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CATH-2 and LL-37 increase mannose receptor expression, antigen presentation and the endocytic capacity of chicken mononuclear phagocytes

Marina D. Kraaij, Albert van Dijk, Henk P. Haagsman*

Division of Molecular Host Defence, Dept. of Infectious Diseases & Immunology, Utrecht University, Utrecht, The Netherlands

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ABSTRACT

Cathelicidins display in vitro and in vivo immunomodulatory activities and are part of the innate immune system. Previously, we found that in ovo treatment with chicken cathelicidin CATH-2 partially protects young broilers against respiratory E. *coli* infection. To determine the cellular aspects of this protection, we investigated immunomodulatory effects of CATH-2 and the human cathelicidin LL-37 on primary chicken peripheral blood mononuclear cells (PBMCs).

Treatment of chicken PBMCs with L-CATH-2, D-CATH-2 or LL-37 increased the percentage of mononuclear phagocytes, but decreased that of B cells. L-CATH-2, D-CATH-2 and LL-37 treatment of chicken PBMCs also enhanced the expression levels of mannose receptor MRC1 and antigen presentation markers MHCII, CD40 and CD86 on mononuclear phagocytes, indicating increased antigen presentation capacity. Concomitantly, L-CATH-2, D-CATH-2 and LL-37 neutralized LPS-induced cytokine production, while increasing the endocytic capacity.

We conclude that L-CATH-2, D-CATH-2 and LL-37 can modulate the immune response of primary chicken immune cells by increasing mannose receptor expression, antigen presentation, endocytosis and neutralizing LPS-induced cytokine production and as a result augment activation of the adaptive immune system.

1. Introduction

Host defense peptides are small and cationic peptides, which are conserved in many organisms, including mammals, birds and plants (Zasloff, 2002). Host defense peptides are important effectors in innate host defense (Yang et al., 2004; Zasloff, 2002). Two major families of host defense peptides are the defensins and the cathelicidins. Cathelicidins are short, cationic peptides with an amphipathic structure (Cuperus et al., 2013). LL-37 is the only cathelicidin in humans, a 37-aa cationic peptide, which can be produced by neutrophils, macrophages, NK cells and epithelial cells (Vandamme et al., 2012). There are four chicken cathelicidins, namely CATH-1, CATH-B1, CATH-2 and CATH-3 (Goitsuka et al., 2007; Lynn et al., 2004; van Dijk et al., 2005; Xiao et al., 2006). Cathelicidins have the ability to directly kill a wide range of bacteria (van Dijk et al., 2009; Veldhuizen et al., 2014). Cathelicidins play a role in the innate immune system a.o. by activating and recruiting a variety of immune cells (Zanetti, 2005). For example, mouse cathelicidin CRAMP and porcine PR39 can induce chemotaxis in neutrophils (Huang et al., 1997; Kurosaka et al., 2005). CATH-2 increased MCP-1 production in human peripheral blood mononuclear cells (PBMCs) (van Dijk et al., 2009), whereas IL-1β and CCL2 production was increased by CATH-1 in RAW264.7 mouse macrophages (Bommineni et al., 2014). LL-37 can direct human macrophages towards a pro-inflammatory phenotype (van der Does et al., 2010) and enhance their phagocytic capacity (Wan et al., 2014). Similarly, the synthetic peptide IDR-1018 has been shown to modulate macrophage differentiation (Pena et al., 2013). LL-37 has also been shown to influence adaptive immunity through modulation of dendritic cell differentiation (Bandholtz et al., 2006; Davidson et al., 2004). In addition, administration of these peptides can provide enhanced protection against infections (Bommineni et al., 2010; Cuperus et al., 2016; Lee et al., 2005). In a murine lung infection model, LL-37 cleared a *Pseudomonas aeruginosa* infection without direct antimicrobial effects and upregulated the neutrophil response (Beaumont et al., 2014). However, overexpression of LL-37 leads to the growth of larger tumors and tumorigenesis (von Haussen et al., 2008). CATH-1 provided partial protection against MRSA showing a delayed onset of disease and a better survival rate in a mouse model (Bommineni et al., 2010). In addition, in a zebrafish model, apart from having a protective effect against bacterial infection, CATH-2 also increased the number of phagocytic cells, indicating an immunostimulatory mechanism (Schneider et al., 2016). Much less is known about the effect of chicken cathelicidins on chicken cells. We previously showed that CATH-2 dose-dependently induced the transcription of IL-8, MCP-3 and RANTES, but not of IL-1β in a chicken

* Corresponding author at: Department of Infectious Diseases & Immunology Utrecht University Yalelaan 1 3584CL Utrecht The Netherlands.
E-mail address: h.p.haagsman@uu.nl (H.P. Haagsman).

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macrophage cell line and inhibited LPS-induced IL-1β and nitric oxide production (van Dijk et al., 2016). And since a protective effect against respiratory E. *coli* after in ovo treatment with CATH-2 was observed (Cuperus et al., 2016), we investigated the effects of CATH-2 and LL-37 in primary chicken cells.

