Cathelicidins and the regulation of the innate immune system

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ISBN: 978-90-393-6595-3

The research described in this thesis was financially supported by the Immuno Valley ALTANT ASIA2 program of the Dutch Ministry of Economic Affairs and a research grant from the Canadian Cystic Fibrosis Foundation.

Research described in this thesis was performed at:
- the Department of Infectious Diseases and Immunology, Division of Molecular Host Defence, Faculty of Veterinary Medicine, Utrecht University, Utrecht, the Netherlands.
- the Department of Infectious Diseases and Immunology, Division of Immunology, Faculty of Veterinary Medicine, Utrecht University, Utrecht, The Netherlands
- the Host Microbe Interactomics Group, Animal Sciences, Wageningen University, Wageningen, The Netherlands
- the Department of Physiology and Pharmacology, The University of Western Ontario, London, Ontario, Canada

Printing of this thesis was financially supported by the Department of Infectious Diseases and Immunology, Division of Molecular Host Defence, Faculty of Veterinary Medicine, Utrecht University, Utrecht, the Netherlands and Infection & Immunity Utrecht, Utrecht, the Netherlands.

Cover Design: purchased from iStock
Layout: Proefschrift-aio.nl
Printing: Proefschrift-aio.nl

Cathelicidins and the regulation of the innate immune system

Cathelicidines en de regulatie van het aangeboren immuunsysteem

(met een samenvatting in het Nederlands)

Proefschrift

ter verkrijging van de graad van doctor aan de Universiteit Utrecht op gezag van de rector magnificus, prof.dr. G.J. van der Zwaan, ingevolge het besluit van het college voor promoties in het openbaar te verdedigen op maandag 26 september 2016 des middags te 2.30 uur

door

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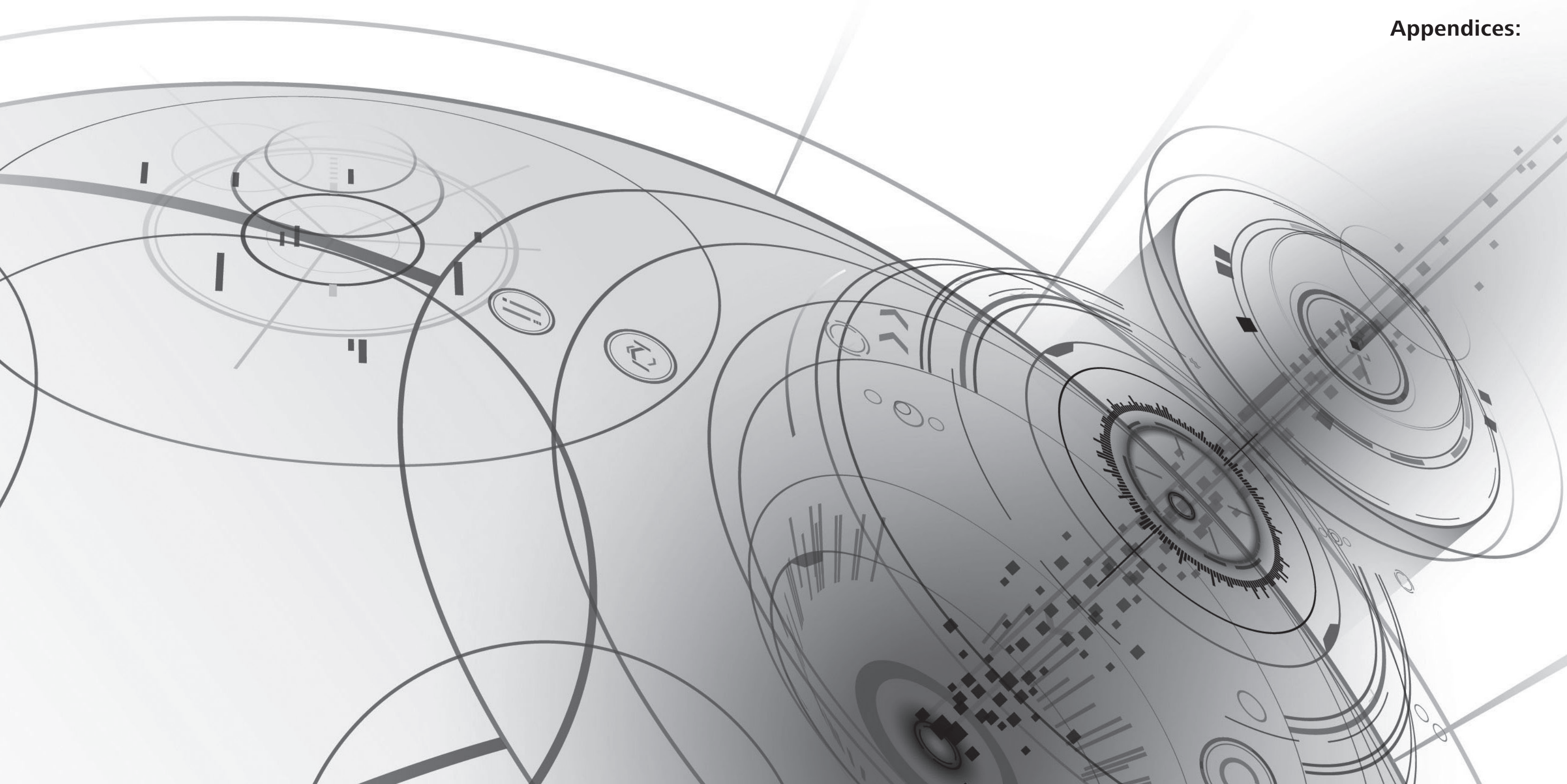

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Chapter 1

General Introduction

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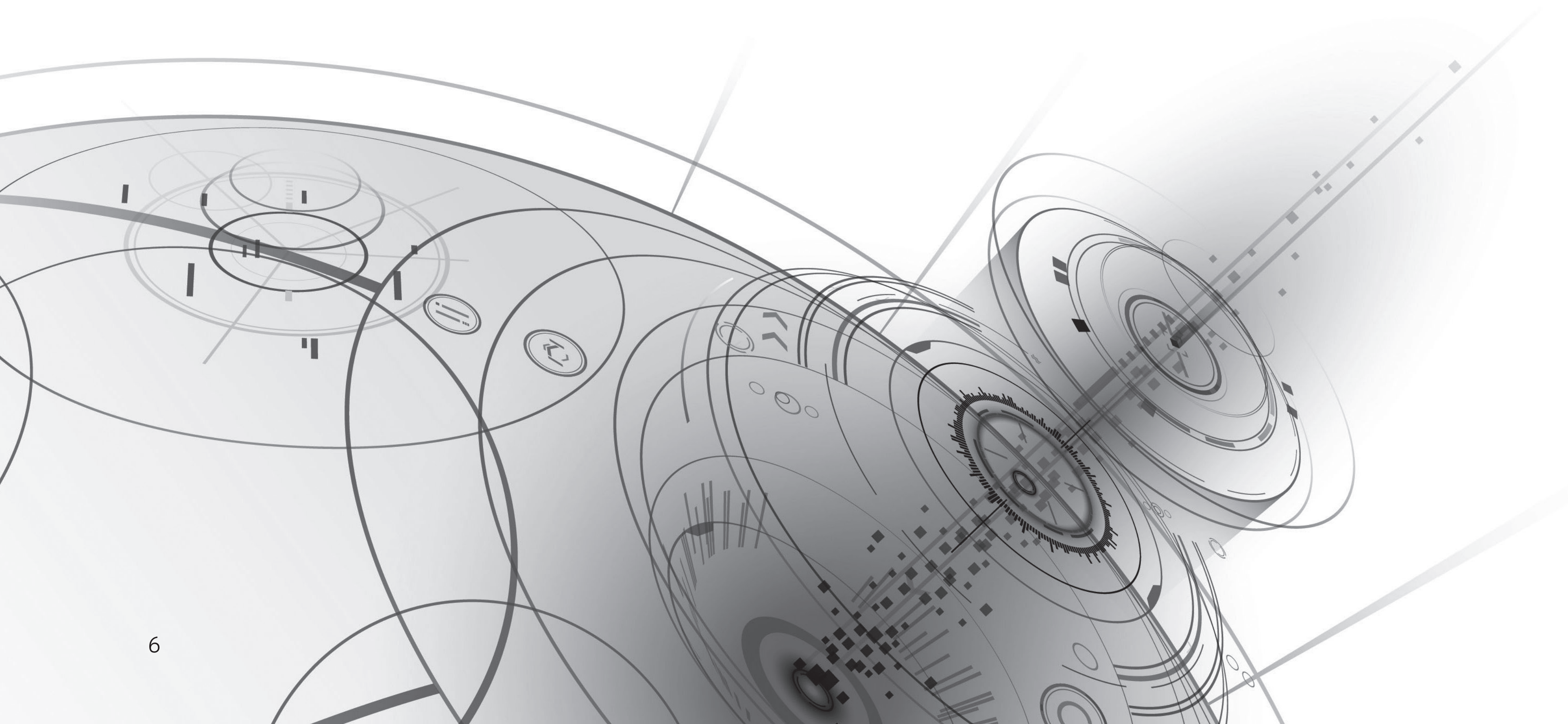

Host responses during infection

Early immune response

During infections, a quick immune response is essential to rapidly eliminate infectious microbes. The initiation of this response takes place at the site of infection, where tissue resident macrophages and dendritic cells (DCs) (1), but also epithelial cells (2) and fibroblasts (3), sense microbe-associated molecular patterns (MAMPs) and damage-associated molecular patterns (DAMPs). MAMPs are highly conserved microbial components, while DAMPs are host-derived components released upon tissue damage and cell death. MAMPs and DAMPs can activate pattern-recognition receptors (PRRs), which results in the local production of pro-inflammatory cytokines and chemokines, such as TNFα, interleukin (IL)-6, CXCL1 and CXCL2 (4). Released chemokines form a chemotactic gradient for the recruitment of neutrophils to the site of infection (5). Once arrived, neutrophils are activated by host- and microbe-derived inflammatory signals and use their broad antimicrobial arsenal to counter the infectious microbes. This arsenal includes the content of azurophilic and specific granules, which contain membrane active antimicrobial peptides, such as defensins and cathelicidins, and enzymes, such as lysozyme and serine proteases. These granule components are released into phagosomes containing phagocytosed microbes, but are also released into the extracellular environment (6). In addition, intracellular and extracellular reactive oxygen species (ROS) and reactive nitrogen species (RNS) are produced to promote bacterial killing (7). Furthermore, neutrophils promote the recruitment of macrophages to the site of infection by releasing chemokines, such as CCL2 and CCL3, as well as chemotactic granule components, such as cathelicidins (6, 8). The recruitment of macrophages is important for the removal of damaged tissue and apoptotic neutrophils. To prevent additional inflammation, lipid mediators, such as lipoxin, resolvin and protectin, are released and increase CCR5 expression on apoptotic neutrophils to sequester CCL3 and CCL5, which prevents additional neutrophil recruitment (4, 6). In addition, lipoxins promote non-inflammatory phagocytosis of apoptotic neutrophils by macrophages (9).

Macrophages

Macrophages have an important function in the onset of inflammation, but are also vital in the prevention of excessive inflammation by the non-inflammatory removal of damaged tissue and apoptotic neutrophils. To acquire these diverse functions at the right moment, macrophage differentiation states are strongly influenced by the microenvironment.

Tissue localization and influx

Macrophages represent a variety of different subsets that are divided both by function and anatomical localization. Specialized tissue macrophage subsets include Kupffer cells in the liver, alveolar macrophages in the lung and osteoclasts in the bone (10). These cell types have specific functions for the tissue they are located in. For instance, osteoclasts are involved in bone-resorption, while Kupffer cells are involved in the processing of heme and iron derived from red blood cells (11). In addition to these tissue-resident macrophages, which can be maintained by self-renewal (12), inflammatory monocytes (marked by high LyC6 expression in mice) are recruited from the bloodstream to the site of infection to differentiate into macrophages. This recruitment depends on chemotactic signals such as CCL2, which can be produced by almost all nucleated cells and promotes the migration of LyC6$^+$ monocytes from the bone marrow into the bloodstream. Other chemokines, such as CCL5, are important in the transendothelial chemotaxis of monocytes into the infected tissue (13). From the infected tissue, monocytes can further traffic to lymph nodes and either deliver antigens to DCs or differentiate into monocyte-derived DCs for antigen-presentation and activation of CD4$^+$ and CD8$^+$ T-cells (14).

Differentiation and polarization

Development of different macrophage subsets depends on various factors. The first signal in macrophage differentiation is activation of the CSF-1 receptor by factors such as M-CSF, which activates the PU.1 transcription factor. PU.1 is found in all macrophages and acts as a basis for further differentiation into distinct phenotypes (11, 15). The second signal can be a tissue-specific signal, which results in the activation of specific transcription factors, such as LXRα in splenic marginal zone macrophages, PPARγ in lung alveolar macrophages and NR4A1 in thymic macrophages (11, 16). After differentiation, most tissue-resident macrophages have an anti-inflammatory phenotype (10, 12), which can be altered by various inflammatory signals. IFNγ, for instance, causes activation of STAT1, which promotes an antimicrobial and pro-inflammatory 'M1' phenotype with high expression of NOS2, MHC class II and IL-12. On the other hand, stimulation of macrophages with IL-4 induces STAT6 activation and differentiation into a 'M2' phenotype with high arginase-1 and macrophage mannose receptor-1 (Mrc1) expression (15). This 'M2' phenotype is more associated with anti-inflammatory responses and promotion of wound healing. Although these phenotypes represent specific macrophage subsets, it is generally accepted that macrophage polarization is a flexible process and that macrophages are more likely to operate somewhere along the spectrum between the defined M1 and M2 phenotypes (10).

Macrophage activation

Activation of PRRs by MAMPs plays an important role in macrophage activation during infection. (12, 15). PRRs are either located on the cell surface, in endosomal compartments or in the cytosol (17-19). Cell surface PRRs include dectin-1, which

recognizes β-glucan (20) and C-type lectin receptors, such as DC-SIGN, which is involved in mannose detection (21). Furthermore, many Toll-like receptors (TLRs) are located at the cell surface, with several additional ones located in endosomes (17). Most cytosolic PRRs are involved in nucleic acid detection, with at least 13 distinct cytosolic proteins involved in DNA recognition (18). In addition, cytosolic NOD1 and NOD2 are involved in the recognition of peptidoglycan fragments (22-24) and activation of cytosolic receptors, such as NLRP3, IPAF and AIM2, results in inflammasome activation, which is important for the cleavage of pro-IL-1β and subsequent release of IL-1β into the extracellular environment (25). Due to the diversity of conserved molecules that can be detected by PRRs, they are able to induce an immune response against a wide variety of pathogens.

MAMPs and TLRs

The TLR family is a well-studied group of PRRs that can be located on the cell surface or in endosomal compartments and are involved in the detection of different MAMPs (Fig. 1). Their name originates from the *Drosophila melanogaster* Toll protein, which is involved in dorsal-ventral development during embryogenesis and anti-fungal responses (26, 27). All TLRs are type I transmembrane proteins with an N-terminal part that consists of a horseshoe-shaped extracellular domain with 19-27 leucine-rich repeats (LRRs). Variations in this ectodomain are important for the distinction of different ligands by different TLRs (28, 29). The C-terminal cytoplasmic region contains a Toll/Interleukin receptor (TIR) domain, which is similar to the IL-1 receptor signaling domain, and is involved in the recruitment of adaptor proteins after TLR activation (30). TLRs located on the cell surface include TLR1, TLR2, TLR4, TLR6 and TLR10, which are mainly involved in the detection of lipid-containing MAMPs (17, 31). TLR5 is also expressed on the cell surface and recognizes bacterial flagellin (17, 32, 33). Endosomal TLR3, TLR7, TLR8, TLR9 and TLR13 are involved in the detection of nucleic acids (34-37), while mouse specific endosomal TLR11 and TLR12 are involved in the detection of profilin from *Toxoplasma gondii* (38-41).The localization of the TLRs to either the cell surface or endosomal compartments depends on specific amino acid motifs in the transmembrane region. Furthermore, the delivery of TLRs to the correct cellular compartments depends on proteins like UNC93B1, for the trafficking of endosomal TLRs, and gp96 and PRAT4A, for the trafficking of several endosomal and cell surface TLRs (42, 43).

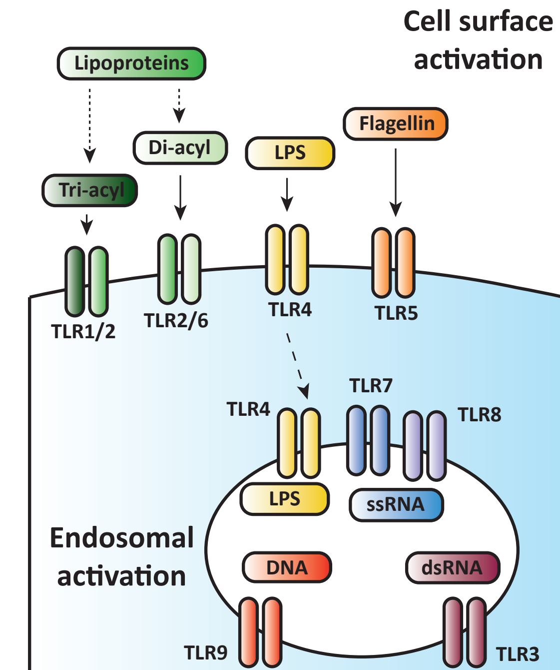

Figure 1: TLR localization and TLR ligands

TLR4

LPS

TLR4 is a cell-surface TLR involved in the detection of lipopolysaccharides (LPS) from Gram-negative bacteria. LPS is part of the bacterial outer membrane (OM), where it is abundantly present in the outer leaflet, in contrast to the phospholipids present in the inner leaflet of the OM (44). LPS molecules consist of three regions (45). The highly variable O-antigen, which consists of a polymer of oligosaccharides, reaches out from the bacterial membrane and plays an important role in inhibition of antibody binding (46, 47), serum-mediated killing (48, 49) and phagocytosis (50-52). Depending on the length of the O-antigen, LPS is either termed smooth (long O-antigen) or rough (short O-antigen) (53). The core-region of the LPS molecule is more conserved than the O-antigen and consists of an outer and inner core. The outer core is a more variable region consisting of hexose sugers, which can include glucose, galactose or *N*-acetyl glucosamine. The inner core can contain heptose and always contains at least one 2-keto-3-deoxyoctonate (KDO) sugar residue. Core region sugars can be modified by the addition of negative groups, such as phosphate groups, to allow the binding of Ca^{2+} and Mg^{2+} ions to the membrane, which is important for membrane structure

and function (54). The third part of the LPS is the lipid A-part, which consists of a phosphorylated diglucosamine backbone that is linked to the KDO from the inner core and is attached to 4-7 acyl chains (54, 55). The lipid A together with the charged inner core is crucial in the activation of the TLR4.

TLR4 activation

Binding of LPS to TLR4 induces the formation of a TLR4 dimer, which consists of 2 TLR4 molecules, 2 MD-2 molecules and 2 LPS molecules. MD-2 is crucial for the LPS binding and contain a hydrophobic pocket for the Lipid A acyl chains. The charged phosphate group of the lipid A backbone interacts with charged residues on both MD-2 and TLR4. The TLR4 dimerization that is induced by the LPS binding is thought to promote the juxtaposition of the intracellular TIR domain, which is followed by the recruitment of adaptor proteins for downstream signaling (55, 56). The number of acyl chains in the LPS molecule plays an important role in the level of TLR4 activation. Six acyl chains appear ideal for the activation of the human TLR4, in which the lack of a sixth acyl chain hampers TLR dimerization (55, 57). However, the mouse and chicken TLR4-MD2-complexes are activated by both penta- and hexa-acylated LPS, demonstrating the species-specificity of LPS-induced TLR4 responses (58, 59).

The TLR4-MD-2-complex depends on several accessory proteins to increase the sensitivity towards LPS. One of these accessory proteins is CD14, which can be found either as a glycosylphosphatidylinositol (GPI)-anchored membrane protein (mCD14) (43) or as a secreted soluble protein (sCD14) (60). CD14 is important for the delivery of smooth LPS, and to a lesser extent rough LPS, to the TLR4-MD-2-complex (61-63). Another accessory protein involved in LPS delivery to this complex is the LPS-binding protein (LBP). LBP acts as a catalyst to remove LPS from membrane structures and deliver it to CD14 for subsequent transfer to the TLR4-MD-2-complex (64). Several other components, including albumin and high-density and low-density lipoproteins (HDL and LDL), are also involved in either the promotion of LPS delivery to TLR4 or the sequestering of LPS to prevent TLR4 activation (60, 64, 65).

TLR2

Lipid-containing MAMPs

TLR1, TLR2, TLR6 and TLR10 are a group of evolutionary closely related TLRs (66). Similar to TLR4, they are primarily involved in the detection of lipid-containing MAMPs, such as lipoproteins or lipoteichoic acid (LTA) (67). TLR activating lipoproteins are found on both Gram-positive and Gram-negative bacteria and are characterized by the presence of 2 acyl chains connected by ester bonds to a glycerol backbone, which is attached to a cysteine via a sulfur atom. An additional lipid chain can be attached via an amide bond on the cysteine to obtain a tri-acylated protein (68-70). The most abundant lipoprotein in Gram-negative bacteria is the tri-acylated Braun lipoprotein (BLP) (68), which activates cells in a TLR2-dependent manner (71, 72). Based on this lipoprotein, a synthetic tri-acylated lipoprotein (Pam_3CSK_4) with potent TLR2 activating capacity has been developed (73). Several di-acylated lipoproteins, such as *Mycoplasma salivarium*-derived LP44 and *Mycoplasma fermentans*-derived M161Ag, were used as a model for the development of di-acylated synthetic analogs, such as FSL-1 and MALP-2 (73). Interestingly, LPS from *P. gingivalis* is also able to induce TLR2 activation, in contrast to other LPS types (54, 74). TLR2 can also be activated by Gram-positive bacteria via LTA, which can be found in the peptidoglycan cell well (67). Its minimal active structure has been determined to be two acyl chains attached to a glycerophosphate backbone (75).

TLR2 activation

Cellular activation by lipoproteins is dependent on TLR1, 2 and 6, which can form heterodimers, leading to TLR1/2 and TLR2/6 complexes (76, 77). The TLR1/2 complex is responsible for the recognition of tri-acylated lipoproteins, such as Pam_3CSK_4. This lipoprotein activates the TLR1/2 complex by inserting the two ester-bound acyl-chains in the TLR2 molecule, while the amide-bound acyl-chain is inserted in the TLR1 molecule (76). The TLR2/6 complex is responsible for the recognition of di-acylated lipoproteins, such as Pam_2CSK_4. Similar to the TLR1/2 complex, Pam_2CSK_4 inserts its two glycerol-bound acyl-chains into the hydrophobic TLR2 pocket. The lack of the third amide-bound acyl-chain is compensated by the increased hydrophobic area of the TLR6 (77). Upon lipoprotein binding, heterodimerization is promoted, which is suggested to promote intracellular TIR-domain dimerization and recruitment of intracellular adaptor proteins for downstream signaling (67). While TLR10 is evolutionary grouped with TLR1, 2 and 6, its ligand and function are not completely understood. However, a recent study has shown that TLR10 might function in inhibiting TLR2 signaling, potentially by forming heterodimers with TLR2 (31).

Similar to activation of TLR4, TLR2 activation can be influenced by accessory molecules. CD36 can enhance TLR2/6 activation by the synthetic lipoprotein MALP-2 (78) and plays a role in LTA and *S. aureus*-induced immune responses (79). Furthermore, CD14 is involved in the sensing of MALP-2, Pam_2CSK_4 and LTA (63) as well as several *M. tuberculosis*-derived lipoproteins (80). Finally, activation of TLR2 can be promoted by the LBP-mediated delivery of lipoproteins and LTA to the TLR2-complexes (81, 82).

