Interspecies cathelicidin comparison reveals divergence in antimicrobial activity, TLR modulation, chemokine induction and regulation of phagocytosis

Maarten Coorens*, Maaike R. Scheenstra*, Edwin J. A. Veldhuizen & Henk P. Haagsman

Cathelicidins are short cationic peptides initially described as antimicrobial peptides, which can also modulate the immune system. Because most findings have been described in the context of human LL-37 or murine CRAMP, or have been investigated under varying conditions, it is unclear which functions are cathelicidin specific and which functions are general cathelicidin properties. This study compares 12 cathelicidins from 6 species under standardized conditions to better understand the conservation of cathelicidin functions. Most tested cathelicidins had strong antimicrobial activity against *E. coli* and/or MRSA. Interestingly, while more physiological culture conditions limit the antimicrobial activity of almost all cathelicidins against *E. coli*, activity against MRSA is enhanced. Seven out of 12 cathelicidins were able to neutralize LPS and another 7 cathelicidins were able to neutralize LTA; however, there was no correlation found with LPS neutralization. In contrast, only 4 cathelicidins enhanced DNA-induced TLR9 activation. In conclusion, these results provide new insight in the functional differences of cathelicidins both within and between species. In addition, these results underline the importance not to generalize cathelicidin functions and indicates that caution should be taken in extrapolating results from LL-37- or CRAMP-related studies to other animal settings.

Cathelicidins are cationic peptides with an important function in the early vertebrate host response against invading pathogens. They are secreted at mucosal surfaces and, during infection, by leukocytes and epithelial cells upon interaction with microbes. Cathelicidins have both direct antimicrobial activity as well as immunomodulatory functions. The importance of cathelicidins in innate host defense has been demonstrated in mice lacking cathelicidin expression. These mice have an increased susceptibility for various pathogens. Cathelicidins have been shown to have therapeutic potential. Overexpression of cathelicidin in a lung xenograft model has been shown to promote *P. aeruginosa* and *S. aureus* killing, while exogenous cathelicidin treatment has been successfully used to inhibit *M. haemolytica*, *E. coli* and *S. aureus* infections. Cathelicidins are found in most vertebrate species and contain a highly conserved cathelin-domain, which is cleaved off during secretion, releasing the active peptide. Although these active peptide sequences are highly variable between species, many cathelicidins have the ability to adopt an amphipathic α-helical structure. Most cathelicidins have been described in the context of their antimicrobial activity, while various other functions have been identified for a limited number of cathelicidins including induction of chemokine expression, intrinsic chemotactict activity, neutralization of LPS-induced TLR4 activation and LTA-induced TLR2 activation, enhancement of DNA-induced plasmacytoid DC and macrophage activation, promotion of wound healing, influencing DC and macrophage differentiation and regulation of phagocytosis. However, even the most well-described functions are often tested under different conditions, making it difficult to compare properties between cathelicidins. In addition, because several functions have only been described for a limited number of species, it is unclear which functions are cathelicidin specific and which functions are general cathelicidin properties.
Table 1. Cathelicidin sequences and antimicrobial activity. C_{max}; cathelicidin concentration (μM) resulting in a delay in bacterial growth with minimal 10 hours. Max: if no C_{max} was reached, hours growth delay was depicted with 20 μM cathelicidin.

| Peptide | Sequence | Length | Charge | C_{max} | MHB | DME | MHB | DME |
|---------|----------|--------|--------|---------|------|-----|------|-----|
| LL-37   | LLGDFRRKSEIKGKEFKRVQRKDFLRNLYVTES | 37    | +6     | 10μM    | 4.5 hr | 0.5 hr | 9.5 hr |
| CRAMP   | GLLRKGGKEIKGKGGKQKFRQIKVLPQFEQ | 34    | +6     | 9.0 hr  | 2.5 hr | 0.5 hr | 6.5 hr |
| L9CATH  | RLKELITGQGKIEKIRRGEKRLKDNLQPEEKS | 38    | +6     | 3.0 hr  | 1.5 hr | 0.5 hr | 4.5 hr |
| eCATH-1 | KRRHWFPLSFQEFLEQRRFRDRQFP | 27    | +4     | 9.0 hr  | 2.0 hr | 1.5 hr | 3.5 hr |
| eCATH-3 | KRRHFWSLQHRQMRIDKSEXHHRIRTRPQLLAS | 40    | +10    | 0.0 hr  | 1.5 hr | 0.0 hr | 1.5 hr |
| chCATH-1| RNVKVVWLPVITTVIAGYNLRKIKK | 26    | +8     | 10μM    | 9.5 hr | 2.5μM | 1.25μM |
| chCATH-2| RFGRFLKIRRFRPVPVTQGSARF-NH₂ | 26    | +9     | 5μM     | 10μM   | 2.5μM | 0.3μM |
| chCATH-3| RVKRFWLPVTAIVALGYNLRKIKK | 29    | +7     | 20μM    | 5.5 hr | 5μM   | 0.3μM |
| MPM-23  | RIIHLLWRQPQPRQKFPVTYVVR | 23    | -6     | 9.5 hr  | 3.5 hr | 20μM | 10μM |
| MPM-36  | Ac-GRFRRLKRRKIRLKKIGKVLKIPPVGSLPCG | 36   | +13    | 5μM     | 2.5μM | 10μM | 0.3μM |
| PR-39   | RRRPRPYPYLPFRPPPPFLPPFRDQPPFGFPFRPFPFPFPFP | 39 | +10    | 20μM    | 7.0 hr | 0.5 hr | 10μM |

cathelicidins, it is unclear which properties are peptide-specific and which are related to general functions of cathelicidins. In this study, 12 cathelicidins from 6 different species were selected to assess their ability to exert various well-known cathelicidin functions. Our results show that various functions, including antimicrobial activity and LPS neutralization, are prevalent for most, although not for all, cathelicidins. In contrast, direct chemokine induction and enhancement of DNA activation of RAW264.7 cells were only observed for a few cathelicidins and only at relatively high concentrations. In total, these results provide novel insights in the functional differences between cathelicidins and could prove useful in the development of new cathelicidin-based anti-infective therapies.

