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RESEARCH ARTICLE

Chicken CATH-2 Increases Antigen Presentation Markers on Chicken Monocytes and Macrophages

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Abstract: ***Background*:** Cathelicidins are a family of Host Defense Peptides (HDPs), that play an important role in the innate immune response. They exert both broad-spectrum antimicrobial activity against pathogens, and strong immunomodulatory functions that affect the response of innate and adaptive immune cells.

***Objective*:** The aim of this study was to investigate immunomodulation by the chicken cathelicidin CATH-2 and compare its activities to those of the human cathelicidin LL-37.

***Methods*:** Chicken macrophages and chicken monocytes were incubated with cathelicidins. Activation of immune cells was determined by measuring surface markers Mannose Receptor C-type 1 (MRC1) and MHC-II. Cytokine production was measured by qPCR and nitric oxide production was determined using the Griess assay. Finally, the effect of cathelicidins on phagocytosis was measured using carboxylate-modified polystyrene latex beads.

***Results*:** CATH-2 and its all-D enantiomer D-CATH-2 increased MRC1 and MHC-II expression, markers for antigen presentation, on primary chicken monocytes, whereas LL-37 did not. D-CATH-2 also increased the MRC1 and MHC-II expression if a chicken macrophage cell line (HD11 cells) was used. In addition, LPS-induced NO production by HD11 cells was inhibited by CATH-2 and D-CATH-2.

***Conclusion*:** These results are a clear indication that CATH-2 (and D-CATH-2) affect the activation state of monocytes and macrophages, which leads to optimization of the innate immune response and enhancement of the adaptive immune response.

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1. INTRODUCTION

Cathelicidins are short cationic peptides with an important role in host defense. They have broad antimicrobial activity as shown by their ability to kill a wide range of bacteria, viruses and fungi [1-4]. Besides direct antimicrobial activity, cathelicidins also play a more immunomodulatory role in the innate immune system through, amongst others, activation and chemotaxis of a variety of immune cells [5] and their role is still expanding [6-9]. However, it is unclear if chicken cathelicidins share all of these activities with their mammalian counterparts.

In human, the only cathelicidin gene hCAP-18, has been shown to yield at least three different mature peptides, of which LL-37 is the most commonly studied [9]. This cathelicidin is produced by different cell types, including neutrophils, macrophages, and NK cells [10]. In chicken there are four different cathelicidins: CATH-1, -2, and -3, and CATH-B1 [11-14]. Multiple reports have described immunomodulatory effects of cathelicidins *in vivo*. For example, LL-37 upregulated the neutrophil response and cleared an infection in a murine *Pseudomonas aeruginosa* lung infection model without the peptide showing direct antimicrobial effects [15]. The chicken cathelicidin CATH-1 was used in a MRSA infection model in mice, where it provided partial protection [16]. In addition, chicken CATH-2 increased the number of phagocytic cells in a zebrafish model leading to a protective effect against bacterial infection. These studies suggested a more immuno-stimulatory mechanism of action for this peptide [17]. Finally, *in ovo* treatment with the all-D enantiomer of CATH-2 (D-CATH-2) showed a protective effect against a subsequent *E. coli* infection 7 days after hatch, indicating

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again that immunomodulatory effects and not direct antimicrobial killing was involved [18].

The involvement of immune cells and specifically macrophages was best shown in a study using a so-called innate defense-regulator peptide, IDR-1. This peptide, which does not have direct antimicrobial activity, could protect mice from infections, but was inactive when these mice were depleted of monocytes and macrophages [19]. In line with this, we have shown that CATH-2, D-CATH-2 and LL-37 have an effect on the mononuclear phagocyte population within chicken PBMCs [20].

In order to better understand the *in vivo* immunomodulatory activity of CATH-2 the current study investigated the effect of CATH-2 on primary chicken monocytes and a chicken macrophage cell line. The all D-enantiomer D-CATH-2 was included based on its stability towards proteases and its described effect *in ovo* [18], while LL-37 was included as a well described immunomodulatory peptide of human origin. The study indicates that CATH-2 has clear effects on macrophage and monocyte antigen presentation markers which could be an important feature of these peptides as part of the innate immune response of chickens towards pathogens.

