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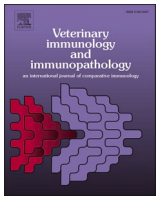

Research paper

The cathelicidin CATH-2 efficiently neutralizes LPS- and *E. coli*-induced activation of porcine bone marrow derived macrophages

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ABSTRACT

Infectious diseases in pigs cause monetary loss to farmers and pose a zoonotic risk. Therefore, it is important to obtain more porcine specific immunological knowledge as a measure to protect against infectious diseases, for example by exploring immunomodulators that are usable as vaccine adjuvants. Cathelicidins are a class of host defence peptides (HDPs) able to directly kill microbes as well as exert a diverse range of effects on the immune system. The peptides have shown promise as immunomodulatory peptides in many applications, including vaccines. However, it is currently unknown what the precise effect of these peptides is on porcine immune cells and whether peptides of other species might also have a strong immunomodulatory effect on porcine macrophages. Mononuclear bone marrow cells of pigs, aged 5–6 months, were cultured into M1 or M2 macrophages and stimulated with LPS or whole bacteria in the presence of host defence peptides (HDPs).

CATH-2 and LL-37 strongly inhibited LPS-induced activation of M1 macrophages, the inhibition of LPS-induced activation of M2 macrophages by HDPs was milder, showing that the peptides have selective effects on different cell types. Upon stimulation with whole bacteria, only CATH-2 could effectively inhibit macrophage activation, showing the potent anti-inflammatory potential of this peptide. These results show that porcine peptides are not necessarily the most active in a porcine system, and that CATH-2 is effective in a porcine system as an anti-inflammatory immune modulator, which can be used, for example, in inactivated pathogen vaccines.

1. Introduction

In 2019, around 850 million pigs were kept in farming worldwide (FAOstat, 2019). Considering the threats to public health of both zoonotic disease and antibiotic resistance, there is a clear need for alternatives to antibiotics in livestock farming (Aarestrup, 2012; Duarte et al., 2021; Luiken et al., 2020). Since it is better to prevent than to cure, vaccination is the most effective method to reduce antibiotics use. However, not all porcine pathogens have broadly effective vaccines available. For instance, *Streptococcus suis*, while being one of the globally most damaging porcine pathogens, currently has no commonly available vaccine (Hernandez-Garcia et al., 2017; Segura et al., 2020, 2017).

While traditionally effective vaccines consist of live attenuated or inactivated pathogens, many modern experimental vaccines are based on subunit technology or DNA/RNA injection. Many of these have shown great efficacy in various animals and humans, for instance in the case of nucleotide injection for COVID-19 vaccines (Corbett et al., 2020; Polack et al., 2020), subunit vaccine for porcine circovirus 2 (PCV2) (Grau-Roma et al., 2011) or acellular vaccination for classical swine fever (Madera et al., 2016). However, many subunit vaccines lack the innate reactogenicity of whole cell or attenuated formulations. These, however, can in turn be too reactogenic and cause systemic inflammatory side effects (Coffman et al., 2010; Nanishi et al., 2020). For these reasons, vaccines can benefit greatly from a diverse selection of immune modulators to fine-tune the immune response. Cathelicidins are a promising option as immune modulator during vaccination.

Cathelicidins are host defence peptides (HDPs) from the vertebrate innate immune system, abundantly present in skin, respiratory and digestive tracts. These HDPs are constitutively expressed at low levels by epithelial cells and released in high concentrations by degranulation of

Abbreviations: TLR, Toll-like receptor; pDC, plasmacytoid dendritic cell; NFκB, nuclear factor κB; BMDM, bone marrow derived macrophage; PCV2, porcine circovirus 2; RPMI-1640, Roswell Park Memorial Institute medium 1640; MHB, Mueller Hinton broth; TSA, tryptic soy agar; BSA, bovine serum albumin; ELISA, enzyme linked immunosorbent assay; FSC, forward scatter; SSC, side scatter.

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Table 1
Peptides' sequence and length/charge.

Peptide	Sequence	Length	Charge
CATH-2	RFGRFLRKIRRFRPKVTITIQGSARF-NH_2	26	+9
PMAP-23	RIIDLLWRVRRPQKPKFVTVWVR- NH_2	23	+6
LL-37	LLGDFFRKSKEKIGKEFKRIVQRIKDFLRNLVPRTES- NH_2	37	+6
PR-39	RRRPRPPYLPRPRPPPFFPPRLPPRIPPGFPPRFPPRFP- NH_2	39	+10

immune cells in response to microbes or injury (Hancock et al., 2016; Scheenstra et al., 2020; van Harten et al., 2018). HDPs function as direct antimicrobials and can modulate the immune system, which includes up- or downregulating of immune related genes, acting as opsonizing or chemoattracting agents, and neutralizing inflammatory signals (Hancock et al., 2016; van Harten et al., 2018). HDPs have been investigated successfully as vaccine components in the past (Garlapati et al., 2011; Kovacs-Nolan et al., 2009; Mookherjee et al., 2020; Prysliak et al., 2017; Schulze et al., 2017; Yu et al., 2016), but not yet in pigs. Moreover, not much is known yet about HDP effects on porcine immune cells.