2. Materials and methods

2.1. Peptides

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

2.2. Cell surface marker staining

Whole blood was collected from ~76 week old healthy chickens and PBMCs were isolated using Ficoll gradient and frozen until use. In a 48-wells Plate 500,000 PBMCs, diluted in RPMI1640 media (Lonza, Basel, Switserland) containing 10% fetal calf serum (Bodinco, Alkmaar, The Netherlands) and 1% penicillin/streptomycin (Life Technologies, Carlsbad, CA, USA), were incubated with different concentrations of peptide for 4 or 16 h at 41 °C. Next, cells were harvested and incubated for 30 min with antibodies CD45-APC (clone LT40), CD3-PE (clone CT3), KUL-01-FITC (CD206; clone KUL01) (Supplementary Fig. 1), MHCII-PE (clone 2G11) (all Southern Biotech, Birmingham, AL, USA) at 4 °C and with primary antibodies Bu-1 (clone AV20; Southern Biotech), CD40 (clone AV79) and CD86 (clone IAH:F853:AG2) (AbD Serotec, Kidlington, UK) and after washing, incubated for 30 min at 4 °C with secondary BV421-labelled antibody (Biolegend, San Diego, CA, USA). Afterwards, cells were washed and analyzed using flow cytometry (FACSCantoII, BD Biosciences, San Jose, CA, USA) and FlowJo. In addition, KUL-01- cells were isolated from PBMCs taking the flow-through after positive selection of KUL-01+ cells by MACS (Miltenyi, Bergisch Gladbach Germany) using a two-step isolation with KUL-01 antibody and IgG1 beads (Miltenyi).

2.3. Cytotoxicity assays

After 4 or 16 h incubation with the peptide, the PBMCs were stained with the Annexin V apoptosis kit (BD Biosciences) according to manufacturer's protocol and analyzed using flow cytometry and FlowJo. In addition, a WST-1 assay was done by incubating $1 \times 10\hat{}6$ PBMCs for 4 or 16 h in the presence of peptide. Medium was removed and cells were incubated with WST-1 (Roche, Basel, Switzerland) for 45 min and supernatant was measured in a Fluostar Omega plate reader (Isogen life science, Utrecht, The Netherlands) at 450 nm. Furthermore, lactate dehydrogenase (LDH) release was measured in supernatants from the same experiments using a cytotoxicity detection kit (Roche) according to manufacturer's protocol.

2.4. Confocal microscopy

PBMCs were incubated for 4 h with 10 μM of D-CATH-2 at 41 °C. After harvesting, cells were stained with KUL-01-FITC as described above. Cells were visualized using Leica SPE-II confocal microscope at the Center for Cell Imaging, University Utrecht, at a 100 x magnification.

2.5. Proliferation assay

In an activation assay, PBMCs were labelled with CFSE according to manufacturer's protocol (Biolegend). In short, cells were incubated with 5 μM of CFSE in PBS for 10 min at 37 °C. To quench the staining, RPMI medium containing 10% FCS was added. In a 96-wells plate, 100,000 PBMCs were added to Concanavalin A (Con A; Sigma-Aldrich, Saint Louis, MO, USA) in concentrations of 0–40 μg/ml in the presence of 5 μM L-CATH-2, or pre-incubated with L-CATH-2 for 1 h, before Con A was added. The cells were incubated for 5 days and analyzed using flow cytometry and FlowJo.

2.6. Endocytosis

In a 96-wells plate, 200,000 PBMCs were incubated with 1 mg/ml FITC-dextran (Sigma-Aldrich) in the presence of different concentrations of peptide for 1 h at 41 °C and on ice. Cells were washed four times with cold PBS and analyzed using flow cytometry. Endocytosis was determined for dextran uptake at 41 °C and corrected for uptake by cells kept on ice.

2.7. Quantitative PCR

Total RNA from PBMCs, incubated for 4 h with peptide or with peptide and 100 ng/ml LPS (E. *coli* 0111:B4; Invivogen, San Diego, CA, USA), was isolated using a high pure RNA tissue kit (Roche). cDNA was synthesized using iScript cDNA synthesis kit (Bio-Rad, Hercules, CA, USA). qPCR was performed using the following primers and probes for GAPDH (forward: GCCGTCCTCTCTGGCAAAG; reverse: TGTAAAC CATGTAGTTCAGATCGATGA; probe: AGTGGTGGCCATCAATGATCCC; accession number K01458.1), IL-1β (forward: GCTCTACAT GTCGTGTGTGATGAG; reverse: TGTCGATGTCCCGCATGA; probe: CCACACTGCAGCTGGAGGAAGCC; accession number Y15006.1) and IL-10 (forward: CATGCTGCTGGGCCTGAAG; reverse: ACGTCTCC TTGATCTGCTTGATG; probe: CGCTGTCACCGCTTCTTCACCTGC; accession number AJ621254.1) and IQ supermix (Bio-Rad). Reactions were performed using CFX Connect qPCR with CFX Manager 3.0 (Bio-Rad) as follows: 3 min at 95 °C (denaturation); 40 cycles: 10 s at 95 °C, 30 s at 60 °C and 30 s at 72 °C. The Ct values were normalized using the housekeeping gene GAPDH and the results were expressed as a fold difference from levels in control PBMCs.

2.8. Statistical analysis

Statistical analysis was performed using Graphpad Prism 6; statistical significance was evaluated by *t*-test and one-way ANOVA. A p-value of < 0.05 was considered statistically significant

3. Results

3.1. L-CATH-2, D-CATH-2 and LL-37 promote secondary necrosis of apoptotic PBMCs

We first investigated the toxicity of L-CATH-2, D-CATH-2 and LL-37 on freshly isolated chicken PBMCs by means of Annexin V/PI staining, LDH release and WST-1 assays. At 10 μM peptide and higher, a decrease in the percentage of AnV-/PI- and AnV + / PI- cells was observed, with an increase in AnV + /PI + cells (Fig. 1A,C,E). No decrease in WST activity was shown for L-CATH-2, D-CATH-2 and LL-37 indicating that the metabolic activity of the cells was not affected (Fig. 1B,D,F). Although the LDH release was increased with L-CATH-2 and D-CATH-2 above a concentration of 5 μM, no effect was shown in the presence of LL-37 (Fig. 1B,D,F). These data suggest enhanced toxicity of L-CATH-2 and D-CATH-2 towards chicken PBMCs at 10 μM and higher.

