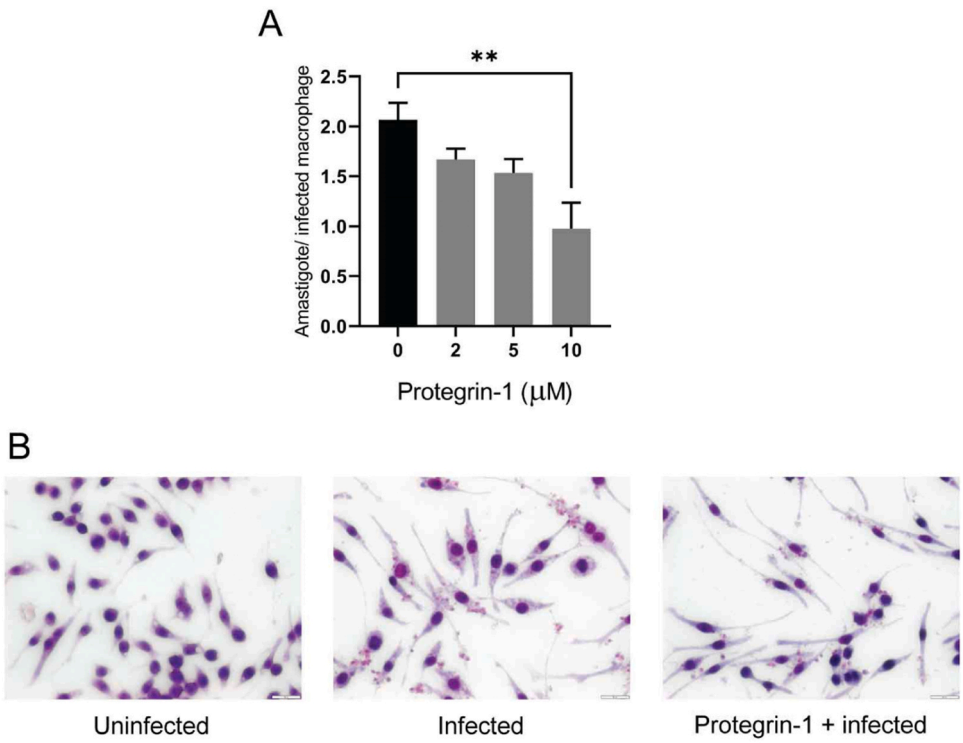

Fig. 8. Protegrin −1 limits L. infantum amastigote survival. RAW 246.7 cells were stimulated with the peptide for 24 h before infecting it with L. infantum promastigotes for another 24 h followed by washing and Giemsa staining. (**A**) parasitic burden was determined by calculating amastigotes per infected macrophage in 200 cells. (**B**) Microscopic images of uninfected and infected (untreated or peptide treated) RAW246.7 cells. Data is plotted as the mean ± SEM of three independent experiments (in duplicate). Data points that are significantly different from each other are denoted by * ($p < 0.05$); ** ($p < 0.005$).

prevented LPS from attaching to macrophages (Fig. 5**E**) which explained the reduced activation of macrophages. Overall this data suggests an LPS neutralization model where Protegrin-1 binds to the phosphate residues of the lipid A part of LPS (Javed et al., 2023b) thereby preventing it, most likely by steric hindrance, from binding to TLR4/MD2 on immune cells, thus avoiding the pro-inflammatory response.

Since multiple factors come into play in a real-time bacterial infection, including the recognition of endotoxins by the host's defense system, the anti-inflammatory role of AMPs like Protegrin-1 becomes important. The ability of Protegrin-1 to kill bacteria with limited LPS-induced macrophage activation was tested by demonstrating its simultaneous bactericidal and endotoxin neutralizing activity (Fig. 4). This shows the potential advantage of LPS neutralizing AMPs over conventional antibiotics in treating Gram-negative infections, because it could be useful in avoiding septic shock during infection treatment. This so called 'silent killing' capacity has been shown before for CATH-2 and Polymyxin antibiotics (Coorens et al., 2017; Javed et al., 2023b). However, curbing the innate immune-recognition of LPS could also prevent the 'necessary' immune response towards certain infections and may be considered as a potential downside of the peptide.

One of the important function of macrophages during infection is controlled phagocytosis. Apart from LL-37, whose ability to enhance *E. coli* phagocytosis has been demonstrated thoroughly (Wan et al., 2014), little is known about the ability of cathelicidins to modulate the bacterial uptake activity of macrophages. This aspect of Protegrin-1 was assessed in this study by observing its effect on phagocytosis of non-opsonized GFP-labeled *E. coli* by macrophages. Results showed that the peptide inhibited bacterial uptake (Fig. 6**B-D**). This effect could actually reduce the bactericidal capacity of macrophages towards an infection but could also suggest a protective role of peptide to pre-emptively avoid the phagosomal escape of intracellular bacteria as seen in case of *S. aureus* (de Jong et al., 2019). In a similar type of study, a set of cathelicidins were shown to variably modulate the RAW264.7 phagocytosis of latex beads with half of them showing a similar inhibitory effect (Coorens et al., 2017c). Nevertheless, it is hard to conclude the peptide's function in this regard from these assays as macrophage uptake of extracellular components is a complex process (Underhill and Goodridge, 2012). In the similar line, to further test the indirect antibacterial and anti-inflammatory activity of Protegrin-1, the intracellular *E. coli* survival in the peptide-treated macrophages was determined. It was observed that Protegrin-1 pre-treatment reduced the intracellular survival in porcine macrophages after 1 h in dose dependent manner (Fig. 7**A**). Furthermore, survival of cell surface adhered bacteria was also inhibited significantly ($p<0.05$).

5. Conclusion

In conclusion, this study showcases a spectrum of immunomodulatory and indirect antimicrobial functions of Protegrin-1 thereby indicating the potential of its protective effects against (Gram-negative) bacterial infections in addition to its direct killing capacity. The shown activities of Protegrin-1 in LPS and bacteria-challenged macrophage models not only describes the possible immune balancing role of Protegrin-1's release in porcine bacterial infections but also emphasizes the potential of its anti-inflammatory utilization to manage infections. Moreover its activity to hinder Leishmania infection broadens the antimicrobial spectrum of this AMP. Further research in this regard would not only help to discover untapped immunomodulatory functions of Protegrin AMPs but also to design new peptides based on the natural scaffolds with optimized activities.

CRediT authorship contribution statement

Trishana Oedairadjsingh: Methodology, Investigation, Data curation. **Irene Ludwig:** Methodology, Investigation, Data curation. **Ali Javed:** Writing – original draft, Methodology, Investigation, Data

curation. **Femke Broere:** Validation, Supervision. **Weingarth Markus:** Conceptualization. **Thomas Wood:** Methodology, Investigation, Data curation. **Nathaniel Martin:** Visualization, Resources. **Edwin Veldhuizen:** Writing – review & editing, Visualization, Supervision, Resources.

Declaration of Competing Interest

The authors declare that they have no conflicts of interest.

Data availability

Data will be made available on request.

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Appendix A. Supporting information

Supplementary data associated with this article can be found in the online version at doi:10.1016/j.molimm.2024.07.011.

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