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Novel insights in antimicrobial and immunomodulatory mechanisms of action of PepBiotics CR-163 and CR-172

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ABSTRACT

Objectives: Our group recently developed a new group of antimicrobial peptides termed PepBiotics, of which peptides CR-163 and CR-172 showed optimized antibacterial activity against *Pseudomonas aeruginosa* and *Staphylococcus aureus* without inducing antimicrobial resistance. In this study, the antibacterial mechanism of action and the immunomodulatory activity of these two PepBiotics was explored.
Methods: RAW264.7 cells were used to determine the ability of PepBiotics to neutralize Lipopolysaccharide (LPS)-and Lipoteichoic acid (LTA)-induced activation of macrophages. Isothermal titration calorimetry and competition assays with dansyl-labeled polymyxin B determined binding characteristics to LPS and LTA. Combined bacterial killing with subsequent macrophage activation assays was performed to determine so-called 'silent killing'. Finally, flow cytometry of peptide-treated genetically engineered *Escherichia coli* expressing Green Fluorescent Protein (GFP) and mCherry in the cytoplasm and periplasm, respectively, further established the antimicrobial mechanism of PepBiotics.
Results: Both CR-163 and CR-172 were shown to have broad-spectrum activity against ESKAPE pathogens and *E. coli* using a membranolytic mechanism of action. PepBiotics could exothermically bind LPS/LTA and were able to replace polymyxin B. Finally, it was demonstrated that bacteria killed by PepBiotics were less prone to stimulate immune cells, contrary to gentamicin and heat-killed bacteria that still elicited a strong immune response.
Conclusions: These studies highlight the multifunctional nature of the two peptide antibiotics as both broad-spectrum antimicrobial and immunomodulator. Their ability to kill bacteria and reduce unwanted subsequent immune activation is a major advantage and highlights their potential for future therapeutic use.

1. Introduction

The use of antimicrobial peptides (AMPs) as an alternative to antibiotics to treat and protect against bacterial infections has shown promising potential [1]. These peptides have direct microbicidal effects against both Gram-positive and Gram-negative bacteria; furthermore, antiviral and antifungal activities have been described [2–5]. In addition, AMPs can display a multitude of immunomodulatory activities ranging from neutralization of bacterial products, such as Lipopolysaccharide (LPS) and Lipoteichoic acid (LTA) [6], to direct effects on host immune cells, such as macrophages and dendritic cells, leading to cell activation and proliferation [7,8]. The number of potential immunomodulatory functions is rapidly increasing, and several recent review articles provide an informative overview of these findings [9–11].

AMPs constitute an ancient part of innate immunity and can be found in all vertebrates, but also in plants and fungi. Several classes of naturally occurring AMPs have been described, of

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which the families of defensins and cathelicidins are the largest and best documented. However, besides these natural peptides, a multitude of potential AMPs have been developed with optimized sequences and structures. New AMPs can be developed by either testing random peptide libraries against microbes in high-throughput settings or based on rational design using parameters such as charge, amphipathicity, hydrophobicity, and peptide length [12–14]. Both methodologies have indeed resulted in new peptides with increased antimicrobial activity in vitro, although the minimal inhibitory concentrations (MICs) of designed AMPs are still in the same micromolar range as seen for naturally occurring AMPs.

In our recent work, new AMPs were designed based on existing cathelicidin peptides, resulting in a family of peptides called PepBiotics, with potential for use in the treatment of *Pseudomonas aeruginosa* and *Staphylococcus aureus* infections in cystic fibrosis (CF) [15]. Although the design of new peptides was based on similar criteria as described above, the peptides were additionally optimized for activity in a physiologically relevant environment of high salt and low pH (as found in the CF lung). Two PepBiotics, CR-163 and CR-172, were shown to maintain high activity in this environment. Furthermore, it was shown that these PepBiotics had low toxicity in vitro and in vivo and that induction of antimicrobial resistance towards these PepBiotics did not occur. However, the mechanism of action of PepBiotics has not yet been examined.

In this study, the broad-spectrum and fast membrane permeabilization activities of CR-163 and CR-172 are described. In addition, we report that both PepBiotics bound strongly to LPS, which led to immunologically silent killing of bacteria. The combined antimicrobial and LPS neutralization activities contribute to the applicability of PepBiotics to fight clinically important bacterial infections in humans effectively with less development of excessive inflammatory responses that may lead to pathological reactions, such as sepsis.

2. Methods

2.1. Peptides

The peptides used in this study were synthesized by China Peptides (Shanghai, China) using Fmoc-chemistry. All peptides were purified by reverse phase high-performance liquid chromatography to a purity of >95%.

2.2. Bacterial strains

The bacterial strains used in this study were *Escherichia coli (*ATCC25922), *Enterococcus faecium, P. aeruginosa, Acinetobacter baumannii, S. aureus* (all clinical isolates, donated by the Department of Medical Microbiology of Utrecht University Medical Center), and *Klebsiella pneumoniae* (ATCC® BAA-1705™). All strains were cultured in Mueller Hinton broth (MHB). Recombinant *E. coli* expressing mCherry in the periplasm and Green Fluorescent Protein (GFP) in the cytoplasm (PerimCherry/cytoGFP) was prepared as previously described [16] and cultured in Lysogeny Broth (LB) medium supplemented with 100 µg/mL ampicillin.