3.2. Increased percentage of KUL-01+ cells but decreased percentage Bu-1+ cells with L-CATH-2, D-CATH-2 and LL-37

Next we studied the effect of L-CATH-2, D-CATH-2 and LL-37 on the leukocyte population (CD45) and populations within: T-cells (CD3),

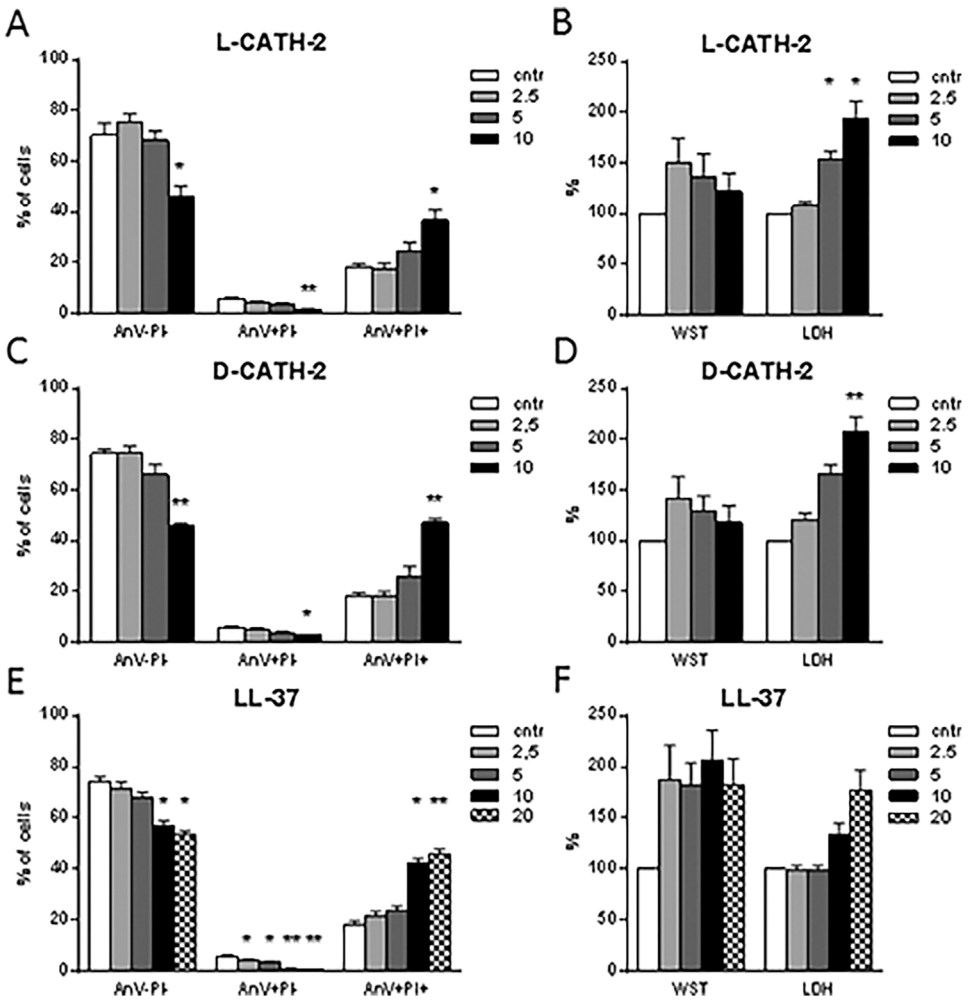

Fig. 1. L-CATH-2, D-CATH-2 and LL-37 promote secondary necrosis of apoptotic PBMCs.
PBMCs were incubated with different concentrations of L-CATH-2, D-CATH-2 (0–10 μM) and LL-37 (0–20 μM) for 4 h. The effect of (A) L-CATH-2, (C) D-CATH-2 and (E) LL-37 on the percentage of AnnexinV/PI cells was determined (n = 4). The effect of (B) L-CATH-2, (D) D-CATH-2 and (F) LL-37 on the relative percentage of WST activity and LDH release was determined (n = 4). Depicted are means ± SEM. *$p < 0.05$, **$p < 0.01$.

mononuclear phagocytes (KUL-01) and B-cells (Bu-1). No effect was observed of L-CATH-2, D-CATH-2 and LL-37 treatment on the percentage of CD45+ and CD3+ cells (Fig. 2A,B,E,F,I,J). L-CATH-2 dose-dependently increased the percentage of KUL-01+ cells and decreased percentage of Bu-1+ cells at a peptide concentration of 10 μM (Fig. 2C,D). A similar effect was observed with D-CATH-2: a dose-dependent increase of the percentage KUL-01+ cells, significant already at 5 μM, and a dose-dependent decrease in the percentage of Bu-1+ cells (Fig. 2G,H). The effect of LL-37 on the KUL-01+ (increased) and Bu-1+ (decreased) cells was only shown at the highest concentration of peptide, indicating that the human LL-37 does not affect the chicken PBMCs in an equal manner compared to the chicken CATH-2 (Fig. 2 K,L). In addition, we investigated the effect of the peptides after 16 h of incubation showing that the percentage of KUL-01+ cells was still increased after L-CATH-2 incubation and that the percentage of Bu-1+ cells was decreased by L-CATH-2, D-CATH-2 and LL-37 (Supplementary Fig. 2A).

To see if the effect of the increased percentage of KUL-01+ and decreased percentage of Bu-1+ was due to immunomodulation by peptides and not to cytotoxicity, we isolated KUL-01- cells and treated them with peptides for 4 h. The observed decreased percentage of Bu-1+ cells in the KUL-01 depleted cell population suggests toxicity for this particular cell population. L- and D-CATH-2 stimulation of KUL-01 depleted PBMCs however induced KUL-01+ expression (Supplementary Fig. 3). This confirms that the earlier observed increase of the percentage of KUL-01+ cells is not a relative increase due to peptide cytotoxicity and indicates that peptide treatment induced mannose receptor MRC1 (recognized by the KUL-01 antibody) in a subset of KUL-01 negative cells.