2. MATERIALS AND METHODS

2.1. Peptides

CATH-2 and D-CATH-2 (amino acid sequence: RFGRFLRKIRRFRPKVTITIQGSARF-NH_2) were synthesized by Fmoc-chemistry (CPC Scientific) and LL-37 was synthesized by Fmoc-chemistry at the Academic Centre for Dentistry Amsterdam (ACTA).

2.2. Cell Surface Marker Staining

Chicken macrophage HD11 cells, (gift from Prof. Jos van Putten, Utrecht University, the Netherlands) were cultured in a 24-wells plate containing 250,000 cells at 37 °C in RPMI1640 media (Lonza, Switzerland) containing 10% fetal calf serum (Bodinco, The Netherlands) and 1% penicillin/streptomycin (Life Technologies, CA, USA). For the monocyte culture, whole blood was collected from ~76 week-old healthy chickens and PBMCs were isolated using a Ficoll gradient and frozen until use. PBMCs were cultured overnight in a 24-wells plate containing $5x10^6$ cells in RPMI1640 media containing 10% fetal calf serum, 10 U/ml penicillin and 10 mg/ml streptomycin. The next day, the wells were washed three times to remove all non-attached cells.

HD11 cells and monocytes were incubated with different concentrations of peptide for 4 or 24 h. In some experiments, monocytes were also stimulated with 100 ng/ml LPS (*E. coli* 0111:B4; InvivoGen, CA, USA) or 100 ng/ml PAM_3CSK_4 (InvivoGen) for 24 h. Next, cells were harvested and incubated for 30 min with antibodies KUL-01-FITC (mannose receptor MRC1/CD206; clone KUL01), MHCII-PE (clone 2G11) (both Southern Biotech, Birmingham, USA) on ice, and subsequently incubated for 30 min on ice with secondary BV421-labelled antibody (Biolegend, CA, USA). Afterwards, cells were washed and analyzed using flow cytometry (FACSCanto-II, BD Biosciences, CA, USA) and FlowJo software (Ashland, OR, USA).

2.3. Intracellular Staining of Cytokines

Monocytes were incubated with 10 μM peptide for 16 h in the presence of 100 ng/ml LPS and 1 μg/ml GolgiPlug (BD Biosciences). After harvesting, cells were incubated with KUL-01-FITC for the cell surface staining. The intracellular staining was performed according to the manufacturer's protocol (BD Cytofix/Cytoperm Fixation/ Permeabilization kit; BD Biosciences). In short, after the cell surface staining, cells were incubated with Fixaton/ Permeabilization solution for 20 min. Next, antibodies to IL-1β, IL-6 and IFN-γ (Bioconnect, Toronto, Canada) with secondary AF405-labelled antibody (Invitrogen, CA, USA) were used to study intracellular cytokine production. Cells were washed and analyzed using flow cytometry and FlowJo. The relative cytokine production was determined by correcting for the staining control and expressed as a fold difference from levels in control HD11 cells.

2.4. Phagocytosis Assay

In a 96-wells plate, 50,000 HD11 cells per well were incubated with carboxylate-modified polystyrene latex beads (Sigma-Aldrich, Missouri, USA) in a 1:10 ratio for 1 h at 41 °C in the presence of different concentrations of peptide. Cells were washed four times with cold PBS containing 1% FSC and 0.01% NaN_3 and analyzed using flow cytometry. Phagocytosis was determined by correcting the uptake of latex beads at 41 °C for the uptake at 4 °C.

2.5. Griess Assay

NO production was determined using the Griess Assay. In a 48-wells plate, 250,000 HD11 cells per well were incubated overnight in the presence of different concentrations of peptide and 100 ng/ml LPS. Supernatant was harvested and 50 μl of the supernatant was mixed with 50 μl 1% sulfanilamide (5% phosphoric acid) and incubated for 5 min at RT in the dark. Then 50 μl of 0.1% *N*-(1-naphthyl) ethylenediamine dihydrochloride was added and incubated for 5 min at RT in the dark. Absorbance was measured at 550 nm and the amount of NO production was determined by a standard of sodium nitrate (Sigma-Aldrich).