2.3. Cell culturing

Murine macrophage RAW264.7 cells (ATCC TIB-71) were cultured in Dulbecco's Modified Eagle Medium (DMEM) (Gibco, Thermo Fisher Scientific, Waltham, MA) supplemented with 10% fetal calf serum (FCS) (Bodinco B.V., Alkmaar, The Netherlands) and 10 U mL penicillin/10 mg/mL streptomycin at 37°C, 5.0% CO_2. Cells were seeded in 96-wells plates at 5×10^4 cells/well, and after adherence for 24 h, used in stimulation experiments.

2.4. Colony count assay

An o/n bacterial culture was diluted 1:100 in 10 mL MHB and incubated for 3 h at 37°C to achieve logarithmic growth phase. The bacterial suspension was diluted to 2×10^6 colony forming units per mL (CFU/mL). Bacteria and PepBiotics were mixed 1:1 in 96-wells in a polypropylene round-bottom plate (Corning Costar, Glendale, AZ) and incubated for 3 h at 37°C. After incubation, dilution series of bacteria were plated out on Tryptic Soy Agar (TSA) plates and incubated at 37°C for 24 h to quantify viable bacteria.

2.5. Kinetic colony count assay

Various concentrations of PepBiotics were incubated 1:1 with mid-logarithmic phase bacteria (2×10^6 CFU/mL in MHB). At various time points (1, 5, 10, 20, 30, and 60 min), aliquots were taken of the bacteria-PepBiotics mixture, further diluted (10-, 100-, and 1000-fold), and plated on TSA plates. After 16 h incubation at 37°C, surviving bacteria were enumerated.

2.6. Isothermal calorimetry titration

The experiments were performed on a NanoITC (TA Instruments, New Castle, DE). *Pseudomonas aeruginosa*-LPS was diluted to 10 µM in 75% (v:v) phosphate-buffered saline (PBS) and *S. aureus*-LTA was diluted to 12.5 or 25 µM in 75% PBS before experiments. The 164 µL chamber was then filled with either the LPS or LTA solution and the syringe was filled with 50 µL 200 µM PepBiotics solution, also dissolved in 75% PBS. Two µL of PepBiotics solution was injected into the chamber every 300 s, except the first injection which consisted of 0.96 µL. The experiments were performed at 37°C while stirring at 300 Rotations per minute (RPM). The data was analyzed with the NanoAnalyze Software.

2.7. Dansyl-polymyxin B replacement assay

The chicken cathelicidin CATH-2, a scrambled version of CATH-2, or PepBiotics were diluted in 5 mM 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES). *Pseudamonas aeruginosa*(PA)-LPS was diluted to 60 µg/mL in 5 mM HEPES, mixed 1:1 with the peptide solution in a 96-wells plate, and incubated for 30 min at 37°C. Dansyl-polymyxin B, prepared and quantified as described by Schindler and Teuber [16], was subsequently added to the peptide/LPS mixture at a final concentration of 4 µM. Excitation/emission was recorded at 340/490 nm using a FLUOstar® Omega microplate reader.

2.8. ODN-1826 uptake by macrophages

RAW264.7 cells were seeded at a density of 1×10^5 cells/well and adhered o/n. Subsequently, cells were incubated for 24 h with 20 nM oligodeoxynucleotide- (ODN)1826 (InvivoGen, Toulouse, France) and premixed with PepBiotics (0–5 µM) in cell culture medium. The supernatant was used in the Griess assay to determine the Nitric oxide (NO) concentration. *Pseudomonas aeruginosa*-LPS (50 ng/mL) was used as positive control for NO production by RAW264.7 macrophages.

2.9. Flow cytometry-based viability testing

PerimCherry/cytoGFP *E. coli* were grown overnight in LB medium containing 100 µg/mL ampicillin. The next day, subcultures were grown to mid-log phase (OD ~ 0.5) in the presence of 0.1% arabinose (to induce GFP expression), and washed and resuspended to an OD ~ 1 in RPMI supplemented with 0.05% human serum albumin (RPMI-HSA). All further incubations were done in

Table 1
Minimal bactericidal concentration (MBC) values of CR-163 and CR-172 against ESKAPE pathogens and *Escherichia coli*.

	CR-163 MBC (μM)	CR-172 MBC (μM)
***E**nterococcus faecium*	5	2.5–5
***S**taphylococcus aureus*	5	2.5–5
***K**lebsiella pneumoniae*	5	5
***A**cinetobacter baumannii*	2.5–5	2.5–5
***P**seudomonas aeruginosa*	2.5–5	2.5–5
***E**scherichia coli*	10–40	10–40

RPMI-HSA. Bacteria with an OD ~ 0.05 were mixed with 1 μM Sytox Blue Dead Cell Stain (ThermoFisher Scientific, Waltham, MA) and exposed to a concentration range of PepBiotics for 30 min at 37°C. For the in-time experiments, bacteria were mixed with the PepBiotics (concentrations indicated in figure legends) every 5 min for 30 min and incubated at 37°C. After the incubations, bacteria were diluted ten times, after which the Sytox blue, mCherry, and GFP intensity were analyzed by flow cytometry (MACSQuant). Data was analyzed in FlowJo, where the percentage of mCherry and GFP-negative or Sytox-positive bacteria was determined by gating on the buffer control.