HDPs have been studied for immunomodulatory functions in multiple other species (Coorens et al., 2017a). The chicken cathelicidin CATH-2 has a strong affinity for LPS and inhibits Toll like receptor-4 (TLR-4) activation in mouse and human cells (Scheenstra et al., 2019; van Dijk et al., 2009, 2016a). Additionally, this cathelicidins can enhance uptake of nucleic acids and thereby enhance TLR-9 signalling in mouse macrophages (Coorens et al., 2015) and porcine plasmacytoid dendritic cells (pDCs) (Baumann et al., 2014). When administered *in ovo*, the all-D-analog of CATH-2 reduced mortality and infection morbidity in chickens from *E. coli* infection up to 7 days after hatch (Cuperus et al., 2016). This indicates an antimicrobial effect that persists beyond the longevity of the HDP itself and suggest that HDPs modulate the immune response. Although HDPs are natural effectors of the immune system, the immune modulatory functions of HDPs act cross-species (Baumann et al., 2017; Coorens et al., 2017a; Pelillo et al., 2014; Scheenstra et al., 2019; Schneider et al., 2016).

Eleven cathelicidins have been identified in pigs, in contrast to humans where only one cathelicidin, LL-37 has been identified. However, only very little is known about the effect of cathelicidins in pigs or on porcine cells. Porcine cathelicidins PMAP-23, PMAP-36 and PR-39 were shown to enhance nucleotide uptake and subsequent activation of porcine pDCs (Baumann et al., 2014). PR-39 induces IL-8 and to a lower extent TNFα expression by the porcine alveolar macrophage cell line 3D4/31 but does not inhibit LPS-induced activation like many other cathelicidins. In porcine 3D4/31 cells, PMAP-23 could only induce IL-8 production (Veldhuizen et al., 2014, 2017).

In this study, we aim to investigate the effects of HDPs on primary bone marrow derived porcine macrophages. Four HDPs were selected based on their described immunomodulatory effects: chicken CATH-2, human LL-37 and porcine PMAP-23 and PR-39, (Baumann et al., 2014; Coorens et al., 2017a, b; Cuperus et al., 2016; Delfino et al., 2004; Kandler et al., 2006; Mookherjee et al., 2006a, b; Schneider et al., 2016; Van Dijk et al., 2016b; Veldhuizen et al., 2014, 2017). The results indicate that especially CATH-2 shows strong anti-inflammatory capacities in porcine cells, both after LPS-induced activation as well as activation with live *E. coli*. Therefore, CATH-2 could potentially be used in bacterial vaccines where overstimulation of immune cells is problematic.

2. Materials and methods

2.1. Peptides

All peptides were synthesized by Fmoc chemistry on a chemical synthesizer (China peptides, Shanghai, China), and C-terminally amidated to improve stability (Table 1). Purity was >95 % for each peptide. After lyophilization, the peptides were reconstituted in MilliQ water and further diluted in appropriate culture medium.

2.2. Primary cell isolation, culture, and stimulation

Porcine bone marrow derived macrophages were isolated as previously described (Gao et al., 2018) from bone marrow of 16 pigs in total, either Great Yorkshire, Large White or F1 of Large White and Nordic Landrace-, aged 5–6 months (70−80 kg) (van Beek SPF Varkens, Lelystad, The Netherlands). The animals are raised as SPF experimental animals, but not housed behind a barrier after arrival. The animals were allowed to acclimatize between 7 and 20 days, before being put under terminal anaesthesia. The animals were then used as educational models for complex surgical techniques for medical doctors and operating room staff. After training, bone marrow was harvested by hip bone puncture by a trained veterinarian. All animals were used and kept under licence of the Central Laboratory Animal Committee of the Netherlands under the advice and guidelines of the animal ethical committee of Utrecht University. Mononuclear cells from bone marrow were isolated by Ficoll (GE Healthcare, Chicago, Illinois) density centrifugation. Mononuclear cells were seeded at a density of 5×10^4 cells per well in a 96-wells plate, then cells from the same individual were cultured into M1- or M2-macrophages by exposure to 40 ng/mL recombinant porcine GM-CSF (BioTechne, Minneapolis, MN) or 30 ng/mL recombinant murine M-CSF (Peprotech, Rocky Hill, NJ), respectively in RPMI-1640 with 10 % FCS and 100 U/mL penicillin/streptomycin (RPMI+/+) for 6 days. Then, the cells were stimulated with 100 ng/mL of LPS-EB from *E. coli* (Invivogen, San Diego, CA) for 24 h. When peptides were included, peptides were pre-mixed with LPS before addition to the cells. Supernatant was isolated and stored at −20 °C for ELISA, and cells were detached with 0.5 mM EDTA in PBS, then analysed by flow cytometry.

2.3. Bacterial stimulation

E. coli ATCC 25922 was grown overnight in Mueller Hinton Broth (MHB) medium at 37 °C, then inoculated in 10 mL fresh MHB and grown to mid-log phase for 2 h at 37 °C on an orbital shaker. Bacterial density was measured at 620 nm and bacteria were diluted to 2.0×10^6 CFU/mL. Bacteria were killed by either 250 μM peptides, with 100 U/mL of penicillin/100 μg/mL streptomycin for 3 h at 37 °C or by heating to 95 °C for 1 h. Live bacteria used were kept on ice until stimulation. Efficiency of the different bacterial killing methods, as well as quantification of the viable bacteria used in the stimulation experiments were verified by plating out on TSA plates.