Materials and Methods

Reagents. TLR ligands: LPS Escherichia coli (E. coli) O111:B4 (TLR-4), LTA S. aureus (TLR-2), and ODN-1826 (TLR-9) were obtained from Invivogen (Toulouse, France). Chicken CATH-2 (chCATH-2) and PMAP-36 were synthesized by Fmoc-chemistry at the Academic Centre for Dentistry Amsterdam (Amsterdam, The Netherlands) (Table 1). Purity of all peptides was >95% as analyzed by HPLC-MS.

Cell and bacterial culture. E. coli O78 (Zoetis Animal Health, Kalamazoo, MI, USA), E. coli ATCC 25922, Staphylococcus aureus ATCC 29213, and methicillin resistant Staphylococcus aureus (MRSA) (WKZ-2, human clinical isolate) were grown overnight from a glycerol stock in Mueller Hinton Broth (MHB) (Becton Dickinson, USA). Before use, bacteria were grown to mid-log phase in MHB for 2 hours at 37 °C, 200 RPM. Murine RAW264.7 macrophages were obtained from the American Type Culture Collection (ATCC, Manassas, VA, USA) and cultured in DMEM (14966-029; Thermo Fisher Scientific, Waltham, MA, USA) supplemented with 10% FCS (Bodinco B.V., Alkmaar, the Netherlands) (DMEM + FCS) at 37°C, 5.0% CO2. Cells were seeded in 96-wells plates at 5 × 104 cells/ml or 12-wells plates at 2 × 105/ml for adherence overnight prior to stimulation.

Antimicrobial activity. Peptides (0.63 to 40 μM) and bacteria mid-log cultures (2 × 106 CFU/ml) were prepared in MHB or DMEM + FCS and mixed (100 μl peptide + 100 μl bacterial culture) in a Bioscreen C analyzer plate (Oy Growth Curves Ab Ltd, Helsingfors, Finland). Plates were placed in a Bioscreen C analyzer (Oy Growth Curves Ab Ltd) and bacteria were grown for 16 h at 37 °C with 200 RPM. The OD was measured every 15 minutes using a wideband filter (450–580 nm) to measure bacterial growth. The time needed grow above an OD of 0.6 was determined for all concentrations. Activity of peptides was expressed as the C_{max}, the concentration of peptide required to delay the growth for 10 h compared to the control. Or, for peptides with low activity (no C_{max}), activity was expressed as the hours of growth delay at 20 μM. Unlike the classic MIC and MBC determination used in microbiology, this method also enabled comparison of peptides with low antibacterial activity.

In order to compare our C_{max} with MBC values, wells without visible bacterial growth were plated out on TSA plates and incubated o/n at 37°C to determine the presence of viable bacteria.

Sytox green assay. Peptide dilutions and bacteria suspensions were prepared as described above for testing the antimicrobial activity in the Bioscreen C analyzer. Peptide dilutions and bacteria were mixed 1:1 and incubated for 30 minutes at 37°C. Next, bacteria were washed once with PBS and transferred to black 96-wells assay plates (Corning, OH, USA). Bacteria were incubated with 1 mM sytox green (Life technologies) (λ ex 504 nm and λ em 523 nm) for 5 minutes after which fluorescence was measured using a FLUOstar Omega microplate reader (BMG Labtech GmbH, Ortenberg, Germany).
Cytotoxicity. WST-1 reagent was obtained from Roche (Basel, Switzerland). RAW264.7 cells were incubated with cathelicidins for 24 h, after which the supernatant was removed and replaced with 10% WST-1 reagent in culture medium. After 20 minutes, absorbance was measured at 450 nm with a FLUOstar Omega microplate reader (BMG Labtech GmbH, Ortenberg, Germany) and was corrected for absorbance at 630 nm. Non-treated control cells were defined as 100% mitochondrial activity.

In addition, cells were detached after peptide exposure and stained with propidium iodide (PI) (BD biosciences, San Jose, CA, USA). Percentages of PI positive (i.e. dead) cells were determined with flow cytometry (BD FACSCanto II flow cytometer (BD Biosciences)) and analyzed with FlowJo software (Ashland, OR, USA).

TLR stimulation. RAW264.7 cells were stimulated with 100 ng/ml LPS. E. coli O111:B4, 1 μg/ml LTA S. aureus or 2.5 nM ODNA in 1826 in the presence of various concentrations of different cathelicidins. TNFα release was determined after 2 h for LPS and TLR stimulation and 24 h for ODNA stimulation. CXCL10, CCL5 and IL-10 release were all determined after 24 h stimulation. As a control, RAW264.7 cells were stimulated for 2 h with 10^6 CFU/ml live or heat-killed (70 °C, 0.5 h) E. coli O78, followed by a double wash with cell culture medium and subsequent 22 h incubation in cell culture medium supplemented with 250 μg/ml gentamicin.

ELISA. ELISA Duoset kits for mouse TNFα, CCL5, CXCL10 and IL-10 were obtained from R&D systems (Minneapolis, MN, USA) and ELISAs were performed according to the manufacturer’s protocol. Samples were stored at –20 °C until analysis and, if needed, diluted in 1% BSA in PBS, pH 7.4. Absorbance at 450 nm was determined in a FLUOstar Omega microplate reader (BMG Labtech GmbH) and corrected for absorbance at 570 nm. Results were analyzed with MARS data analysis software (BMG Labtech GmbH).

Phagocytosis assay. Red fluorescent (λex 575 nm and λem 610 nm) carboxylate-modified polystyrene latex beads (0.5 μm; Sigma Aldrich, St. Louis, MO, USA) were washed three times with PBS and resuspended in culture medium. Peptide dilutions were prepared in culture medium and added to RAW264.7 cells, directly followed by the latex beads (ratio 10 beads to 1 cell). Cells were incubated for 30 minutes at 37 °C, 5% CO2 (energy-dependent uptake) or 0 °C (non-specific adherence), after which cells were washed extensively with ice-cold PBS supplemented with 1% FCS and 0.01% NaN3 to remove all free beads. After washing, cells were scraped and resuspended in FACS buffer (PBS supplemented with 0.5% BSA). Samples were measured with the BD FACSCanto II flow cytometer (BD Biosciences, San Jose, CA, USA) and analyzed with FlowJo software (Ashland, OR, USA). Mean fluorescence intensity (MFI), corrected for non-specific adherence, was used as an indicator for the number of beads taken up.

Statistics. Results are presented as the mean ± standard error of the mean (SEM) of at least three independent experiments. Statistical significance was assessed with Two-way ANOVA followed by the Bonferroni Post-Hoc test in Prism software, version 6.02 (GraphPad Prism, La Jolla, CA, USA). All samples were compared to 0 μM controls. *p < 0.05; **p < 0.01; ***p < 0.001.