TLR5

Flagellin

TLR5 is important for the detection of bacterial flagellin (32, 83). Flagellin is part of the bacterial flagellum, which is important for bacterial motility (84). The flagellum consists of a basal body, a torsion hook and a hollow helical filament (85). The helical filament is composed of polymerized flagellin proteins, which consist of a highly variable part (domain D2 and D3) that is projected to the outside of the flagellum, and a highly conserved part (domain D0 and D1) that is buried inside the flagellum (86, 87). The conserved region is the immunogenic part and is only detected by TLR5 once flagellin monomers are separated from the flagellum (88-90). The release of these monomers into the microenvironment is not completely understood, but could be the results of depolymerization of the flagellum or leakage of flagellin from the flagellum structure (86).

TLR5 activation

Activation of TLR5 is induced by the highly conserved flagellin D1-domain. First, a flagellin monomer is bound by a TLR5 monomer, forming a complex, after which two TLR5-flagellin-complexes interact to form a complex of 2 TLR5 molecules bound to 2 flagellin molecules. The TLR5 dimerization is thought to cause the juxtaposition of the intracellular TIR-domain, leading to the recruitment of adaptor proteins for the downstream signaling (91). Interestingly, although flagellin is highly conserved, not all flagellated bacteria induce TLR5 activation. Proteobacteria from the ε and α classes, such as *Campylobacter jejuni* contain specific changes in the flagellin domain that is recognized by TLR5, evading its activation (92, 93).

TLR3, 7 and 8

RNA

TLR3, 7 and 8 are expressed in intracellular endolysosomal compartments and are sensors for RNA. Because of their endosomal localization, their activation depends on active uptake of RNA through phagocytosis or endocytosis (94). TLR3 senses double-stranded RNA (dsRNA), which can be found directly in dsRNA viruses (95) or during the replication cycle of ssRNA or DNA viruses (96). It has been shown that TLR3 can be activated in DCs after phagocytosis of virus-infected cells (97). In addition, synthetic dsRNA consisting of at least 40-50 bp of polyinosinic:polycytidylic acid (Poly(I:C)) is also a potent activator of TLR3 (34, 98, 99). TLR7 and TLR8 sense single-stranded RNA (ssRNA) and short dsRNA (100, 101) and can be activated by viruses (35, 102-104) and bacteria (105, 106). The RNA sequence is an important factor in the immunogenicity of the RNA. It has been suggested that sequences rich in guanine and uridine (GU-rich) are effective in the activation of both human TLR7 and TLR8, while adenosine-uridine-rich (AU-rich) sequences are only effective in activation of human TLR8 (107). In addition, several synthetic imidazoquinoline analogs have been shown be potent activators of TLR7 (108). Finally, while not much is known yet about the activation of murine TLR13, recent studies have shown that this receptor is activated specifically by bacterial 23S ribosomal RNA (37, 109, 110).

TLR3, 7 and 8 activation

Activation of TLR3, 7 and 8 is dependent on homodimerization of two TLR molecules. Interaction of two TLR3 molecules with one dsRNA molecule stabilizes the protein-protein interaction for TLR3 dimerization (99). Initial structural studies on TLR8 dimerization were done with the imidazoquinoline CL097 and showed a 2:2 interaction for proper dimerization (111). However, the recently elucidated structure of TLR8 in the context of RNA, showed the presence of two binding sites on each TLR8 molecule. The first site binds a monomeric uridine nucleoside, while the second site interacts with a dimer (or longer) oligonucleotide containing a purine base (112). The crystal structure for ligand-bound TLR7 remains to be determined, although modeling has suggested that amino acid differences between TLR7 and TLR8 could explain the differences in ligand specificity (113).

While RNA sequence and structure are important factors for the activation of these RNA-sensing TLRs, activation of TLR3 and TLR7 has also been shown to depend on endosomal acidification and the activation of proteases and nucleases (106, 114). Protease activation is important for the release of RNA from phagocytosed pathogens (105), while nucleases are important for the partial degradation of ssRNA for TLR7 activation (112). In addition, proteases are important for the cleavage of both TLR3 and TLR7, which is required for proper activation of both receptors (115-119). Finally, RNA-induced TLR activation can also be enhanced by accessory proteins, with CD14 enhancing TLR3 and TLR7 activation (120, 121).

TLR9

DNA

Similar to other nucleic acid-sensing TLRs, the DNA-sensing TLR9 is located in endosomal compartments (94) and requires phagocytosis or endocytosis for the delivery of DNA to endosomes (36). Stimulation with dsDNA or ssDNA can lead to TLR9 activation (36, 122) and modifications in the DNA bases or the DNA backbone can alter the immunogenicity of the DNA. For instance, DNA with a phosphodiester backbone can induce TLR9 activation, but addition of unmethylated cytosine-guanine (CpG)-islands can increase its immunogenicity (123, 124). Because eukaryotic DNA, in contrast to prokaryotic DNA, contains mostly methylated CpG-islands, this is suggested to be a

way to distinguish between different DNA sources (122). For potent TLR9 activation, synthetic oligodeoxynucleotides (ODNs) are often designed with phosphorothioate backbones, in which one of the oxygen atoms of the phosphate group is exchanged for a sulfur atom. This modification results in nuclease resistance, which is a beneficial property for the use of ODNs as adjuvants (125, 126).

TLR9 activation

The crystal structure of the TLR9 dimer shows that two TLR9 molecules bind two short phosphodiester ODNs. Interestingly, an inhibitory ODN was shown to also bind the TLR9, but prevent dimerization, suggesting that TLR9 dimerization also depends on the bridging function of the ligand for protein-protein interaction between two TLR9 molecules (127). In contrast to the phosphodiester ODNs, phosphorothioate-modified ODNs appear to form aggregates with TLR9 ectodomains, which could hint at a different mechanism for activation by these ODNs (124).

For proper activation, TLR9 requires cleavage of the N-terminal ectodomain by proteases in acidified endomoal compartments (119, 128). However, this N-terminal part does have a signaling function as it has to be present for proper activation (124). Interestingly, ODNs can also interact with the uncleaved full-length ectodomain, however, this does not promote efficient dimerization (127). In addition to being important for the TLR cleavage, proteases facilitate the degradation of bacteria to promote DNA release for TLR9 activation (129). Furthermore, while the lower pH activates proteases, it also increases the binding affinity of DNA towards TLR9 (127, 130). Finally, TLR9 activation can also be enhanced by accessory molecules, including CD14 and HMGB1 (121, 131, 132). The latter can enhance TLR9 activation through binding of the RAGE receptor, which co-localizes with TLR9 in endosomal compartments (132, 133).

TLR signaling

After interaction with their ligands, TLRs depend on their cytosolic TIR-domain for the activation of downstream signaling pathways, which are roughly defined as Myeloid differentiation protein 88 (MyD88)-dependent and TIR-domain containing adaptor inducing interferon β (TRIF)-dependent (134) (Fig. 2).

MyD88-dependent signaling

All TLRs, except TLR3, utilize MyD88 for activation of downstream signaling. MyD88 can either directly interact with the TLR-TIR-domain or needs MyD88 adaptor-like (Mal) as an adaptor molecule, as is the case for TLR2, TLR4, TLR7 and TLR9 (135, 136). Interaction between the TLR and MyD88 promotes the recruitment of serine/threonine kinase IRAK-4, which is autophosphorylated upon interaction with MyD88. This in turn

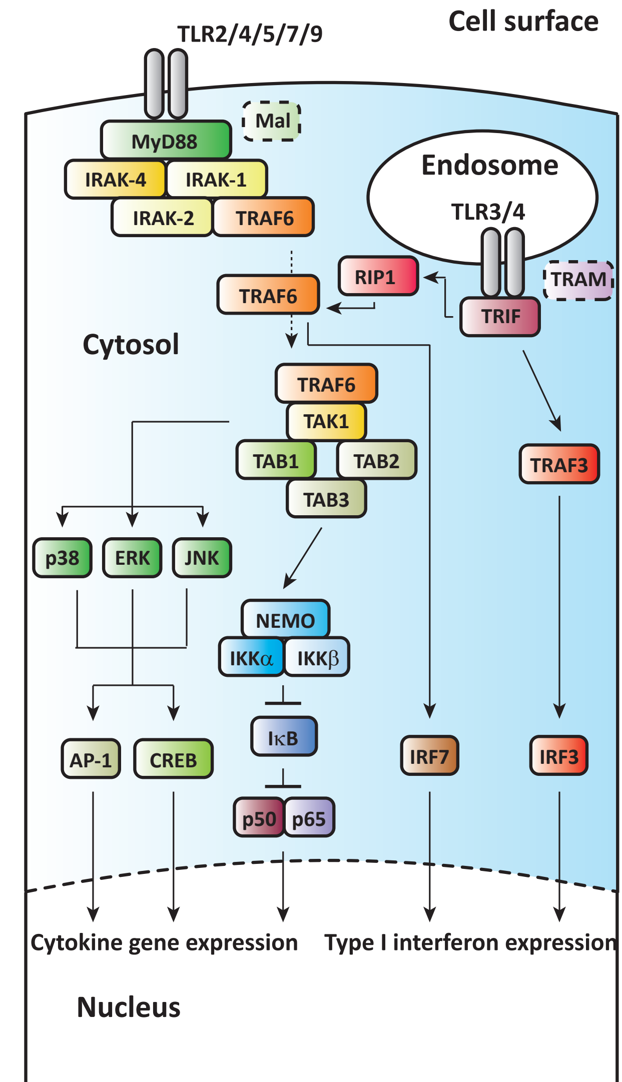

Figure 2: Downstream signaling after TLR activation

recruits IRAK-1 and IRAK-2 to form a protein complex termed the 'myddosome' (137, 138). While IRAK-1 and IRAK-2 appear partially redundant, IRAK-1 is thought to be important for early NF-κB responses, while IRAK-2 is thought to be more important for a longer, sustained TLR response (139, 140). Activation of the IRAK-proteins induces recruitment and activation of the E3 ubiquitin ligase TRAF6. Upon activation, TRAF6 is released into the cytosol to form a complex with TAK1, TAB1 and TAB2/3, which

activates the IKK complex, containing NEMO, IKKα and IKKβ (134, 141-143). The active IKK-complex causes phosphorylation and degradation of IκB, followed by the release of NF-κB, allowing nuclear translocation of NF-κB and initiation of cytokine gene expression. Furthermore, TRAF6 activates IRF5, which is also important for pro-inflammatory cytokine production (144). TAK1 has an additional role in the activation of MAPKs and subsequent activation of AP-1 and CREB (134, 145, 146). Finally, signaling through the MyD88-IRF7 axis has been shown to be important for type I interferon production in plasmacytoid DCs (pDCs) following activation of TLR7 and TLR9 (147-149).

TRIF-dependent signaling

The TRIF-dependent pathway can only be activated by TLR3 and TLR4. TLR3 is directly bound by TRIF, while TLR4 depends on the TRIF-related adaptor molecule (TRAM) for interaction with TRIF (134, 150). Although TLR4 first activates the MyD88-dependent signaling pathway, this is followed by receptor internalization and TRIF-dependent signaling from the endosomal compartment (151). TRIF activates TRAF6 and TRAF3, followed by TRAF6-mediated recruitment of RIP1 kinase for NF-κB activation, while TRAF3 activates IRF3 for type I interferon production (134, 152-154).

Avian Toll-like receptors

Similar to mammalian species, a repertoire of TLR molecules has also been found in avian species (Table 1). However, several differences have been observed in both the TLR repertoire as well as the conservation of functions. Similar to the human TLR family, the avian TLR family consists of multiple lipoprotein-sensing TLRs. These receptors are termed TLR2 type 1 (TLR2t1), TLR2 type 2 (TLR2t2), TLR1-like A (TLR1LA) and TLR1-like B (TLR1LB) (28, 155). TLR2t1 and TLR2t2 can form heterodimers with TLR1LA and TLR1LB for both the detection of di- and tri-acylated lipoproteins. Only the combination of TLR2t1 with TLR1LA appears to be less active compared to the other combinations (156, 157). Interestingly, the activation of TLR2t2 and TLR1LA can be enhanced by the presence of mammalian CD14, suggesting a possible role for a CD14 molecule in avian TLR activation as well (157). However, although an avian CD14 ortholog has been described, its functionality in modulating TLR activation is still unknown (158).

Chicken TLR3, TLR4 and TLR5 functions appear to be conserved compared to their mammalian counterparts. Chicken TLR3 is activated in endosomal compartments and also responds to Poly(I:C), which results in the expression of IFNβ (159-163). The chicken TLR4 responds to penta- and hexa-acylated LPS in a similar manner as murine TLR4 and activation of chicken TLR4 can also be enhanced by the presence of human CD14 in a Hela 57A transfection model. Interestingly, no activation of a TRIF-dependent pathway

Table 1: Comparison of avian and mammalian TLRs

Ligand	Mammalian TLRs	Avian TLRs
Di-acylated lipoproteins	TLR2-TLR6	TLR2t1-TLR1LB, TLR2t2-TLR1LA, TLR2t2-TLR1LB
Tri-acylated lipoproteins	TLR2-TLR1	TLR2t1-TLR1LB, TLR2t2-TLR1LA, TLR2t2-TLR1LB
LPS	TLR4	TLR4
Flagellin	TLR5	TLR5
Proteinase K	-	TLR15
dsRNA	TLR3	TLR3
ssRNA	TLR7 / TLR8	TLR7
DNA	TLR9	TLR21

leading to IFNβ production can be detected upon stimulation of chicken macrophages with LPS (59). Furthermore, the function of chicken TLR5 appears to be conserved compared to mammalian TLR5 and is important for the detection of flagellin in chickens (164).

In addition to TLR3, avian species appear to have a functional TLR7 for RNA detection, but lack a functional TLR8 (165). Chicken cells are activated by mammalian TLR7 agonists, such as R848, and this activation appears to occur in endosomal compartments (166-168). Overexpression of the pigeon TLR7 in HEK293T cells has been shown to result in NF-κB activation after stimulation with R848 and pigeon PBMCs respond to R848 by expressing pro-inflammatory cytokines (169). Together, this suggests a similar function for avian and mammalian TLR7.

TLR15 and TLR21 are two additional TLRs that can be found in chickens, but are absent in mammals. TLR15, which is phylogenetically unrelated to the previously mentioned TLRs, appears to be activated by yeast-derived proteases and some bacterial proteases (170-172). Activation occurs by proteolytic cleavage of the extracellular N-terminal domain, which promotes NF-κB activation in overexpressing Hela 57A cells (172). In addition, activation of TLR15 in HD11 macrophages promotes pro-inflammatory cytokine expression (171). The TLR21 in chickens appears to be the functional counterpart for the mammalian TLR9. Similar to TLR9, TLR21 is activated by DNA in endosomal compartments, which induces a pro-inflammatory response in HD11 chicken macrophages and TLR21-transfected HEK-cells (162, 173).

TLRs in infections

TLRs are crucial for the detection of a wide variety of pathogens and initiation of an immune response in many different species (174). Much of the knowledge on the importance of TLR activation has been obtained by the use of TLR knockout mice. For instance, TLR2$^{-/-}$ mice are more susceptible to infections with Gram-positive *S. aureus* and *S. pneumoniae* (175), which can activate TLR2 via LTA derived from the bacterial cell wall (81, 176, 177). Loss of TLR4, on the other hand, renders mice more susceptible to infections by Gram-negative bacteria, such as *S. typhimurium, E. coli, P. aeruginosa* and *F. tularensis* (178-182). The importance of TLR5 is exemplified by the higher susceptibility of humans having a SNP mutation in the TLR5 gene for *L. pneumoniae* infection (183) and the higher susceptibility of TLR5$^{-/-}$ mice for *E. coli* urinary tract infections (184). TLR5 activation also plays an important role in lung inflammation and is activated on both alveolar macrophages and lung epithelial cells by *L. pneumoniae* and *P. aeruginosa* (185-188). Interestingly, *S. typhimurium* has been shown to actually make use of TLR5 on CD11c^{+} intestinal lamina propria cells to disseminate to the mesenteric lymph nodes (189). Furthermore, TLR9 has been shown to play an important role in immune activation during polymicrobial sepsis and *E. coli* infection (190, 191), while TLR9$^{-/-}$ mice and TLR11$^{-/-}$ mice have been shown to be more susceptible to *S. typhimurium* infections (192, 193).

While many effects can be observed upon loss of a single TLR, most pathogens express multiple MAMP s and activated multiple TLRs during infections (194, 195). Therefore, some effects are only observed when activation of multiple TLRs is disrupted. For instance, the loss of TLR2 alone often has a limited effect on Gram-negative infections, while loss of both TLR2 and TLR4 results in a lower inflammatory response and higher susceptibility to infections compared to the loss of TLR4 alone (179, 180). A similar effect can be observed for the susceptibility of mice towards *S. typhimurium and P. aeruginosa* infections. While loss of TLR5 does not increase the susceptibility of mice to infections by these pathogens, a higher susceptibility is obtained when both TLR4 and TLR5 expression is lost, compared to loss of TLR4 alone (186, 196, 197).

The effects observed in these double-KO mice show that while much is known about the activation of single TLRs, new challenges can be found in understanding TLR activation in the context of intact pathogens expressing multiple MAMPs. It will be interesting to elucidate which TLR signaling pathways are activated in the context of different pathogens and how cross-talk among TLRs, but also cross-talk with other PRRs and microenvironmental components, can shape the inflammatory responses against invading pathogens.

Cathelicidins

Part of the immune response against invading pathogens is the release of cathelicidins. These short cationic peptides can be secreted from various cell types, including leukocytes and epithelial cells (198-203) and are crucial in the protection against infections (204-206). Although they were initially described as antimicrobial peptides, many new functions involving the regulation of immune activation have been discovered since (Fig. 3).

Structure and synthesis

Cathelicidins are short cationic and amphipathic peptides expressed in a wide variety of vertebrate species (207). They are synthesized as prepropeptides in neutrophils, containing a signal peptide, a conserved cathelin-domain and a C-terminal peptide. First, the N-terminal signal peptide is cleaved of by a signal peptidase and the still inactive propeptide is stored in secondary (or specific) granules (6, 208-210). Upon neutrophil activation, cathelicidins are released into the extracellular environment together with the content from azurophilic granules, which contain elastase and proteinase-3. These are required for the cleavage of the C-terminal active mature peptide from the cathelin-domain (203, 211, 212).

In contrast to the highly conserved cathelin-domain, the sequence and structure of the mature peptides is highly variable (213). Nevertheless, most mature peptides assume an amphipathic α-helical shape in a biological membrane environment. Several non-α-helical peptides include cathelicidins rich in specific amino acids, such as tryptophan-rich or proline and arginine-rich cathelicidins. Furthermore, several cathelicidins contain cysteines to form intramolecular disulphide bridges (211). Within the group of α-helical cathelicidins, differences in charge density and hydrophobicity can affect their higher order structure in solution and their interaction with biological membranes. For instance, the human cathelicidin LL-37 is disordered in aqueous solutions, but forms an amphipathic α-helical conformation under physiological conditions. This α-helical formation is dependent on salt, pH and cathelicidin concentration (214) and promotes the oligomerization of LL-37 in solution (215, 216). In addition, the α-helical conformation of LL-37 is further stabilized upon membrane interaction (214, 217). This is in contrast with many other α-helical cathelicidins, which only adopt the α-helical structure in a membranous environment and remain monomeric in solution (218).

Cathelicidins are important in the initial host defense against invading pathogens and can be detected at sites of host-microbe interaction, including the lung, the intestine, the skin and sites of infection (199, 219-221). The expression of cathelicidins

can be increased by the presence of pathogens, such a Group A Streptococcus in skin infection, or by tissue damage (219). In addition, the active form of vitamin D_3, 1,25-dihydroxyvitamin D_3 (Vit D3), can activate the Vit D_3 receptor (VDR), which increases cathelicidin expression. Other components, such as the short-chain fatty acids (SCFA) butyrate and phenylbutyrate, have also been shown to increase cathelicidin expression (222, 223).

Cathelicidin functions

Cathelicidins were initially described as antimicrobial peptides, with broad-spectrum antimicrobial activity. However, many more functions have been identified over the last couple of years. This includes: recruitment of leukocytes due to direct chemotactic activity, induction of chemokine release, modulation of macrophage and DC differentiation, enhancement of phagocytosis, promotion of wound healing and regulation of TLR activation (224-226). A couple of functions will be discussed here in more detail.

Antimicrobial activity

Most cathelicidins have a broad-spectrum antimicrobial activity against both Gram-positive and Gram-negative bacteria, fungi (227-232), and in some cases viruses (233). Most α-helical cathelicidins kill bacteria by disrupting the bacterial membrane (234-237). Due to their positive charge, they are most likely attracted to the negatively charged components on the bacterial surface, such as LPS on Gram-negative bacteria or lipoteichoic acids on Gram-positive bacteria (213, 238). This is followed by membrane perturbation, which has been visualized by live imaging LL-37-mediated *E. coli* killing. LL-37 first disrupts the bacterial OM, which is followed by the slower permeabilization of the inner membrane (IM) (235). This order of events was also detected for the porcine PMAP-36 (236). The permeabilization of the IM by cathelicidins involves interaction with both phospholipid headgroups and the hydrophobic regions within the IM (237). Various models have been suggested to describe the mechanisms of membrane disruption by different antimicrobial peptides. In the "barrel-stave model", peptides obtain an α-helical structure and penetrate the membrane by forming a transmembrane pore. In the "carpet-model", peptides accumulate on the membrane surface due to electrostatic interaction. Once a sufficient concentration is reached, the peptides disrupt the membrane bilayer in a detergent-like manner, leading to the formation of micelles and disruption of membrane integrity. In the "toroidal-pore model", peptides insert into the membrane, which bends in on itself and forms a worm-hole like structure, with the hydrophilic part of the peptides, as well as the phopholipid head groups of the membrane, lining the lumen of the pore (237, 239). It is becoming increasingly clear that most likely multiple membrane-disruptive mechanisms can be utilized by cathelicidins

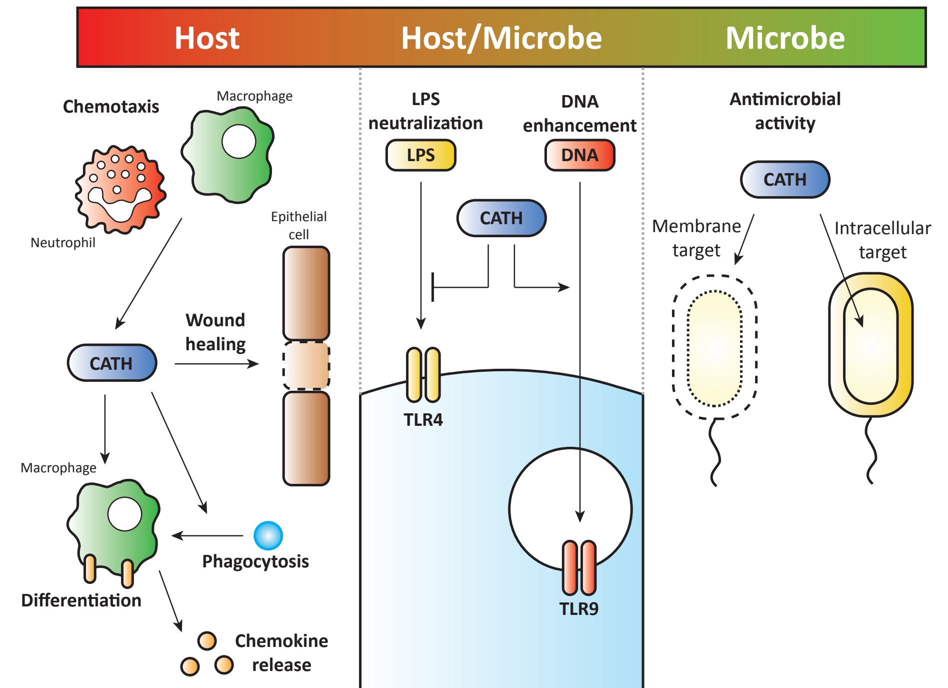

Figure 3: Overview of previously described cathelicidin functions

and that it depends strongly on the membrane lipid composition, environmental factors and peptide concentrations, which mechanism is predominantly used in a specific situation (240, 241).

In addition to membrane disruption, several cathelicidins act on intracellular targets to kill bacteria. Proline-arginine-rich peptides are thought to interact with a receptor or docking molecule to translocate into the interior of the bacteria. It was shown that the IM protein SbmA, which is predicted to be a part of an ABC transporter, might play a role in this (242). Once internalized, the peptides can target several processes, including protein-folding (243, 244), DNA synthesis, RNA transcription and RNA translation (237, 245, 246).