Kinetics of permeabilization were also studied in a continuous measurement setup. For this, bacteria were incubated with PepBiotics in the presence of 1 μM Sytox Green Nucleic Acid stain (Thermofisher). Fluorescence was continuously measured in a microplate reader (CLARIOstar, Labtech) at 37 °C under non-shaking conditions.

2.10. Silent killing

RAW264.7 cells were seeded as described in cell culturing. *Pseudomonas aeruginosa* (final density: 1×10^6, or 1×10^7 CFU/mL in DMEM) was treated in the following ways: i) heat treatment: 1 h at 96 °C; ii) 1 mg/mL gentamicin: 1 h at 37°C; iii) 20 μM CR-172, 1 h at 37 °C; iv) 20 μM CR-163, 1 h at 37 °C; and v) 20 μM CATH-2, 1 h at 37°C. Effectivity of the lethal treatments was checked by counting colonies of surviving bacteria. Non-viable *P. aeruginosa*, or viable control *P. aeruginosa*, were added to RAW264.7 cells, and after 2 h incubation, TNF-α production was determined in the supernatant by ELISA, according to the manufacturers' protocol (R&D systems, Minneapolis, MN).

2.11. Statistical analysis

The results were statistically tested for significant differences using ordinary one-way ANOVA and tested for normality using the Shapiro-Wilk test. The software GraphPad Prism v8.4.2 was used for the tests, and P values <0.05 were considered statistically significant.

3. Results and discussion

3.1. Antimicrobial activity

In order to elaborate on the described antimicrobial activity of CR-163 and CR-172 against *P. aeruginosa* and *S. aureus,* colony count assays were performed against additional ESKAPE pathogens *E. faecium, K. pneumoniae, A. baumannii*, and *E. coli*. Both PepBiotics were active against these bacteria with MBC values of 2.5–5 μM, indicating that these PepBiotics have a broad spectrum of activity that extends beyond the major CF pathogens (Table 1, Supplementary Fig. S1). Only *E. coli* had a lower susceptibility, with MBC values of 10–40 μM for both PepBiotics.

To investigate the characteristics of antimicrobial activity further, killing kinetics of CR-163 and CR-172 were determined against *P. aeruginosa* and *S. aureus* (Fig. 1). At 5 μM (MBC), both CR-163 and CR-172 reduced the number of viable bacteria below detectable levels within 5 min. Lower concentrations did not completely eradicate all bacteria but showed substantial decrease of viable bacteria, usually within the first 30 min. Similar results were observed for killing kinetics against *S. aureus*, although 5 μM CR-163 was not sufficient to reach MBC in every single experiment (two of three), leading to an average viability just above the detection limit.

3.2. LPS and LTA binding

In our earlier studies, we described that both CR-163 and CR-172 were able to neutralize LPS- and LTA-induced activation of macrophages [15]. Isothermal Titration Calorimetry (ITC) was utilized to determine if a direct interaction between PepBiotics and LPS/LTA underlies this neutralization. As shown in Fig. 2A, heat was released upon injection of PepBiotics in a *P. aeruginosa* LPS solution, indicative of exothermic binding. This was also demonstrated to a lower extent for PepBiotics binding to *S. aureus* LTA. Interestingly, two control PepBiotics, CR-161 and CR-169, that were not able to neutralize LPS and LTA in the earlier study, also bound LPS and LTA. As indicated in Supplementary Table S1, all four peptides bound exothermically (negative enthalpy ΔH) to LPS, with a Kd in the μM range. Only CR-161 showed a positive entropy upon binding to LPS and LTA, indicating that hydrophobic interactions might contribute to LPS binding for this PepBiotic, but not for CR-163, CR-169, and CR-172.

3.3. Competition with dansyl-polymyxin B

To further study the binding of PepBiotics to LPS from *P. aeruginosa*, competitive binding experiments were performed using dansyl-labeled polymyxin B (PmB). Dansyl fluorescence is strongly influenced by its environment, having much stronger fluorescence when bound to LPS. As shown in Fig. 2B, CATH-2, which is known to have strong LPS-binding characteristics, can almost completely outcompete dansyl-PmB binding to LPS, leaving approximately 10% dansyl fluorescence (compared to the no-peptide/medium control) at 5–10 μM. On the contrary, a scrambled version of CATH-2 was only able to lower the dansyl fluorescence to approximately 50%, showing that, not surprisingly, the extent of LPS binding is sequence dependent. CR-172 was equally as active as CATH-2, while CR-163 showed a less pronounced effect (25% fluorescence at 10 μM). Two PepBiotics, CR-161 and CR-169, that showed comparable LPS binding in the ITC studies but lower LPS-neutralizing capabilities, both also showed lower capability to outcompete the binding of dansyl-PmB to LPS. Overall, as previously described, a correlation between neutralization of LPS-induced macrophage activation by PepBiotics with their capability to compete with dansyl-PmB was observed in this assay.

3.4. ODN-1826 uptake

CATH-2 can stimulate DNA uptake by macrophages and thereby increase TLR9 activation, leading to a higher production of NO [17]. The potential of CR-163 and CR-172 to have similar immunomodulatory activity was studied (Fig. 3). Addition of 2 μM CATH-2 significantly increased NO production compared with ODN-1826 alone. However, PepBiotics (1–5 uM) did not show any significant effect on ODN-1826–induced NO production.