2.6. Statistical Analysis

Statistical significance was assessed with one-way ANOVA followed by the Dunnett Post-Hoc test in GraphPad Prism software, version 6.02. A p-value of <0.05 was considered statistically significant.

3. RESULTS

3.1. CATH-2 and D-CATH-2 Increased Antigen Presentation Capacity of Primary Chicken Monocytes

Chicken monocytes were incubated with chicken CATH-2, D-CATH-2 and human LL-37. After 4 h of incubation, CATH-2 and D-CATH-2 dose-dependently increased the expression of MRC1 approximately 12- and 15-fold, respectively, while LL-37 had no effect on expression

(Figure **1A**). Similarly, CATH-2 and D-CATH-2 could induce MHC-II expression, albeit to a lower extend (1.2- and 2-fold increased expression, respectively: Figure **1B**). This indicates that CATH-2 and its all-D enantiomer D-CATH-2 can affect the antigen presentation capacity of monocytes.

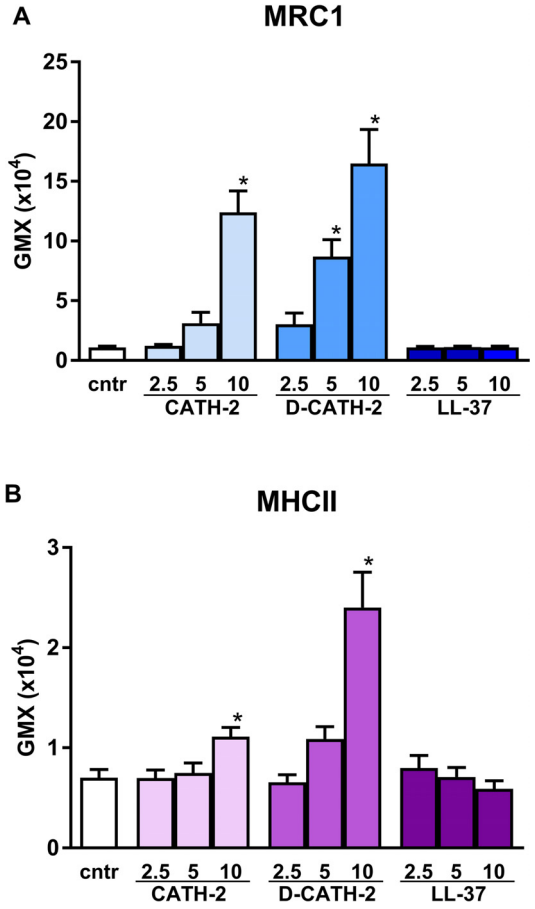

Figure 1. CATH-2 and D-CATH-2 increase the antigen presentation capacity of primary monocytes. Primary chicken monocytes were incubated with different concentrations of CATH-2, D-CATH-2 and LL-37 (0-10 μM) for 4 h. The effect on the expression of (**A**) MRC1 and (**B**) MHC-II was determined using flow cytometry. Depicted are the mean ± SEM of at least 7 independent experiments. *Indicates significant difference ($p<0.05$) compared to the control.

The effects of TLR4 agonist LPS and TLR1/TLR2 agonist PAM_3CSK_4 alone or in combination with cathelicidins (2.5 μM) for 24 h on expression of MRC1 and MHC-II in primary chicken monocytes was also studied. In the absence of peptides, no significant effects were observed for either TLR ligand (white bars, Figures **2A** and **2B**). Similarly, LPS or PAM_3CSK_4 did not lead to increased expression of MRC1 or MHC-II in the presence of 2.5 μM CATH-2, D-CATH-2 or LL-37. The only significant difference found was for MRC1 expression for all groups containing D-CATH-2 compared to samples without peptide present, confirming the results from Figure **1** where D-CATH-2 by itself increased MRC1 expression.