After killing, the bacterial solutions were pre-mixed with peptides with the exception of peptide-killed bacteria (as they already had peptides in solution), and added to macrophages in 1:100 dilution, leading to a final concentration of 2.5 μM peptides and/or an MOI of 2. After 2 h of stimulation, supernatant containing the bacteria was removed and the cells were washed with RPMI+/+ and incubated a further 22 h. Then, the cells and supernatant were harvested for analysis by flow cytometry and ELISA respectively.

2.4. Flow cytometry

Macrophages were detached by vigorous pipetting after a 5 min incubation with 0.5 mM EDTA in PBS at 37 °C. After detachment, the cells were washed and kept in 0.5 % w/v BSA in PBS on ice. After washing,

the cells were stained for 20 min in the dark. The following antibodies were used: anti CD163-FITC mouse IgG2, anti-human CD14-PB (Bio-Rad, USA), aSWC3a-PE (Invitrogen, USA), recombinant CTLA-4-MuIgG-APC (Ancell, USA) which binds to the CD80/86 complex. Measurements were acquired with a FACS Canto II (BD Biosciences, Franklin Lakes, NJ). Analysis was performed with FlowJo v10 (BD Biosciences).

2.5. ELISAs

ELISAs were performed with porcine specific kits for TNFα, IL-10, IL-1β and IL-8 (R&D Systems, Minneapolis, MN) according to the manufacturer's recommendations.

2.6. Statistics

Statistical analyses were performed using Prism 8 (GraphPad, San Diego, CA), being paired T tests, mixed-model analysis with Tukey's correction for multiple comparisons or Friedman tests with Dunn's correction for multiple comparisons. Results were considered significant if $p < 0.05$ compared to the relevant control.

3. Results

3.1. Porcine bone marrow-derived M1 macrophages strongly increase their cytokine secretion upon LPS stimulation

For this study, bone marrow cells were cultured into M1 macrophages as was previously set up and validated by our group (Gao et al., 2018). The cells were analysed using flow cytometry at day 7 of culture. Macrophages were first gated based on size (forward scatter (FSC)) and granularity (side scatter (SSC)) (Fig. 1A), followed by the expression of the porcine myeloid cell specific marker SWC3α (Fig. 1B). The macrophages were a homogeneous culture, expressing CD163 (Fig. 1C) and CD80/86 (Fig. 1E), and high levels of CD14 (Fig. 1F). Most markers only slightly changed upon LPS stimulation (Fig. 1C–F). One individual showed a potent increase in CD80/86 expression, whereas the increase for the other individuals was less pronounced. LPS stimulation significantly reduced CD14 expression in these cells (Fig. 1F).

In contrast to the marker expression, cytokine expression was strongly induced upon LPS-stimulation. The M1 macrophages produced 10−1000-fold increased amounts of TNFα, IL-10, IL-1β and IL-8 compared to unstimulated controls (Fig. 1G–J).