The antimicrobial potency of cathelicidins can be greatly influenced by microenvironmental factors. For instance, mono- and divalent cations such as Na^+, Ca^{2+} and Mg^{2+} can have a negative impact on the antimicrobial activity of many cationic peptides, including cathelicidins (221, 247-250). Incorporation of Ca^{2+} and Mg^{2+} in the bacterial OM is important for OM stability and reduces the overall negative surface charge to limit

interaction with cationic cathelicidins (54, 207). Furthermore, serum components, such as lipoproteins, have also been shown to inhibit cathelicidin antimicrobial activity (214, 251-256). On the other hand, the presence of carbonate has a positive effect and improves the antimicrobial activity of cathelicidins against *S. aureus*, *E. coli* and *Salmonella* (257). Furthermore, synergism between bactericidal compounds can enhance cathelicidin antimicrobial activity and might be important for activity under physiological conditions. For instance, by combining cathelicidins, they can act synergistically against *E. coli* and *P. aeruginosa* (258). In addition, LL-37 in combination with lactoferrin enhances *E. coli* and *S. aureus* killing, while LL-37 in combination with lysozyme improves *E. faecalis* killing (221). Interestingly, combinational treatment of cathelicidins with conventional antibiotics can improve antimicrobial activity as well (259-261). However, since these results are all obtained *in vitro*, it will depend on the balance of positive and negative regulators at the site of infection, whether cathelicidins exert antimicrobial activity *in vivo*.

Besides the positive and negative effects of the microenvironment on antimicrobial activity, bacteria have developed several resistance mechanisms to counter the antimicrobial activity of cathelicidins and other antimicrobial peptides (262). For instance, both Gram-positive and Gram-negative bacteria can produce proteases, which can degrade cathelicidins into inactive fragments (263-265). Another bacterial defense mechanism is the production of capsular polysaccharides. Various bacteria, including *N. meningitidis, P. aeruginosa* and *K. pneumoniae*, use this mechanism to trap antimicrobial peptides and prevent them from reaching the IM membrane (266-270). In addition, the production of outer membrane vesicles by Gram-negative bacteria under stress could provide a way to remove cathelicidins from the bacterial surface (271). Finally, bacteria can modify their membrane or cell wall components to neutralize the negative charge. For instance, a 4-aminoarabinose (Ara4N) modification to the lipid A phosphate group increases *S. typhimurium* resistance (268). Other modifications include the addition of phosphoethanolamine to the lipid A, which increases resistance of *N. gonorrhoeae* and *N. meningitides* (265), and D-alanylation of teichoic acids in the cell wall of Gram-positive bacteria (270).

Chemokine induction and chemotaxis

Another function that has been attributed to cathelicidins is the induction of chemotaxis. Direct chemotaxis can be induced by both non-α-helical (272, 273) and α-helical cathelicidins and can include the recruitment of neutrophils, eosinophils, mast cells, monocytes and T-cells (8, 274-277). The two receptors that have been implicated in the induction of the monocyte recruitment are the G-protein-coupled receptor (GPCR) FPRL1 and CXCR2 (8, 278). An additional mechanism for cathelicidin-mediated recruitment of leukocytes is the induction of chemokine expression. LL-37, for instance, induces the release of CXCL8 by airway epithelial cells and airway smooth muscle cells. In epithelial cells, CXCL8 release is dependent on the activation of the epidermal growth factor receptor (EGFR)- and ERK-phosphorylation (279, 280), while smooth muscle cell activation depends on Src-phosphorylation through activation of a purinergic receptor, most likely P_2X_7 (281, 282). Similarly, human keratinocytes also respond to LL-37 by increasing CXCL8 production in a Src and P_2X_7-dependent manner (283). In RAW264.7 cells, LL-37 was shown to induce CCL2 and CCL7 release. And this increase in CCL2 was also observed *in vivo* after instillation of LL-37 in mouse lungs (280). Interestingly, a study by Mookherjee et. al. suggested that CCL2 and CCL3 release by human THP-1 cells depends on GAPDH as an intracellular receptor for LL-37 (284). Other cathelicidins, including indolicidin and chicken cathelicidin-2 (CATH-2), have been shown to increase chemokine release. Indolicidin increases CXCL8 release from human lung epithelial cells, while CATH-2 has been shown to increase *CXCLi2*, *CCLi4* and *CCL7* expression in chicken macrophages (273, 285). Nevertheless, most of these studies have focused on the *in vitro* effects of cathelicidins and much still remains to be learned about how these effects play a role in leukocyte recruitment *in vivo*.

TLR regulation

Because cathelicidins are released during host-microbe interaction, they are prone to encounter MAMPs. Investigation on the role of cathelicidins in TLR activation by these MAMPs has led to the identification of regulatory effects on a variety of TLRs.

Lipid MAMPs

Various reports have shown that LL-37 can inhibit LPS-induced TLR4 activation on various cell types, including monocytes (286), macrophages (280), dendritic cells (287), epithelial cells (288, 289) and fibroblasts (290). Furthermore, this inhibitory effect appears to be conserved among many cathelicidins from various species (256, 285, 287, 291-297) and has been observed both *in vitro* and *in vivo* (295, 298-300) against various smooth and rough LPS types, including *E. coli* LPS (280), *S. minnesota* LPS (285), *P. aeruginosa* LPS (301) and the TLR2 activating *P. gingivalis* LPS (302).

Most cathelicidins appear to inhibit TLR4 activation by directly binding LPS (276, 285, 295, 303, 304) . An NMR study on chicken CATH-1 has modeled how fragments from this cathelicidin can interact directly with LPS (305). In addition, LL-37 and CAP11 were shown to outcompete LBP for LPS binding and inhibited the interaction between LPS and CD14 or TLR4 on macrophages (300, 304, 306, 307). Interestingly, LL-37 also removed LPS from CD14 on the macrophage surface (304), which could explain why post-incubation with LL-37 after LPS stimulation lowers activation of RAW264.7 cells

and HD11 cells (280, 285). In addition to the direct neutralization, several studies have shown that pre-incubation of cells with LL-37, BMAP-27 or CRAMP can also inhibit LPS-induced activation (286, 308, 309), although other studies have shown little to no effect by pre-incubating cells with cathelicidins (300, 301).

Besides the inhibitory effects of cathelicidins on TLR4 activation, it was shown that LL-37 can enhance the internalization of LPS in lung epithelial cells and liver sinusoidal endothelial cells. The LL-37-mediated uptake in lung epithelial cells results in increased activation of TLR4 inside endosomal compartments (310), while the uptake in the liver sinusoidal endothelial cells does not result in cellular activation (311).

In addition to LPS neutralization, LTA-induced TLR2 activation can also be inhibited by cathelicidins (280, 309). LL-37 inhibits LTA-induced TNFα production in bone marrow-derived macrophages and PBMCs (280, 286, 309, 312) and inhibits LTA-induced release of TNFα, IL-6 and IL-12 in DCs (313). Effects on other lipid-containing MAMPs, such as Pam3CSK4, are less evident, with either descriptions of no or partial inhibition (286, 302, 314, 315) and in some cases enhancement of activation, such as the increased activation of bronchial epithelial cells by Pam3CSK4 in the presence of LL-37 (316).

Flagellin

In contrast to the described effects on TLR4 activation by cathelicidins, relatively little is known about the effects of cathelicidins on TLR5 activation by flagellin. It has been shown that flagellin-induced CXCL8 production in keratinocytes can be enhanced by LL-37 via activation of the P_2X_7 receptor, which leads to Src and Akt activation. Interestingly, LL-37 also appeared to enhance the activation of keratinocytes by IL-1β (283, 316). In bronchial epithelial cells, LL-37 can increase IL-6 and CXCL8 release in combination with flagellin (316, 317). In contrast, studies on the role of LL-37 in myeloid cell types have either shown small inhibitory effects or no effect on flagellin-induced activation (302, 313, 315, 318).

Nucleic Acids

The effect of cathelicidins on activation of nucleic acid-detecting TLRs has gained much interest due to the observed importance in various autoimmune diseases. In psoriasis patients, LL-37 was found to increase TLR9-dependent, DNA-induced IFNα production in pDCs (319). This was further expanded by the finding that complexes of extracellular DNA from neutrophil-extracellular traps (NETs), which contain LL-37 and HNP, increase pDC IFNα production in patients suffering from systemic lupus erythematosus (SLE) (320). However, samples from SLE patients in a recent study did not show a correlation between LL-37 levels and disease severity (321). Furthermore, cathelicidins may also

play an important role in the onset of type I diabetes, as it was shown that complexes of self-DNA, anti-DNA-IgG and CRAMP promote pDC activation and IFNα production in a nonobese diabetic (NOD) mouse model, resulting in an auto-reactive T-cell response (322).

The increased activation of TLR9 in pDCs by DNA-cathelicidin-complexes is partially explained by the increased uptake of DNA into the endosomal compartments (319). However, the enhanced DNA response in macrophages and DCs by CRAMP appears to be independent of increased DNA uptake and might be dependent on altered endosomal processing of the DNA and increased binding to the TLR9, as has been suggested for LL-37 (323, 324). In keratinocytes there also appears to be an indirect effect of LL-37 on TLR9 activation, since only pre-incubation with LL-37 enhances DNA-induced TLR9 activation, while complexes of DNA and LL-37, although taken up by the keratinocytes, inhibit the activation (325, 326). Interestingly, one report on the effect of LL-37/DNA-complexes in monocyte activation indicates that uptake of the complex results in cytosolic localization and TLR9-independent IFNα production (327).

Similar to DNA-induced activation, cathelicidins can also enhance RNA-induced activation. In keratinocytes, Poly(I:C)-induced CXCL8 production is enhanced by LL-37 (316, 328). In bronchial epithelial cells, Poly(I:C)-induced and viral dsRNA-induced production of IL-6, TNFα and CXCL8 is enhanced by LL-37, but not by CRAMP (289, 316, 329). The enhanced activation of epithelial cells is dependent on uptake of RNA/LL-37-complexes through activation of FPRL1 and results in activation of TLR3 in endosomal compartments. In order to allow TLR3 activation, LL-37 needs to be released from the complex, presumably due to the lower pH in the endosomal compartments (329, 330). Activation of pDCs by ssRNA can also be enhanced by LL-37 and leads to increased IFNα production due to the enhanced delivery of RNA to endosomal compartments, where TLR7 can be activated. Interestingly, the RNA/LL-37-complex triggers TLR8 activation in mDCs, which results in TNFα and IL-6 production instead of IFNα production (331). In contrast to the enhancement of TLR activation by RNA in all these cell types, LL-37 and CRAMP inhibit Poly(I:C)-induced IL-6 production in murine RAW264.7 macrophages and murine fibroblasts (329, 332), suggesting cell type specific effects.

While many effects of cathelicidins have been observed on TLR activation by nucleic acids or other MAMPs, most studies have identified these effects using purified or synthetic TLR ligands. Further research will be needed to determine which of these effects occur in the context of complete bacteria expressing multiple MAMPs at once.

Antibiotic resistance

While antibiotics have effectively been used for the treatment of bacterial infections in humans and animals, many bacteria have developed antibiotic resistance. This includes methicillin-resistant *S. aureus* (MRSA), which is resistant to penicillin-derived β-lactam antibiotics (333, 334), vancomycin-resistant enterococcus (VRE) (335) and extended-spectrum β-lactamase producing-enterobacteriaceae (ESBL) (336). Awareness on the induction of antibiotic resistance in bacteria has increased over the last years and has led to the development of global programs to reduce antibiotic resistance and promote sensible use of antibiotics (337, 338). This includes the selection of appropriate antibiotics by early screening of the infectious pathogen to prevent antibiotic use that will not be effective (339) and decreasing the amount of antibiotics that is used in the veterinary sector as preventative treatment or as growth promoter (340, 341). Furthermore, since the development of new antibiotics has declined over the years, new initiatives for the development of novel antibiotics are essential (342).

Cathelicidins, as well as other host defense peptides, are interesting candidates for the development of novel antibiotics. One of their key features is their broad-spectrum antimicrobial activity, which makes them broadly applicable against both Gram-positive and Gram-negative infections (227, 343). In addition, where other antibiotics, such as β-lactams, are thought to increase LPS release from bacteria and subsequently increase TLR4 activation (344-346), cathelicidins inhibit LPS-induced TLR4 activation, which can provide protection against inflammation and sepsis (250). Furthermore, the induction of leukocyte recruitment can promote the hosts' own defense against the infection in concert with the antimicrobial activity of the cathelicidins (347).

A number of initial studies have shown the potential protective effect of cathelicidins as novel anti-infectives (348-350). In addition, upregulation of the hosts' own cathelicidin expression could be a useful alternative to protect against infections (351). These initial results provide a good rationale to investigate cathelicidin functions in more detail to better understand their biological role during infections as well as their potential as anti-infectives.

Scope of Thesis

Exploring cathelicidin functions is important to obtain a better understanding on their role in the host response during infections. In addition, elucidation of cathelicidin functions can lead to new insight in the applicability of cathelicidin-based anti-infective therapies. Therefore, this project was aimed at better understanding cathelicidin functions, with an emphasis on their role in TLR activation.

In **Chapter 2**, an overview of the current knowledge on avian cathelicidin biology is provided, especially to introduce the chicken cathelicidin-2 (CATH-2). This cathelicidin has been suggested as a good potential candidate for the development of anti-infective therapies due to its potent antimicrobial activity and immunomodulatory functions. Furthermore, it is used throughout this thesis alongside other cathelicidins to better understand the role of cathelicidins in innate immunity.

In **Chapter 3**, various well-known cathelicidin functions are compared directly for twelve cathelicidins from six different species in order to obtain insight in the conservation of these cathelicidin functions.

In **Chapter 4**, the CATH-2-mediated enhancement of DNA-induced macrophage activation is further investigated to obtain insight in the mechanism of action, which can have important implications in the use of cathelicidins in anti-infective therapies.

In **Chapter 5**, the effects of cathelicidins on TLR activation are investigated in the context of complete and viable Gram-negative bacteria to determine how cathelicidins regulate TLR activation under more physiological circumstances.

In **Chapter 6**, the inhibitory effect of CATH-2 on immune activation by Gram-negative bacteria is demonstrated in an *in vivo* model using *P. aeruginosa*, which demonstrates the potential of CATH-2 as an anti-infective.

In **Chapter 7**, the main findings of this project are summarized and discussed in a broader context.

List of abbreviations

AIM2, absent in melanoma 2; **AP-1***, activator protein-1;* **BLP**, Braun lipoprotein; **CATH-2**, chicken cathelicidin-2; **CpG-islands**, cytosine-guanine islands; **CREB,** cAMP response element-binding protein; **CSF-1,** colony stimulating factor-1; **DAMP**, damage-associated molecular pattern; **DC**, dendritic cell; **EGFR**, epidermal growth factor receptor; **ESBL**, extended-spectrum β-lactamase producting-enterobacteriaceae; **FPRL-1**, formyl-peptide receptor-like-1; **GPCR**, G-protein-coupled receptor; **GPI**, glycosylphosphatidylinositol; **HDL**, high-density lipoprotein; **IFNγ**, interferon gamma; **IκB**, inhibitor of κB; **IKK**, IκB kinase; ***IL****, interleukin;* **IM**, inner membrane; **IPAF**, ICE protease-activating factor; **IRAK**, interleukin-1 receptor-associated kinase; **IRF**, interferon regulatory factor; **KDO**, 2-keto-3-deoxyoctonate; **LBP**, LPS-binding protein; **LDL, l**ow-density lipoprotein; **LPS**, lipopolysaccharide; **LRR**, leucine-rich repeats; **LTA**, lipoteichoic acid; **LXRα**, liver X receptor alpha; **MAMP**, microbe-associated molecular pattern; **MAPK**, m*itogen-activated protein kinases;* **mCD14**, membrane CD14; **M-CSF**, macrophage colony stimulating factor; **MHC**, m*ajor histocompatibility complex;* **Mrc1**, macrophage mannose receptor 1; **MRSA**, methicillin-resistant *S. aureus*; **MyD88**, myeloid differentiation protein-88; **NEMO**, NF-κB essential modulator; **NET**, neutrophil-extracellular traps; **NF-κB**, nuclear Factor-κB, **NLRP3**, NACHT, LRR and PYD domains-containing protein 3; **NOD**, nucleotide-binding oligomerization domain-containing protein; **NOS2**, nitric oxide synthase 2; **NR4A1**, nuclear receptor subfamily 4, group A, member 1; **ODN**, oligodeoxynucleotide; **OM**, outer membrane; **pDC**, plasmacytoid DC; **Poly(I:C)**, polyinosinic:polycytidylic acid; **PPARγ**, peroxisome proliferator-activated receptor gamma; **PRAT4A**, protein associated with Toll-like receptor 4; **PRR**, pattern-recognition receptor; **RIP1**, receptor-interacting serine/threonine-*protein* kinase 1; **RNS**, reactive nitrogen species; **ROS**, reactive oxygen species; **sCD14**, soluble CD14; **SCFA**, short-chain fatty acids; **SLE**, systemic lupus erythematosus; **SNP**, single nucleotide polymorphism; **STAT**, signal transducer and activator of transcription; **TAB**, TGF-β activated kinase 1 binding protein; **TAK**, TGF-β activated kinase; **TIR**, Toll/Interleukin receptor; **TLR**, Toll-like receptor; **TRAF6**, TNFR-associated factor 6; **TRAM**, TRIF-related adaptor molecule; **TRIF**, TIR-domain containing adaptor inducing interferon β; **UNC93B1**, Unc-93 homolog B1; **VDR**, Vit D_3 receptor; **Vit D_3**, 1,25-dihydroxyvitamin D_3; **VRE**, vancomycin-resistant enterococcus.

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Chapter 2

Review on avian cathelicidins

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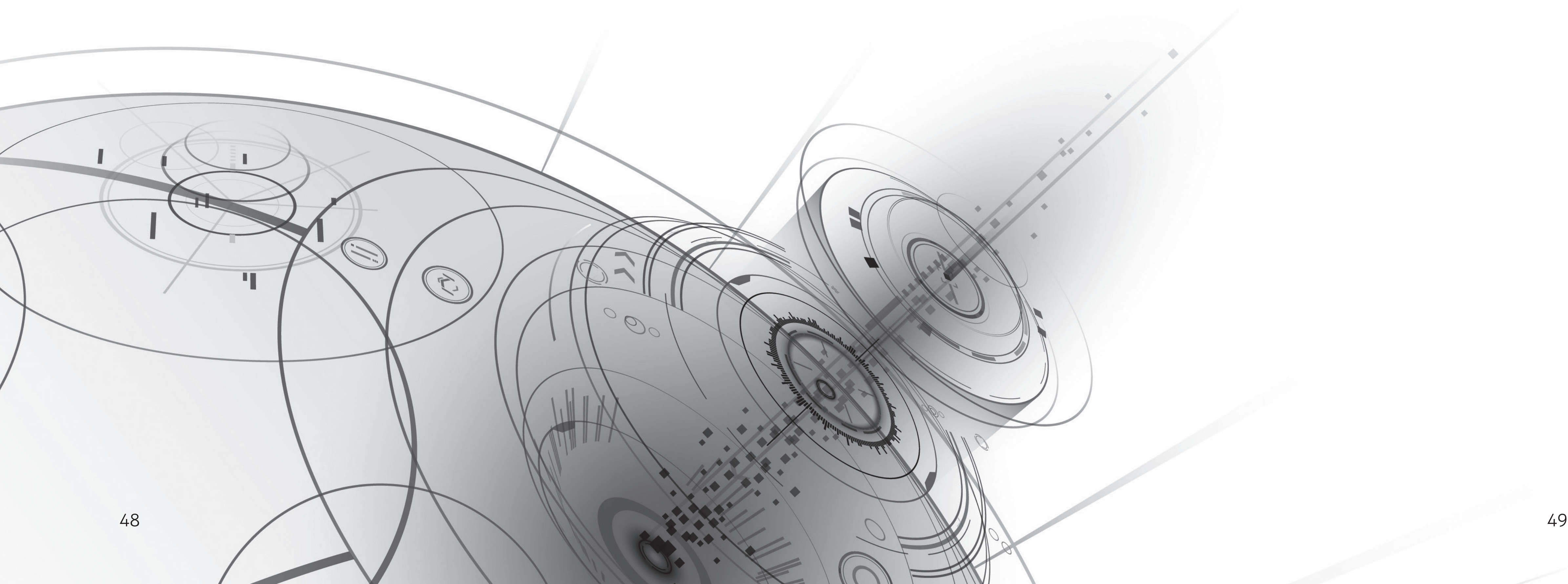
Developmental & Comparitive Immunology (2013) **41**:352-69 (in part)



Abstract

Cathelicidins are important effector molecules of the innate immune system of vertebrates. They display broad-spectrum antimicrobial activities and fulfill an important role in the first line of defense of many organisms. It is becoming increasingly clear that the functions of cathelicidins are not confined to direct antimicrobial actions. Research in mammals has indicated that cathelicidins have many immunomodulatory functions and are also involved in other physiological processes ranging from development to wound healing. During the past five years our knowledge about avian cathelicidins has increased considerably. This review addresses our current knowledge on the evolution, regulation and biological functions of avian cathelicidins.

Introduction

The avian innate immune system possesses a wide spectrum of defense mechanisms against invading pathogens and consists of immune effector cells, enzymes, proteins and peptides that function as a first line of defense. Although many aspects of the immune system are similar to their mammalian counterparts, important differences can be found, such as the presence of heterophils in birds, which are thought to play a similar role in innate immunity as neutrophils do in mammals (1). Similarly, although avian species express cathelicidins, the number of cathelicidin genes and the peptide sequences of the mature cathelicidin peptides are different from many other mammalian and non-mammalian species.

Cathelicidins are small, cationic peptides and are present in a wide variety of organisms. Originally designated as antimicrobial peptides (AMPs) after their ability to kill bacteria *in vitro*, further research showed these peptides possess many additional functions in the regulation of innate immune responses. It is now well established that cathelicidins can cause differentiation, activation and chemotaxis of multiple cell types, inhibit LPS-induced TLR4 activation, enhance DNA-induced TLR9 activation and promote wound healing (2-4). It is clear these peptides form a vital, but often overlooked component of the innate immune system. In the following sections we review the current knowledge on avian cathelicidins compare the avian cathelicidins to their mammalian counterparts.

Avian cathelicidins

Cathelicidins are conserved throughout a wide variety of vertebrates, including mammals, birds and reptiles (2, 5-7). In chickens, four cathelicidins have been described. These are CATH-1, -2, -3 and -B1 (5).

Classes and structures

Cathelicidins are short cationic peptides, which are characterized by a highly conserved cathelin-like domain (2). They are produced as prepropeptides containing an N-terminal signal peptide, the conserved cathelin-like domain and a C-terminal mature peptide. The signal peptide is cleaved off before secretion and the cathelin-like domain is cleaved off by serine proteases once the peptide is secreted (2, 8, 9). The mature peptides found after the protease cleaving steps are quite divers. Most of them, including all avian cathelicidins (5), are α-helical cationic peptides, like SMAP-29 in sheep (10), BMAP-27 and -28 in cows (11) and the PMAP peptides in pigs (12). They have an amphipathic structure that enables interaction with both negatively charged molecules on bacterial surfaces, like lipopolysaccharide (LPS) or lipoteichoic acid (LTA), as well as with hydrophobic lipid structures in bacterial membranes (13, 14). The α-helicity of most of these peptides

is dependent on the environment. Chicken CATH-1, -2a and -3, but also several other α-helical peptides, are largely unstructured in aqueous solution, but can attain α-helical properties when interacting with a membrane-mimicking environment (15-21). Additionally, structural analysis of CATH-1, CATH-2a and CATH-3 showed formation of two α-helical regions, separated by a kink or hinge-region induced by a glycine or proline near the center of the mature peptide, which gives the peptide more flexibility (16, 20, 21). They also have hydrophobic aromatic groups on both sides of the kink, important for the amphipathic character of these peptides (5). Other types of cathelicidins have been described, like short cyclic peptides that have β-sheets and form intramolecular disulfide bridges, and peptides containing a high amount of specific amino acids, like tryptophans or prolines (2, 8, 22). These forms, however, have not been found in avian species.