3.3. L-CATH-2, D-CATH-2 and LL-37 increased expression of KUL-01, MHCII, CD40 and CD86 on KUL-01+ cells

No effect of L-CATH-2, D-CATH-2 and LL-37 on the expression of CD45, CD3 and Bu-1 cells was observed (Fig. 3A,B,D,E,F,H,I,J,L). The KUL-01 expression was increased by L-CATH-2, D-CATH-2 and LL-37 in a dose-dependent manner (Fig. 3C,G,K). Already at a concentration of 2.5 μM there was an augmented effect on KUL-01 expression albeit not significant at this concentration. The increased expression of KUL-01 by D-CATH-2 was also shown by confocal microscopy (Fig. 3 M). At 16 h of incubation the increased expression of KUL-01 was still observed, whereas the Bu-1 expression was not affected by L-CATH-2, D-CATH-2 and LL-37 (Supplementary Fig. 2B). We also investigated the effect of L-CATH-2, D-CATH-2 and LL-37 on antigen presentation by studying the expression of MHCII and co-stimulatory molecules CD40 and CD86 on mononuclear phagocytes. The expression of MHCII and CD40 on KUL-01+ cells was dose-dependently increased after L-CATH-2 incubation (Fig. 4A). D-CATH-2 and LL-37 significantly increased the expression of MHCII, CD40 and CD86 on KUL-01+ cells (Fig. 4B,C). This indicates that L-CATH-2, D-CATH-2 and LL-37 increase the antigen presentation capacity of chicken mononuclear phagocytes.

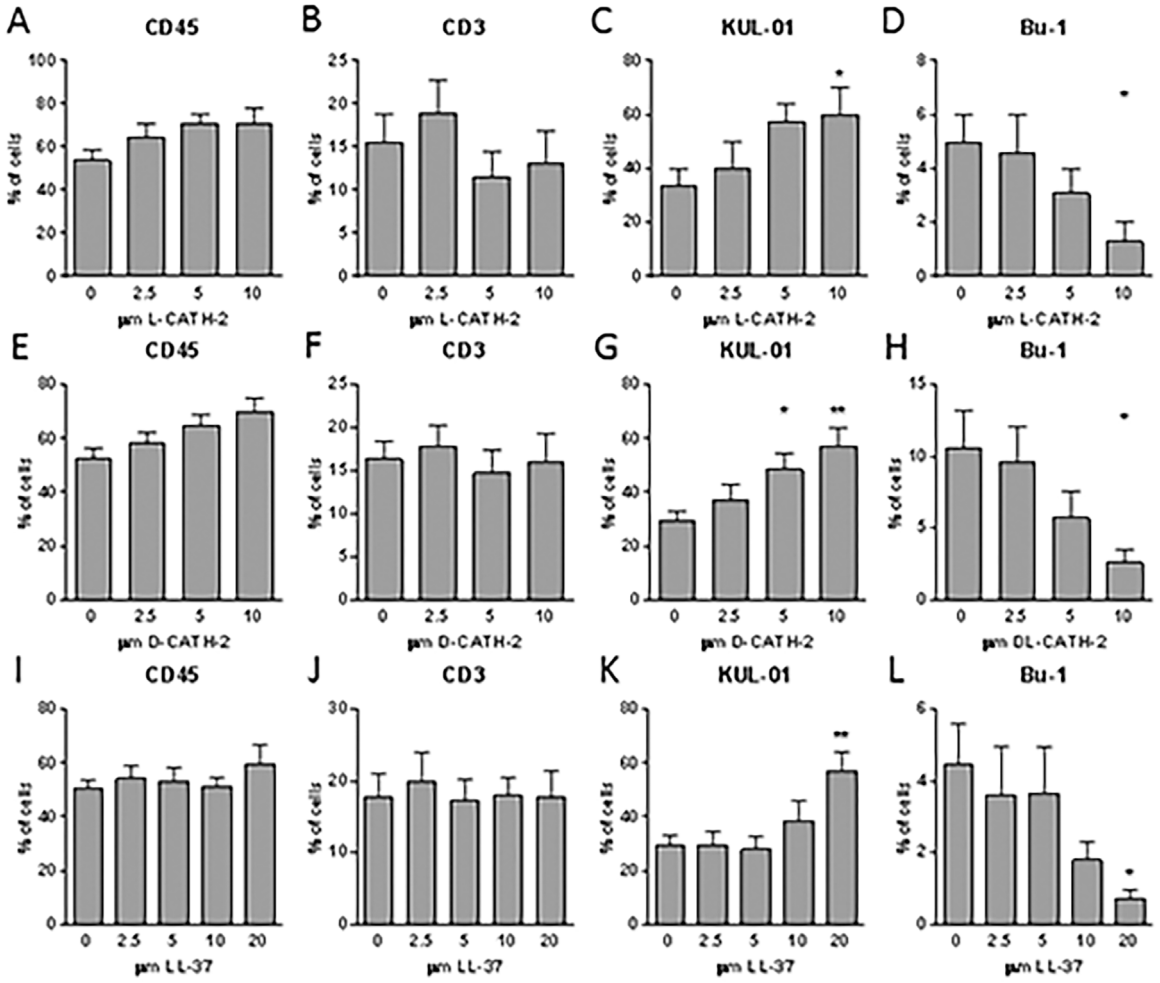

Fig. 2. L-CATH-2, D-CATH-2 and LL-37 increase the percentage of KUL-01 + cells but decrease the percentage Bu-1 + cells.
PBMCs were incubated with different concentrations of L-CATH-2, D-CATH-2 (0–10 μM) and LL-37 (0–20 μM) for 4 h. The effect of L-CATH-2 on the percentage of (A) CD45, (B) CD3, (C) KUL-01 and (D) Bu-1 was determined. The effect of D-CATH-2 on the percentage of (E) CD45, (F) CD3, (G) KUL-01 and (H) Bu-1 was determined. The effect of LL-37 on the percentage of (I) CD45, (J) CD3, (K) KUL-01 and (L) Bu-1 was determined (n = 4-9). Depicted are means ± SEM. *p < 0.05, **p < 0.01.