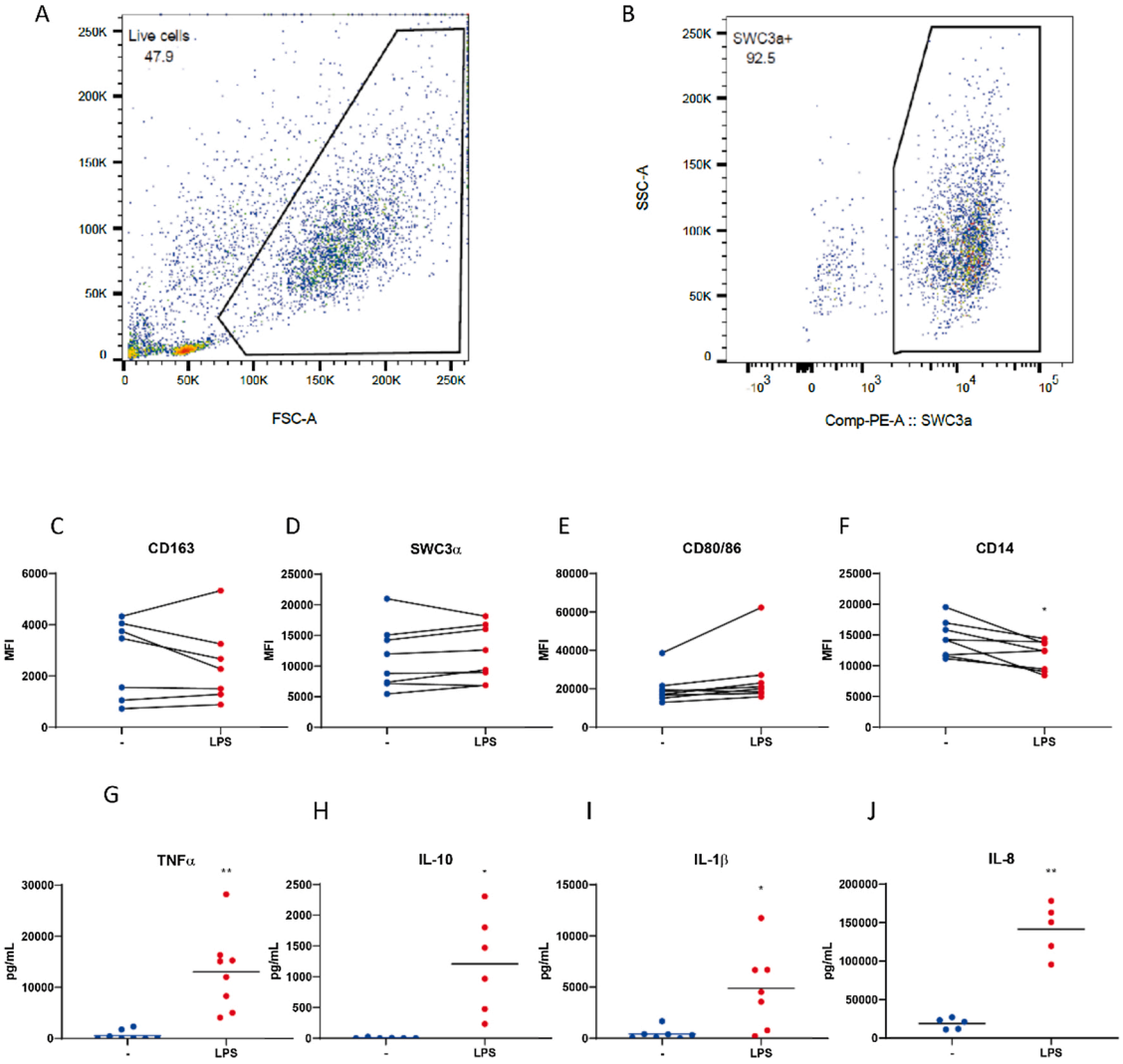

Fig. 1. Porcine SWC3α^{+} M1 macrophages are CD163 and CD14 positive, and express large amounts of cytokines upon LPS stimulation. Porcine M1 macrophages are gated on FSC/SSC profile (**A**), then selected for the myeloid marker SWC3α (**B**). **C-F** The change in surface marker expression upon LPS stimulation was measured by the median fluorescence intensity (MFI) of cells within the SWC3α+ gate. **G-J** The levels of cytokines TNFα, IL-10, IL-1β and IL-8 was measured using ELISA. Individual pigs (N = 3-6) are depicted in the graphs and paired T-test was used for statistical analysis (* = $p < 0.05$, ** = $p < 0.01$, *** = $p<0.001$).

3.2. CATH-2, LL-37, and PMAP-23 inhibit LPS-induced stimulation of porcine M1 macrophages

The immunomodulatory properties of chicken CATH-2, porcine PMAP-23, human LL-37, and porcine PR-39 were tested. None of the peptides alone could induce significant changes on cytokine production or marker expression (Fig. S1). Since pigs are genetically diverse animals, the interindividual variation in marker expression and cytokine production was substantial (Figs. 1 and S1). Therefore, the data showing immunomodulatory capacity by the peptides was normalized to the LPS-stimulated condition of each individual control.

With respect to the cell marker expression, stimulation of M1 macrophages with LPS in combination with LL-37 caused a dose-dependent decrease of CD163 expression compared to LPS alone (Fig. 2A), not seen