Evolution

Similar to mammalian cathelicidins, the signal peptide and cathelin domain are highly conserved in the four chicken peptides. The signal peptides of CATH-1 and CATH-2 show 94% similarity at the amino acid level, while the cathelin domains of these genes are 56% homologous (23, 24). In contrast, the mature peptides are highly diverged, e.g. mature CATH-2 shows less than 10% homology to other chicken cathelicidins (23). CATH-1 and -3 are highly similar, with a >90% identity considering the complete prepro-sequence, in addition, the intron sequences of these genes show a high similarity (24). Similar high identity was found for quail CATH-1 and -3, suggesting these two genes are the result of a recent duplication (25). So far, CATH-B1 has only been described in the chicken and shares a low homology with other chicken and avian cathelicidins (26). With the genomes of turkey and zebra finch sequenced and increasing sequence information accessible for other birds, it is expected we will soon learn if this gene is present in other avian species (27, 28).

The four chicken cathelicidin genes are tightly clustered in a 7.5 kB region at the proximal end of chromosome 2 (24). All avian cathelicidins described so far consist of four exons separated by three introns. The first three exons encode the signal peptide and the cathelin domain, with the mature peptide encoded in the fourth exon.

When avian cathelicidins are compared with mammalian proteins, the highest sequence similarity is found with neutrophilic granule proteins (NGP) of rabbits and mice. These little researched cathelicidin-like sequences, sometimes called '15 kDa protein AMP' have been found in rabbits and rodents and more recently also in the bovine genome (29, 30). NGPs are negatively charged, in contrast to the cationic 'classic' cathelicidins and have not been described in primate genomes. More proof for the close relationship between NGP and avian cathelicidins comes from the fact that both NGPs and chicken

cathelicidins are located in close proximity to the Kelch-like 18 (KLHL18) gene, while the 'classic' cathelicidins are found more than 500 kb away (24). There is shared synteny for the cathelicidin gene cluster across mammalian species and the chicken. The cluster is located in a region flanking the highly conserved KLHL18 gene in both classes of animals. Chicken, pheasant and quail cathelicidin orthologs show a high degree of similarity (25, 31). The same was observed for cathelicidins discovered in different snake species (32). In contrast, large differences were found among mammalian cathelicidins, even for animal species belonging to the same clade.

Sites of production

CATH-1, -2 and -3 are expressed in a wide range of tissues, including the respiratory tract, gastrointestinal tract and multiple lymphoid organs. Expression for all three genes was found to be high in lung, bone marrow, bursa and cecal tonsils (23, 33, 34). CATH-1 and -3 also showed high expression in the testis and abundant expression for CATH-2 was found in the uropygial gland. Little to no expression of these cathelicidins was found in esophagus, crop, skin and brain (23, 33). A limited expression analysis was performed for pheasant CATH-1 and was comparable to its chicken ortholog, i.e. high expression being found in bone marrow, bursa, lung and testis (31).

In contrast, CATH-B1 was reported to be selectively expressed in the bursa of Fabricius (26), although recent studies have shown low levels of CATH-B1 transcription in other organs, including jejunum, colon, thymus and peripheral blood leukocytes (PBLs) (33, 35). In the bursa, CATH-B1 transcripts are confined to secretory enterocytes of the interfollicular bursal epithelium. CATH-B1 mRNA was not detected in the neighboring M cells, yet CATH-B1 peptide was found in the M cell region. Most likely, CATH-B1 is produced by secretory enterocytes and secreted into the bursal lumen, where it is taken up by M cells via pinocytosis (26).

Localization studies using a CATH-2 specific antibody showed CATH-2 protein in heterophils, whereas it was not found in monocytes, lymphocytes or thrombocytes (9). Looking at sections of multiple gastrointestinal tissues, CATH-2 protein expression was equally restricted to heterophils. No expression in intestinal epithelial cells was seen, either in control or infected intestinal tissues (9, 36). These findings suggest that CATH-2 is exclusively produced by heterophils, in contrast to human LL-37 and murine CRAMP, which are also expressed by epithelial cells of multiple organs.

CATH-2 protein expression was investigated in developing heterophils in the bone marrow. The peptide was first seen in the promyelocyte stage and was abundantly present in myelocytes, metamyelocytes and mature heterophils (9). mRNA expression of CATH-2 was

observed both in bone marrow precursors and mature heterophils. A similar pattern was observed for LL-37 protein in developing human neutrophils, where mRNA expression mainly occurred in myelocyte precursors and was markedly reduced in more mature stages (37). This indicates that CATH-2 is expressed during early stages of granulocyte development, which leads to accumulation of the peptide in the mature cells.

Regulation of cathelicidin production

To understand cathelicidin functions, it is important to study the stimuli which regulate the expression of these peptides. In the following sections we will outline what is known about the regulation of avian cathelicidins during embryonic and adult life.

Developmental expression

Meade et al. (2009) investigated the mRNA expression of chicken cathelicidins during embryonic development (38). CATH-1 to -3 expression was found in the earliest samples of embryonic day (ed) 3, while the first evidence of CATH-B1 expression was found at ed9. The cell types in which cathelicidins are expressed during embryonic development are unknown. The first primitive white blood cells arise in the yolk sac at ed3, and are expected to express CATH-1 to -3 (39). Although the bursal anlage is first seen at ed4, the interfollicular bursal epithelium known to express CATH-B1 in adult chickens does not appear before ed14, suggesting that CATH-B1 might be expressed by other cell types during embryonic development (40). Following the cathelicidin expression during embryonic development, a biphasic expression pattern was seen for CATH-1 to -3, similar to the pattern observed for avian β-defensin (AvBD) AvBD2, -6 and -7. For the ed12 samples, mRNA of the head and abdomen region was tested separately. No preferential expression of any of the chicken cathelicidin genes for either of these regions was observed (38). In another study, CATH-B1 expression was not found in the bursa of 1-day old Ross chicks, but was detected at 11 days of age (41). In contrast, during a more extensive study of the post-hatch regulation of chicken cathelicidins, CATH-B1 was already expressed at day 2. The expression of this cathelicidin and CATH-1 peaked at day 4 and decreased thereafter. Different patterns of expression were seen in different organs, as a trend towards increasing expression with age was seen in the lung for all cathelicidins, albeit without significant differences between investigated ages. In the cecum, an abrupt increase by >10-fold in expression of CATH-1 to -3 was seen on day 28 (33). Expression of cathelicidins early in embryonic development was also shown in mice, where the murine cathelicidin CRAMP was already found as early as ed12, which is before the establishment of the granulocytic lineage (42).

Regulation of expression

The induction of cathelicidin expression by pathogenic microorganisms is well described in mammals, but little is known about avian cathelicidin expression. *S. typhimurium* induces CATH-1 expression in cecal tonsils of 1-day old infected chicks at 3 days post challenge (43). In other studies *S. typhimurium* infection did not significantly change cathelicidin expression in PBLs or jejunum (36, 44). In the last study, an accumulation of CATH-2 containing heterophils in the lamina propria of the jejunum was observed. These apparent differences could be explained by the fact that older chickens were used and tissues were sampled at earlier time points. It is well known that Salmonella infections can be severe in young chickens, while clinically apparent disease is rare in adult animals (45).

Similar to defensins, *Campylobacter jejuni* is able to decrease cathelicidin expression. Oral challenge with *C. jejuni* of 4 week old broiler chickens led to significantly decreased CATH-2 and -3 mRNA expression in PBLs at 6 hours p.i. (35). A decrease of CATH-2 levels was also found in the jejunum of *C. jejuni* infected broiler chicks at 48 hours p.i. (36). The parasitic poultry pathogen *Eimeria praecox* may employ the same strategy, as CATH-3 expression was downregulated in the jejunum of *E. praecox* infected chicks (46).

A study of Zhang et al. (2011) suggests that cathelicidin expression may be regulated by Vitamin D_3, an important modulator of the immune system (47). This has previously been described in mammals and Vitamin D receptor elements (VDREs) in cathelicidin promotors were shown to be involved (48). After four weeks of feed supplemented with Vitamin D_3, CATH-1 expression was increased in the bursa and thymus of young chickens, although VDREs were not detected in the promotor region of CATH-1.

CATH-B1 expression in the bursa is increased by treatment with probiotics or organic acids in young broiler chickens (41). For CATH-1, probiotics alone could not increase transcription, but the treatment impeded the increased expression of CATH-1 induced by *S. typhimurium* (43).

Avian cathelicidin functions

Since the initial discoveries of cathelicidins (49), many cathelicidins from different species have been studied. The peptides showed antimicrobial activity against many pathogens, including Gram-positive and -negative bacteria, fungi and even enveloped viruses (8, 14, 50). Three models have been proposed by which these cationic peptides can induce pore formation to kill pathogens: the barrel-stave model, the toroidal model and carpet model. In the barrel-stave model peptides penetrate perpendicular to the membrane, their hydrophobic side interacting with the lipid-bilayer and their hydrophilic side directed

to the lumen of the pore formed. In the toroidal model peptides cause inward folding of the membrane, inducing pore formation. The carpet model represents coating of the membrane surface until a threshold is reached at which micelles are formed, which are removed from the membrane, creating pores (8, 14, 51, 52). Recently, other mechanisms of microbial killing have also been proposed, including intracellular targeting of DNA and RNA synthesis, protein synthesis or protein folding (8, 53). Dependent on peptide concentrations, different antimicrobial mechanisms could be used by one peptide (8, 53, 54). However, where initially direct bacterial killing was thought to be the main function of these peptides, other functions, mainly involving immune activation and regulation, have been found for many of these peptides (55). Many of these antimicrobial and immunomodulatory functions have been described for human LL-37 (56), but avian cathelicidins have also shown to exhibit both antimicrobial and immunomodulatory effects. The following paragraphs will summarize the current knowledge on the biological functions of avian cathelicidins.

Antimicrobial activity

A wide variety of bacteria, both Gram-positive and Gram-negative, has been shown to be susceptible to killing by avian cathelicidins. Quail CATH-2 and -3 and pheasant CATH-1 show MIC values in the range of 1-10 µM for most Gram-positive and Gram-negative bacteria, which is lower compared to the human LL-37 (25, 31). MIC values for all four chicken cathelicidins are also in the same order of magnitude as the other avian cathelicidins (9, 16, 24, 26). Also, fungi like *Candida albicans* are susceptible to avian cathelicidins, showing MIC values in the range of 1-5 µM (9, 25, 31) and CATH-2b even inhibits biofilm formation (57). The killing of the bacteria appears to be very fast, ranging from 10-30 minutes for killing of *S. enteritidis* (58) and 30-60 minutes for killing of *E. coli* (16, 20). However, the mechanisms responsible for bacterial killing are still a matter of discussion. Nevertheless, a lot is known about peptide properties required for bacterial killing.

Several studies have shown that the presence of an α-helical region is important in bacterial killing. Removal of the N-terminal α-helix of CATH-2a and -2b results in the loss of antimicrobial activity (20, 58). This was also observed with an α-helical synthetic peptide, where remodeling of the N-terminal α-helix, disrupting helix formation, resulted in the loss of antibacterial activity (59). Removal of the C-terminal α-helix of CATH-2a mostly reduces killing compared to the full-length peptides, but still contains some antibacterial activity (20). In addition, truncation of CATH-2b, where only the N-terminal α-helix is present, showed an increase in antibacterial activity (58, 60). However, in the presence of 100 mM NaCl, this effect was mostly lost, while the full-length CATH-2a and -2b were largely unaffected (20, 60). Other full-length avian cathelicidins also retain antimicrobial activity in the presence of 100 mM NaCl (25, 31), indicating the importance of α-helical regions for functionality. Nevertheless, a higher percentage of α-helicity does not guarantee better antibacterial effects. Amino acid substitutions increasing total α-helicity of CATH-1 or truncated LL-37 variants, do not increase the antibacterial activity (21, 61). Thus, while α-helicity is important for high efficacy in bacterial killing, increasing the percentage of α-helicity throughout the peptide does not necessarily lead to better activity.

A second important region in many α-helical AMPs, is the kink or hinge-region formed around the center of the peptide (19, 59), which induces flexibility and is thought to be important for the insertion in the bacterial membrane causing pore-formation (19, 59). Removal or substitution of these glycines or prolines at the center of an α-helical peptide, including CATH-2b, can indeed greatly reduce antibacterial activity (58, 59, 62). Interestingly, a CATH-2a truncation containing only the first 14 amino acids, which is the entire N-terminal α-helix, showed a great reduction in antibacterial activity (20). If a one amino acid longer peptide was tested, some of the antibacterial activity was restored and a peptide comprising the first 18 amino acids (N-terminal α-helix including the hinge-region), showed comparable activities to the full length peptide (±2 µM for most Gram-positive and Gram-negative bacterial strains). A C-terminal truncation of CATH-2b which leaves the hinge region and N-terminal α-helix intact also shows very good antibacterial activity (58). Additionally, the C-terminal α-helix of CATH-2a and -2b without the hinge region shows no antibacterial activity, while the C-terminal α-helix of CATH-2a with the hinge-region shows better antibacterial activity in the range of the 1-20 µM. Therefore, it appears that although it is thought that the hinge region is important for insertion of the tail of the peptide into the bacterial membrane, this region also has antibacterial properties by itself (20).

Hydrophobicity is thought to be important for the interaction of the cathelicidins with bacterial membranes (53, 63). Membrane interaction of CATH-1 has been investigated by determining its structure in a DPC-micelle model (18). This model showed that the α-helical and hydrophobic center of the peptide formed an oligomeric structure in the lipid bilayer, probably by interacting with the acyl groups of the lipids, while the polar residues of the peptide could interact with the phosphate groups on the outside of the lipid bilayer. This indicates that pores are formed by these oligomeric structures, through which water and ions can freely enter and exit the cell, disrupting the osmotic balance and hampering bacterial survival (18). Interestingly, for human LL-37 it was shown that its hydrophobic residues also interact with the membrane surface, but would not form a pore by aligning itself through the membrane (64), showing that although very alike in structure, mechanisms of action can differ between different cathelicidins. Loss of the first tryptophan of CATH-1 reduces antibacterial activity, indicating the importance of the hydrophobic residue in this peptide (16). Loss of the more hydrophobic C-terminal

in CATH-2a analogs also results in reduced bacterial killing (20), although the C-terminal truncation of CATH-2b (C1-15) resulted in higher antibacterial activity (58). Interestingly, when using an N-terminal truncation, only leaving the hydrophobic C-terminal α-helix, antibacterial activity is almost completely lost, probably because the first interaction with the bacteria is through the polar part of the CATH-2a and -2b (20, 58). Substitution of phenylalanines with more hydrophobic tryptophans in the C1-15 peptide (60), resulted in enhanced bacterial killing and also better resistance against a salty environment. Additionally, substitution of a tyrosine by an alanine in a Cecropin A-Magainin-2 fusion peptide greatly reduced antibacterial activity (59).

The cationic nature of most cathelicidins is probably important for the initial interaction with the bacterial surface (53). The highly cationic CATH-2a and -2b and N-terminal analogs thereof, show good antimicrobial killing, while N-terminal truncations remove most of the cationic charges and reduce the antibacterial activity (20, 58). Increasing the charge of CATH-2a did not result in large changes in bacterial killing, which is in line with results found for other α-helical peptides where increased charge did not directly correlate with increased anti-microbial activity (61, 65).

Immunomodulation

Next to direct killing of bacteria, most cathelicidins also have immunomodulatory effects. These effects have been extensively studied for the human cathelicidin LL-37, but also avian cathelicidins have been a subject of investigation in terms of their immunomodulatory properties. The first report on immunomodulation by LL-37 showed that LL-37 could block LPS-induced IL-1β mRNA transcription (66). After this initial report, many other studies were performed showing a wide array of effects induced by LL-37, or effects modulated by the presence of LL-37. These include effects on TLR activation (67-73), effects on cell differentiation and activation (74-76) and leukocyte migration (47, 77). Investigation of immunomodulation by avian cathelicidins has recently been reported, but is mainly limited to inhibition of TLR activation and direct immune activation.

Similar to LL-37, CATH-1 and -2 have the ability to block LPS-induced macrophage activation, inhibiting TNFα, IL-1β, MCP-1 and NO production in RAW264.7 mouse macrophages (16, 20, 24). C-terminal truncations of CATH-2a and -2b result in reduced inhibition of LPS activation, indicating the importance of the hydrophobic C-terminal part in LPS inhibition. N-terminal truncations of CATH-2a and -2b also result in loss of LPS inhibition, although less if a large part of the hinge-region is left intact (20, 58). Interestingly, peptides which have the best LPS-inhibiting potential are also most toxic, measured by erythrocyte hemolysis and cytotoxicity towards Caco-2 cells (20). However, toxicity is rather low when testing at MIC values. Truncations of CATH-1 lacking either the N-terminal tryptophan or the two C-terminal tyrosines, showed greatly reduced LPS-binding capacity of the peptide, but also greatly reduced hemolytic and cytotoxic effects (16, 21). Also for LL-37, hydrophobicity is positively correlated with LPS inhibition, but also with cytotoxicity (61, 78). Structural analysis of CATH-1 analogs in LPS showed that indeed the tryptophan at the N-terminus and the tyrosines in the C-terminal segment are in very close proximity to LPS, indicating a key role in the binding of LPS and thereby possibly in blocking activation of TLR4 (15). However, binding of LPS may only be one out of several mechanisms by which cathelicidins can block LPS-induced immune responses, since pre- or post-incubation of cells with LL-37 still shows inhibition of LPS-activation (79, 80). Another possibility is competition between LPS and peptide for binding of LBP or CD14, thereby outcompeting the LPS to activate macrophages (81).

Cathelicidins also have direct immune-stimulatory effects. Human LL-37 was shown to induce *CCL7* and *IL10* gene expression and CCL2 secretion in mouse RAW 264.7 macrophages (82), while CATH-2b induces CCL2 secretion from human peripheral blood mononuclear cells (58). Also, LL-37 has been shown to act as a chemoattractant for T-cells, neutrophils and monocytes, though the receptor through which LL-37 works remains a matter of debate (47, 72, 77, 83).

In conclusion, while various functions described for mammalian cathelicidins appear to be conserved in avian cathelicidins, such as the antimicrobial activity, LPS-neutralization and even chemokine induction, further research will be needed to better understand the similarities as well as the differences between avian and, for instance, mammalian cathelicidins. This will ultimately be needed to understand their biological functions and their role in the protection against infections.

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Chapter 3

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

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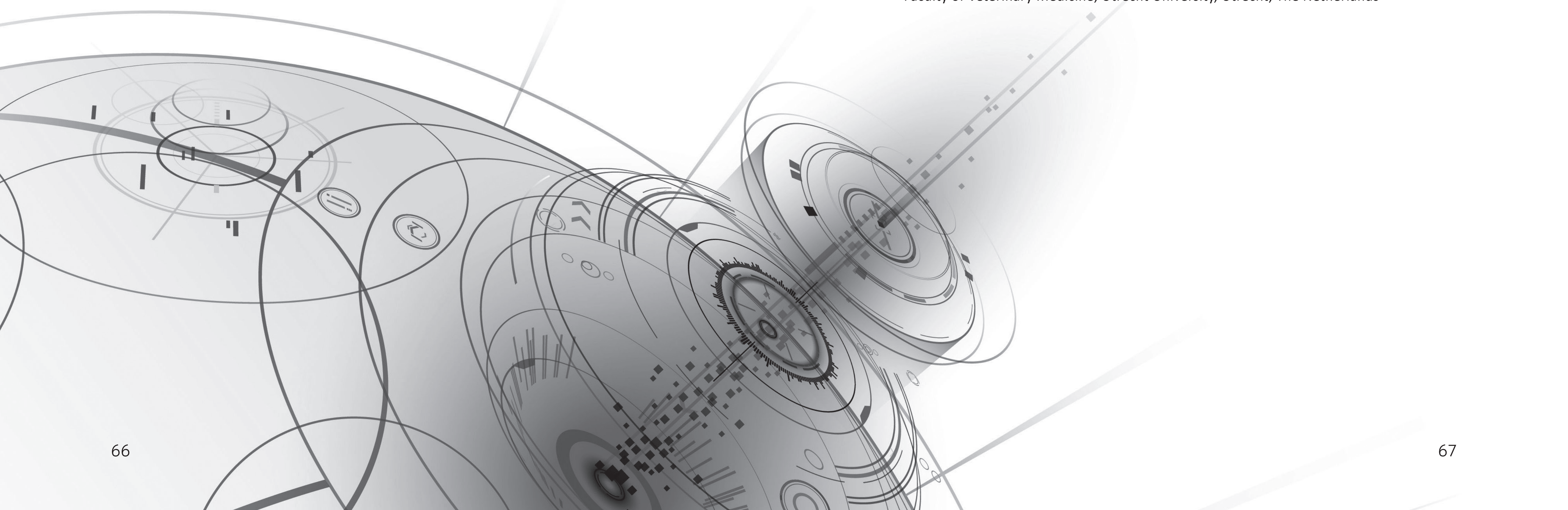


Abstract

Cathelicidins have been shown to be crucial in the innate host defense against invading pathogens and have been shown to exert antimicrobial activity as well as variety of immune regulatory functions. However, since most findings have been described in the context of the human LL-37 or murine CRAMP, it is unclear which functions are specific for these cathelicidins and which functions are more general cathelicidin properties. In addition, many effects have been investigated under varying conditions, which makes it difficult to directly compare cathelicidin functions. This study provides an interspecies cathelicidin comparison to better understand the conservation of cathelicidin functions. The results show that antimicrobial activity and LPS neutralization are most conserved among the functions tested in this study. In addition, while physiological conditions limit the antimicrobial activity of most cathelicidins against *E. coli*, the antimicrobial activity against MRSA under these conditions is enhanced. Furthermore, chemokine and cytokine induction appears to be low and only occurs at high cathelicidin concentrations. In total, these results provide new insight in the conservation of cathelicidin functions and demonstrate the diversity in cathelicidins properties within and between species.

Introduction

Cathelicidins are cationic peptides with an important function in the early vertebrate host response against invading pathogens (1). They are secreted at mucosal surfaces and sites of infection by leukocytes and epithelial cells upon interaction with microbes and have both direct antimicrobial activity as well as immunomodulatory functions (2-7). The importance of cathelicidins in innate host defense has been demonstrated in knockout mice lacking cathelicidin expression, which have an increased susceptibility towards various pathogens (8-11). In addition, 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 (12), while exogenous cathelicidin treatment has been successfully used in to inhibit *M. haemolytica*, *E. coli* and *S. aureus* infections (13-15).

Cathelicidins are conserved within most vertebrate species and although the active peptide sequences are highly variable, most peptides retain the ability to form an amphipathic α-helical structure (16). While most of these peptides have been described in the context of their antimicrobial activity, various other functions have been identified for a number of cathelicidins (17). These include induction of chemokine expression (18), intrinsic chemotactic activity (19), inhibition of LPS-induced TLR4 activation and LTA-induced TLR2 activation (18, 20, 21), enhancement of DNA-induced plasmacytoid DC and macrophage activation (22, 23), promotion of wound healing (24), effects on DC and macrophage differentiation (25, 26) and regulation of phagocytosis (27). 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 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 conserved functions for most, but not all, cathelicidins. In contrast, chemokine induction and enhancement of DNA-induced TLR9 activation appear to be less conserved and are only induced at relatively high cathelicidin concentrations. These findings demonstrate the variability in cathelicidin functions and the differences in potency with respect to these functions. In total, this study provides 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: LTA *S. aureus*, LPS *Escherichia coli* (*E. coli*) O111:B4 and ODN-1826 were obtained from Invivogen (Toulouse, France). Chicken CATH-2 (chCATH-2) and PMAP-36 were synthesized by Fmoc-chemistry at China Peptides (CPC scientific, Sunnycale, CA) and all other cathelicidins were synthesized by Fmoc-chemistry at the Academic Centre for Dentistry Amsterdam (Amsterdam, The Netherlands) (Table S1).