3.4. L-CATH-2, D-CATH-2 and LL-37 neutralized LPS-induced cytokine production

We also wanted to investigate if the three peptides could have a functional effect on chicken cells, since it has been shown previously that CATH-2 can neutralize LPS-induced cytokine production in human PBMCs(van Dijk et al., 2009). Therefore, we studied the effect of L-CATH-2, D-CATH-2 and LL-37 on the cytokine producing capacity of chicken PBMCs. L-CATH-2 slightly increased IL-1β mRNA levels (1.5-fold) and increased IL-10 mRNA levels (3-fold). D-CATH-2 treatment increased IL-10 mRNA expression levels to the same extent, whereas LL-37 decreased IL-1β and IL-10 mRNA levels (Fig. 5A). However, after LPS stimulation L-CATH-2, D-CATH-2 and LL-37 neutralized the LPS-increased IL-1β and IL-10 mRNA production (Fig. 5B,C).

3.5. L-CATH-2, D-CATH-2 and LL-37 increased PBMC endocytosis

Another important cell function is endocytosis and we investigated this by determining dextran uptake by PBMCs in the presence of the three peptides. L-CATH-2 showed a significant increase in endocytosis already at a peptide concentration of 2.5 μM (Fig. 5D). D-CATH-2 and LL-37 also showed a dose-dependent increase in endocytic capacity (Fig. 5D).

3.6. Pre-incubation with L-CATH-2 decreased PBMC proliferation

Proliferative capacity was determined in order to investigate the effect of L-CATH-2 on Con A-stimulated PBMCs. Proliferation was observed with Con A-stimulated PBMCs, however, the presence of L-CATH-2 did not affect the percentage of proliferation (Fig. 5E). When L-CATH-2 was pre-incubated 1 h before the addition of Con A, a decrease in proliferation was shown and this was reversed when L-CATH-2 was washed away after pre-incubation and then cells were stimulated with Con A (Fig. 5E).

4. Discussion

In this study we investigated the effects of chicken CATH-2 and human cathelicidin LL-37 on primary chicken cells. Apart from examining the influence of the physiological CATH-2 peptide, we also investigated the D-form of CATH-2 (D-CATH-2) for a potential therapeutic use, as we previously have observed prophylactic protective effects against bacterial infections of this peptide in both a zebrafish- and a chicken infection model (Cuperus et al., 2016; Schneider et al., 2016). A concern was the potential toxicity of peptides towards cells. To this end we measured the percentage of AnnexinV + /PI + cells, metabolic activity and LDH release of PBMCs after incubation with L-CATH-2, D-CATH-2 and LL-37. For fowlicidin 1–3 toxicity was reported in the range of 6–40 μM showing 50% killing of mammalian erythrocytes