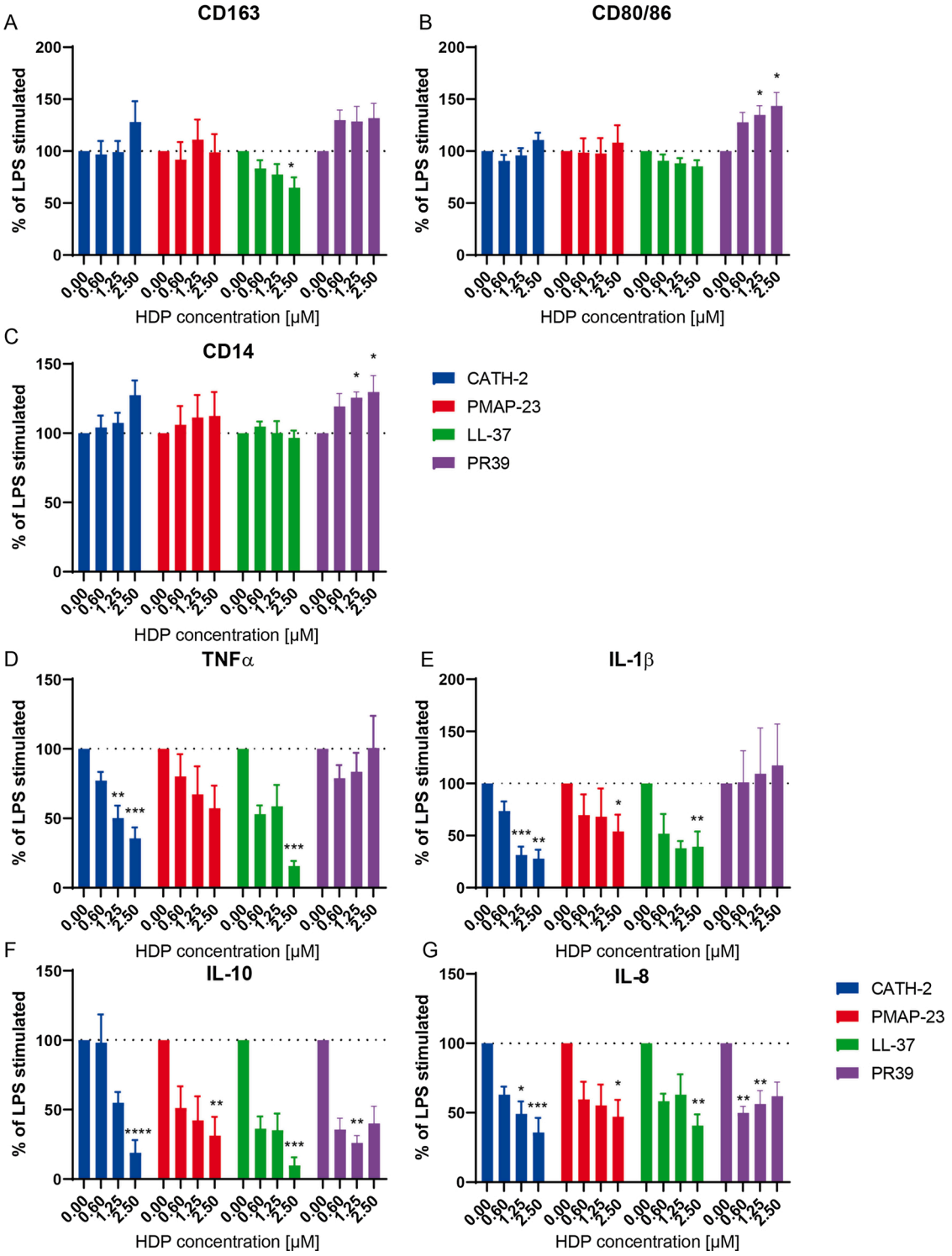

Fig. 2. CATH-2, LL-37, and PMAP-23 inhibit LPS-induced stimulation of porcine M1 macrophages. M1 macrophages were stimulated with 100 ng/mL LPS in the presence and absence of HDPs. Changes in surface markers CD163, CD80/86 or CD14 (A-C) and cytokine expression of TNFα, IL-1β, IL-10 and IL-8 (D-G) were determined and normalized to the LPS-only control of that individual pig. Significant difference to the control was calculated by Friedman test with Dunn's correction for multiple comparison (* = $p < 0.05$, ** = $p < 0.01$, *** = $p<0.001$, **** = $p<0.0001$). (N = 5-7 individuals, results are shown as mean ± SEM).

for any of the other HDPs tested. LPS-stimulation together with PR-39 caused a significant increase of CD80/86 (Fig. 2B) and CD14 expression (Fig. 2C). PMAP-23 and CATH-2 showed a tendency towards higher surface marker expression for CD163 and CD14, but this did not reach statistical significance.

The effect of the peptides on LPS-induced cytokine expression was much stronger compared to the relative smaller differences in surface marker expression. LL-37, CATH-2, and, to a lesser extent PMAP-23, strongly inhibited the LPS-induced activation of M1 macrophages, demonstrated by a marked dose dependent decrease for all tested cytokines. For example, 2.5 μM CATH-2 reduced the LPS-induced TNFα production to 35 %, while 2.5 μM LL-37 even reduced it to 15 % of the original expression (Fig. 2D). PR39 strongly inhibited the expression of IL-10 (to 40 %) and IL-8 (to 62 %), but the expression of TNFα and IL-1β was unaffected by the peptide. This indicates a different mode of action of PR39 compared to CATH-2, LL-37, and PMAP-23 (Fig. 2A–G).

3.3. HDPs inhibit LPS induced activation in M2 macrophages

CD163 is reduced in both human (van der Does et al., 2010) and porcine M1 macrophages (Fig. 2A) after exposure to LL-37. Since anti-inflammatory M2 macrophages are characterized by a higher CD163 expression (Gao et al., 2018), the effect of the peptides was also tested on porcine M2 macrophages but, contrary to M1 macrophages, none of the peptides affected the CD163 expression (Fig. 3A). In contrast, LL-37 reduced CD80/86 expression by 25 % at 2.5 μM (Fig. 3B). CD14 expression was also much higher in LPS stimulated M2 macrophages when co-stimulated with LL-37 and CATH-2, with the latter even doubling the average MFI (Fig. 3C).

Next, when observing cytokine expression in M2 macrophages, the LPS induced cytokine expression was reduced by the peptides. M2 macrophages are characterized by a high production of IL-10 (Gao et al., 2018) (Fig. S2). CATH-2 and LL-37 significantly inhibited LPS-induced TNFα production with 50 %, although only at the highest HDP concentration tested (2.5 μM) (Fig. 3D). For PMAP-23 a small increase of the TNFα production was observed for some individuals, whereas in other individuals, expression was not changed. PR-39 had no effect on TNFα release. With respect to IL-10 expression only CATH-2 seemed to inhibit at 2.5 μM, but this failed to reach significance (Fig. 3E). Overall, no significant peptide effects were observed in IL-1β release in M2 macrophages (Fig. 3F). Finally, addition of 2.5 μM PMAP-23 or PR-39 significantly increased the LPS-induced IL-8 secretion by 132 % and 84 % on average, respectively (Fig. 3G). Taken together, the inhibitory effect of the peptides on M2 macrophages are present, but less pronounced than in M1 macrophages which often showed a larger relative reduction of cytokine production.