Finally, intracellular levels of IL-1β, IL-6 and IFN-γ were determined for LPS-stimulated monocytes in the presence or absence of 10 μM of peptides using FACS analysis. Comparable to the antigen presenting markers, no significant effect of LPS and/or cathelicidins was observed (data not shown). Thus, chicken monocytes are not particularly sensitive for TLR2 or TLR4 stimulation with regards to marker expression and cytokine expression.

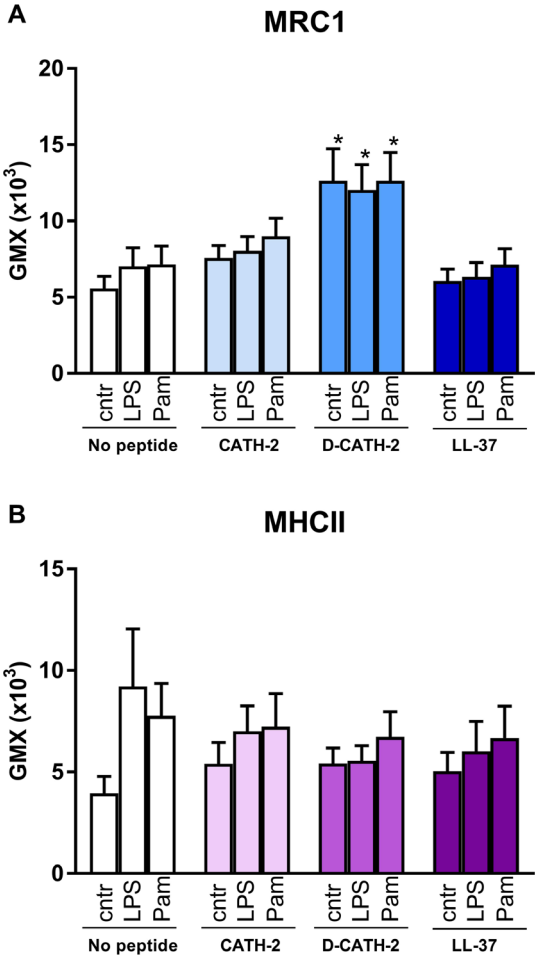

Figure 2. No effect of LPS and/or PAM_3CSK_4 on peptide-induced stimulation of antigen presentation capacity of monocytes. Primary chicken monocytes were incubated with 2.5 μM CATH-2, D-CATH-2 and LL-37 for 24 h in the presence of 100 ng/ml LPS or PAM_3CSK_4 (Pam). The effect of CATH-2, D-CATH-2 and LL-37 on the expression of (**A**) MRC1 and (**B**) MHC-II was determined using flow cytometry. Depicted are mean ± SEM of at least 8 independent experiments. *Indicates significant difference ($p<0.05$) compared to the no peptide control (white bars).

3.2. D-CATH-2 Increased the Antigen Presentation Capacity of HD11 Cells

In order to confirm our results obtained in primary chicken monocytes, we also studied the effects of CATH-2, D-CATH-2 and LL-37 on the chicken macrophage cell line HD11. As observed for primary monocytes D-CATH-2 induced an increase in MRC1 and MHC-II expression in HD11 cells after 4 h exposure Surprisingly, unlike in primary monocytes, CATH-2 did not affect the expression of these cell surface markers in HD11 cells (Figures **3A** and **B**). An increased incubation time did also not result in