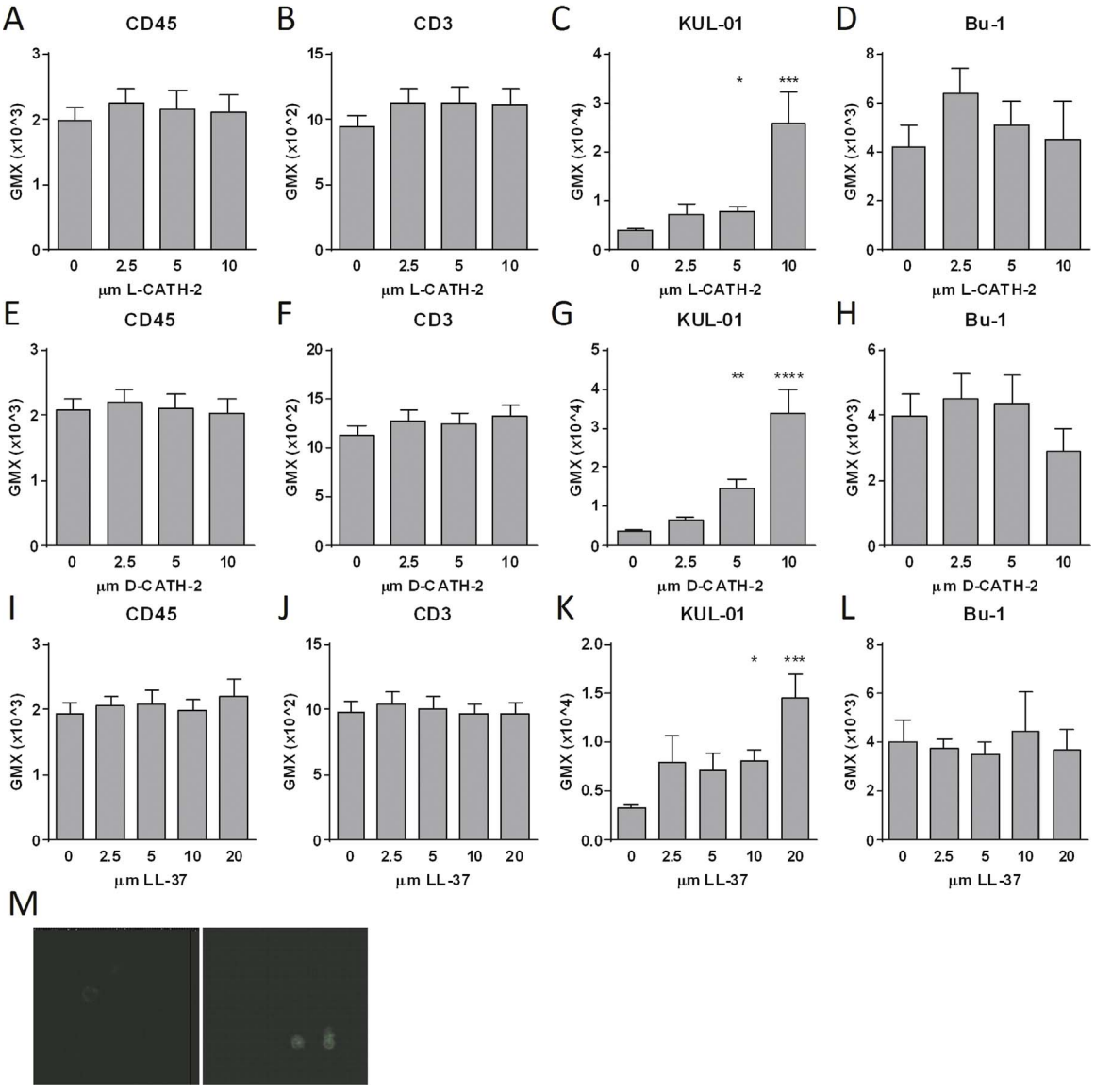

Fig. 3. L-CATH-2, D-CATH-2 and LL-37 increase KUL-01 expression.
PBMCs were incubated with different concentrations of L-CATH-2, D-CATH-2 (0–10 µM) and LL-37 (0–20 µM) for 4 h. The effect of L-CATH-2 on the expression of (A) CD45, (B) CD3, (C) KUL-01 and (D) Bu-1 was determined. The effect of D-CATH-2 on the expression of (E) CD45, (F) CD3, (G) KUL-01 and (H) Bu-1 was determined. The effect of LL-37 on the expression of (I) CD45, (J) CD3, (K) KUL-01 and (L) Bu-1 was determined (n = 4-9). (M) KUL-01 staining shown by confocal microscopy with control (left) and D-CATH-2 (right) incubated cells. Depicted are means ± SEM. *p < 0.05, **p < 0.01, ***p < 0.001, ****p < 0.0001.

(Xiao et al., 2006). CATH-2 had only marginal toxic effects towards human PBMCs, whereas LL-37 showed toxicity at 40 µM to these cells as measured by WST activity (van Dijk et al., 2009). We observed no significant decrease in WST activity with all three peptides, which is in agreement with these earlier observations. However, the percentage of AnnexinV + /PI + cells as well as LDH release was increased, indicating a potential toxicity of L-CATH-2, D-CATH-2 and LL-37 against chicken PBMCs. In human neutrophils, LL-37 promotes secondary necrosis and this has also been described for CRAMP (Li et al., 2009; Zhang et al., 2008). We also see this conversion of AnnexinV + /PI- into AnnexinV + /PI + chicken PBMCs with LL-37, L-CATH-2 and D-CATH-2, indicating that secondary necrosis of apoptotic primary chicken cells is promoted by these peptides.

The endocytic capacity of chicken PBMCs was enhanced by L-CATH-2, D-CATH-2 and LL-37, which is in line with the enhanced uptake by cathelicidins of LPS or DNA via endocytic pathways (Coorens et al., 2015; Shaykhiev et al., 2010; Suzuki et al., 2016), indicating a more active transport of molecules and particles into the cells due to the peptides. Activation of PBMCs by Con A was not affected by L-CATH-2, only pre-incubation of the peptide with PBMCs showed decreased proliferative capacity. Thus L-CATH-2 needs to have an intrinsic effect on PBMCs first. Interestingly, after washing away the peptide, the reduction in proliferation was reversed, suggesting that the peptide needs to be present to exert its effect on PBMCs to maintain their diminished activation capacity in response to Con A.

Previously it has been shown that LL-37 can block LPS-induced pro-inflammatory cytokines in both human monocytes and T cells (Mookherjee et al., 2009). In human PBMCs and mouse RAW264.7 cells, chicken cathelicidins CATH-1, CATH-2, CATH-3 and bovine cathelicidin BMAP-28 all can inhibit LPS-induced cytokine expression, including IL-1β and IL-6 (Bommineni et al., 2007; van Dijk et al., 2009; Xiao et al., 2006; D'Este et al., 2012). Although with L-CATH-2 and D-