Cell and bacterial culture

E. coli O78 (Zoetis Animal Health, Kalamazoo, MI, USA) 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 2-3 hours at 37 °C on a shaker, 200 RPM. Murine RAW264.7 macrophages (ATCC-TIB-71) were obtained from the American Type Culture Collection (ATCC, Manassas, VA, USA) and cultured in DMEM (Thermo Fisher Scientific, Waltham, MA, USA) supplemented with 10% FCS (DMEM+FCS) (Bodinco B.V., Alkmaar, the Netherlands) at 37 °C, 5.0% CO_2. Cells were seeded in 96-wells plates at $5x10^5$ cells/ml or 12-wells plates at $2x10^5$/ml for adherence overnight prior to stimulation.

Antimicrobial activity

Peptides and bacterial mid-log cultures were diluted to the correct concentrations in MHB or DMEM+FCS. Bacteria (10^6 CFU/ml) and peptide dilutions were added to Bioscreen C analyzer plates (Oy Growth Curves Ab Ltd, Helsingfors, Finland) and incubated for 16 h at 37 °C, 200 RPM in a Bioscreen C analyzer (Oy Growth Curves Ab Ltd). The OD was measured every 15 minutes using a wideband filter (450-580 nm) and the time required to reach an OD of 0.6 was determined for all concentrations. When no complete inhibition was obtained at 20 μM, percentage of growth delay was calculated.

WST-1 assay

WST-1 reagent was obtained from Roche (Basel, Switzerland). RAW264.7 cells were incubated with cathelicidins for 24 h, after which media 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.

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 ODN-1826 in the presence of various concentrations of different cathelicidins. TNFα release was determined after 2 h for LPS and LTA stimulation and 24 h for ODN-1826 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 22h incubation in cell culture medium supplemented with 250 μg/ml gentamicin. TNFα release was determined after 2 h, while CXCL10, CCL5 and IL-10 release were determined after 24 h.

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. Data were analyzed with MARS data analysis software (BMG Labtech GmbH).

Phagocytosis assay

Peptide dilutions were prepared in culture medium shortly before use. Red fluorescent (λ_{ex} 575 nm and λ_{em} 610 nm) carboxylate-modified polystyrene latex beads (0.5 mm; 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 the cells, directly followed by the latex beads (ratio 10 beads to 1 cell). Cells were incubated for 0.5 h at 37 °C, 5% CO_2 (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% NaN_3, to remove all free beads. After washing, cells were scraped and resuspended in FACS buffer (PBS supplemented with 0.5% FCS). 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 5 software (Graphpad, La Jolla, CA, USA). All samples were compared to 0 μM controls. * = $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$.

Results

Antimicrobial activity

Cathelicidins selected for this study are the human LL-37, murine CRAMP, dog K9CATH, equine CATH (eCATH)-1, -2 and -3, chicken CATH (chCATH)-1, -2, and -3, and porcine PMAP-23 and -36 and PR-39 (Table S1). To directly compare the antimicrobial activity of these cathelicidins, *E. coli* and MRSA growth inhibition was determined under standard MHB culture conditions in the presence of various cathelicidin concentrations (Table 1, Fig. S1A-B). A delay in *E. coli* growth of at least 70% was observed for all cathelicidins, except canine K9CATH (only 25% delay at 20 μM) and eCATH-3 (0% delay at 20 μM). chCATH-2 and PMAP-36 were most potent in inhibiting *E. coli* growth, reaching complete inhibition at 5 μM, while the other active cathelicidins reached complete or almost complete growth inhibition at 20 μM (Fig. S1A). Compared to *E. coli*, MRSA appeared more resistant to most cathelicidins, except for chCATH-1, -2 and -3, which were more active against MRSA, and PMAP-23, -36 and eCATH-1, which showed similar activity against both MRSA and *E. coli* (Fig. S1B).

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 a negative impact on antimicrobial activity (28-30), growth inhibition of *E. coli* and MRSA was also assessed in DMEM+FCS, which better represents physiological conditions (Table 1, Fig. S1C-D). Under these conditions, almost all cathelicidins were less potent against *E. coli*, with only chCATH-1, -2 and -3, PMAP-36 and PR-39 inhibiting at least 40% of growth at 20 μM. Interestingly, in contrast to all other cathelicidins, PMAP-36 was more active in DMEM+FCS compared to MHB. Furthermore, while these conditions had an inhibitory effect on *E. coli* killing for all cathelicidins except PMAP-36, MRSA growth inhibition was enhanced for all cathelicidins in DMEM+FCS compared to MHB. This was most pronounced for chCATH-3 and PMAP-36, which completely inhibited MRSA growth at nM concentrations.

Cytotoxicity

Since cathelicidins have membrane-perturbing properties that could affect the host's cell membrane, cytotoxicity of the cathelicidins was assessed. This was done by determining mitochondrial activity of RAW264.7 cells with the WST-1 assay after 24 h exposure to different cathelicidin concentrations (Fig. 1). No or limited cytotoxicity (>80% mitochondrial activity) was observed for cathelicidins at concentrations up to 5 μM. However, PMAP-36 showed strong cytotoxicity at 20 μM, reducing mitochondrial activity to approximately 20%, while chCATH-1 and chCATH-3 showed minor cytotoxicity at 20 μM, reducing mitochondrial activity to 71% and 79% of the control, respectively. All other cathelicidins only had marginal or no effect on mitochondrial activity.

Table 1: Cathelicidin antimicrobial activity

	MHB				DMEM+FCS			
	E. coli		MRSA		*E. coli*		MRSA	
	MIC	Max	MIC	Max	MIC	Max	MIC	Max
LL-37	**20**		-	7%	-	37%	**20**	
CRAMP	-	74%	-	3%	-	17%	-	64%
K9CATH	-	25%	-	2%	-	10%	-	47%
eCATH-1	-	88%	-	69%	-	29%	**20**	
eCATH-2	-	70%	-	14%	-	19%	-	35%
eCATH-3	-	0%	-	1%	-	12%	-	13%
chCATH-1	**10**		**2.5**		-	91%	**1.25**	
chCATH-2	**5**		**2.5**		**10**		**1.25**	
chCATH-3	-	95%	**5**		-	45%	**0.6**	
PMAP-23	-	92%	-	92%	-	30%	**10**	
PMAP-36	**5**		**10**		**10**		**0.3**	
PR-39	**20**		-	4%	-	60%	**10**	

MIC: minimal inhibitory concentration for complete growth inhibition (μM)

Max: percentage of growth delay at 20 μM

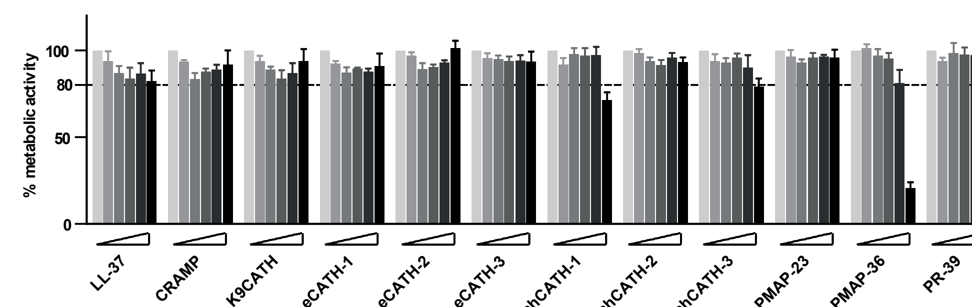


Figure 1: Cytotoxicity. RAW264.7 cells were incubated for 24 hours with 0 μM, 0.08 μM, 0.31 μM, 1.25 μM, 5 μM, or 20 μM of the different cathelicidins, after which they were incubated with WST-1 reagent for 20 minutes to measure the mitochondrial activity. Dotted line represents 80% metabolic activity. N = 4.

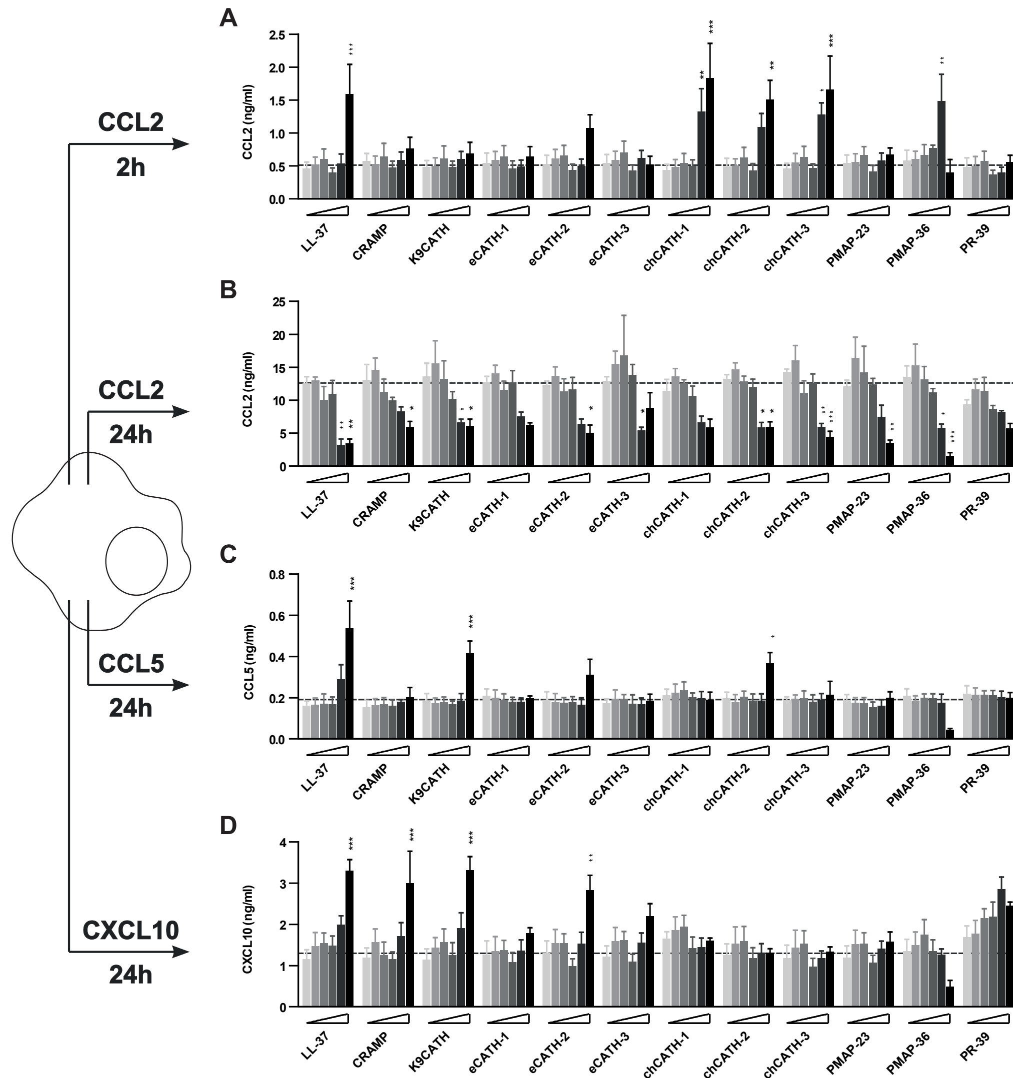


Figure 2: Chemokine and cytokine release. RAW264.7 cells were incubated for 2 h or 24 h with 0 μM, 0.08 μM, 0.31 μM, 1.25 μM, 5 μM, or 20 μM of different cathelicidins, after which the supernatants were harvested and tested for release of CCL2 at 2 h (A) and 24 h (B), CCL5 at 24 h (C) and CXCL10 at 24 h (D). Dotted line represents average cytokine release of control samples. Statistical differences were determined by Two-way ANOVA with Bonferroni post-hoc test. N = 3.

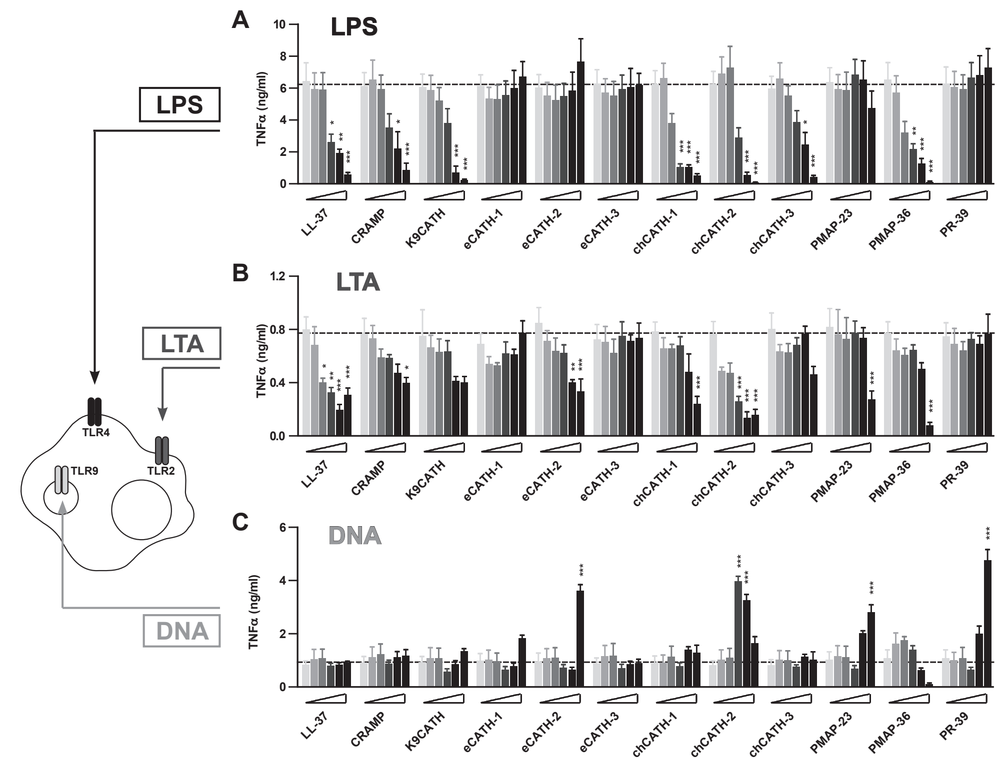


Figure 3: Effects on activation of TLR-2, -4, and -9. 100 ng/ml LPS (TLR4) (A), 1 μg/ml LTA (TLR2) (B) or 2.5 nM ODN-1826 (TLR9) (C) was mixed with 0 μM, 0.08 μM, 0.31 μM, 1.25 μM, 5 μM, or 20 μM of different cathelicidins. RAW264.7 cells were stimulated for 2 hours (A and B) or 24 hours (C) with these mixtures. After incubation, supernatants were harvested and levels of TNFα were measured. Dotted line represents average cytokine release of control samples. Statistical differences were determined by Two-way ANOVA with Bonferroni post-hoc test. N = 3.

Chemokine and cytokine release

To determine the effect of cathelicidins on chemokine induction, RAW264.7 cells, which have previously been shown to increase chemokine production upon stimulation with LL-37 (18), were stimulated with cathelicidins at various concentrations for 2 h or 24 h, after which CCL2, CCL5, CXCL10, TNFα and IL-10 release were determined. Release of CCL2 after 2 h stimulation was only enhanced (3-4 fold) at 5 μM by chCATH-1, chCATH-3 and PMAP-36 and at 20 μM by LL-37 and chCATH-1, -2 and -3 (Fig. 2A). None of the other peptides had a significant effect on CCL2 release after 2 h stimulation. In contrast, all cathelicidins inhibited CCL2 release at 24 h, although inhibition by eCATH-1, chCATH-1 and PR-39 was non-significant (Fig. 2B). Interestingly, compared to CCL2 release after 2 h, CCL5 release after 24 h was affected by another set of cathelicidins, with 20 μM LL-37, K9CATH and chCATH-2 showing a 2-3 fold increase in CCL5 release (Fig. 2C). CXCL10 release after 24 h was enhanced 2-3 fold by LL-37, CRAMP, K9CATH and eCATH-2, but none of the other cathelicidins, at 20 μM (Fig. 2D). In addition, release of TNFα (2 h) and IL-10 (24 h) were determined after cathelicidin stimulation. However, the observed cytokine levels were too close to the detection limit to properly analyze (Fig. S2A-B). Furthermore, although significant differences in chemokine release were detected after stimulation with various cathelicidins, the release appears marginal in comparison to TNFα, IL-10 and CCL5 release after stimulation with viable or heat-killed *E. coli* (Fig. S2C). Only the increase in CXCL10 release by cathelicidins was found to be in the same range as the increase after *E. coli* stimulation.

Effects on activation of TLR-2, -4, and -9

Inhibition of LPS-induced TLR4 activation was tested by stimulation of RAW264.7 cells for 2 h with 100 ng/ml LPS from *E. coli* O111:B4 in the presence of different cathelicidins at various concentrations (Fig. 3A). Most cathelicidins, including LL-37, CRAMP, K9CATH, chCATH-1, -2 and -3, and PMAP-36 significantly inhibited LPS-induced activation at a concentration of 1.25 μM or 5 μM. In contrast, all the equine cathelicidins as well as PMAP-23 and PR-39, were unable to neutralize LPS, even at 20 μM. To test the neutralization of LTA, RAW264.7 cells were stimulated with 1 μg/ml *S. aureus* LTA for 2 h in the presence of various cathelicidin concentrations (Fig. 3B). 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. It should be noted that the inhibition of LPS- and LTA-induced activation with 20 μM PMAP-36 could be caused by cytotoxic effects of the peptide at this concentration.

In addition to the inhibition of TLR2 and TLR4 activation, cathelicidins have also been described to enhance DNA-induced TLR9 activation (22, 23). Therefore, the effect of cathelicidins on DNA-induced TNFα release in RAW264.7 cells was analyzed (Fig. 3C). Interestingly, only chCATH-2 increased the DNA-induced activation after 24 h at concentrations of 1.25 μM and 5 μM. Other cathelicidins only showed enhancement at 20 μM, such as eCATH-2, PMAP-23 and PR-39, or no enhancement at all. To confirm that the observed activation was indeed enhancement of DNA-induced activation and not a result of TNFα release due to macrophage activation by cathelicidins, TNFα release was determined after 24 h stimulation with cathelicidins only. However, none of the cathelicidins induced TNFα release after 24 h, indicating the increased activation is indeed enhancement of DNA-induced activation (Fig. S2D).

Phagocytosis

While the above-described functions of cathelicidins are well-known, little is known about their effects on phagocytosis (27). Therefore, the effect of cathelicidins on phagocytosis was analyzed by using a fluorescent bead-assay designed for flow cytometry. In order to ensure active uptake, fluorescence was corrected for non-specific binding of beads to the cells at 0 °C. All cultured RAW264.7 took up the latex beads, resulting in ±90% bead-positive cells after 30 minutes (Fig. 4A). Histograms exemplify changes in bead phagocytosis at different concentrations of K9CATH (Fig. 4B), eCATH-2 (Fig. 4C) and chCATH-3 (Fig. 4D). The bead internalization was quantified for all cathelicidins at 0.3, 1.25 and 5 μM by determining the average mean fluorescence intensity (MFI) (Fig. 4E). CRAMP, K9CATH, chCATH-1 and -2, PMAP-23 and PR-39 reduced bead-uptake in a dose-dependent manner. In contrast, eCATH-2 was the only peptide that increased the uptake by almost 50%, albeit not significant. PMAP-36 showed a mixed response, with a moderate, although non-significant, increase in uptake at a concentration of 0.31 μM and inhibition of uptake at a concentration of 5 μM. LL-37, eCATH-1 and -3, chCATH-3 and PR-39 did not induce a significant change in 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, making it difficult to compare properties of cathelicidins. In this study, 12 cathelicidins were selected and compared for various well-known functions to determine the conservation of these functions between cathelicidins. 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,

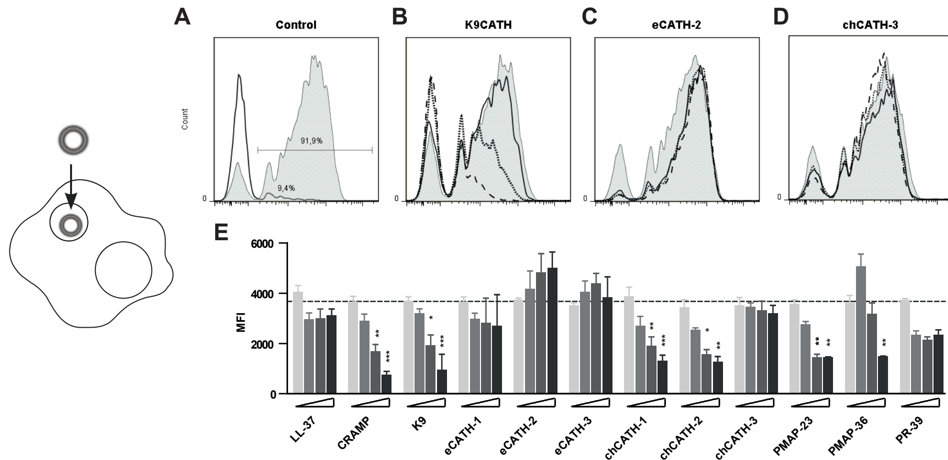


Figure 4: Phagocytosis. RAW264.7 cells were incubated with 0 μM, 0.31 μM, 1.25 μM, or 5 μM of the different cathelicidins together with red fluorescent latex beads (10 beads to 1 cell) and incubated for 0.5 h at 37 °C, 5% CO_2 (energy dependent uptake) or at 0 °C (non-specific adherence). Histograms show (A) control bead uptake at 37 °C (tinted line) and 0 °C (solid line), or (B-D) uptake in presence of different concentrations of indicated cathelicidins; 0 μM (tinted line), 0.31 μM (solid line), 1.25 μM (dotted line) or 5 μM (dashed line). (E) Uptake was quantified by determining the MFI after correction for 0 °C control. Dotted line represents average MFI in the absence of cathelicidins. Statistical differences were determined by Two-way ANOVA with Bonferroni post-hoc test. N = 3.

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.

Antimicrobial activity of cathelicidins has been extensively tested over the years and is a function that has previously been demonstrated for all cathelicidins tested in this study (31-40). Our results indicate that most cathelicidins have similar antimicrobial activity against *E. coli* (complete or almost complete killing at 5-20 μM), but diverge in their antimicrobial potency against MRSA, with especially chicken cathelicidins showing potent antimicrobial activity. Interestingly, when testing the antimicrobial activity under more physiological conditions, i.e. DMEM+FCS, lower activity was observed against *E. coli* for all cathelicidins except PMAP-36, while MRSA inhibition is enhanced for all cathelicidins. The lower antimicrobial activity against *E. coli* is most likely caused by the inhibitory effects of salts and serum components in DMEM+FCS, which have been described in previous studies to lower cathelicidin antimicrobial activity (29, 30, 34, 41). However, while salt and serum have also been suggested to limit antimicrobial activity against Gram-positive bacteria (41, 42), the use of DMEM+FCS increased the activity

Table 2: Summary of cathelicidin functions

Peptide	*E. coli*		MRSA		TLR activation			Chemokines			Phagocytosis
	MHB	DMEM	MHB	DMEM	LPS	LTA	DNA	CCL2 (2h)	CCL5 (24h)	CXCL10 (24h)	
LL-37	+++	-	-	+++	↓↓	↓↓	-	↑	↑	↑	-
CRAMP	+	-	-	+	↓↓	↓	-	-	-	↑	↓↓
K9CATH	-	-	-	+	↓↓	-	-	-	↑	↑	↓↓
eCATH-1	++	-	+	+++	-	-	-	-	-	-	-
eCATH-2	+	-	-	-	-	↓↓	↑	-	-	↑	-
eCATH-3	-	-	-	-	-	-	-	-	-	-	-
chCATH-1	+++	++	+++	+++	↓↓	↓	-	↑↑	-	-	↓↓
chCATH-2	+++	+++	+++	+++	↓↓	↓↓	↑↑	↑	↑	-	↓↓
chCATH-3	++	+	+++	+++	↓↓	-	-	↑↑	-	-	-
PMAP-23	++	-	++	+++	-	↓	↑	-	-	-	↓↓
PMAP-36	+++	+++	+++	+++	↓↓	-	-	↑	-	-	↓↓
PR-39	+++	+	-	+++	-	-	↑	-	-	-	-

+++ = 100% inhibition ≤ 20 μM, **++** = > 80% inhibition at 20 μM, **+** = > 40% inhibition at 20 μM

↑↑ = significant increase ≤ 5 μM, ↑ = significant increase at 20 μM

↓↓ = significant decrease ≤ 5 μM, ↓ = significant decrease at 20 μM

of all cathelicidins against MRSA. This could be caused by the presence of carbonate, which can increase bacterial susceptibility to cathelicidin-mediated bacterial killing (43). Although carbonate also 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 *E. coli* killing than on *S. aureus* killing, with Ca^{2+}, for instance, being important for the structural integrity of the outer membrane of Gram-negatives (44). 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 (30, 45). While most cathelicidins have antimicrobial activity, it is evident that, if measured under similar conditions, the activities greatly differ, especially with respect to MRSA killing. Furthermore, these results suggest that depending on the pathogen, physiological conditions can have either a net-inhibitory or net-enhancing effect on antimicrobial activity.