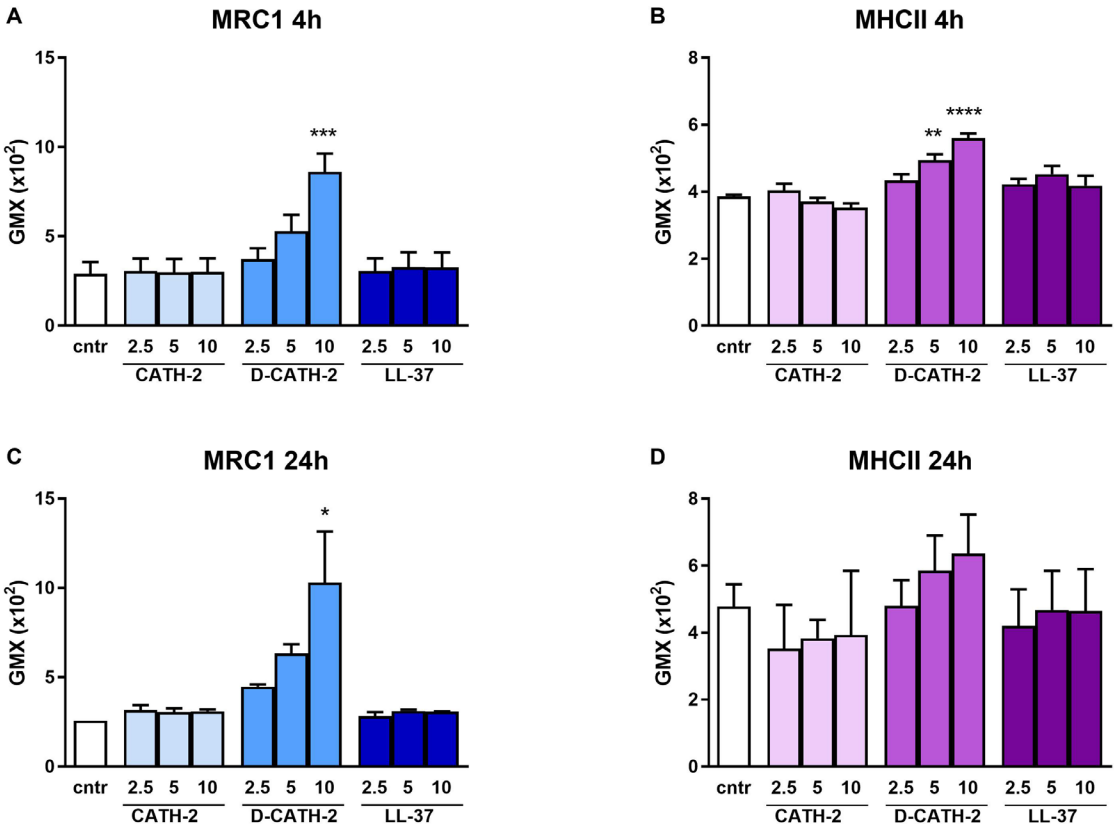

Figure 3. D-CATH-2 increases MRC1 and MHC-II expression in HD11 cells. HD11 cells were incubated with different concentrations of CATH-2, D-CATH-2 (0-10 µM) and LL-37 (0-10 µM) for (**A, B**) 4 or (**C, D**) 24 h. The effect of CATH-2, D-CATH-2 and LL-37 on the expression of MRC1 and MHC-II was determined (n=2-4). Depicted 'h' are mean ± SEM. * Indicates significant difference ($p<0.05$) compared to the control.

significant effects on MRC1 and MHC-II expression for CATH-2 (Figures **3C** and **D**), indicating that the difference observed between monocytes and macrophages for CATH-2 stimulation is not likely due to kinetic differences. As expected, LL-37 also failed to induce an increased expression of MRC1 or MHC-II in HD11 cells.

3.3. Cathelicidins Inhibited LPS-Induced NO Production and Increased Phagocytosis in HD11 Cells

To determine possible functional implications of incubating immune cells with CATH-2, D-CATH-2 and LL-37, the effect on LPS-induced NO production and phagocytic capacity of HD11 cells was investigated. LPS stimulation led to 25-fold higher NO levels compared to unstimulated cells (Figure **4A**). There was a significant decrease in the LPS-induced NO production with CATH-2 and D-CATH-2 (Figure **4A**), with the latter showing a strong effect at the lowest concentration tested (2.5 µM), while CATH-2 required higher concentrations for full activity. As a second functional assay the effect of cathelicidins on phagocytic activity of macrophages was tested (Figure **4B**). Interestingly, LL-37 had a clear dose-dependent effect on the phagocytosis of beads showing a 3-, and 5-fold increase in uptake at 5 µM and 10 µM of LL-37. CATH-2 and D-CATH-2 had no effect on phagocytosis at any concentration. These assays show that human and chicken cathelicidins seem to affect different functions of chicken macrophages.