3.4. CATH-2 inhibits M1 inflammation in whole bacterial cell stimulation as well as LPS stimulation

Since whole cell vaccines contain, besides LPS (for Gram-negative bacteria), a large mixture of other immune stimulatory components, we investigated the effect of *E. coli* stimulation of macrophages in the presence of peptides. Additionally, since HDPs can kill microbes directly, we assessed the effect of stimulation of macrophages with *E. coli* killed by HDPs, as well as by other methods such as heat treatment or incubation with antibiotics. Viable *E. coli* efficiently stimulated the M1 macrophages, as was shown by increased (15,000 pg/mL) TNFα secretion compared to the unstimulated control (800 pg/mL; Fig. 4A). Using the same initial bacterial density, non-viable *E. coli* (both heat-killed, and penicillin/streptomycin killed) also significantly induced TNFα production compared to the non-stimulated control, although to a somewhat lower level than viable *E. coli.*

In contrast to heat and antibiotic treatment, addition of 2.5 μM CATH-2 together with live *E. coli*, also rendering the bacteria non-viable, reduced TNFα levels almost to non-stimulated levels. PMAP-23 had no effect on bacterially induced TNFα production, while incubation of viable *E. coli* with LL-37 led to a slight induction in TNFα production (Fig. 4A).

Next, we wanted to know if peptides could similarly affect the activation of M1 macrophages by non-viable bacteria, since whole cell vaccines often contain inactivated microbes. Therefore, we incubated the macrophages with the heat and penicillin/streptomycin killed bacteria in the presence of CATH-2, PMAP-23, and LL-37. The already lower effect of heat-killed bacteria on TNFα levels could not be further reduced (Fig. 4B). Basically, when CATH-2 was present, TNFα levels were similar irrespective of the killing method of the bacteria. Contrary to the immunomodulatory effects observed for CATH-2, LL-37 was not effective in reducing TNFα induction by any of the tested bacterial viability states and showed an increase to almost 2000 pg/mL, when LL-37 was added together with live bacteria (Fig. 4C). Additionally, no effect on TNFα production was observed when LL-37 was added to non-viable (heat or antibiotic treated) bacteria showing a similar TNFα levels as bacteria killed by antibiotics alone. Finally, PMAP-23 showed no inhibitory effects on bacterial stimulation of M1 macrophages irrespective of the viability of the bacteria or the method of killing of the non-viable E. *coli* (Fig. 4D).

4. Discussion

In the past, inactivated bacterial vaccines have led to good protection but they possess a risk of overactivation of the immune response as compared to subunit vaccines (Nanishi et al., 2020). On the other hand, subunit vaccines can result in possibly too little activation, and are prone to weaning protection, as has happened in the past with the vaccine for whooping cough (Gilberg et al., 2002; Mooi et al., 2001). In this case, overactivation caused by either whole cell vaccines or bacterial outer membrane vesicles could be tempered by formulation with HDPs (Balhuizen et al., 2021). Similarly, experimental vaccines for pigs could benefit from immunomodulatory peptides such as CATH-2.

Many porcine immune cell experiments are performed with alveolar macrophages, either directly isolated from pigs, or with the alveolar macrophage-derived cell line 3D4/31. However, alveolar macrophages do not produce NO in response to stimulation (Zelnickova et al., 2008), indicating that these cells might not be ideal for *in vitro* experiments. Therefore, a bone marrow-derived macrophage, culture system was recently set up by our group (Gao et al., 2018). These cells were responsive to stimulation by bacterial components, especially on the level of cytokine expression. The cells could be polarized into M1 and M2 macrophages, which responded comparable to human M1 and M2 macrophages (Fleetwood et al., 2007; Gao et al., 2018; van der Does et al., 2010). Additionally, bone marrow mononuclear cells can be easily cryopreserved, and large quantities can be stored from one individual. However, compared to cell lines, the variability in this system is higher. This is due to genetic variance in the outbred individuals, which makes the obtained results better translatable to the whole population, as genetic variability is present there, too.

Little is known about HDP activity in pigs. Previously obtained results focused particularly on porcine cathelicidins (Holani et al., 2016; Scheenstra et al., 2019; Veldhuizen et al., 2017) and porcine pDCs (Baumann et al., 2014). However, cathelicidins are known for broad activity across species barriers (Coorens et al., 2017a; Hancock et al., 2016; van Harten et al., 2018). It is therefore important to assess in depth the effect of HDPs without being limited to the target species' native HDPs. Here, we assessed CATH-2 and LL-37 alongside PMAP-23 and PR-39 in a porcine macrophage system. Surprisingly, the porcine peptides had the lowest immunomodulatory effect, despite them being part of the natural innate immune system of pigs. It must be noted that not all pig HDPs were tested here, as pigs express 11 different cathelicidins in addition to a broad repertoire of defensins (Sang and Blecha, 2009). In this work, CATH-2 actually functioned as the most potent anti-inflammatory HDP for pig cells, despite the fact that CATH-2 is a