Results

Antibacterial activity of cathelicidins. Twelve cathelicidins from 6 different species were selected for this study, namely: human LL-37, murine CRAMP, dog K9CATH, equine CATH (eCATH)-1, -2 and -3, chicken CATH (chCATH)-1, -2, and -3, porcine PMAP-23 and -36 and PR-39 (Table 1). Antimicrobial activity against E. coli and MRSA was directly compared under standardized conditions in MHB, a medium optimized for bacterial growth. Bacterial growth delay was defined as the delay in hours for peptide-treated bacteria to reach an OD of 0.6, compared to the non-treated control. Cmax is the minimal concentration needed to delay growth for a minimum of 10 hours compared to the non-treated control (Table 1 and Fig. 1A).

Ten out of 12 cathelicidins strongly delayed the growth of E. coli, with a growth delay of 9 h or more, with chCATH-2 and PMAP-36 as most potent, with a Cmax of 5 μM. Only K9CATH and eCATH-3 showed no or very little activity (Fig. 1B). MRSA was more resistant to most peptides than E. coli. Only chCATH-1, -2, and -3 were more active against MRSA than against E. coli. In addition to the three chicken cathelicidins, PMAP-23 and -36 had a Cmax of 20 μM and 10 μM respectively and eCATH-1 delayed MRSA growth with 7.5 hours at 20 μM. However, none of the other peptides were active against MRSA in MHB (Fig. 1C).

While these testing conditions are widely used to determine antimicrobial activity, they poorly represent physiological conditions. Since it has been shown that serum components and salts can have an inhibitory effect on antimicrobial activity, growth inhibition of E. coli and MRSA was also assessed in DMEM + FCS, which better represents physiological conditions. Under these conditions only chCATH-1, -2, PMAP-36 and PR-39 were able to delay the growth of E. coli at least 7 hours. Interestingly, PMAP-36 was the only peptide with an increased activity against E. coli in DMEM + FCS compared to MHB (Fig. 1D). In contrast to E. coli, antimicrobial activity against MRSA was enhanced for all 12 cathelicidins in DMEM + FCS. This was most pronounced for chCATH-2 and -3 and PMAP-36, with a Cmax of 0.31 μM, which is the lowest concentration tested (Fig. 1E). In addition, while PR-39 had no effect on MRSA in MHB, it was more potent in DMEM + FCS with a Cmax of 10 μM. In order to rule out bacterial strain specific effects, peptides were also tested against E. coli ATCC 25922 and S. aureus ATCC 29213. Very similar results, with a maximal 2 fold difference in Cmax were observed in this strain variability was observed (Supplementary Fig. S1).

The capacity of peptides to induce membrane leakage was tested using the permeability marker sytox green. For E. coli, all peptides that caused growth delay also caused membrane permeability, with the exception of PR-39 which showed only a minimal permeability at its Cmax concentration (Supplementary Fig. S2). For MRSA,
fluorescence levels were generally very low even at \( >C_{\text{max}} \) levels, hampering a good comparison between growth delay and permeabilization for this strain.

**Cathelicidin induced chemokine and cytokine release by RAW264.7 cells.** To determine the direct effect of cathelicidins on chemokine induction, RAW264.7 cells were used, which have previously been shown to increase chemokine secretion upon stimulation with LL-37\(^{18} \). Since cathelicidins have membrane-perturbing properties which might affect the host’s cell membrane\(^{15,31-35} \), first the possible cytotoxicity of the cathelicidins to RAW264.7 cells was assessed. No or very limited cytotoxicity was observed for most cathelicidins. chCATH-1 and PMAP-36 showed some cytotoxicity, but only at the highest concentration (see Supplementary Fig. S3). Next,
RAW264.7 cells were stimulated with the cathelicidins at various concentrations for 2 h and 24 h, after which release of CCL2 (MCP-1), CCL5 (RANTES), CXCL10 (IP-10), TNF-α and IL-10 was determined. After 2 h stimulation, CCL2 secretion was 3–4 fold enhanced by chCATH-1, chCATH-3 and PMAP-36 at 5 μM and by LL-37 and chCATH-1, -2 and -3 at 20 μM. None of the other peptides had a significant effect on CCL2 secretion after 2 h stimulation (Fig. 2A). In contrast, all cathelicidins inhibited CCL2 secretion after 24 h stimulation, although inhibition by eCATH-1, chCATH-1 and PR-39 was non-significant (Fig. 2B). CCL5 secretion after 24 h stimulation is 2–3 fold induced, however by a different set of cathelicidins than found for CCL2, e.g. LL-37, K9CATH and chCATH-2 (20 μM) (Fig. 2C). LL-37, CRAMP, K9CATH and eCATH-2 (20 μM) were able to induce CXCL10 secretion with a 2–3 fold increase after 24 h stimulation (Fig. 2D). Secretion of TNF-α (2 h) and IL-10 (24 h) was also determined after cathelicidin stimulation. Some induction was observed at higher concentrations; however, the measured cytokine levels were very low. (see Supplementary Fig. S4A,B). Various cathelicidins significantly induce cytokine and chemokine secretion. This increase appears marginal in comparison to TNF-α, CCL5 and IL-10 secretion after stimulation with viable or heat-killed E. coli (see Supplementary Fig. S4C), only the induction of CXCL10 secretion by cathelicidins was in the same range as after E. coli stimulation.

Figure 2. Cathelicidin induced chemokine and cytokine release by RAW264.7 cells. RAW264.7 cells were incubated for 2 h and 24 h with cathelicidins (0.08 μM, 0.31 μM, 1.25 μM, 5 μM, and 20 μM), after which the supernatants were harvested and tested for release of CCL2 at 2 h (A) and 24 h (B), CCL5 at 24 h (C) and CXCL10 at 24 h (D). Dotted line represents average cytokine release of control samples. Results are presented as average +/− SEM (N = 3). Statistical differences were determined by Two-way ANOVA with Bonferroni post-hoc test.
Effects of cathelicidins on TLR-2, -4, and -9 activation. RAW264.7 cells were stimulated for 2 h with 100 ng/ml LPS from *E. coli* O111:B4 in the presence of different cathelicidins at various concentrations to study the capability of LPS neutralization (Fig. 3A). Seven out of 12 cathelicidins significantly neutralized LPS at a concentration of 1.25 μM or 5 μM and thereby inhibited LPS-induced activation. None of the equine cathelicidins nor PMAP-23 and PR-39 were capable to neutralize LPS. Next, RAW264.7 cells were stimulated with 1 μg/ml *S. aureus* LTA for 2 h in the presence of various cathelicidin concentrations, to test LTA neutralization (Fig. 3B). Also seven out of 12 cathelicidins were able to neutralize LTA; interestingly, while some cathelicidins, such as chCATH-2 and LL-37, potently inhibited both LPS- and LTA-induced activation, others only significantly inhibited either LPS, such as K9CATH and chCATH-3, or LTA, like eCATH-2.