4. DISCUSSION

In this study, immunomodulatory effects of CATH-2, D-CATH-2 and LL-37 on chicken immune cells were investigated using chicken monocytes and HD11 cells. The antigen presentation capacity of primary chicken monocytes was increased after incubation with D-CATH-2 and CATH-2, as shown by the increased expression of the mannose receptor MRC1 and MHC-II molecule. Recently, it was demonstrated by our research group that these markers are upregulated in the mixed population of mononuclear phagocytes when looking within the entire PBMC population [20] and here we confirm that in a specific monocyte/macrophage cell population. A similar effect was described for a chicken CATH-1 derivative (fowlicidin 16-26) on mouse macrophages [21], indicating that this could be a feature of more chicken cathelicidins. No effect on antigen presentation markers on chicken monocytes or macrophages was observed for LL-37. In contrast, this human cathelicidin increases surface markers on human dendritic cells and monocytes, indicating that this could at least partially be a species-specific effect of cathelicidins [22, 23]. The increased antigen presentation capacity by CATH-2 suggests that the presence of the peptide helps to prepare the cell for an enhanced adaptive immune response leading to an optimal reaction for fighting an infection.

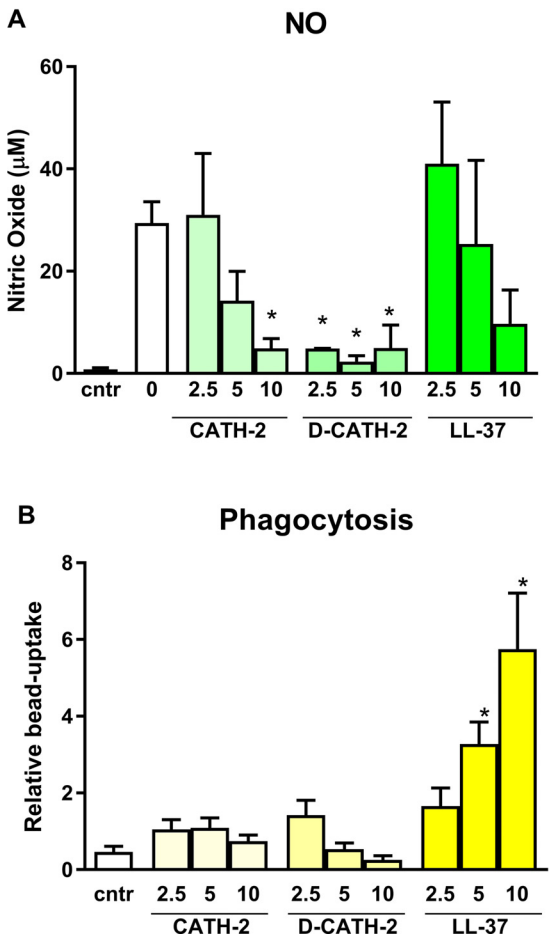

Figure 4. Effect of cathelicidins on LPS-induced NO production and phagocytic capacity of macrophages. HD11 cells were incubated with different concentrations of CATH-2, D-CATH-2 (0-10 μM) and LL-37 (0-10 μM). (**A**) NO production was determined in the supernatant of HD11 cells after 16 h of incubation with peptide in the presence of 100 ng/ml LPS (n=3-5). * indicates significant difference ($p<0.05$) compared to LPS exposure in the absence of peptide (white bar) (**B**) HD11 cells were incubated with peptide and latex beads for 1 h to determine phagocytosis. The relative phagocytosis compared to control samples is shown. Depicted are the mean ± SEM of 3-4 independent experiments. * Indicates significant difference ($p<0.05$) compared to the control.