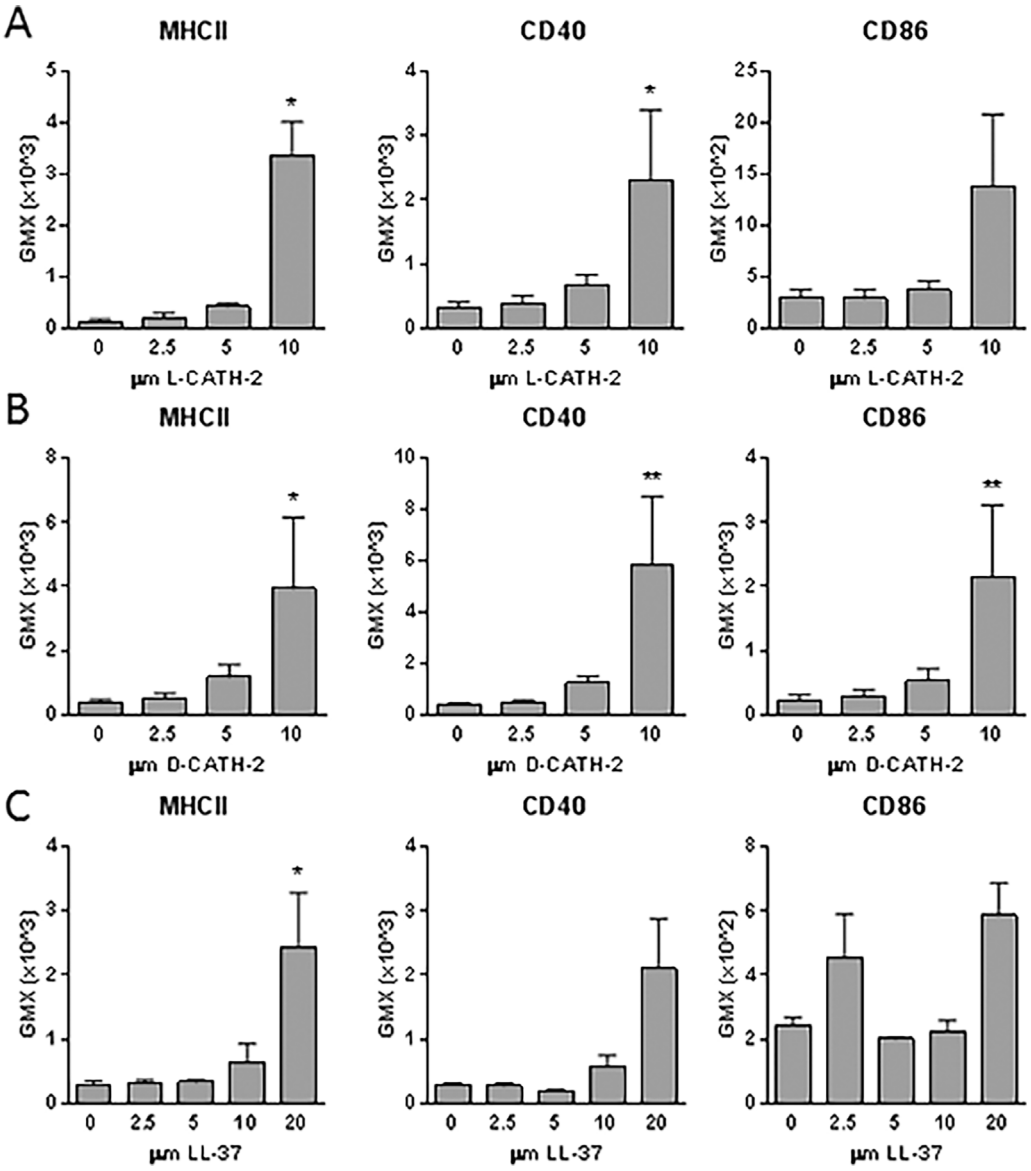

Fig. 4. L-CATH-2, D-CATH-2 and LL-37 increase expression of MHCII, CD40 and CD86 on KUL-01 + cells.
PBMCs were incubated with different concentrations of L-CATH-2, D-CATH-2 (0–10 μM) and LL-37 (0–20 μM) for 4 h. The effect of (A) L-CATH-2, (B) D-CATH-2 and (C) LL-37 on the expression of MHCII (left), CD40 (middle) and CD86 (right) on KUL-01+ cells was determined (n = 3-9). Depicted are means ± SEM. *p < 0.05, **p < 0.01, ***p < 0.001.

CATH-2 alone IL-1β is increased, the LPS-induced IL-1β and IL-10 mRNA levels were neutralized by L-CATH-2, D-CATH-2 and LL-37. This indicates that L-CATH-2, D-CATH-2 and LL-37 are able to reduce the LPS-induced cytokine response in chicken mononuclear phagocytes.

In human PBMCs, different cell populations, including monocytes, T cells and B cells, respond to LL-37 (Mookherjee et al., 2009). In chicken PBMCs, L-CATH-2, D-CATH-2 and LL-37 increased the percentage of mononuclear phagocytes and decreased the percentage of B cells, with no effect on the T cells. The decrease in B cells appears to be due to a toxic effect of the three peptides, since already at low concentration hardly any B cell is present. Thus B cell functions, such as the production of antibodies, are inhibited when L-CATH-2, D-CATH-2 and LL-37 are given to PBMCs in vitro. The expression of KUL-01 was increased by L-CATH-2, D-CATH-2 and LL-37. Interestingly, the KUL-01 antibody recognizes a homologue of the macrophage mannose receptor MRC1 (Staines et al., 2014). In mammals, MRC1 (CD206) is a pattern recognition receptor involved in antigen uptake and presentation that recognizes terminal mannose residues on a.o. *Candida albicans* (Martinez-Pomares et al., 1998), *Pneumocystis carinii* (O'Riordan et al., 1995) and *Leishmania donovani* (Chakraborty et al., 2001). Importantly, mammalian MRC1 is also thought to regulate the levels of mannosylated glycoproteins (such as lysosomal hydrolases, tissue plasminogen activator) released during an inflammatory response. Only during resolution of inflammation, MRC1 expression is highly upregulated, enabling the removal of host tissue-damaging glycoproteins from circulation (Gazi and Martinez-Pomares, 2009; Lee et al., 2002). Although little is known about the biological functions, it is tempting to speculate that avian MRC1 may similarly contribute to the resolution of inflammation in birds.