Since, LL-37, CRAMP, chCATH-2, and PMAP-36 have been described to enhance DNA-induced activation, the effect of all cathelicidins on DNA-induced TNFα release in RAW264.7 cells was analyzed (Fig. 3C). chCATH-2 was most potent to induce DNA activation after 24 h with an optimal concentration of 1.25 μM. eCATH-2, PMAP-23 and PR-39 (20 μM) were also able to enhance DNA-induced TLR-9 activation. All other peptides had no effect on the DNA-induced TLR-9 activation. To ensure that the increase of TNFα secretion was indeed enhancement of DNA-induced activation and not a result of direct induction by cathelicidins, TNFα release was determined after 24 h cathelicidin stimulation (see Supplementary Fig. S2D). None of the cathelicidins induced TNFα release after 24 h, (if anything, most cathelicidins caused a small reduction of TNFα secretion) indicating the increased activation is indeed enhancement of DNA-induced activation.

Effects of cathelicidins on phagocytosis by RAW264.7 cells. While the above-described functions of cathelicidins are well-known, relatively little is known about the influence of cathelicidins on phagocytosis. Therefore, RAW264.7 cells were incubated with fluorescent beads to study the effect of cathelicidins on phagocytosis. The uptake was corrected for fluorescence measured after incubation on ice instead of 37 °C, which inhibits the active process of phagocytosis. After 30 minutes, 90% of the RAW264.7 cells took up latex beads (Fig. 4A). Histograms exemplify changes in bead phagocytosis with different concentrations of K9CATH.
(Fig. 4B), eCATH-2 (Fig. 4C) and chCATH-3 (Fig. 4D). Effect of all cathelicidins are depicted by the average mean fluorescence intensity (MFI) in Fig. 4E.

CRAMP, K9CATH, chCATH-1 and -2, and PMAP-23 reduced bead-uptake in a dose-dependent manner, whereas PMAP-36 only reduced phagocytosis at 5 μM. In contrast, eCATH-2 was the only peptide that increased the uptake by almost 50%, although not significantly. However, as shown in Fig. 4C, RAW264.7 cells are not be able to take up more beads per cell. LL-37, eCATH-1 and -3, chCATH-3, and PR-39 did not affect bead uptake at any of the used concentrations.

Discussion

The current knowledge on functions of cathelicidins is mostly based on results from experiments with the human cathelicidin LL-37 and, to a lesser extent, murine CRAMP. In addition, even the most extensively described functions are often tested under different conditions, which makes it difficult to compare properties of cathelicidins. In this study, 12 cathelicidins were selected and compared in different assays to determine the conservation of the different functions between cathelicidins (results are summarized in Table 2). The cathelicidin selection included a number of well-known cathelicidins that have already been tested for various functions, such as LL-37, CRAMP, PR-39 and chCATH-2. In addition, several cathelicidins of which very little is known, such as the equine cathelicidins and K9CATH, were selected and compared to the better studied peptides. Furthermore, chCATH-1 and -3 were included to complement chCATH-2 and PMAP-23 and -36 were selected to represent the α-helical porcine cathelicidins. In fact, all selected cathelicidins are α-helical cathelicidins, except for PR-39, which is a proline-rich peptide forming a polyproline helix.

Antimicrobial activity of cathelicidins has been extensively tested over the years and has been demonstrated for all cathelicidins included in this study.35,38–46. The antimicrobial capacities were tested under the same conditions and expressed as growth delay instead of the more classical MIC/MBC determination using broth dilution assays. The advantage of our methodology is that activity of less potent peptides that do not establish a real MBC value can still be determined and compared. As can be seen from Fig. 1A our C_{max} value does not correspond to MBC (since 20 μM LL-37 is below the MBC but reached our 10 hour delay criterium). However, by plating out wells without visible growth, it was shown that real MBC values were consistently close (0–4 fold difference) to the C_{max} indicating that the order of activity would be similar in classic broth dilution tests (results not shown). The only exception was PR-39 with a 16 fold higher MBC than C_{max} against S. aureus in DMEM. The fact that DMEM medium makes S. aureus susceptible to PR-39 is surprising since this peptide is thought to require active uptake by the SbmA transporter, only present in Gram – bacteria. The results obtained in this work, might
for both Gram-positive and Gram-negative bacteria. On the other hand, DMEM also contains carbonate, which can increase bacterial susceptibility to cathelicidin-mediated bacterial killing. Although carbonate has been described to increase the susceptibility of E. coli towards cathelicidins, the presence of salts and serum might have a stronger inhibitory effect on the cathelicidins than carbonate on E. coli. For example, Ca²⁺ is important for the structural integrity of the outer membrane of Gram-negatives. In contrast, the increased susceptibility by MRSA due to the carbonate probably has more influence than the inhibitory effects of salt and serum on the cathelicidins. In addition, additive or synergistic effects between serum components and cathelicidins might be another cause for the more efficient killing of S. aureus in DMEM + FCS. These results suggest that, while most cathelicidins have antimicrobial activity, the efficacy is strongly dependent on the pathogen and the physiological conditions.

Murine RAW264.7 cells were used in all mammalian cell related assays. RAW264.7 cells have been used extensively to identify and describe a wide variety of cathelicidin functions, such as cytokine and chemokine induction, LPS-neutralization, LTA-neutralization, and DNA-enhancement, which were shown for multiple cathelicidins from various species, including human, mouse, pig, cow and chicken. Although use of a murine cell line can obscure possible species-specific effects or cell-specific effects, the results can be used as a basis for further studies on cathelicidin-mediated effects in this cell line or as a comparison with primary cells from different species.