Primary monocytes were relatively insensitive towards stimulation with LPS and PAM_3CSK_4, and the presence of CATH-2 or LL-37 could not enhance MRC1 and MHC-II expression. This is in line with the observation that chickens have decreased sensitivity towards LPS compared to mammals [24, 25]. On the other hand, LPS was capable of inducing NO production in chicken macrophages, which could be neutralized by the chicken cathelicidins. The capacity to neutralize LPS has been described for more cathelicidins [26, 27] and is partly attributed to direct binding of the peptide to LPS [28, 29]. Interestingly, no effect of LPS was observed on intracellular cytokine levels of monocytes. Several studies have actually shown that LPS induced cytokine levels in chicken immune cells including macrophages and monocytes, however, these were all measured on a transcriptional level [30, 31]. The current results indicate that this might actually not be reflected on a translational level. Alternatively, the incubation time of 24 h might have resulted in tolerance to LPS and in that way early responses towards LPS might have been overlooked. However, a full kinetic study was beyond the scope of the current study and will be addressed in follow-up studies.

The effect of cathelicidins on the functionality of chicken macrophages indicated that phagocytosis was strongly increased by LL-37, while there was no effect observed for CATH-2 and D-CATH-2. This corresponds well to a study where a similar enhancing effect of LL-37 on phagocytic capacity of human macrophages was described [32]. However, related studies showed that LL-37 had no effect on phagocytosis of mouse bone-marrow-derived macrophages [26], and actually decreased the phagocytic activity of mouse RAW264.7 cells [33]. This suggests that the effect exerted by LL-37 on this cell function is dependent on the species and type of cells.

Interestingly, some differences were observed in immunomodulatory activity between CATH-2 and D-CATH-2. The latter showed higher capacity to induce antigen presentation markers (Figure **3**) and in LPS neutralization activity (Figure **4**). This could be due to higher stability of the D-enantiomer, either towards proteases, or a more general stability during the incubation times of the experiments. However, since L- and D-CATH-2 are mirror images of each other, especially receptor based interactions, but also more general membrane interactions of CATH-2, could be affected by structural differences due to the incorporation of D-amino acids into the molecule. However, the activity-spectrum of both CATH-2 peptides was largely overlapping, while LL-37 showed an almost opposite set of activities, with no effect on antigen marker presentation, but very strong effects on phagocytosis.

The enhancement of antigen presentation markers adds onto a long list of immunomodulatory functions for this (and other) cathelicidins. For example, CATH-2 was shown to enhance cytokine production in HD-11 cells and using truncated and mutated versions of CATH-2, some structural features could be linked to this activity [30]. In addition CATH-2 was shown to enhance uptake of bacterial DNA and subsequent TLR21 activation [34], but also showed an inhibitory effect of the immune response towards non-viable bacteria, reducing the potential damaging effects of a pro-inflammatory response [35, 36]. Which of these activities is important for the described protective effect of CATH-2 *in vivo* [18] is unclear, but the observed activities in *in vitro* systems highlight the complexity and also the potential of CATH-2 in prevention and possibly treatment of microbial infections. Furthermore, a better understanding of structure-activity relationships of these peptides might help in development of optimized peptide for therapeutic use, while for prophylactic effects of peptides it is, especially in chicken, probably more cost-effective to determine ways to upregulate natural production of cathelicidins or other host defence peptides such as defensins.

CONCLUSION

Overall, this study shows that both CATH-2 and D-CATH-2 increase antigen presentation markers on both

primary monocytes and HD11 cells. These indicators of an increased inflammatory response could lead to more T cell activation and thus the potential for a better adaptive immune response against infection. This novel finding adds another functionality to the chicken cathelicidin repertoire in immunity.

ETHICS APPROVAL AND CONSENT TO PARTICIPATE

Not applicable.

HUMAN AND ANIMAL RIGHTS

No Animals/Humans were used for studies that are the basis of this research.

CONSENT FOR PUBLICATION

Not applicable.

AVAILABILITY OF DATA AND MATERIAL:

Not applicable.

FUNDING

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CONFLICT OF INTEREST

The authors declare no conflict of interest, financial or otherwise.

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