Similar to the extensive research on antimicrobial activity, LPS neutralization has been observed for at least 13 different cathelicidins from 9 different species (18, 36, 38, 46-55) and is thought to be one of their main functions. However, no data were available yet

on the LPS neutralizing activity of canine, equine and porcine cathelicidins. Our results show that only 7 out of the 12 cathelicidins selected for this study potently inhibit LPS-induced macrophage activation, including K9CATH and PMAP-36. Interestingly, none of the equine cathelicidins was able to neutralize LPS, which could indicate that horses may depend on other host defense molecules to inhibit LPS-induced activation. In addition, 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 inhibits LTA-induced activation and K9CATH and chCATH-3 only show potent inhibition of LPS-induced activation. In addition, neutralization of LPS and LTA does not appear to correlate with the antimicrobial activity against *E. coli* and MRSA, respectively. These results further underline the divergence in cathelicidin functions, both between and within species, and show that, while antimicrobial activity and LPS neutralization are commonly regarded as intrinsic properties of cathelicidins, the functions of the various cathelicidins may differ between 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 (18, 56-58). Our results indicate that several cathelicidins induced a 2-4 fold increase in chemokine expression in RAW264.7 cells at 20 μM, but that only LL-37 was able to increase the expression of all three chemokines tested. However, chemokine and cytokine induction 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 (57, 59).

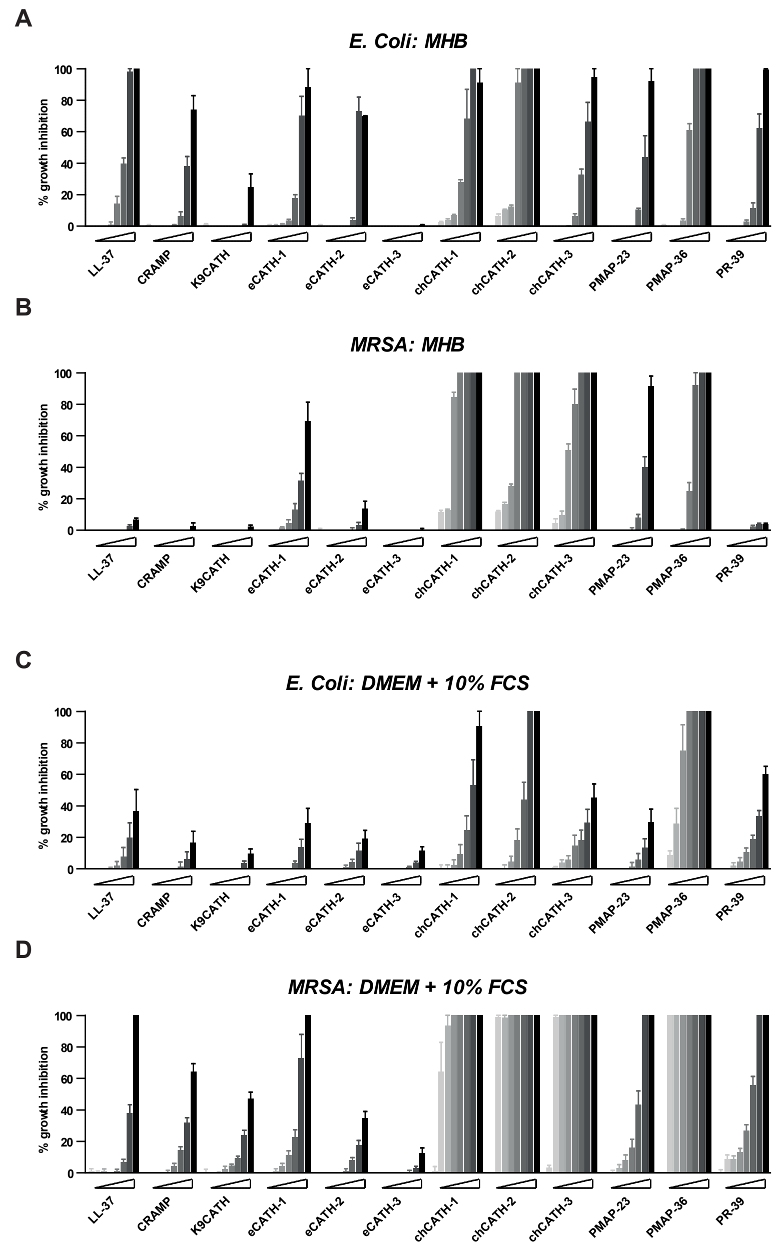
In addition to the induction of chemokine release, it has been shown previously that cathelicidins are chemotactic themselves (19, 60, 61), which could be another explanation for the observed induction of chemotaxis by CRAMP and LL-37 in other studies (62, 63). In addition, in a recent study it was shown that LL-37 can increase neutrophil influx in a murine lung model, but only in the context of an infection and without the alteration of cytokine or chemokine expression (64). Further research will be needed to understand to what extent direct chemotaxis and chemokine induction play a role in leukocyte recruitment during steady state situations and in the context of an infection.

Although not conserved for all cathelicidins, antimicrobial activity and LPS neutralization appear to be major cathelicidin functions, while induction of chemokine release was limited and enhancement of pro-inflammatory DNA-induced macrophage activation appears to be CATH-2 specific. Interestingly, an initial analysis of the effect of cathelicidins on phagocytosis showed that 6 out of 12 cathelicidins reduced latex bead

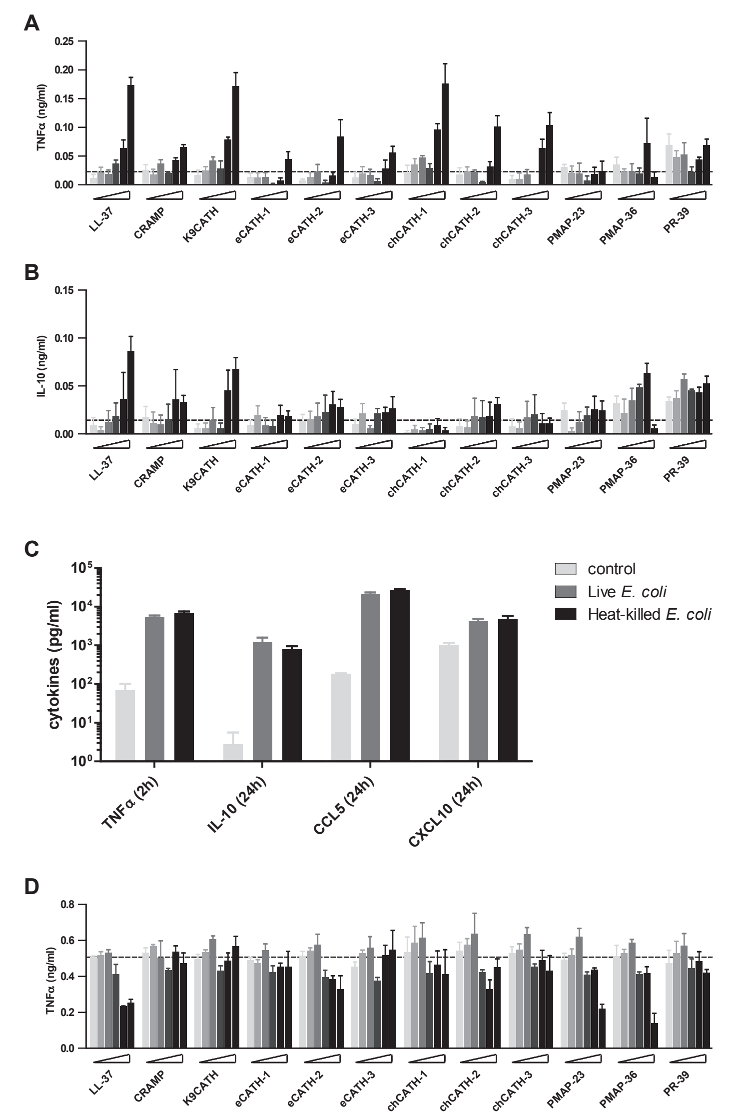
internalization. However, since uptake of extracellular components is a complicated process, it is not possible to draw conclusions about functions *in vivo* based on these initial observations only (65). Nevertheless, since various cathelicidins do inhibit bead uptake, it will be interesting to study their effects on internalization of beads or bacteria under more physiological conditions in future studies.

Finally, while these results are interesting from a biological point of view, elucidation of cathelicidin functions is also important for the development of cathelicidin-based anti-infectives. 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 (66). Especially chCATH-2 appears to be an interesting candidate with very strong antimicrobial activity against both *E. coli* and MRSA under physiological conditions and limited resistance induction in bacteria (67). In addition, since sepsis is a major problem in patients suffering from bacterial infections (68), 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.

In conclusion, the results presented in this study, and summarized in Table 2, underline the importance of not generalizing cathelicidin functions and indicate that caution should be taken in the extrapolation of these functions, for instance from murine CRAMP KO-models to the human situation or other animal settings. 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 conserved cathelicidin functions.



Supplemental Figure 1: Antimicrobial activity. Antimicrobial activity of cathelicidins was determined in (A-B) MHB or (C-D) DMEM+FCS. 10^6 CFU/ml of *E. coli* (A-C) or MRSA (B-D) were incubated with various concentrations of indicated cathelicidins (0.31 µM, 0.63 µM, 1.25 µM, 2.5 µM, 5 µM, 10 µM, or 20 µM) for 16 h with constant shaking (200 RPM). Values represent the percentage of growth delay. N = 3 or more.



Supplemental Figure 2: Chemokine and cytokine release. RAW264.7 cells were incubated with 0 µM, 0.08 µM, 0.31 µM, 1.25 µM, 5 µM, or 20 µM of the different cathelicidins, after which the supernatants were tested for (A) TNFα after 2 h and (B) IL-10 after 24 h. N = 3. (C) Stimulation of RAW264.7 cells with 10^6 CFU/ml live or heat-killed (70 °C, 30 min) *E. coli* O78 for 2 h, after which cells were washed three times and incubated for another 22 h in culture medium containing 250 µg/ml gentamicin. TNFα release was determined after 2 h and IL-10, CCL5 and CXCL10 release was determined after 24 h. N = 3. (D) RAW264.7 cells were incubated for 24 h with 0 µM, 0.08 µM, 0.31 µM, 1.25 µM, 5 µM, or 20 µM of the different cathelicidins, after which the supernatants were tested for TNFα concentrations. N = 3.

Supplemental Table 1: Cathelicidin sequences and characteristics

Peptide	Sequence	Length	# positive AA	# Negative AA	Net charge	# Aromatic AA	# Hydrophobic AA
LL-37	LLGDFFRKSKEKIGKEFKRIVQRIKDFLRNLVPRTES	37	11	5	6	4	16
CRAMP	GLLRKGGEKIGEKLKKIGQKIKNFFQKLVPQPEQ	34	9	3	6	2	17
K9CATH	RLKELITTGGQKIGEKIRRIGQRIKDFFKNLQPREEKS	38	11	5	6	2	15
chCATH-1	RVKRVWPLVIRTVIAGYNLYRAIKKK	26	8	0	8	3	16
chCATH-2	RFGRFLRKIRRFRPKVTITIQGSARF-NH_2	26	9	0	9	4	13
chCATH-3	RVKRFWPLVPVAINTVAAGINLYKAIRRK	29	7	0	7	3	19
eCATH-1	KRFGRLAKSFLRMRILLPRRKILLAS	26	9	0	9	2	15
eCATH-2	KRRHWFPLSFQEFLEQLRRFRDQLPFP	27	7	3	4	6	13
eCATH-3	KRFHSVGSLIQRHQQMIRDKSEATRHGIRIITRPKLLLAS	40	12	2	10	1	17
PMAP-23	RIIDLLWRVRRPQKPKFVTVWVR	23	7	1	6	3	13
PMAP-36	Ac-GRFRRLRKKTRKRLKKIGKVLKWIPPIVGSIPLGCG	36	13	0	13	2	21
PR-39	RRRPRPPYLPRPRPPPFFPPRLPPRIPPGFPPRFPPRFP	39	10	0	10	6	29

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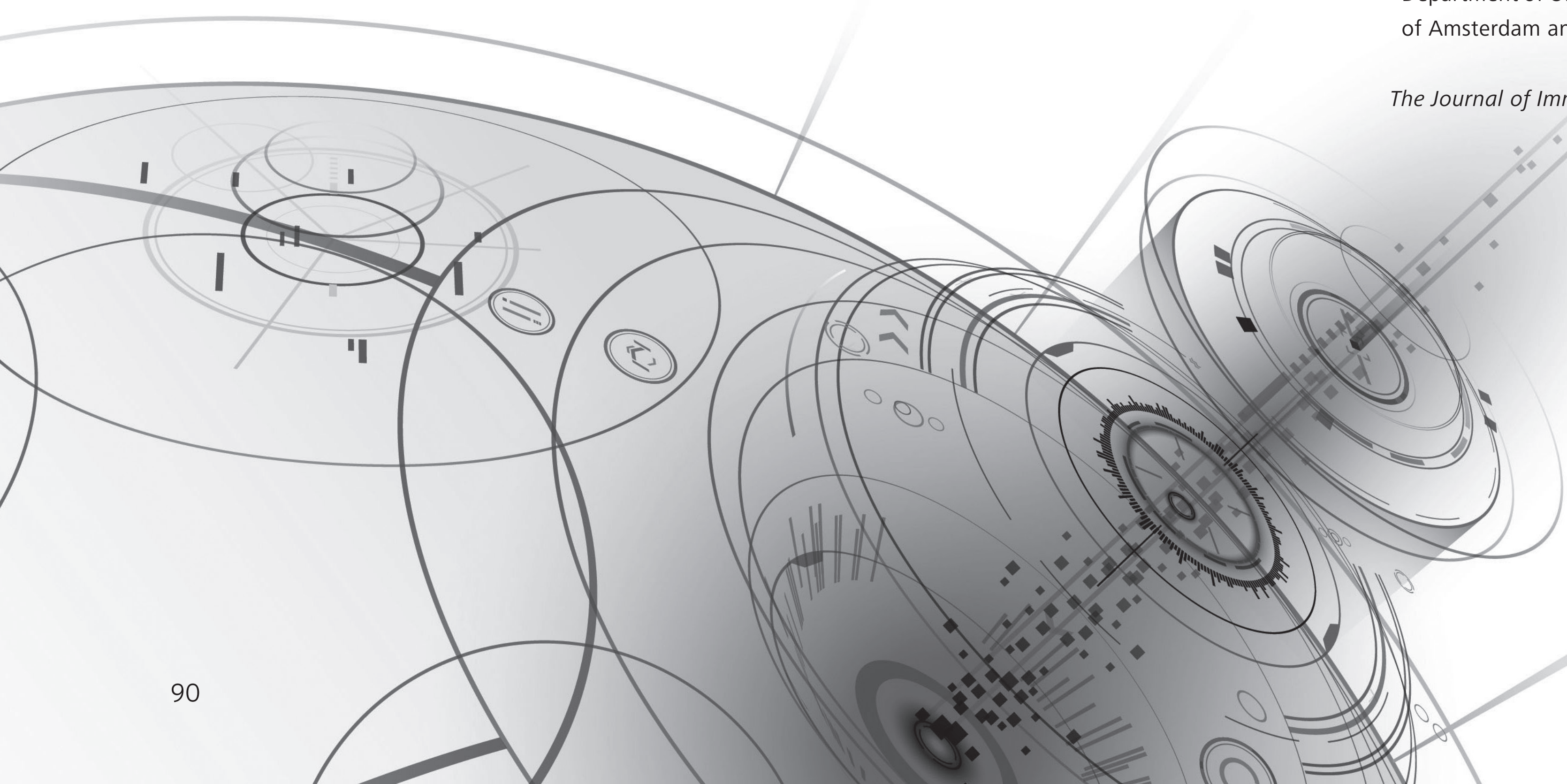

Chapter 4

Importance of endosomal cathelicidin degradation to enhance DNA-induced chicken macrophage activation

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The Journal of Immunology (2015) **195**:3970-3977

Abstract

Cathelicidins are essential in the protection against invading pathogens through both their direct antimicrobial activity and their immunomodulatory functions. While cathelicidins are known to modulate activation of several Toll-like receptors, little is known about their influence on DNA-induced TLR activation in macrophages. In this study, we explored the effects of cathelicidins on DNA-induced activation of chicken macrophages and elucidated the intracellular processes underlying these effect. Our results show that chicken cathelicidin-2 (CATH-2) strongly enhances DNA-induced activation of both chicken and mammalian macrophages due to enhanced endocytosis of DNA/CATH-2 complexes. After endocytosis, DNA is liberated from the complex due to proteolytic breakdown of CATH-2, after which Toll-like receptor 21 is activated. This leads to increased cytokine expression and nitric oxide production. Through the interaction with DNA, CATH-2 can play an important role in modulating the immune response at sites of infection. These observations underline the importance of cathelicidins in sensing bacterial products and regulating immune responses.

Introduction

Host Defense Peptides (HDPs) are a group of short cationic peptides with an essential role in the innate host defense system (1). HDPs, which are also known as antimicrobial peptides, are mainly produced by leukocytes and epithelial cells at sites of infection and/or mucosal surfaces (2). They are known for their broad-spectrum antimicrobial activity and their more recently discovered immunomodulatory functions (3). Their importance in innate host defense has been clearly demonstrated in several *in vivo* knock-out models, where loss of HDP expression was shown to result in an increased susceptibility to infections (4-6). Additionally, administration of HDPs was shown to have a protective effect in multiple *in vivo* infection models (7-9). Due to this strong protective activity, therapeutic use of HDPs as anti-infectives has gained great interest in both human and veterinary medicine (10).

Another important molecule in the regulation of infection and inflammation is extracellular DNA. During infections, DNA can be released from various sources into the extracellular microenvironment and can subsequently activate DNA-receptors to induce immune activation (11). Bacteria secrete DNA during biofilm formation (12) or can release DNA after being killed by antimicrobial components (13, 14). Host cells either secrete DNA actively, i.e. neutrophils undergoing NETosis (15), or release DNA passively, due to tissue damage (16). Moreover, administration of synthetic DNA is often used during vaccination therapies to boost vaccination efficiency (17). Interestingly, the potential of extracellular DNA to induce an inflammatory response depends greatly on other extracellular components, such as HDPs. For example, in psoriasis patients, complex formation between HDPs and DNA has been shown to induce a strong inflammatory response by enhancing DNA uptake in plasmacytoid DCs (pDCs). This subsequently increases TLR9 activation, which leads to more IFNα production (18-20). While these papers show the strong potential of HDPs in regulating DNA-induced immune activation, little is known about the role of HDPs in DNA-induced activation in other cell types, such as macrophages.

In this study, we focused on elucidating the role of cathelicidins in chicken innate immune activation by extracellular DNA. This has led to the identification of chicken cathelicidin-2 (CATH-2) as a potent enhancer of DNA-induced macrophage activation in both avian and mammalian macrophages. Enhancement of DNA-induced activation results from enhanced DNA/CATH-2 complex endocytosis and subsequent TLR21 activation. The endosomal degradation of CATH-2, which releases the DNA from the complex and enables it to activate the TLR21, was found to be essential in this process. Ultimately, this leads to the amplification

of DNA-inducible macrophage responses, such as cytokine expression and NO production.

Elucidation of the role of CATH-2 in DNA-induced macrophage activation provides new insight in both the evolutionary conservation of cathelicidin functions and the role of cathelicidins in innate immunity. Moreover, these results provide useful information for the development of multifunctional HDP-based anti-infective therapies.

Materials and methods

Reagents and stimulation

CATH-2 (21) and D-CATH-2 were synthesized by Fmoc-chemistry (CPC Scientific, Sunnyvale, USA). Truncated peptides (Table 1) and N-terminal labeled peptides, as well as LL-37 and CRAMP, were synthesized by Fmoc-chemistry at the Academic Centre for Dentistry Amsterdam (ACTA), Amsterdam, The Netherlands. ODN-1826, ODN-2216, ODN-2006, ODN-M362, inhibitory ODN (ODN-TTAGGG), control ODN (ODN-2088 control), *E. coli* DNA and *S. minnesota* LPS were obtained from Invivogen, Toulouse, France. 3'-labeled ODN-2006-Alexa Fluor 488 (DNA-AF488) and ODN-2006 for ITC experiments were obtained from Eurofins MWG Operon, Huntsville, USA. Endocytosis inhibitors chlorpromazine, ethylisopropyl amiloride (EIPA), Cytochalasin B, Methyl-β-cyclodextrin (MβCD), filipin and nocodazole were obtained from Sigma Aldrich, St. Louis, USA. Endosome acidification inhibitors that were used are chloroquine (Sigma Aldrich), bafilomycin A1 (Invivogen) and NH_4Cl (Merck, Kenilworth, USA). All experiments described concerning DNA and peptide stimulation were performed by pre-mixing the DNA and the peptide of interest in culture medium, followed immediately by stimulation of cells with this mixture.

Cell cultures

Chicken macrophage cell lines HD11 (22) and MQ-NCSU (23), as well as murine macrophage cell line RAW264.7 (24), were a kind gift from Prof. Jos van Putten, Utrecht University, Utrecht. HD11 cells were cultured in RPMI 1640 (Life Technologies, Carlsbad, USA) complemented with 10% FCS (GE Healthcare Europe GmbH, Eindhoven, the Netherlands), MQ-NCSU cells were cultured in DMEM (Life Technologies) complemented with 10% FCS and 1% Non-Essential Amino Acids (Life Technologies), RAW264.7 cells were cultured in DMEM complemented with 10% FCS. All cell lines were kept at 37 °C, 5.0% CO_2. Chicken PBMCs were obtained from healthy adult chickens. Blood was diluted in PBS and blood cells were separated by Ficoll density gradient centrifugation. PBMCs were collected at the interphase and washed with RPMI 1640. For primary monocyte selection, PBMCs were seeded at $1x10^7$ cells/well in a 48-wells plate in RPMI 1640 complemented with 10% FCS and 1% Penicillin/Streptomycin (Life Technologies). After

Table 1: Sequences truncated CATH-2 peptides

Peptide	Sequence
C1-27	RFGRFLRKIRRFRPKVTITIQGSARFG
C1-26* (CATH-2)	RFGRFLRKIRRFRPKVTITIQGSARF-NH2
C1-15*	RFGRFLRKIRRFRPK-NH2
C12-26*	FRPKVTITIQGSARF-NH2
C1-21*	RFGRFLRKIRRFRPKVTITIQ-NH2
C4-21*	RFLRKIRRFRPKVTITIQ-NH2
C5-21*	FLRKIRRFRPKVTITIQ-NH2
C7-21*	RKIRRFRPKVTITIQ-NH2
C8-21*	KIRRFRPKVTITIQ-NH2
C9-21*	IRRFRPKVTITIQ-NH2
C10-21*	RRFRPKVTITIQ-NH2
C11-21*	RFRPKVTITIQ-NH2

overnight adherence at 41 °C, 5.0% CO_2, cells were washed three times with RPMI 1640 and incubated with fresh RPMI 1640 complemented with 10% FCS and 1% Penicillin/Streptomycin. After an additional 3 day incubation, cells were used for stimulation.