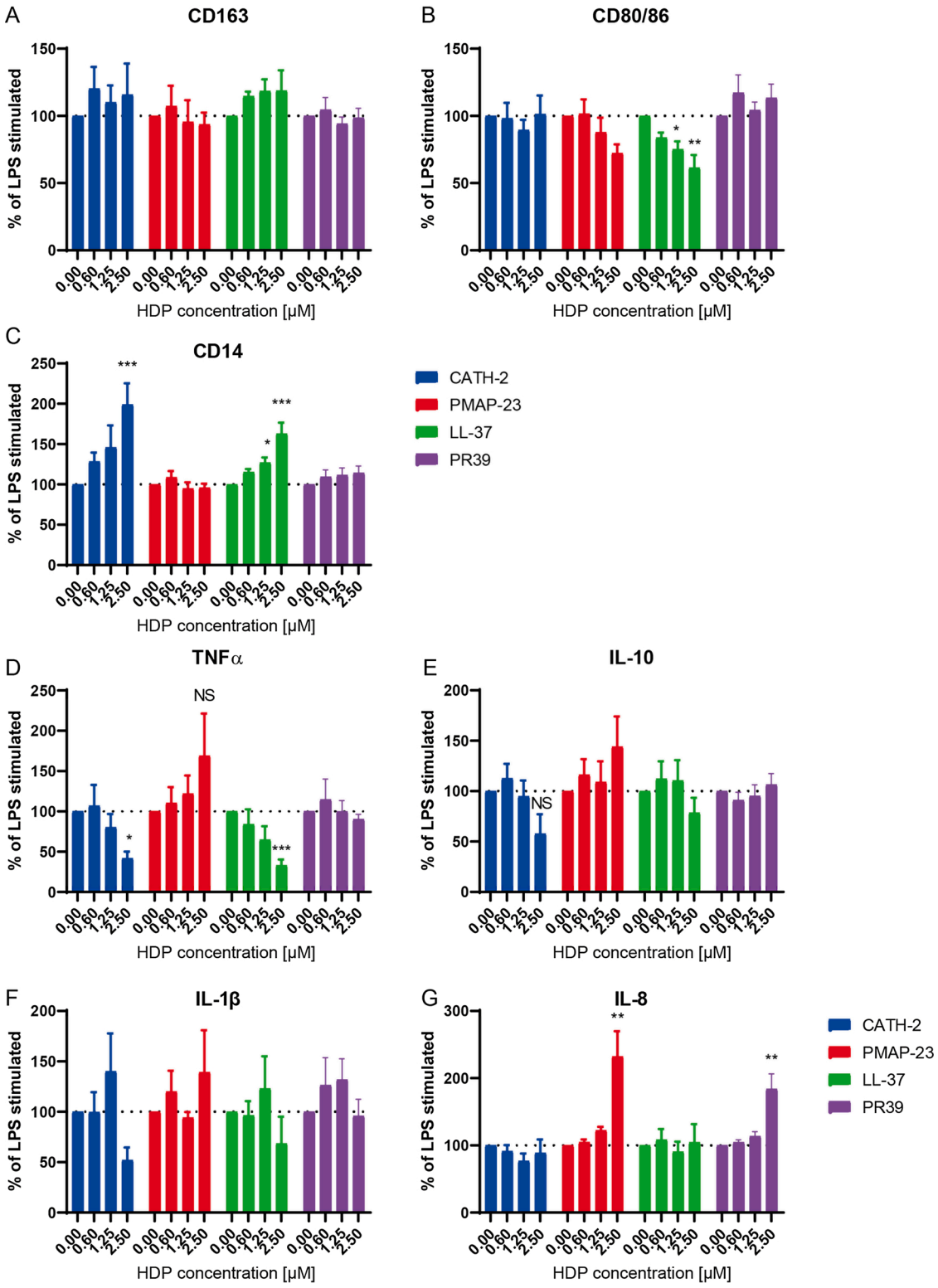

Fig. 3. HDPs show mild inhibition of LPS-induced stimulation of porcine M2 macrophages. M2 macrophages were stimulated with 100 ng/mL LPS in the presence and absence of HDPs. Changes in surface markers CD163(A), CD80/86(B) or CD14(C) and cytokine expression of TNFα(D), IL-1β(E), IL-10(F) and IL-8(G) were determined and normalized to the LPS-only control of that individual pig. Surface marker and cytokine expression levels are normalized to the LPS-only control of that individual pig. Significance was calculated by Friedman test of the raw data with Dunn's correction for multiple comparisons (* = $p < 0.05$, ** = $p < 0.01$, *** = $p<0.001$, **** = $p<0.0001$). (N = 5-7, results are shown as mean ± SEM).