3.5. Bacterial membrane damage

To further characterize the antibacterial mechanism of the two PepBiotics on Gram-negative bacteria, a fluorescent reporter system was used. PepBiotics were incubated with Per-

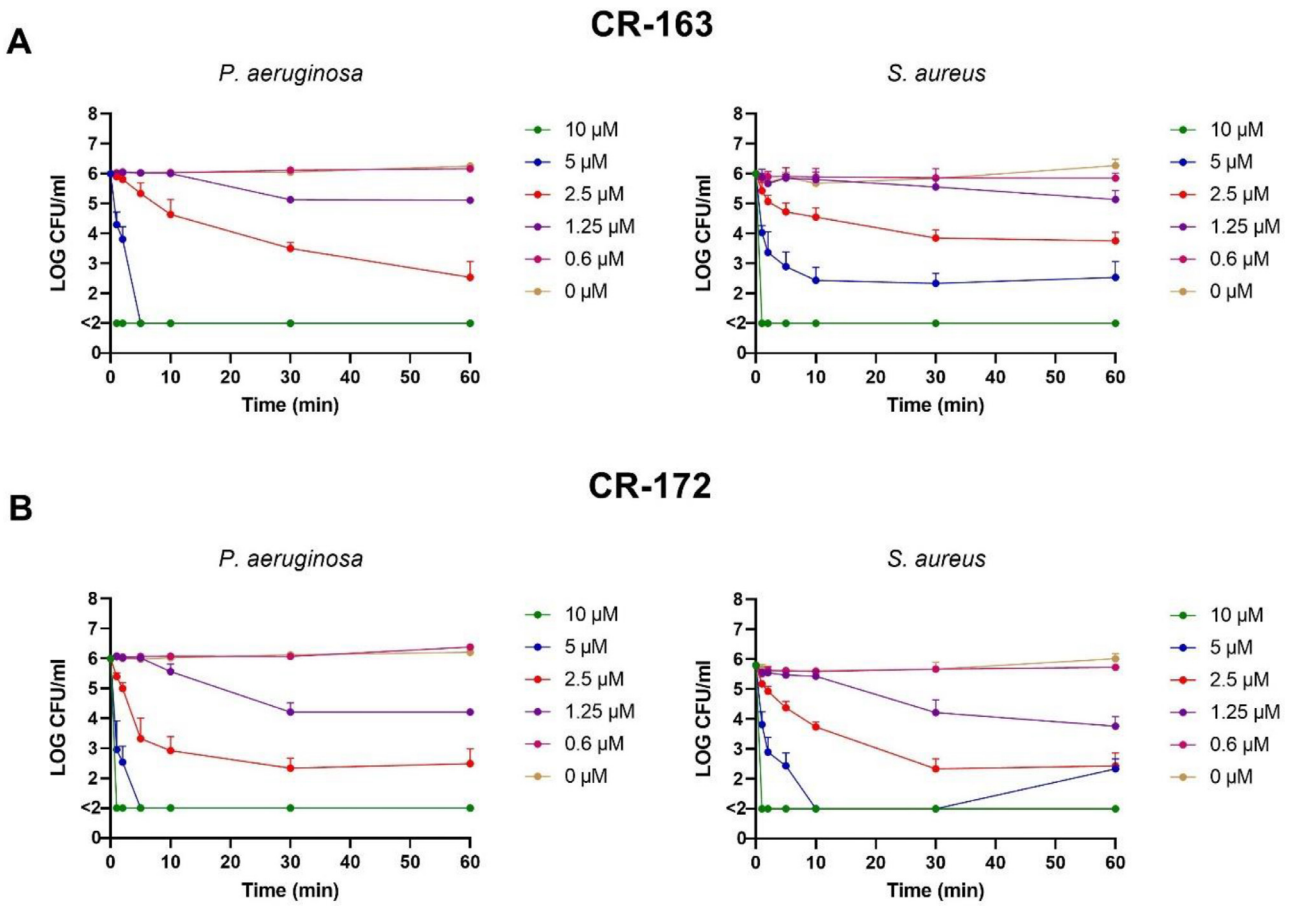

Fig. 1. PepBiotics rapidly kill *Staphylococcus aureus* and *Pseudomonas aeruginosa*. Incubation of 1 × 10^6 CFU/mL *P. aeruginosa* or *S. aureus* with PepBiotics CR-163 (A) and CR-172 (B). Samples were collected at different time points and surviving bacteria were counted after 24 h of growth on TSA plates. Shown are mean ± SEM of three independent experiments.

imCherry/cytoGFP *E. coli* (genetically engineered *E. coli* expressing mCherry in the periplasm and GFP in the cytoplasm) in a medium containing Sytox-blue. Using flow cytometry, the release of mCherry and GFP, indicative of outer membrane (OM) and inner membrane (IM) permeabilization, respectively, and influx of Sytox blue, indicative of small perturbations of the IM, were measured. As shown in Fig. 4A, increasing concentrations of PepBiotics led to the release of mCherry and GFP and an influx of Sytox-blue. For CR-163, maximum activity was observed at 20 µM, where almost all bacteria were Sytox-positive and mCherry- and GFP- negative, corresponding with the observed MBC value for CR-163 against *E. coli*. Using this optimal concentration, the release or uptake of the individual dyes was followed in time, showing that Sytox influx occurred almost instantaneously, followed by the release of mCherry and GFP after approximately 5 min (Fig. 4B). The picture for CR-172 was slightly different: maximum activity was seen at a lower concentration of 2.5 µM, while the distinction between Sytox influx and GFP/mCherry release was not observed. For both PepBiotics, however, it is clear that OM and IM permeabilization occurs rapidly and is likely the main contributor to the antimicrobial mechanism of action. A strange phenomenon was observed for CR-172, where higher PepBiotic concentrations resulted in a lower percentage of permeabilized (GFP–/$mCherry^-$/Sytox+) bacteria (Fig. 4A). Since bacteria were actually non-viable at these higher concentrations, it is currently unclear whether this reflects a different mechanism of action at higher PepBiotic concentrations, or a detection artifact.