Similar to the extensive research on antimicrobial activity, LPS neutralization has been shown in multiple studies for at least 13 different cathelicidins from 9 different species. Therefore, it is thought to be one of the main cathelicidin functions. However, to our knowledge, nothing is known yet about the LPS neutralizing activity of canine, equine and porcine cathelicidins. Our results showed that 7 out of 12 cathelicidins inhibited LPS-induced macrophage activation, including K9CATH and PMA-36, but none of the equine cathelicidins. Also 7 cathelicidins were found to neutralize LTA; however, there appears to be no correlation between LPS neutralization and LTA neutralization. For instance, LL-37 and CATH-2 potently exert both functions, while eCATH-2 only inhibited LTA-induced activation and K9CATH and chCATH-3 only showed potent inhibition of LPS-induced activation. In addition, neutralization of LPS and LTA did not appear to correlate with the antimicrobial activity against E. coli and MRSA, respectively (Table 2). These results showed that, while antimicrobial activity and LPS neutralization are commonly regarded as intrinsic properties of cathelicidins, these functions may differ between the various cathelicidins and species.

The induction of chemokine release by cathelicidins was first detected in RAW264.7 cells and was later also observed in THP-1 cells, primary monocytes and bronchial epithelial cells. Our results indicate that several cathelicidins induced a 2–4 fold increase in chemokine expression by RAW264.7 cells at 20 μM; however, only LL-37 was able to increase the expression of all cytokines and chemokines tested (Table 2). The levels of chemokine and cytokine secretion induced by cathelicidins was generally low, especially compared to stimuli such as live or heat-killed E. coli. This appears to be in line with other studies, where cathelicidin-mediated induction of chemokine release in RAW264.7 or THP-1 cells also appears to be low compared to other stimuli, such as LPS. In addition to the induction of chemokine release, it has been previously shown that cathelicidins can indicate that the peptide possibly switches to a different mechanism of action with initially a bacteriostatic effect, but more thorough studies are required to elucidate the details of these initial observations.

The growth delay results indicate that most cathelicidins, except K9CATH and eCATH-3, have similar antimicrobial activity against E. coli (Cmax of 5–20 μM) or a growth delay of at least 9 hours at 20 μM. However, the antimicrobial potencies against MRSA strongly diverge. Interestingly, antimicrobial activity against E. coli is strongly reduced for all cathelicidins, except PMAP-36, if tested under more physiological conditions, i.e. DMEM + FCS, while activity against MRSA is enhanced for all cathelicidins under these conditions (Table 2). It has been shown in previous studies that salts or serum components of DMEM + FCS can lower cathelicidin antimicrobial activity for both Gram-positive and Gram-negative bacteria. On the other hand, DMEM also contains carbonate, which can increase bacterial susceptibility to cathelicidin-mediated bacterial killing.

Table 2. Summary of cathelicidin functions. ++ = Cmax at ≤20 μM, +++ = >8 hours inhibition at 20 μM, + = >4 hours inhibition at 20 μM. ↑↑ = significant increase ≤5 μM, ↑ = significant increase at 20 μM. ↓↓ = significant decrease ≤5 μM, ↓ = significant decrease at 20 μM.
have a direct chemotactic effect\textsuperscript{19,27,28}. The induction of low chemokine levels could be another explanation for the stimulation of chemotaxis by CRAMP and LL-37\textsuperscript{22,23}. However, LL-37 has been shown to increase neutrophil influx in a murine lung model during inflammation, but without alteration of cytokine or chemokine expression\textsuperscript{24}. Further research will be needed to understand to what extent direct chemotaxis and chemokine induction play a role in leukocyte recruitment during both steady state situations and in the context of an infection.

So far, antimicrobial activity and LPS neutralization, but not cytokine and chemokine induction, appear to be major cathelicidin functions, although not conserved for all cathelicidins. Enhanced DNA-induced TLR9 activation has been described in literature for LL-37, CRAMP, chCATH-2, and PMAP-36. Our results show that enhancement of DNA-induced macrophage activation is not a conserved function of cathelicidins, but only found for eCATH-2, chCATH-2, PMAP-23, and PR39, with chCATH-2 as most potent one (Table 2). LL-37, on the other hand, was shown to form a complex with DNA which enhances binding efficiency and increased IFN\textalpha production in pDCs\textsuperscript{25} and monocytes\textsuperscript{25}. In B-cells, LL-37 enhances the uptake of DNA and promotes IL-6 production\textsuperscript{27}. Endogenous CRAMP has been shown to increase TLR9 activation and TNF\alpha release in macrophages; however, exogenous treatment with CRAMP had no effect on TLR-9 activation\textsuperscript{25}. Together with the results presented in this study, it appears that the presence of exogenous cathelicidins can enhance DNA-induced stimulation; however, in a species- and cell type-specific manner.

Because relatively little is known about the influence of cathelicidins on phagocytosis, an initial analysis on phagocytosis was performed. Six out of 12 cathelicidins reduced latex bead internalization with eCATH-2 as only the only cathelicidin that induced uptake (Table 2). However, since uptake of extracellular components is a complex process, it is not possible to draw conclusions about functions in vivo based on these initial observations only\textsuperscript{28}. Nevertheless, a detailed analysis with more specific inhibitors for phagocytosis, such as cytochalasin D, and live bacteria with or without opsonization, could lead to a more detailed understanding of the role of cathelicidins in the regulation of phagocytosis.

Finally, elucidation of cathelicidin functions is also important for the development of cathelicidin-based antibiotics. Due to the emergence of more multidrug resistant bacteria, new molecules with broad-spectrum antimicrobial activity could be useful to combat infections by antibiotic resistant bacteria, such as MRSA\textsuperscript{79}. Especially chCATH-2 appears to be an interesting candidate with strong antimicrobial activity against both bacteria\textsuperscript{80}. The dual activity of chCATH-2, i.e. antimicrobial activity and neutralization of LPS and LTA, can potentially provide protection against the infection as well as limit excessive inflammation. The latter is most important since sepsis is a major and life threatening problem in patients suffering from bacterial infections\textsuperscript{81}.

In conclusion, this study provides a systematic comparison of 12 cathelicidins from 6 species, showing that physiological conditions can both positively and negatively affect antimicrobial activity and that the antimicrobial activity and LPS/LTA neutralization appear to be the most prevalent cathelicidin functions. However, this study also underlines the importance of not generalizing cathelicidin functions and that caution should be taken in the extrapolation of different functions, for instance the extrapolation from murine CRAMP KO-models to the human situation or other animal models.