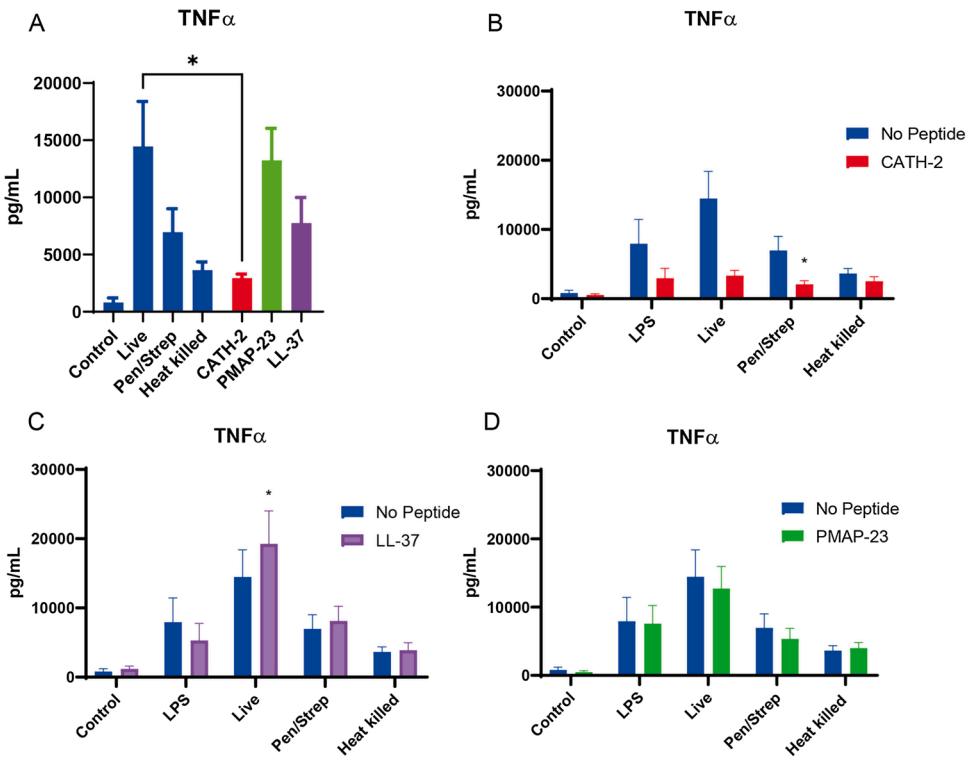

Fig. 4. CATH-2 can efficiently inhibit macrophage stimulation by viable and non-viable bacteria. A. Porcine M1 macrophages were stimulated with LPS or bacteria, either live or killed by heat, antibiotics, or HDPs CATH-2, PMAP-23, and LL-37, then added to porcine M1 macrophages. **B–D** Bacteria or LPS were mixed with peptide or control in various viability states, then TNFα production of M1 macrophages was measured. Significance was calculated by Friedman test with Dunn's correction for multiple comparisons (* = $p < 0.05$, ** = $p < 0.01$, *** = $p<0.001$, **** = $p<0.0001$). (N = 4; results are shown as mean ± SEM).

chicken cathelicidin.

LPS binding and subsequent steric hindrance of TLR-4 binding and activation could be the main mechanism for peptide mediated neutralization. For example, CATH-2 is predicted to bind to LPS in a receptor-blocking manner. Truncations of CATH-2, that are too short to reach this part of LPS, are not able to inhibit LPS activation (van Dijk et al., 2016a). In this study, we found that CATH-2 was able to efficiently inhibit activation of macrophages by whole bacteria as well as LPS separately. Therefore, the modulation of effects that were observed for CATH-2 could be broader than only steric hindrance of TLR-4 activation.

Interestingly, in previous studies, LL-37 has been a better inhibitor of LPS activation of immune cells than CATH-2 (Coorens et al., 2017a; Scheenstra et al., 2019). This was shown by LL-37 having inhibitory effects at lower concentrations, while actually having lower affinity for LPS. However, in whole bacterial cell stimulation, LL-37 does not potently inhibit macrophage responses, especially in the presence of live bacteria. However, when macrophages were stimulated using dead bacteria, LL-37 is again a potent inhibitor. This indicates that although peptides can have similar inhibition profiles using LPS only, the viability state of bacteria is important to predict the immunomodulating effect of HDPs *in vivo*. In line with this: the immunomodulating effect of HDPs in vaccine preparations is likely very different depending on the type of vaccine (subunit, inactivated or attenuated) used, underlining the importance of studying immunomodulatory effects of HDPs in different conditions.

Surprisingly, the inhibitory effects of peptides did not always extend to all cytokines. For example, where CATH-2 had potent inhibitory effects on LPS-induced cytokine production in M1 cells, IL-8 production in M2 macrophages was not affected upon LPS stimulation. Likewise, where PR-39 inhibits IL-10 and IL-8 production in M1 macrophages, it has no effect on IL-1β release or TNFα production. Peptides evidently have differing ways of skewing inflammation, either by inhibiting specific cytokines or activation in general. It is therefore essential to thoroughly investigate peptide effects in a particular system.

In conclusion, HDPs LL-37 and CATH-2 inhibit LPS-induced activation of porcine primary bone marrow derived macrophages. HDPs affect both M1 and M2 macrophages, although the inhibition is more effective for M1 macrophages. Only CATH-2 shows inhibition of live bacterial activation, which makes CATH-2 the most interesting candidate to add in the formulation of whole cell vaccines.

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Declaration of Competing Interest

None.

Appendix A. Supplementary data

Supplementary material related to this article can be found, in the online version, at doi:https://doi.org/10.1016/j.vetimm.2021.110369.

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