For comparison, Sytox-influx was also measured for *P. aeruginosa* over time for both PepBiotics (Supplementary Fig. S2). This showed similar fast IM permeabilization kinetics as those seen for *E. coli*, indicating that the mechanism of action against these bacterial strains is similar. Complete permeabilization was observed at lower concentrations of the PepBiotics, corresponding to the lower MBC for *P. aeruginosa* compared with that of *E. coli* (Table 1).

3.6. Silent killing

The silent killing experiments were performed at two bacterial densities. As described before, addition of non-viable bacteria can still result in strong immune reactions of macrophages, as seen by the TNF-α production when either heat-killed or gentamicin-killed *P. aeruginosa* were added (Fig. 5A,B). In the case of gentamicin-killed bacteria, this TNF-α response was significantly higher than the response associated with the addition of viable bacteria to RAW264.7 macrophages. When *P. aeruginosa* was killed by PepBiotics or by CATH-2, the immune response was significantly lower (at 10^6 CFU/mL) for all peptides, indicative of the phenomenon described as silent killing [18,19]. Viability counts after treatment actually indicated that CR-163, in contrast to CR-172, failed to completely eradicate *P. aeruginosa* in DMEM. Bacterial density was significantly reduced by 1 or 2 LOG, but did not reach the detection limit, indicating that the observed remaining TNF-α production for this peptide can at least partially be explained by the presence of viable bacteria. However, for both CR-163 and CR-172, it