Inhibition assays

Endocytosis inhibition assays were performed by pre-incubation for 1h with chlorpromazine (30 μM), EIPA (80 μM), Cytochalasin B (10 μM), MβCD (5 mM), Filipin (2 μg/ml) or nocodazole (20 μM) and subsequent co-incubation with inhibitors and stimulants for 4h. Viability was assessed by WST-1 assay (Roche, Basel, Switzerland) following manufacturer's protocol. For endosome acidification inhibition, chloroquine (25 μg/ml), Bafilomycin A1 (250 nM) or NH_4Cl (10 mM) were used during stimulation. For inhibition of TLR21, cells were pre-incubated for 5h with inhibitory ODN (ODN-TTAGGG) or control ODN (2 μM). After pre-incubation cells were washed and treated with indicated stimulants.

Griess Assay

NO production was measured by the Griess Assay. $5x10^4$ HD11 cells were seeded in a 96-wells plate and incubated overnight. After 17h stimulation, 50 ml supernatant was mixed with 50 ml 1% sulfanilamide (5% phosphoric acid) and incubated 5 min at RT in the dark. 50 ml 0.1% N-(1-naphthyl)ethylenediamine dihydrochloride was added before another 5 min incubation at RT in the dark. Absorbance was measured at 550 nm. For stimulation with *E. coli* DNA, DNA was diluted in incubation buffer (1M NaCl, 20 mM Tris, 2 mM $MnCl_2$) and treated with DNAse I (Roche) for 0, 1 or 2 min. DNAse activity was stopped with inhibition-buffer (20 mM Tris, 8 mM EDTA) and DNA length was determined by gel electrophoresis.

TNFα ELISA

For quantification of TNFα production, $5x10^4$ RAW 264.7 cells were seeded in a 96-wells plate and incubated overnight. Cells were stimulated for 24h, after which supernatant was collected and stored at -20 °C. Samples were diluted 5x in 1% BSA in PBS, pH 7.4. ELISAs were performed using the TNFα ELISA DuoSet (R&D Systems, Minneapolis, USA) following the manufacturer's protocol.

qPCR

For qPCR experiments, cells were stimulated for 4h after which RNA was isolated with the High Pure RNA Tissue kit (Roche). RNA was converted to cDNA using iScript cDNA synthesis kit (Bio-Rad, Veenendaal, the Netherlands). qPCR was performed using primers, probes (Table S1) and IQ supermix (Bio-Rad). Experiments were performed using a CFX Connect qPCR with CFX Manager 3.0 (Bio-Rad). Cq-values were corrected for PCR efficiency and housekeeping gene expression (28S and GAPDH). When no signal was detected after 40 cycles, samples were given an arbitrary Cq value of 40. Unstimulated samples were set to 1.

Confocal microscopy

Experiments were performed with DNA-AF488. Cells were seeded $4x10^4$/well on 8mm glass coverslips in a 48-wells plate and incubated overnight. After 4h stimulation with 25 nM DNA-AF488 with or without 5 μM peptide, cells were washed twice with RPMI 1640 and fixed in 4% paraformaldehyde in 0.1 M phosphate buffer (Na_2HPO_4 + KH_2PO_4), pH 7.4 for 30 min at RT. Cells were subsequently incubated with 20 mM NH_4Cl in PBS for 15 min at RT. Cells were washed once with PBS and stained with 1:500 WGA-Alexa Fluor 647 (Life technologies) in PBS for 30 min. Cells were washed twice with PBS, once with H_2O and mounted in FluoroSave (Merck Millipore, Billerica, USA) on a coverslide. Confocal imaging was performed on a Leica SPE-II DMI4000 microscope with LAS-AF software (Leica, Wetzlar, Germany) using a 100X HCX PLAN APO OIL CS objective. Z-stacks consist of 10 images taken over 1.17 mm. Averages of 4 frames per image were obtained at a resolution of 2048 x 2048. Image analysis was performed with ImageJ (National Institutes of Health, Bethesda, USA). Z-stacks were converted to average intensity images after which brightness and contrast were adjusted equally on all images.

Flow cytometry

Experiments were performed with DNA-AF488. HD11 cells were seeded $3x10^5$ in 24-wells plates and incubated overnight. After 4h stimulation cells were washed twice with PBS, harvested mechanically with a cell scraper (Corning) in PBS and transferred to BD Falcon tubes. Cells were kept on ice in the dark and were analyzed immediately

with a FACS Calibur (BD Biosciences, San Jose, USA). Data was analyzed using FlowJo software (FlowJo LLC, Ashland, USA).

Gel electrophoresis

For gel electrophoresis, ODN-2006 (1 μg, 8.7 μM) was incubated with various concentrations of peptide for 30 min at RT in RPMI 1640 medium + 10% FCS. After incubation, samples were run on a 1% agarose gel. DNA migration was visualized by staining the gel with Midori Green Advance DNA stain (Nippon Genetics Europe GmbH, Dueren, Germany) and analyzed with a ChemiDoc (Bio-Rad).

Isothermal titration calorimetry (ITC)

ITC experiments were performed using the Low Volume NanoITC (TA instruments – Waters LLC, New Castle, USA). The 50 ml syringe contained 200 μM peptide and the cell contained 250 ml 8 μM ODN-2006. Both peptide and ODN-2006 were diluted in the same 10 mM phosphate buffer (pH 7.4 or pH 4.4) containing Na_2HPO_4-KH_2PO_4 and 100 mM NaCl. Titrations were incremental with 1 ml injections at 300s intervals. Experiments were performed at 37 °C. Data was analyzed with the Nano Analyze software (TA instruments – Waters LLC).

Statistical analysis and graphics

Statistical analysis was performed using SPSS 20 (IBM, Armonk, USA). Data were analyzed by independent samples T-test for comparison of 2 groups, or One-Way ANOVA with Dunnett's Post-Hoc test for more than 2 groups. Levene's test was used to determine homogeneity of variance. Pearsons' correlation test was used to determine linear correlation. For qPCR analysis, data were log-transformed. Prism 5 software (Graphpad, La Jolla, USA) was used for graphical presentation of data.

Results

CATH-2 enhances DNA-induced macrophage activation

To determine the effect of cathelicidins on DNA-induced chicken macrophage activation, HD11 cells were stimulated with DNA in the form of a single-stranded oligodeoxynucleotide (ODN-2006), in the presence of different chicken or mammalian cathelicidins (Fig. 1A). The three chicken cathelicidins CATH-1, -2 and -3 were all able to increase DNA-induced HD11 activation, as measured by means of NO production, with CATH-2 being the most potent. Other cathelicidins with known DNA enhancing abilities, including human LL-37 (18), porcine PR-39 and PMAP-23 (25), and murine CRAMP (19), increased DNA-induced NO production as well. In addition, eCATH-1 and -2 also enhanced activation, while eCATH-3 and canine K9 did not significantly increase activation. Stimulation with cathelicidins only did not induce NO production.

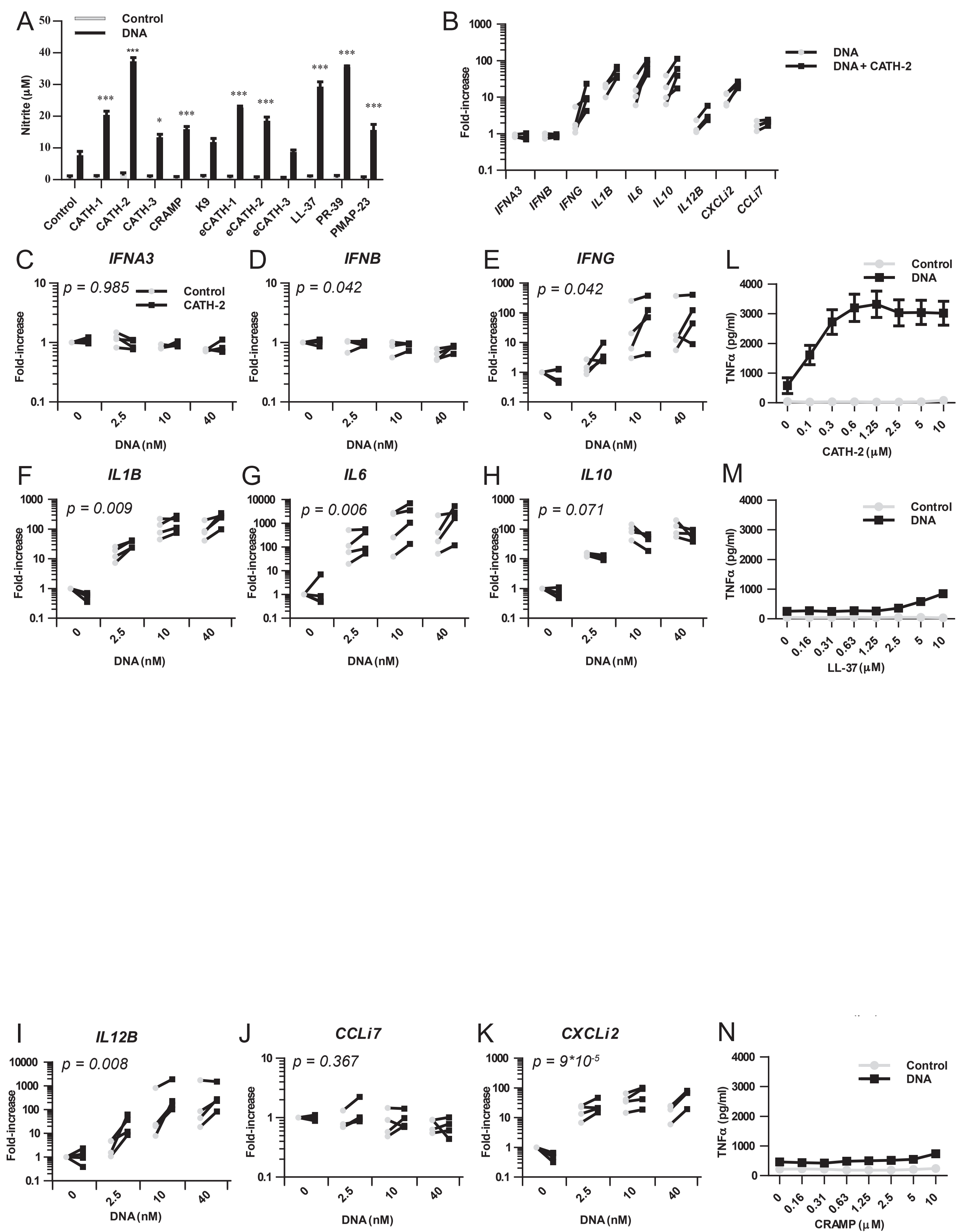


Figure 1: Enhancement of DNA-induced macrophage activation by cathelicidins. (A) NO production by HD11 cells with selected cathelicidins (5 μM) from different species in the presence or absence of 2.5 nM ODN-2006. *p*-values are obtained by One-Way ANOVA with Dunnett's post-hoc test. N = 3, error bars = SEM. (B) qPCR analysis of cytokine expression after HD11 cell stimulation with 2.5 nM ODN-2006 in the presence or absence of CATH-2 (5 μM). Data represents fold-increase in cytokine expression normalized to unstimulated cells. N = 4. (C-K) Expression of (C) *IFNA*, (D) *IFNB*, (E) *IFNG*, (F) *IL1B*, (G) *IL6*, (H) *IL10*, (I) *IL12B*, (J) *CCLi7* and (K) *CXCLi2* in adherent monocytes, isolated from blood of 4 different chickens. Cells were stimulated with ODN-2006 in presence or absence of CATH-2 (5 μM). Data represents fold-increase in cytokine expression normalized to unstimulated cells. Two-way Repeated Measures ANOVA was performed to determine significance of CATH-2 presence. N = 4. (L-N) TNFα production of RAW264.7 macrophages after stimulation with 2.5 nM ODN-1826 in presence of different concentrations of (L) CATH-2, (M) LL-37 or (N) CRAMP. N = 6 for (L) and (M). N = 3 for (N). Error bars = SEM.

Since CATH-2 was the most potent chicken cathelicidin to enhance the DNA-induced activation, this peptide was chosen to further determine the mechanism of enhancement of DNA-induced macrophage activation in more detail.

Thus, to further characterize the activation state of the HD11 cells, gene expression of *IFNA3, IFNB, IFNG, IL1B, IL6, IL10, IL12B, CXCLi2* and *CCLi7* was determined after co-incubation with DNA and CATH-2 (Fig. 1B, Table S2). Co-incubation resulted in enhanced expression of multiple DNA-inducible genes, including *IL1B*, *IL6*, *CXCLi2*, *IL12B* and *IFNG*. In addition, these genes were also slightly enhanced after stimulation with DNA and LL-37, though not significantly. Gene expression of *IFNA3, IFNB* and *CCLi7* was unaffected by stimulation with DNA, cathelicidins or a combination of both. To rule out cell line specific effects, the chicken macrophage cell line MQ-NCSU (Table S2) and primary chicken monocytes (Fig. 1C-K) were screened for cytokine expression. Co-incubation of MQ-NCSU cells with DNA and CATH-2 resulted in a similar expression pattern compared to HD11 cells, while LL-37 was unable to enhance DNA-induced expression. In line with these results, stimulation of primary monocytes with DNA and CATH-2 resulted in enhanced expression of *IFNG, IL1B*, *IL6*, *IL12B* and *CXCLi2*. DNA-induced expression of *IL10* in the presence of CATH-2 was slightly lower, albeit not significant. No changes were observed in expression of type I interferons or *CCLi7* either.

Since mammalian cathelicidins were able to enhance DNA-induced NO production of chicken macrophages, CATH-2 was analyzed for possible cross-species activation. To test this, murine RAW264.7 cells were stimulated with DNA in combination with either CATH-2 (Fig. 1L), LL-37 (Fig. 1M) or CRAMP (Fig. 1N). Again, CATH-2 strongly enhanced DNA-induced activation, measured by TNFα production. LL-37 augmented the activity as well, although not as potently as CATH-2, whereas CRAMP did not enhance activation. Taken together, these results indicate that of the peptides tested, CATH-2 is the most potent enhancer of DNA-induced macrophage activation in both avian and mammalian macrophages.

CATH-2 enhances both ssDNA and dsDNA activity

To determine whether CATH-2 could enhance macrophage activation by different DNA types, HD11 cells were incubated with different ODNs (Fig. 2A-D). ODN-2216 is an A-type ODN, which forms complex G-tetrad structures. ODN-2006 is a B-type ODN, which remains single-stranded, and ODN-M362 is a C-type ODN, which forms dimers (26). Stimulation with only cathelicidins (Fig. 2A) or ODN-2216 with or without cathelicidins (Fig. 2B), did not result in any NO production. Activation induced by ODN-2006 (Fig 2C, Fig. S1A) and ODN-M362 (Fig. 2D) was clearly enhanced by CATH-2. ODN-2006 and ODN-M362-induced activation was also enhanced by LL-37, although higher concentrations of up to 5 μM LL-37 were needed to enhance the activation. In addition, CATH-2 was able to enhance activation of HD11 cells by *E. coli* DNA of different lengths (Fig. 2E-F).

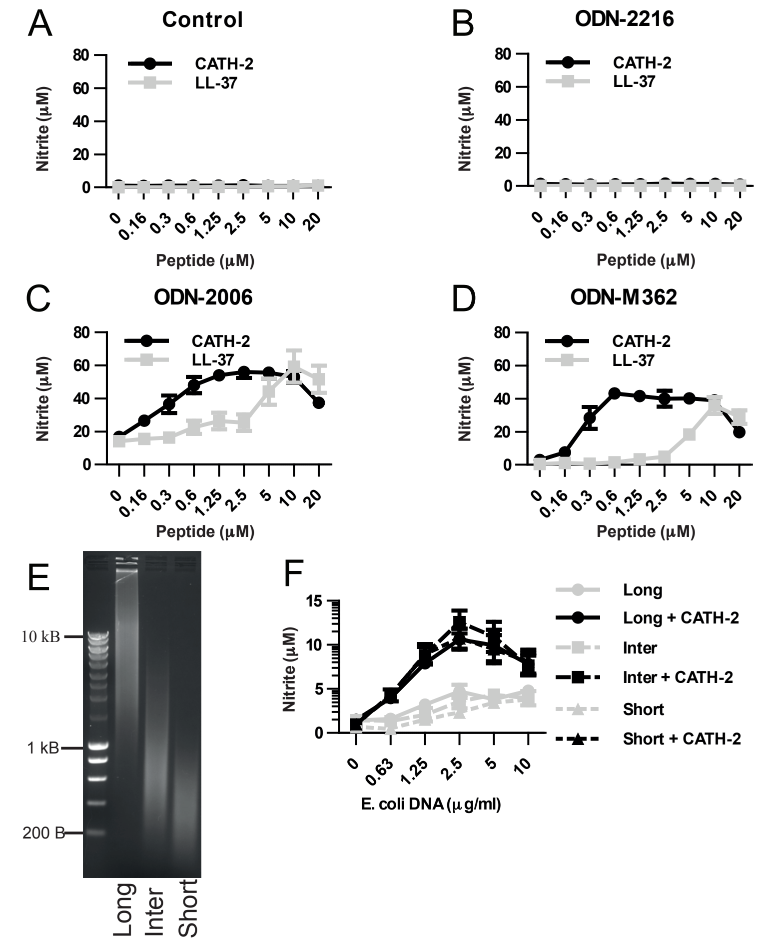


Figure 2: DNA type specific enhancement of macrophage activation. NO production of HD11 cells stimulated with CATH-2 or LL-37 in (A) the absence of DNA or presence of 2.5 nM (B) ODN-2216, (C) ODN-2006 or (D) ODN-M362. N = 3-7, error bars = SEM. (E) Gel electrophoresis of *E. coli* DNA after 0 (Long), 1 (Inter) or 2 (Short) min of DNAse I digestion. Samples were analyzed on 1% agarose gel. Representative of 2 independent experiments. (F) NO production of HD11 cells stimulated with *E. coli* DNA as digested in (E) in presence or absence of CATH-2 (5 μM). N = 3. Error bars = SEM.

Enhanced activation is dependent on increased DNA-uptake

The ability of LL-37 to enhance DNA-induced activation in pDCs is dependent on complex formation between LL-37 and DNA (18). To verify whether complex formation occurs between CATH-2 and DNA, both components were mixed in RPMI 1640 medium supplemented with 10% FCS and run on an agarose gel. Migration of DNA was inhibited in the presence of either CATH-2 or LL-37 (Fig. S1B), suggesting interaction between the two components. To further assess the interaction, ITC analysis was performed. ITC analysis confirmed high affinity binding between DNA and both CATH-2 and LL-37, with K_d-values of 11.3 nM (Fig. S1C) and 110 nM (Fig. S1D), respectively. In addition, the binding between CATH-2 and DNA was enthalpy-driven (ΔH = -97.9 kJ/mol) and showed a loss of entropy (ΔS = -163,4 J/mol*K), which suggests strong ionic interaction between the cationic residues of the peptide and the anionic phosphate groups of the DNA backbone.

To determine whether the enhancement of DNA-induced activation is a result of increased DNA uptake, HD11 cells were stimulated with Alexa-Fluor 488 labeled ODN-2006 (DNA-AF488) and CATH-2, after which DNA uptake was analyzed by confocal microscopy (Fig. 3A-D). Confocal images showed an increased uptake of DNA-AF488 in the presence of CATH-2 (Fig. 3C) compared to DNA-AF488 alone (Fig. 3B), with punctate intracellular localization of the DNA suggesting endosomal uptake. DNA uptake was enhanced to a lesser extent by LL-37 (Fig. 3D). Quantification of the uptake by flow cytometry (Fig. 3E) also confirmed the enhanced DNA uptake by CATH-2 and LL-37. Mouse cathelicidin CRAMP, however, did not significantly alter DNA uptake. To confirm whether CATH-2-enhanced DNA uptake correlates to the enhanced activation, several truncated analogs of CATH-2 were synthesized (Fig. 3F, Table 1). Augmented DNA uptake and DNA-induced activation were strongly correlated (R^2 = 0.762, $p < 0.001$), indicating that the enhanced uptake of DNA is a crucial step in enhancing the DNA-induced activation. In addition, this led to the identification of a core sequence (C7-21*), which appeared critical in enhancing DNA uptake and activation. Interestingly, while the C1-15* fragment is unable to enhance DNA uptake or DNA-induced activation of HD11 cells, ITC analysis indicates it still has a high binding affinity for DNA (K_d = 22 nM) (Fig. S1E), suggesting that complex formation between the anionic DNA and a cationic peptide alone is not sufficient to enhance DNA uptake and DNA-induced macrophage activation.

CATH-2 enhances endosomal activation of TLR21

To determine whether DNA/CATH-2 complexes are actively internalized, internalization of DNA-AF488 was determined at 4 °C. This reduced fluorescence back to background

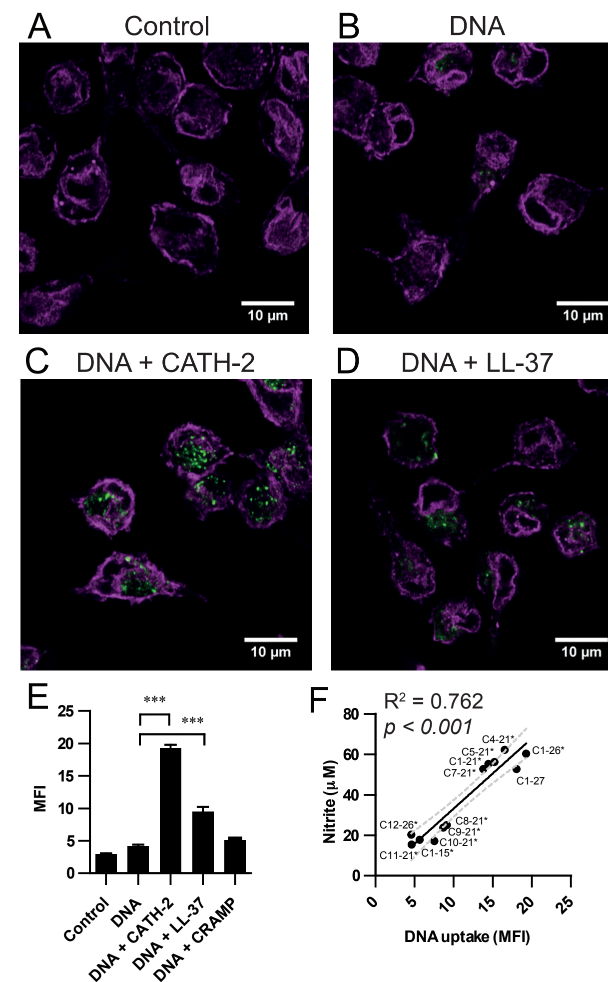


Figure 3: Localization and quantification of uptake of fluorescently labeled DNA in macrophages. Confocal images of 4% paraformaldehyde-fixed HD11 cells, either (A) unstimulated, or stimulated with 25 nM DNA-AF488 (green); (B) no peptide, (C) CATH-2 (5 µM) or (D) LL-37 (5 µM). Membrane staining was performed by WGA-Alexa Fluor-647 (magenta). Images are representative of 3 independent experiments. (E) Quantification of DNA-AF488 (2.5 nM) uptake in HD11 cells after 4h in the presence of different cathelicidins (5 µM) by flow cytometry. *p*-values are obtained by One-way ANOVA with Dunnett's Post-Hoc test. N = 3-4. Error bars = SEM. (F) Quantification of NO production and DNA-AF488 uptake in HD11 cells. Cells are stimulated with 2.5 nM DNA-AF488 and different CATH-2 analogs (5 µM) (Table 1). Uptake is determined by flow cytometry. Correlation was determined by Pearson's correlation test. Graph shows averages of measured values and a best-fit with 95% CI. N = 3.

levels (Fig. S2A), indicating that the complex is actively internalized. To identify whether CATH-2 changed the pathway through which DNA was internalized, HD11 cells were stimulated with several endocytosis inhibitors (Fig. 4A). Uptake of both DNA alone and DNA in complex with CATH-2 was inhibited by chlorpromazine (clathrin-dependent uptake) and EIPA (macropinocytosis), indicating that the endocytic pathways involved in DNA uptake are similar in the presence and absence of CATH-2. In addition, both inhibitors also inhibited IL-1β expression after stimulation with DNA alone or in complex with CATH-2 (Fig. S2B). Analysis of inhibitor toxicity by WST-1 showed no toxic effects at the concentrations used (Fig. S2C).