References
1. Zasloff, M. Antimicrobial peptides of multicellular organisms. Nature 415, 389–395 (2002).
2. Gudmundsson, G. H. et al. The human gene FALL39 and processing of the cathelin precursor to the antibacterial peptide LL-37 in granulocytes. Eur. J. Biochem. 238, 325–332 (1996).
3. Schauber, I. et al. Expression of the cathelicidin LL-37 is modulated by short chain fatty acids in colonocytes: relevance of signalling pathways. Gut 52, 735–741 (2003).
4. Agerberth, B. et al. The human antimicrobial and chemotactic peptides LL-37 and alpha-defensins are expressed by specific lymphocyte and monocyte populations. Blood 96, 3086–3093 (2000).
5. Larrick, J. W. et al. Structural, functional analysis and localization of the human CAP18 gene. FEBS Lett. 398, 74–80 (1996).
6. Di Nardo, A., Vitiello, A. & Gallo, R. L. Cutting edge: mast cell antimicrobial activity is mediated by expression of cathelicidin antimicrobial peptide. J. Immunol. 170, 2274–2278 (2003).
7. Vandamme, D., Landuyt, B., Luyten, W. & Schools, L. A comprehensive summary of LL-37, the factotum human cathelicidin peptide. Cell. Immunol. 280, 22–35 (2012).
8. Chromek, M. et al. The antimicrobial peptide cathelicidin protects the urinary tract against invasive bacterial infection. Nat. Med. 12, 636–641 (2006).
9. Chromek, M., Arvidsson, I. & Karpman, D. The antimicrobial peptide cathelicidin protects mice from Escherichia coli O157:H7-mediated disease. PLoS One 7, e46476 (2012).
10. Nizet, V. et al. Innate antimicrobial peptide protects the skin from invasive bacterial infection. Nature 414, 454–457 (2001).
11. Huang, L. C., Reins, R. Y., Gallo, R. L. & McDermott, A. M. Cathelicidin-deficient (Cnlp −/−) mice show increased susceptibility to Pseudomonas aeruginosa keratitis. Invest. Ophthal. Vis. Sci. 48, 4498–4508 (2007).
12. Bals, R., Weiner, D. J., Meegalla, R. L. & Wilson, J. M. Transfer of a cathelicidin peptide antibiotic gene restores bacterial killing in a cystic fibrosis xenograft model. J. Clin. Invest. 103, 1113–1117 (1999).
13. Brogden, K. A. et al. The ovine cathelicidin SMAP29 kills ovine respiratory pathogens in vitro and in an ovine model of pulmonary infection. Antimicrob. Agents Chemother. 45, 331–334 (2001).
14. Benincasa, M., Skeralvaj, B., Gennaro, R., Pellegrini, A. & Zanetti, M. In vitro and in vivo antimicrobial activity of two alpha-helical cathelicidin peptides and of their synthetic analogs. Peptides 24, 1723–1731 (2003).
15. Bommineni, Y. R. et al. A fowlicidin-1 analog protects mice from lethal infections induced by methicillin-resistant Staphylococcus aureus. Peptides 31, 1225–1230 (2010).
16. Khindoli, D. et al. The human cathelicidin LL-37 - A pore-forming antibacterial peptide and host-cell modulator. Biochim. Biophys. Acta 1858, 546–566 (2016).
17. Zanetti, M. The role of cathelicidins in the innate host defenses of mammals. Curr. Issues Mol. Biol. 7, 179–196 (2005).
18. Scott, M. G., Davidson, D. J., Gold, M. R., Bowdish, D. & Hancock, R. E. The human antimicrobial peptide LL-37 is a multifunctional modulator of innate immune responses. J. Immunol. 169, 3883–3891 (2002).
19. De, V. et al. LL-37, the neutrophil granule- and epithelial cell-derived cathelicidin, utilizes formyl peptide receptor-like 1 (FPRL1) as a receptor to chemotact human peripheral blood neutrophils, monocytes, and T cells. J. Exp. Med. 192, 1069–1074 (2000).
20. Hirata, M. et al. Characterization of a rabbit cationic protein (CAP18) with lipopolysaccharide-inhibitory activity. Infect. Immun. 62, 1421–1426 (1994).
21. Larrick, J. W. et al. Human CAP18: a novel antimicrobial lipopolysaccharide-binding protein. Infect. Immun. 63, 1291–1297 (1995).
22. Lande, R. et al. Plasmacytid dendritic cells sense self-DNA coupled with antimicrobial peptide. Nature 449, 564–569 (2007).
23. Coorens, M., van Dijk, A., Bikker, F., Veldhuijzen, E. J. & Haagsman, H. P. Importance of Endosomal Cathelicidin Degradation To Enhance DNA-Induced Chicken Macrophage Activation. J. Immunol. 195, 3970–3977 (2015).
24. Carretero, M. et al. In vitro and in vivo wound healing-promoting activities of human cathelicidin LL-37. J. Invest. Dermatol. 128, 223–236 (2008).
25. Davidson, D. J. et al. The cationic antimicrobial peptide LL-37 modulates dendritic cell differentiation and dendritic cell-induced T cell polarization. J. Immunol. 172, 1146–1156 (2004).
26. van der Does, A. M. et al. LL-37 directs macrophage differentiation toward macrophages with a proinflammatory signature. J. Immunol. 185, 1442–1449 (2010).
27. van, M. et al. Antimicrobial peptide LL-37 promotes bacterial phagocytosis by human macrophages. J. Leukoc. Biol. 95, 971–981 (2014).
28. Anderson, R. C. & Yu, P. L. Factors affecting the antimicrobial activity of ovine-derived cathelicidins against E. coli 0157:H7. Int. J. Antimicrob. Agents 25, 205–210 (2005).
29. Johansson, J., Guidonsson, G. H., Rottenberg, M. E., Berndt, K. D. & Agerberth, B. Conformation-dependent antibacterial activity of the naturally occurring human peptide LL-37. J. Biol. Chem. 273, 3718–3724 (1998).
30. Bals, R., Wang, X., Zaslav, M. & Wilson, J. M. The peptide antibiotic LL-37/hCAP-18 is expressed in epithelia of the human lung where it has broad antimicrobial activity at the airway surface. Proc. Natl. Acad. Sci. USA 95, 9541–9546 (1998).
31. Xiao, Y. et al. The central kink region of fowlicidin-2, an alpha-helical host defense peptide, is critically involved in bacterial killing and endotoxin neutralization. J. Innate Immun. 1, 268–280 (2009).