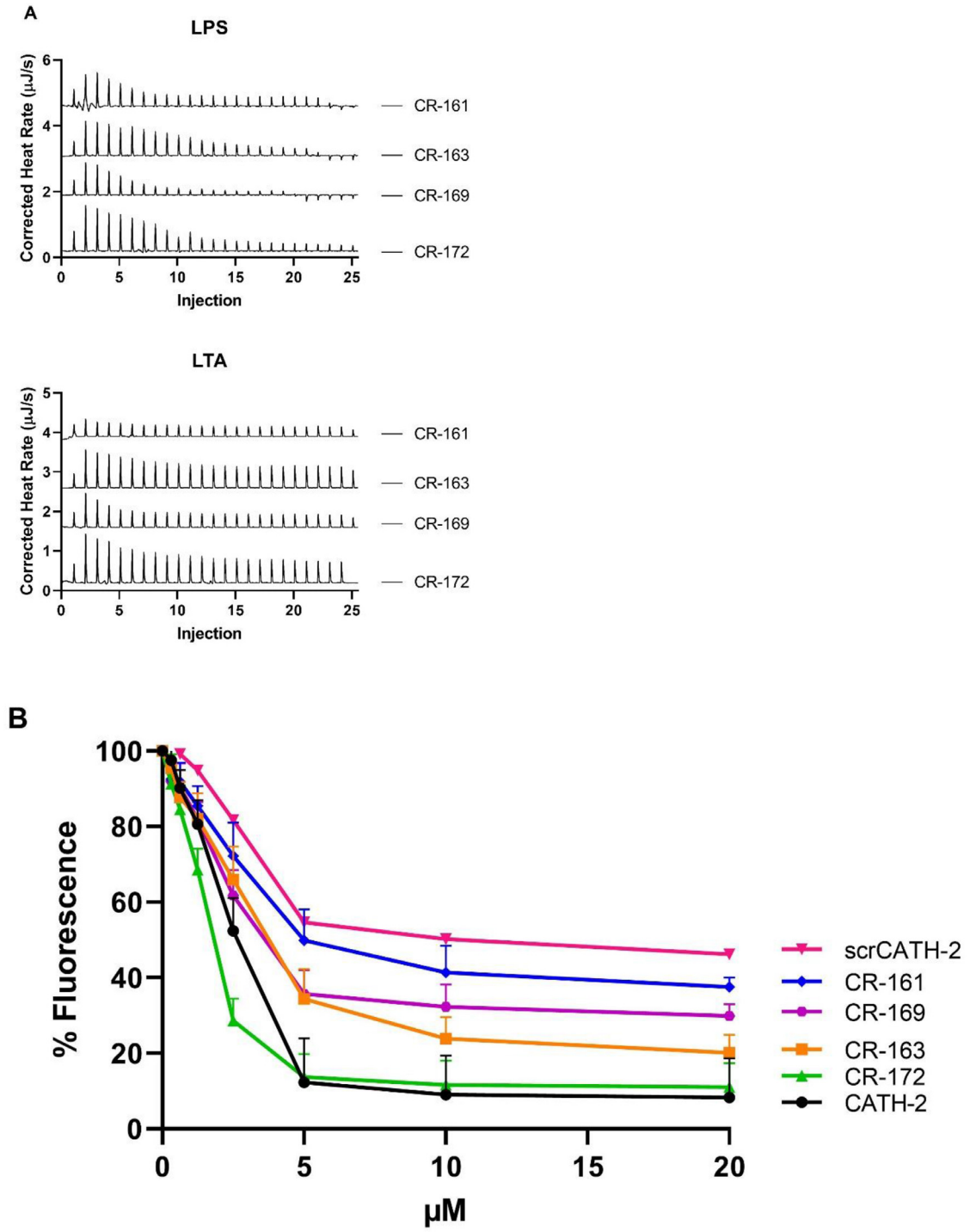

Fig. 2. PepBiotics interact with Lipopolysaccharide (LPS) and Lipoteichoic acid (LTA). A) Isothermal titration calorimetry. Shown are representative thermographs of PepBiotics (200 µL) injections into solutions of *Pseudomonas aeruginosa* LPS (10 µM) or *Staphylococcus aureus* LTA (12.5 µM). B) Dansyl-polymyxin B competition assay. *P. aeruginosa* LPS was incubated with increasing concentrations of peptides and 4 µM dansyl-polymyxin B. Fluorescence was measured at 490 nm after excitation at 340 nm. Shown are mean ± SEM of three independent experiments.

was demonstrated that PepBiotic-mediated killing of *P. aeruginosa* is silent, leading to low immune activation of macrophages.

3.7. Discussion

In this study, CR-163 and CR-172 were further characterised, including their immunomodulatory capabilities towards immune stimulating bacterial products. Antimicrobial activity of CR-163 and CR-172 extended beyond the CF pathogens and included activity against most ESKAPE bacteria. This is of particular interest because these bacteria are characterized as the most important life-threatening multidrug-resistant nosocomial pathogens [20]. Not surprisingly, a relatively large proportion of current research funding in infectious diseases is used for the development of new treatments for infections caused by these ESKAPE pathogens [21].

Bactericidal activity of PepBiotics was quick, killing most bacteria within 30 min of exposure. This might not be surprising,

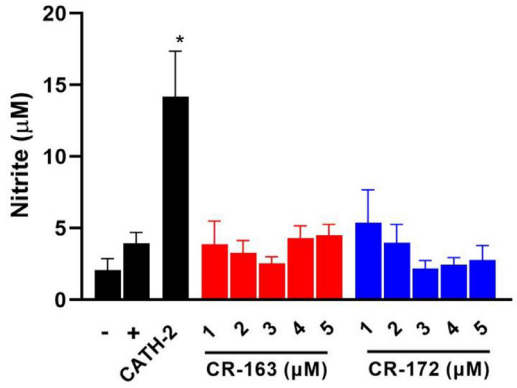

Fig. 3. PepBiotics do not increase Oligodeoxynucleotide (ODN)-induced macrophage activation. RAW264.7 cells were stimulated with 20 nM ODN-1826 in the presence of 2 μM CATH-2, 1–5 μM CR-163 (red), or CR-172 (blue). After 24 h nitrate (representative of NO production) was measured in the cell supernatant using the Griess Assay. '-': No ODN; '+': ODN-1826; CATH-2: 20 nM ODN-1826 + 2 μM CATH-2; red bars: 20 nM ODN-1826 ± 1–5 μM CR-163; blue bars: 20 nM ODN-1826 in the presence of 1–5 μM CR-172. Shown are mean ± SEM of three independent experiments. $^{*}P$ <0.05 compared with ODN-1826 (no peptide control).

considering the similar characteristics of many other antimicrobial peptides [1,22], but it extends the potential use of PepBiotics for infectious diseases other than CF. Only *E. coli* was slightly less susceptible, resulting in two- to four-fold higher MBC values, although substantial (>2LOG) reductions in viable bacteria were seen at 5–10 μM (Supplementary Fig. S1).

Besides development of resistance, the use of antibiotics also bears the danger of inducing excessive inflammation. Antibiotic treatment can result in the release of LPS from the Gram-negative bacterial outer membrane [23–27] or LTA from the membrane of Gram-positive bacteria [28–30]. As a result, a strong increase in inflammation can occur despite the lowered viable bacterial load, which relates to why it has been frequently found in critical care units that the situation for patients treated with antibiotics is worsened [31,32]. Therefore, it is an advantage for new antimicrobial compounds if, upon killing of bacteria, the release or activity of immune-stimulating compounds like LPS and LTA is inhibited.

In this study, we provide evidence using ITC for direct interaction between PepBiotics and LPS or LTA (Fig. 2). Interestingly, binding of PepBiotics to LPS did not always correlate to neutralization of LPS. This was observed in our ITC assays where CR-161 and CR-169, two PepBiotics that are not capable of reducing LPS-induced activation of macrophages [15], bound relatively strongly to LPS in the ITC assays. In the dansyl-PMB replacement assay, a better correlation between LPS binding and inhibition of LPS-induced activation was observed (Fig. 2B), but this assay also showed LPS binding of CR-161 and CR-169. A possible explanation could be that these peptides bind differently or on a different part of LPS. Indeed, the ITC experiments showed that more hydrophobic interactions between CR-161 and LPS were involved compared with the other PepBiotics. It would be interesting to determine what the exact binding mode of these peptides is and whether (slightly) different binding sites on LPS are occupied by CR-161 and CR-169 in comparison with CR-163 and CR-172, which can subsequently affect interactions with toll-like receptor 4 on macrophages.