We observed that the two chicken peptides, L-CATH-2 and D-CATH-2, increase surface expression of MHCII, CD40 and CD86 on mononuclear phagocytes. In agreement, CATH-1 has been reported to enhance the expression of MHCII and CD86 on mouse macrophage RAW264.7 cells (Bommineni et al., 2014). Furthermore, LL-37 enhances HLA-DR and CD86 expression on human dendritic cells, indicating that LL-37 actively takes part in the dendritic cell-derived immune response (Bandholtz et al., 2006; Davidson et al., 2004). Together, the observed increased expression of KUL-01, MHCII and co-stimulatory molecules points to an increased antigen presentation capacity of L-CATH-2 and D-CATH-2 stimulated chicken PBMCs, indicating an enhanced adaptive response against pathogens, which could in part explain the protective effect of D-CATH-2 after in ovo treatment against a respiratory E. *coli* infection.

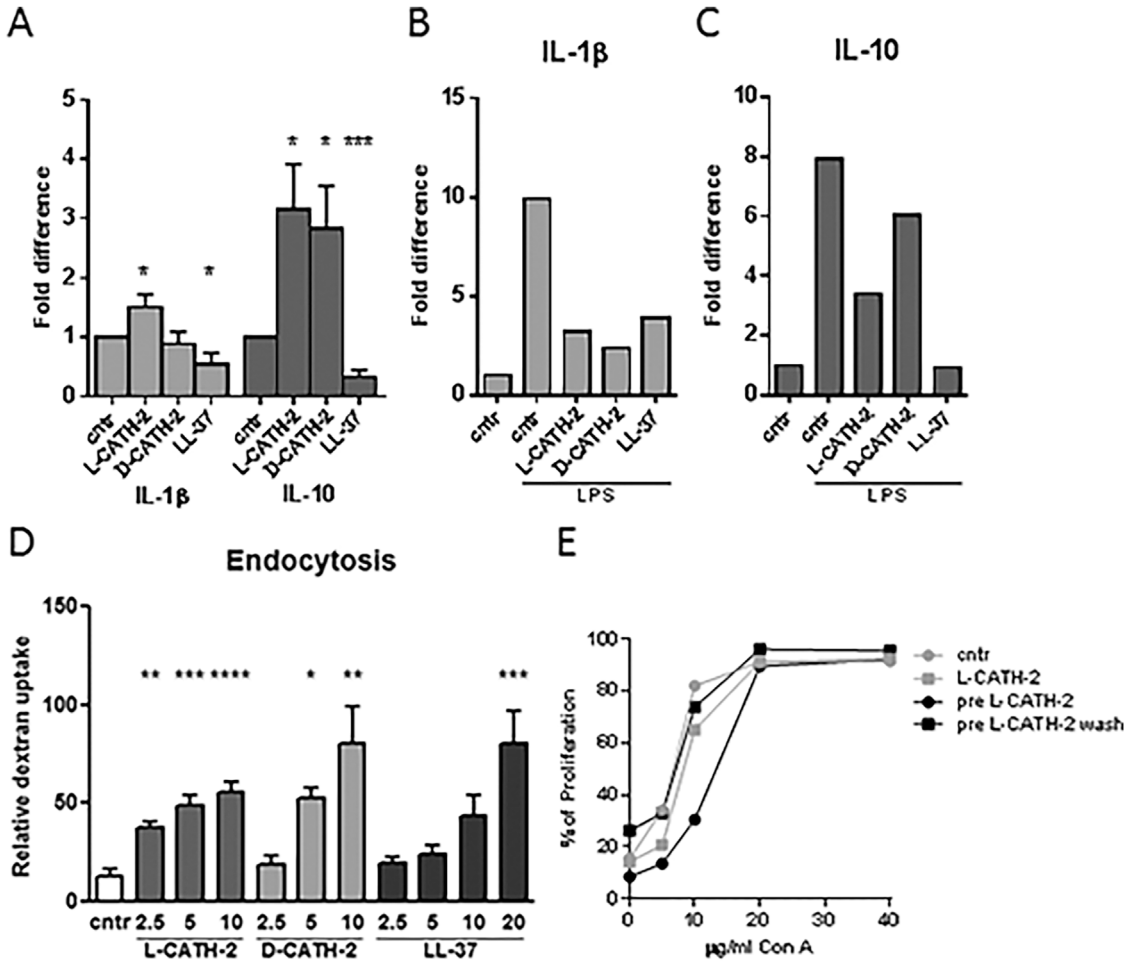

Fig. 5. L-CATH-2, D-CATH-2 and LL-37 neutralize LPS-induced cytokine production and increase endocytosis.
(A) PBMCs were incubated with 10 µM of L-CATH-2 or D-CATH-2 or with 20 µM LL-37 for 4 h. mRNA production of IL-1β and IL-10 was determined and normalized using GAPDH and expressed as a fold difference compared to control cells (n = 5). Depicted are means ± SEM. A representative example of (B) IL-1β and (C) IL-10 mRNA expression is shown after 4 h of L-CATH-2, D-CATH-2 and LL-37 incubation in combination with LPS stimulation. (D) PBMCs were incubated with different concentrations of L-CATH-2, D-CATH-2 or LL-37 in the presence of dextran for 1 h. The relative endocytosis compared with control samples was determined (n = 4). Depicted are means ± SEM. (E) In an activation assay, 100,000 PBMCs were added to different concentrations of Con A in the presence of 5 µM of L-CATH-2 and proliferation was measured after 5 days (n = 6). A representative example is shown of proliferation of PBMCs with different concentrations of Con A for control cells (cntr), L-CATH-2-incubated cells (L-CATH-2), cells pre-incubated with L-CATH-2 before Con A was added (pre L-CATH-2), and cells pre-incubated with L-CATH-2 than washed away before Con A was added (pre L-CATH-2 wash). *p < 0.05, **p < 0.01, ***p < 0.001, p < 0.0001.

Acknowledgements

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.molimm.2017.07.005.

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