32. Xiao, Y. et al. Identification and functional characterization of three chicken cathelicidins with potent antimicrobial activity. J. Biol. Chem. 281, 2858–2867 (2006).
33. Lee, E. K., Kim, Y. C., Nan, Y. H. & Shin, S. Y. Cell selectivity, mechanism of action and LPS-neutralizing activity of bovine myeloid antimicrobial peptide-18 (BMAP-18) and its analogs. Peptides 32, 1123–1130 (2011).
34. Lv, Y. et al. Antimicrobial properties and membrane-activated mechanism of a potential alpha-helical antimicrobial derived from cathelicidin PMAP-36. PLoS One 9, e86364 (2014).
35. Scochi, M. et al. Structural aspects and biological properties of the cathelicidin PMAP-36. FEBS J. 272, 4398–4406 (2005).
36. Baumann, A., Demoulins, T., Python, S. & Summerfeld, A. Porcine cathelicidins efficiently complex and deliver nucleic acids to plasmacytid dendritic cells and can thereby mediate bacteria-induced IFN-alpha responses. J. Immunol. 193, 364–371 (2014).
37. Nakagawa, Y. & Gallo, R. L. Endogenous intracellular cathelicidin enhances Toll-like receptor activation in dendritic cells and macrophages. J. Immunol. 194, 1274–1284 (2015).
38. Agerberth, B. et al. FALL-39, a putative human peptide antibiotic, is cysteine-free and expressed in bone marrow and testis. Proc. Natl. Acad. Sci. USA 92, 195–199 (1995).
39. Agerberth, B. et al. Amino acid sequence of PR-39. Isolation from pig intestine of a new member of the family of proline-arginine-rich antibacterial peptides. Eur. J. Biochem. 202, 849–854 (1991).
40. Gallo, R. L. et al. Identification of CRAMP, a cationic-related antimicrobial peptide expressed in the embryonic and adult mouse. J. Biol. Chem. 272, 13088–13093 (1997).
41. Sang, Y. et al. Canine cathelicidin (KWCATH): gene cloning, expression, and biochemical activity of a novel pro-myeloid antimicrobial peptide. Dev. Comp. Immunol. 31, 1278–1296 (2007).
42. Skerlavaj, B., Scochi, M., Gennaro, R., Risso, A. & Zanetti, M. Structural and functional analysis of horse cathelicidin peptides. Antimicrob. Agents Chemother. 45, 715–722 (2001).
43. Xiao, Y. et al. Structure-activity relationships of fowlicidin-1, a cathelicidin antimicrobial peptide in chicken. FEBS J. 273, 2581–2593 (2006).
44. van Dijk, A. et al. Identification of chicken cathelicidin-2 core elements involved in antibacterial and immunomodulatory activities. Mol. Immunol. 46, 2465–2473 (2009).
45. Bommineni, Y. R. et al. Fowlcidein-3 is an alpha-helical cationic host defense peptide with potent antibacterial and lipopolysaccharide-neutralizing activities. FEBS J. 274, 418–427 (2007).
46. Zanetti, M., Storici, P., Tossi, A., Scochi, M. & Gennaro, R. Molecular cloning and chemical synthesis of a novel antibacterial peptide derived from pig myeloid cells. J. Biol. Chem. 269, 7855–7858 (1994).
47. Tönnies, K., Wang, X., Dinh, N. N., Waring, A. J. & Lehrer, R. I. Activity of LL-37, a cation-associated antimicrobial peptide of human neutrophils. Antimicrob. Agents Chemother. 42, 2206–2214 (1998).
48. Dorschner, R. A. et al. The mammalian ionic environment dictates microbial susceptibility to antimicrobial defense peptides. FASEB J. 20, 35–42 (2006).
49. Ertridge, C., Stewart, J., Bennett-Guerrero, E., McIntosh, T. J. & Powton, I. R. The biological activity of a liposomal complete core lipopolysaccharide vaccine. J. Endotoxin Res. 8, 39–46 (2002).
50. van der Linden, D. S., Short, D., Dittmann, A. & Yu, P. L. Synergistic effects of ovine-derived cathelicidins and other antimicrobials against Escherichia coli O157:H7 and Staphylococcus aureus 1056 MSR5. Biotechnol. Lett. 31, 1265–1267 (2009).
51. Scott, M. G., Vreugdenhil, A. C., Buurman, W. A., Hancock, R. E. & Gold, M. R. Cutting edge: cationic antimicrobial peptides block the binding of lipopolysaccharide (LPS) to LPS binding protein. J. Immunol. 164, 549–553 (2000).
52. Rosenvold, K., Papo, N. & Shi, Y. Endotoxin lipopolysaccharide neutralization by innate immunity host-defense peptides. Peptide properties and plausible modes of action. J. Biol. Chem. 281, 1636–1643 (2006).
53. Nijnik, A., Pistoric, J., Wyatt, A., Tam, S. & Hancock, R. E. Human cathelicidin peptide LL-37 modulates the effects of IFN-gamma on APCs. J. Immunol. 183, 5788–5798 (2009).
54. Singh, D., Qi, R., Jordan, J. L., San Matteo, L. & Kao, C. C. The human antimicrobial peptide LL-37, but not the mouse ortholog, mCRAMP, can stimulate signaling by poly(I:C) through a TLR7-dependent pathway. J. Biol. Chem. 288, 8258–8268 (2013).
55. Bommineni, Y. R., Pham, G. H., Sunkara, L. I., Achanta, M. & Zhang, G. Immune regulatory activities of fowlicidin-1, a cathelicidin host defense peptide. Mol. Immunol. 59, 55–63 (2014).
56. Som, A. et al. Identification of synthetic host defense peptide mimics that exert dual antimicrobial and anti-inflammatory activities. Clin. Vaccine Immunol. 19, 1784–1791 (2012).
57. Czerni, C. D., Siegwardtott, T., Schmidchen, A. & Bodenssell, M. Antimicrobial and chemoattractant activity, lipopolysaccharide neutralization, cytotoxicity, and inhibition by serum of analogs of human cathelicidin LL-37. Antimicrob. Agents Chemother. 49, 2845–2850 (2005).
58. Di Nardo, A. et al. Cathelicidin antimicrobial peptides block dendritic cell TLR4 activation and allergic contact sensitization. J. Immunol. 178, 1829–1834 (2007).