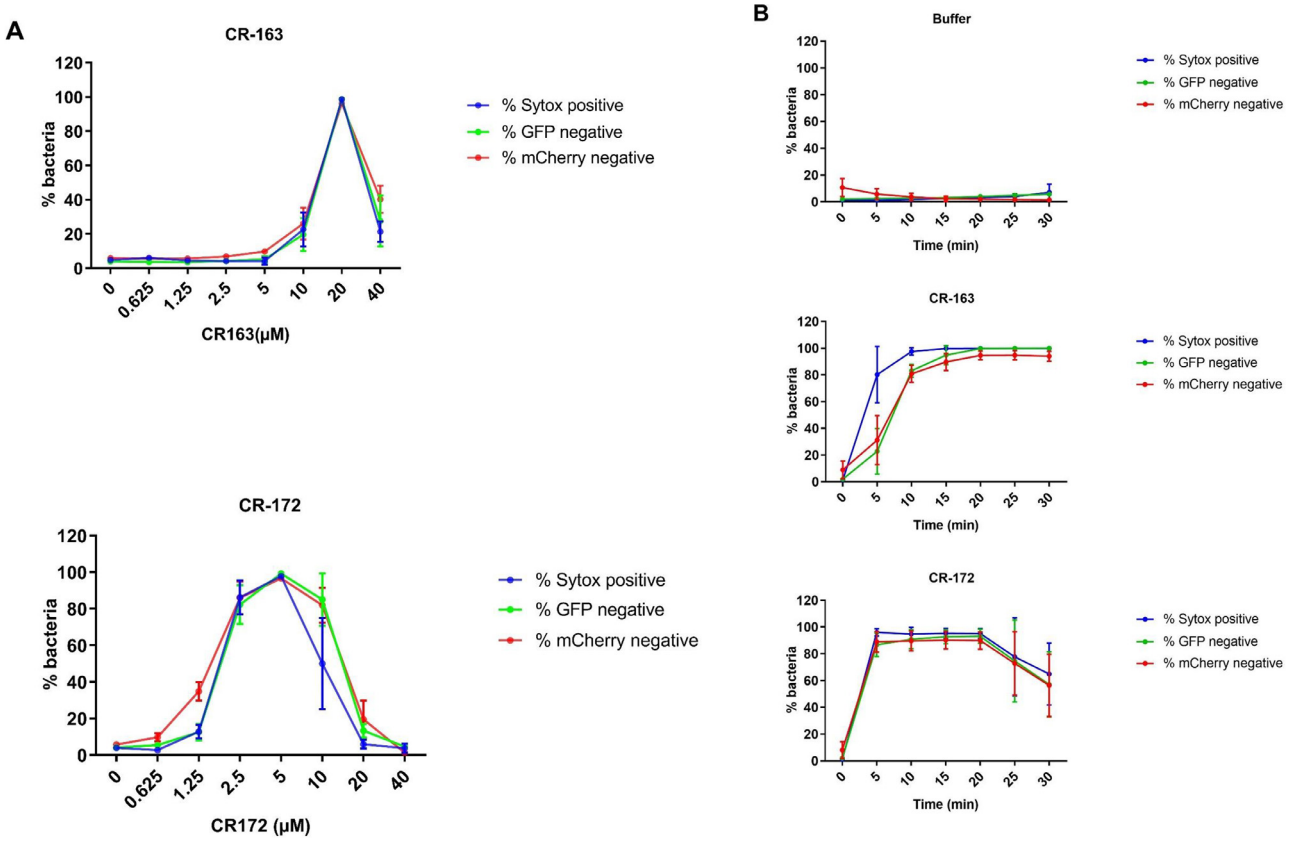

Fig. 4. PepBiotics permeabilize bacterial membranes of *Escherichia coli* at their MBC value. A) PerimCherry/cytoGFP *E. coli*, a strain expressing mCherry in the periplasm and Green Fluorescent Protein (GFP) in the cytoplasm, was used to assess outer membrane (OM) and inner membrane (IM) damage. Bacteria were incubated with a concentration range of CR-163 or CR-172 and after 30 min, mCherry release, GFP release, and influx of Sytox were measured using FACS. Shown are mean ± SD of three independent experiments. B) Kinetics of GFP and mCherry release and Sytox influx were measured at the MBC of CR-163 and CR-172. Shown are mean ± SD of three independent experiments.