59. Ghiselli, R. et al. Neutralization of endotoxin in vitro and in vivo by Bac(71–35), a proline-rich antibacterial peptide. Shock 19, 577–581 (2003).
60. Giacometti, A. et al. Cathelicidin peptide sheep myeloid antimicrobial peptide-29 prevents endotoxin-induced mortality in rat models of septic shock. Am. J. Respir. Crit. Care Med. 169, 187–194 (2004).
61. Giacometti, A. et al. The antimicrobial peptide BMAP-28 reduces lethality in mouse models of staphylococcal sepsis. *Crit. Care Med.* **32**, 2485–2490 (2004).
62. Larrick, J. W. et al. A novel granulocyte-derived peptide with lipopolysaccharide-neutralizing activity. *J. Immunol.* **152**, 231–240 (1994).
63. Nagaoka, I. et al. Cathelicidin family of antibacterial peptides CAP18 and CAP11 inhibit the expression of TNF-alpha by blocking the binding of LPS to CD14 (+) cells. *J. Immunol.* **167**, 3329–3338 (2001).
64. van Dijk, A. et al. Immunomodulatory and Anti-Inflammatory Activities of Chicken Cathelicidin-2 Derived Peptides. *PLoS One* **11**, e0147919 (2016).
65. Wei, L. et al. Identification and Characterization of the First Cathelicidin from Sea Snakes with Potent Antimicrobial and Anti-inflammatory Activity and Special Mechanism. *J. Biol. Chem.* **290**, 16633–16652 (2015).
66. Yu, H. et al. Novel Cathelicidins from Pigeon Highlights Evolutionary Convergence in Avian Cathelicidins and Functions in Modulation of Innate Immunity. *Sci. Rep.* **5**, 11082 (2015).
67. Bowdish, D. M., Davidson, D. J., Scott, M. G. & Hancock, R. E. Immunomodulatory activities of small host defense peptides. *Antimicrob. Agents Chemother.* **49**, 1727–1732 (2005).
68. Mookherjee, N. et al. Intracellular receptor for human host defense peptide LL-37 in monocytes. *J. Immunol.* **183**, 2688–2696 (2009).
69. Mookherjee, N. et al. Systems biology evaluation of immune responses induced by human host defence peptide LL-37 in mononuclear cells. *Mol. Bioyst* **5**, 483–496 (2009).
70. Tjabringa, G. S., Nijaber, D. K., Drijfhout, J. W., Rabe, K. F. & Hiemstra, P. S. Human cathelicidin LL-37 is a chemotactic peptide for eosinophils and neutrophils that acts via formyl-peptide receptors. *Int. Arch. Allergy Immunol.* **140**, 103–112 (2006).
71. Hua, H. J., Ross, C. R. & Blecha, F. Chemotactic properties of PR-39, a neutrophil antibacterial peptide. *J. Leukoc. Biol.* **61**, 624–629 (1997).
72. Soehnlein, O. et al. Neutrophil secretion products pave the way for inflammatory monocytes. *Blood* **112**, 1461–1471 (2008).
73. Kurosaka, K., Chen, Q., Yarovinsky, F., Oppenheim, J. J. & Yang, D. Mouse cathelin-related anti-microbial peptide chemotaxis leukocytes using formyl peptide receptor-like 1/ mouse formyl peptide receptor-like 2 as the receptor and acts as an immune adjuvant. *J. Immunol.* **174**, 6257–6265 (2005).
74. Beaumont, P. E. et al. Cathelicidin host defence peptide augments clearance of pulmonary Pseudomonas aeruginosa infection by its influence on neutrophil function in vivo. *PLoS One* **9**, e90029 (2014).
75. Schmidt, N. W. et al. Liquid-crystalline ordering of antimicrobial peptide-DNA complexes controls TLR9 activation. *Nat. Mater.* **14**, 696–700 (2015).
76. Chamilo, G. et al. Cytosolic sensing of extracellular self-DNA transported into monocytes by the antimicrobial peptide LL37. *Blood* **120**, 3699–3707 (2012).
77. Hurtado, P. & Peh, C. A. LL-37 promotes rapid sensing of CpG oligodeoxynucleotides by B lymphocytes and plasmacytoid dendritic cells. *J. Immunol.* **184**, 1425–1435 (2010).
78. Underhill, D. M. & Goodridge, H. S. Information processing during phagocytosis. *Nat. Rev. Immunol.* **12**, 492–502 (2012).
79. Nikaido, H. Multidrug Resistance in Bacteria. *Annu. Rev. Biochem.* **78**, 119–146 (2009).
80. Veldhuizen, E. J., Brouwer, E. C., Schneider, V. A. & Fluit, A. C. Chicken cathelicidins display antimicrobial activity against multiresistant bacteria without inducing strong resistance. *PLoS One* **8**, e61964 (2013).
81. Cohen, J. The immunopathogenesis of sepsis. *Nature* **420**, 885–891 (2002).

**Acknowledgements**

This work was financially supported by the Immuno Valley ALTANT ASIA2 program of the Dutch Ministry of Economic Affairs.

**Author Contributions**

M.C., M.S., E.V., and H.H. designed the experiments. M.C. and M.S. performed the experiments. M.C., M.S., E.V., and H.H. analyzed the data and wrote the manuscript.

**Additional Information**

**Supplementary information** accompanies this paper at http://www.nature.com/srep

**Competing financial interests:** The authors declare no competing financial interests.

**How to cite this article:** Coorens, M. et al. Interspecies cathelicidin comparison reveals divergence in antimicrobial activity, TLR modulation, chemokine induction and regulation of phagocytosis. *Sci. Rep.* **7**, 40874; doi: 10.1038/srep40874 (2017).

**Publisher's note:** Springer Nature remains neutral with regard to jurisdictional claims in published maps and institutional affiliations.

This work is licensed under a Creative Commons Attribution 4.0 International License. The images or other third party material in this article are included in the article’s Creative Commons license, unless indicated otherwise in the credit line; if the material is not included under the Creative Commons license, users will need to obtain permission from the license holder to reproduce the material. To view a copy of this license, visit http://creativecommons.org/licenses/by/4.0/

© The Author(s) 2017