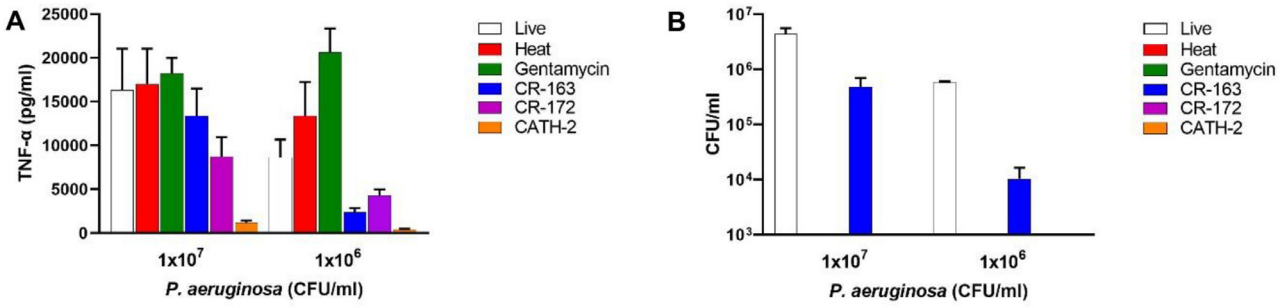

Fig. 5. PepBiotics kill *Pseudomonas aeruginosa* with limited macrophage activation. *Pseudomonas aeruginosa* was treated with gentamicin, heat treatment, CATH-2 (20 μM, control), CR-163 (20 μM), or CR-172 (20 μM) and subsequently added to RAW264.7 macrophages. A) Production of TNF-α was measured using ELISA after 2 h of treatment, as indicated. B) Viability of *P. aeruginosa* after treatment was checked by plating on TSA plates. Shown are mean ± SEM of three independent experiments.

When macrophages were exposed to whole bacteria, PepBiotics were able to reduce pro-inflammatory cytokine production, unlike the conventional antibiotic gentamicin or heat-treatment of bacteria. This dual LPS neutralizing and antibacterial effect is not uncommon among antimicrobial compounds [33,34], and leads to the phenomenon of silent killing [18,19]. At higher bacterial densities, this effect was moderate when compared with CATH-2, which is very effective at silent killing. How great the actual silent killing effects of PepBiotics might be in vivo, and their contribution to clearance of infection without collateral tissue damage, may therefore be dependent on the ratio of PepBiotics to bacterial density at the place of infection. Many more AMPs can neutralize the immune response against dead bacteria, even if they are not very active in killing bacteria themselves. An example is the human cathelicidin LL-37, which is capable of neutralizing LPS of non-viable bacteria but is not a strong killer under physiological conditions [19]. An AMP could act as a sensor that detects if a bacterium is viable and subsequently neutralizes an unnecessary immune response if the bacterium has been killed by other components of the immune system.

LPS binding of PepBiotics suggested that the antibacterial activity will have a membranolytic origin. Most naturally occurring AMPs act on bacterial membranes, permeabilizing them through pore formation, or by destabilizing the membranes to such an extent that bacteria lose their integrity [35,36]. Only for a limited group of AMPs, most notably the proline-arginine–rich peptides, such as mammalian peptides PR-39, BAC5, and BAC7, and insect AMPs, such as apidaecins [37,38], have intracellular targets been defined, often leading to inhibition of protein synthesis of the bacterium. Exposure of CR-163 and CR-172 to *E. coli* resulted in a rapid leakage of periplasmic and cytoplasmic bacterial content. Similarly, as measured by Sytox influx, *P. aeruginosa* were permeabilized quickly, indicating that it is not a bacterial strain-specific antimicrobial mechanism of PepBiotics.

Interestingly, the kinetics of permeabilization showed that, for CR-172, IM and OM permeabilization leading to the leakage of (intra)cellular content occurred almost at the same time. For CR-163, IM destabilization preceded the leakage of proteins, suggesting that, at least for this PepBiotic, it was a sequential mechanism; the PepBiotic first permeabilized the OM, which led to interactions between CR-163 and the IM. It is likely that this stepwise mechanism of action is also happening with CR-172, but the kinetics are too fast for us to distinguish between IM and OM permeabilization with our current experimental set-up. This indicates that, even though PepBiotics likely bind LPS first (because it is exposed on the outer leaflet of the OM of *E. coli*), it can quickly reach and permeabilize the IM. Although binding affinities to IM phospholipids for PepBiotics were not determined, it is likely that these are relatively strong. For CR-163, it was shown, using vesicles composed of phosphatidylcholine (PC) and phosphatidylglycerol (PG) in isothermal titration calorimetry and static ^{31}P solid-state NMR studies, that the negatively charged PG molecule interacted with the peptide while PC did not contribute to binding [15]. Overall, the cationic and amphipathic characteristics of PepBiotics seem to be ideally suited to interact and disrupt both OM and IM of Gram-negative bacteria.

The cost of producing peptides for therapeutic use is still considered an important hurdle that needs to be overcome. Indeed, compared with regular antibiotics, AMPs have high production costs, which may limit cost-effective production of AMP-based drugs. However, the price of chemical solid-phase–synthesis of peptides has dropped dramatically; also, recombinant production of AMPs is increasingly successful. These new developments in the production of AMPs were recently nicely reviewed [39]. The fact that production costs are seen as less as a problem is also reflected in the number of clinical trials that are currently performed with AMP-based drugs [40]. Most of these are for topical use against infections, and therefore would require small amounts of peptide; however, it is clear that AMPs, or peptide-based drugs, are a serious option for the treatment of microbial infections.

In conclusion, this study extends our knowledge of the functional properties of CR-163 and CR-172, and now includes: (i) broad-spectrum antimicrobial activity against ESKAPE pathogens, (ii) LPS binding characteristics, and (iii) the ability to silence LPS-induced immune responses after killing bacteria. These characteristics will expand the possibilities for the use of PepBiotics as alternatives to common antibiotics.

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Ethical approval

Not required

Competing interests

None declared

Supplementary materials

Supplementary material associated with this article can be found, in the online version, at doi:10.1016/j.jgar.2022.07.009.

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