Activation of chicken macrophages by endocytosed DNA occurs through activation of the endosomal located TLR21, which acts as a functional homolog of the mammalian TLR9 (27). Up to date, this has been the only DNA-receptor identified in chickens. Activation of both TLR9 and TLR21 depends on endosomal acidification, which is thought to be necessary for proper cleavage of the receptor. To determine whether the DNA/CATH-2 complex could still activate the endosomal TLR21, Bafilomycin A1 (Fig. 4B), NH_4Cl or chloroquine (Fig. S2D-E), were used to inhibit the endosomal acidification. This resulted in inhibition of HD11 activation by both DNA alone or in complex with CATH-2, indicating activation is TLR21-dependent. In contrast, activation of TLR4 on the cell surface by LPS was unaffected by the inhibition of endosomal acidification. In line with these results, inhibition of TLR21 activation by pre-incubation with an inhibitory ODN also reduced activation by both DNA and DNA/CATH-2 stimulation, while not affecting LPS-induced activation (Fig. 4C). A control ODN, which neither activates nor inhibits TLR21 activation, did not affect DNA-induced activation in the presence or absence of CATH-2. Together, these results indicate TLR21-dependent activation of HD11 cells by DNA/CATH-2 complexes.

Degradation of CATH-2 is essential for macrophage activation by DNA

While the activation of the HD11 cells is enhanced by increasing DNA uptake due to complex formation with CATH-2, it is interesting to note that this complex formation is not hindering the interaction between DNA and the TLR21. This also appears to be the case in other studies investigating the role of HDPs on TLR9 activation (18, 28, 29). Nevertheless, very little is known about the intracellular stability of HDP/DNA complexes and how this influences endosomal TLR activation. To determine the stability of DNA/CATH-2-complexes in the endosomal environment, binding affinity between CATH-2 and DNA was determined by ITC analysis at pH 4.4 (Fig. S2F). While a difference in affinity was detected between pH 7.4 (11 nM) and pH 4.4 (32 nM), the acidic endosomal environment itself appears to be insufficient to destabilize the DNA/

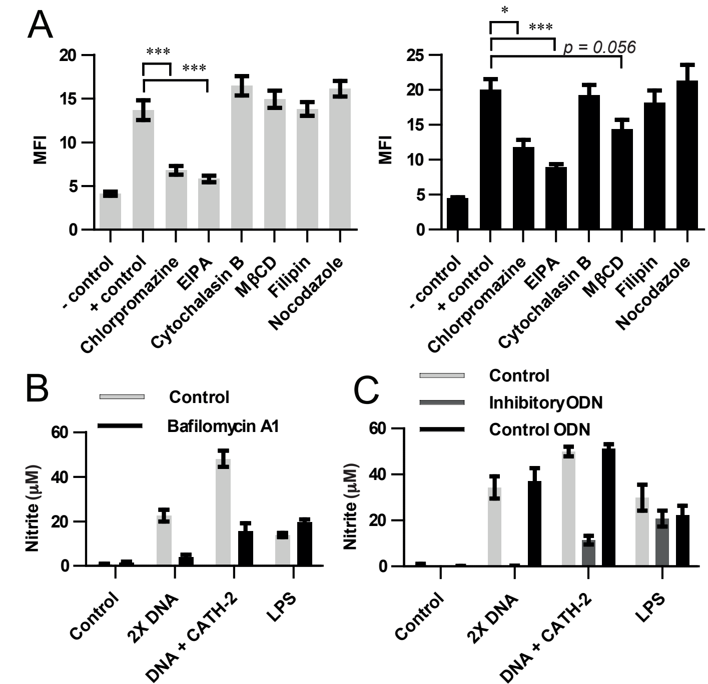


Figure 4: Endocytosis pathways involved in DNA uptake and TLR21 dependency of CATH-2 enhancement of DNA-induced activation. (A) Quantification of DNA-AF488 uptake in HD11 cells in the absence (left) or presence (right) of CATH-2 (5 μM) and different endocytosis inhibitors. Fluorescence was analyzed by flow cytometry after 4h. Stimulation with DNA alone was performed with 10 nM DNA-AF488, while stimulation with DNA and CATH-2 was performed with 2.5 nM DNA-AF488. *p*-values are obtained by One-way ANOVA with Dunnett's Post-Hoc test. N = 4-6. Error bars = SEM. (B-C) NO production of HD11 cells after stimulation with 5 nM ODN-2006, 2.5 nM ODN-2006 + 5 μM CATH-2 or LPS (100 ng/ml) in combination with (B) Bafilomycin A1 (250 nM), or (C) after pre-incubation with 2 μM inhibitor or control ODN. N = 3-5. Error bars = SEM.

CATH-2 complex. A process that accompanies the decrease in endosomal pH is the activation of endosomal proteases. To test whether proteolytic breakdown of CATH-2 plays a role in induction of TLR21 activation, CATH-2 activity was compared to a proteolytic resistant full D-amino acid analog of CATH-2. Intracellular DNA localization (Fig. 5A) as well as quantity of DNA uptake (Fig. 5B) in HD11 cells was similar between CATH-2 and D-CATH-2. In addition, binding affinity between D-CATH-2 and DNA (11 nM) (Fig. S2G) was comparable between D-CATH-2 and the natural CATH-2. However, DNA-induced activation of HD11 cells as measured by NO production (Fig. 5C) or *IL1B* expression (Fig. S2H) was completely inhibited in the presence of D-CATH-2, indicating

that peptide degradation is critical for TLR21 activation. To determine if D-CATH-2, but not CATH-2, remained in complex with the intracellular DNA, both D-CATH-2 and CATH-2 were synthesized with an N-terminal Dabcyl-label, which is able to quench Alexa-Fluor-488 fluorescence. This means that close interaction between DNA-AF488 and Dabcyl-CATH-2 would lead to a loss of intracellular fluorescence. When measuring intracellular fluorescence by flow cytometry, co-incubation with DNA and Dabcyl-D-CATH-2 resulted in a complete loss of fluorescence, in contrast to stimulation with DNA and Dabcyl-CATH-2, which showed similar intracellular fluorescence, compared to the unlabeled CATH-2 (Fig. 5D). Importantly, the N-terminal-Dabcyl group did not affect the activity of either CATH-2 or D-CATH-2 to alter DNA-induced activation (Fig. 5E).

To further verify the endosomal degradation of CATH-2, endosomal acidification inhibitors were used to inhibit Dabcyl-CATH-2 degradation, which should result in a loss of intracellular fluorescence (Fig. 5F). Indeed, addition of Bafilomycin A1 or NH_4Cl for 4h resulted in low levels of intracellular Alexa-Fluor-488 fluorescence. Subsequent washing, removing the inhibitors and extracellular labelled DNA-peptide complexes, resulted in a time-dependent increase in intracellular fluorescence, i.e. the presence of free labelled DNA in the acidified endosomes. Similar experiments with the Dabcyl-D-CATH-2 did not show an increase in fluorescence due to sustained quenching of the DNA-AF488 by the protease resistant peptide. Taken together, these results show the importance of CATH-2 degradation from the CATH-2/DNA complex to activate the TLR21 and enhance macrophage activation.

Discussion

HDPs have been shown to be multifunctional in their regulation of inflammatory responses. One of their previously described functions is the enhancement of DNA-induced IFNα production in pDCs (18, 29). To our knowledge, this study is the first description of enhancement of DNA-induced immune activation by cathelicidins in a non-mammalian species. In addition, to our knowledge, we provide first evidence for the necessity of intracellular cathelicidin degradation for endosomal TLR activation.

Our results show that cathelicidins of various species can enhance the immunogenicity of DNA by increasing the DNA uptake in chicken macrophages. This enhanced response against DNA/HDP complexes has also been reported for other mammalian cell types, such as pDCs, B-cells and monocytes (18, 25, 29-31), but appears to be limited to professional phagocytes. Keratinocyte responses towards DNA are even inhibited when DNA is presented in complex with LL-37 (32, 33). Of the previously described responses, macrophage activation by DNA/CATH-2 complexes appears to be most similar to B-cell

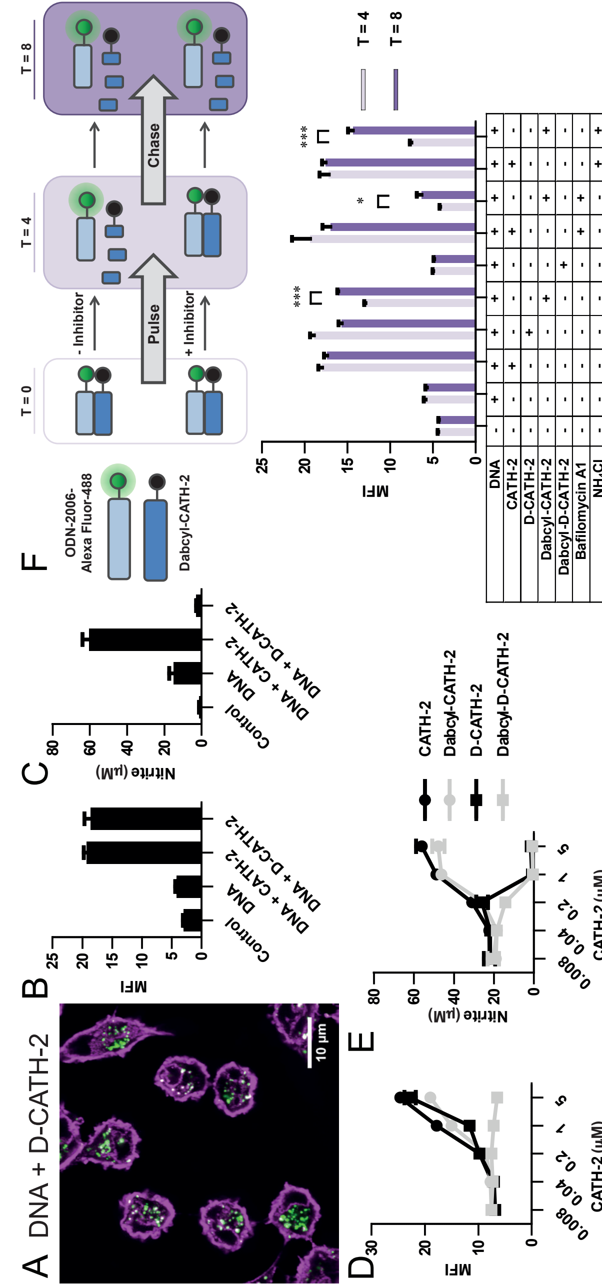


Figure 5: CATH-2 degradation in endosomal compartments of macrophages. (A) Confocal imaging of HD11 cells fixed with 4% paraformaldehyde after stimulation with 25 nM DNA-AF488 (green) and D-CATH-2 (5 μM). Membranes are stained with WGA-647 (magenta). Shown is a representative image of 3 independent experiments. (B-C) Quantification of (B) DNA uptake with flow cytometry and (C) NO production after stimulation of HD11 cells with 2.5 nM DNA-AF488 in the presence of 5 μM CATH-2 or D-CATH-2. N = 3. Error bars = SEM. (D) Quantification of intracellular fluorescence in HD11 cells by flow cytometry after stimulation with DNA-AF488 (2.5 nM) in presence of several concentrations of CATH-2 or D-CATH-2 with or without a Dabcyl-label. N = 4-5. Error bars = SEM. (E) NO production by HD11 cells after stimulation with DNA-AF488 (2.5 nM) in presence of several concentrations of CATH-2 or D-CATH-2 with or without a Dabcyl-label. N = 3. Error bars = SEM. (F) Endosomal CATH-2 degradation over time. HD11 cells are stimulated for 4h (T = 4) with DNA-AF488 (2.5 nM), Dabcyl-labeled or unlabeled CATH-2 (5 μM) and with or without acidification inhibitor (Bafilomycin A1 or NH4Cl). After 4h cells are washed and incubated for an additional 4h (T = 8). Flow cytometric analysis of intracellular fluorescence is determined at T = 4 and T = 8. *p*-values are obtained by 1-sided independent sample T-tests. N = 4. Error bars = SEM.

activation by DNA/LL-37 complexes (30). B-cells also respond by upregulation of pro-inflammatory cytokines, such as IL-6, while reported responses of monocytes and pDCs are limited to enhanced type I interferon production (18, 25, 31), which was unaffected in our study. Interestingly, while many cathelicidins were able to enhance DNA-induced activation in chicken macrophages, other studies have shown that increasing DNA uptake might not be the only way by which cathelicidins can enhance endosomal TLR activation (34, 35).

In mammalian models, cellular activation in response to DNA/HDP-complexes has so far been attributed to either activation of a TLR9-independent pathway, such as the activation of a cytosolic receptor in monocytes (31), or alternative downstream signaling of TLR9, leading to IRF7-phosphorylation and IFNα production instead of NF-kB-phosphorylation and pro-inflammatory cytokine production (18, 25, 28). In chickens, comparatively little is known yet about cellular responses towards DNA. So far, no cytosolic DNA-receptors have been identified and research on the downstream signaling of the TLR21 is limited to activation of NF-kB (27, 36). It will therefore be interesting to see whether TLR21 signaling is limited to NF-kB activation, or whether, like mammalian TLR9, alternative downstream pathways can be activated. Nevertheless, the increased pro-inflammatory response towards DNA/CATH-2 complexes observed in this study was induced in both chicken and mouse macrophages, suggesting that, regardless of alternative downstream pathways for TLR9 and possibly TLR21, macrophages of both species appear to respond in a NF-kB-dependent manner when presented with DNA/CATH-2 complexes. Further research will be needed to ascertain whether the different responses between cell types are due to differences in endosomal processing (37), downstream TLR signaling (38) or other processes.

While the immunogenic capacity of DNA/HDP complexes is well established, little is known about the intracellular fate of these complexes. This study shows that once endocytosed, proteolytic breakdown of CATH-2 from its complex with DNA is essential for the DNA to be able to interact with the TLR21 and induce macrophage activation. However, studies on other endosomal TLRs and their ligands shows the potential importance of endosomal degradation of cathelicidins. A recent report on activation of TLR3 by RNA/LL-37 complexes in epithelial cells indicated that interaction between LL-37 and RNA was lost during endosomal acidification (39). Although the stability of LL-37/RNA complexes at low pH are unclear (39, 40), proteolytic breakdown could be the reason for the loss of RNA/LL-37 interaction and increased TLR3 activation. Additionally, increased LPS uptake was detected in epithelial cells in the presence of LL-37 (41). This in turn led to an increase of intracellular TLR4 activation. While LL-37 normally inhibits LPS-induced TLR4 activation at the cell surface, it is very well possible that due to endosomal LL-37 degradation, LPS is

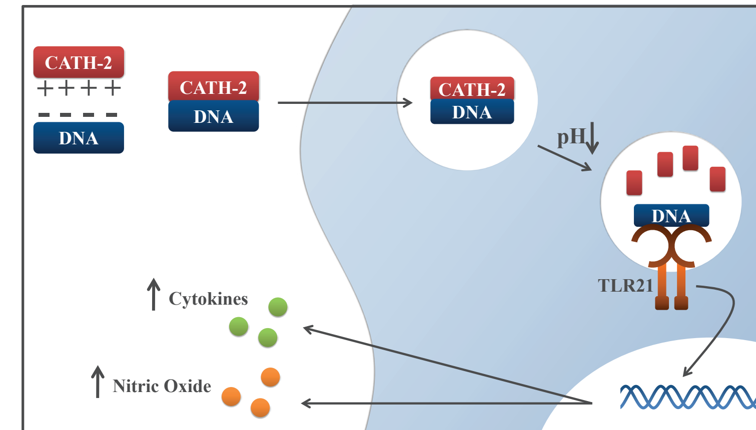


Figure 6: Model for regulatory role of CATH-2 in DNA-induced macrophage activation. Extracellular binding of DNA to CATH-2 due to ionic interaction promotes the uptake of DNA in endosomal compartments, which is followed by endosomal acidification resulting in protease activation and subsequent CATH-2 degradation. After CATH-2 degradation, DNA is free to bind the TLR21, resulting in enhanced NO production and cytokine gene expression.

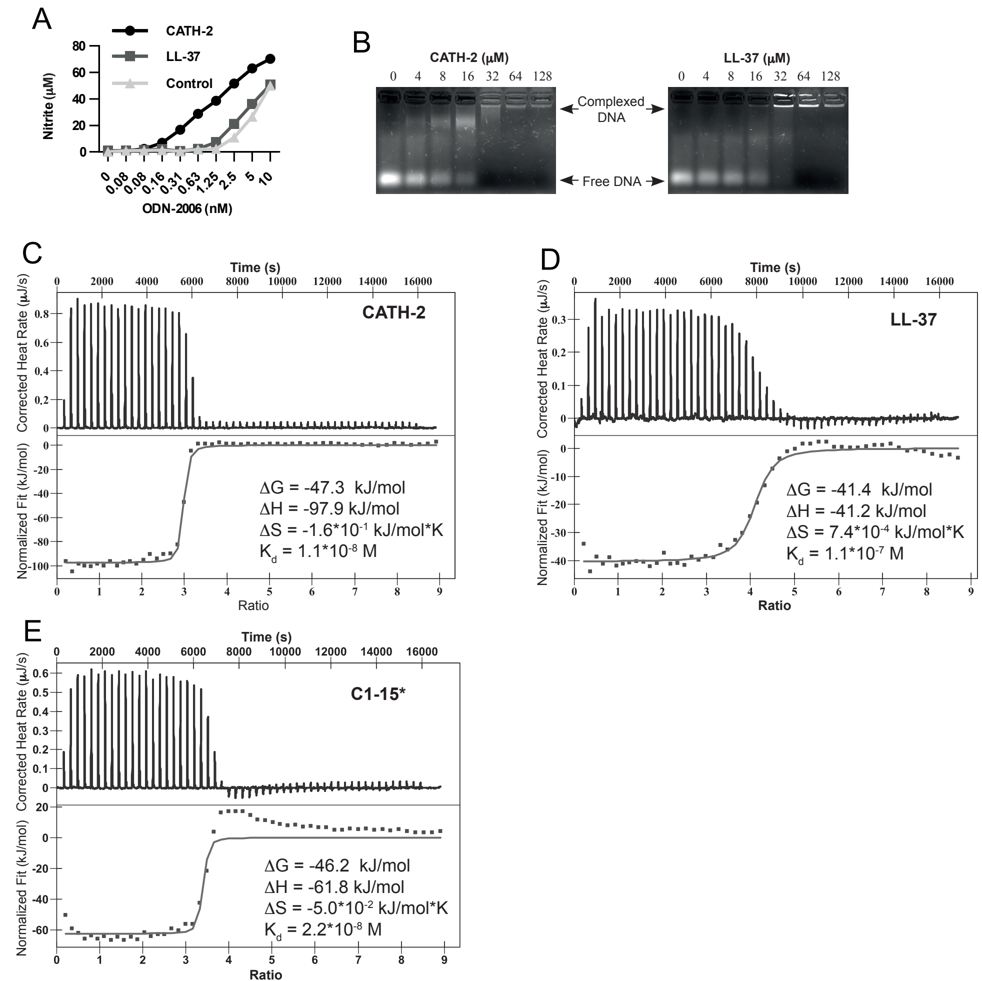
released from a complex with LL-37, which enables it to activate TLR4 inside endosomes. The elucidation of cathelicidin effects on DNA-induced TLR activation *in vitro* has revealed a number of potential cathelicidins functions during infections. In addition, animal studies have provided evidence for these functions *in vivo* as well. Interestingly, while most of these studies have been focusing on the strong activation of pDCs by DNA/HDP-complexes, experiments have demonstrated that not only IFNα, but also other pro-inflammatory cytokines are upregulated *in vivo* after stimulation with DNA/HDP-complexes (29, 42). The *in vitro* pro-inflammatory macrophage responses described in this paper could play a role in the production of pro-inflammatory cytokines in *in vivo* models as well.

While interesting from a biological point of view, functions like the enhancement of DNA-induced activation can prove useful in HDP-based anti-infective therapies as well. In fact, HDP-based peptides have been shown to enhance vaccination efficiency in several *in vivo* models, in which the vaccination cocktail contains synthetic DNA (43-47). While little is known about the effects of these specific HDPs on DNA-induced immune activation, it is possible that part of their efficacy comes from increasing the pro-inflammatory DNA-induced immune response. Moreover, therapeutic use of HDPs as antimicrobials, could affect inflammatory responses due to the presence of extracellular DNA during infections and inflammation (11, 12). These inflammatory responses can be adjusted through peptide modifications to improve vaccination efficacy.

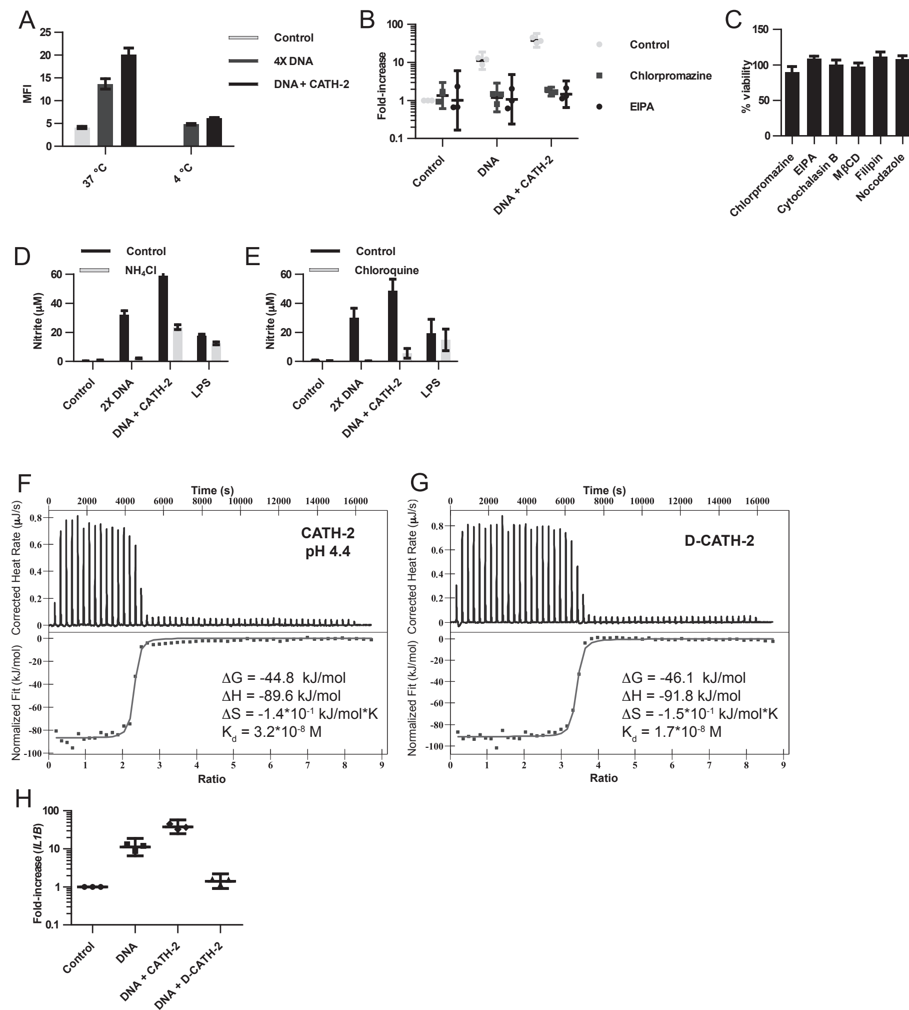
In conclusion, this study provides a detailed analysis of the mechanism by which cathelicidins enhance DNA-induced macrophage activation in chickens, but also mammalian macrophages (Fig. 6). We show that DNA and cathelicidins form a complex, which leads to enhanced endocytosis of the DNA. After endocytosis, proteases in the acidified endosome degrade cathelicidin from the complex, which is needed to liberate the DNA and allow for interaction between the DNA and TLR21 (Fig. 6). These results help in better understanding the role of cathelicidins in chicken innate host defense but also give a possible explanation for the beneficial effects of HDPs in vaccinations and potential immunoregulatory effects of other HDP-based anti-infective therapies.

Acknowledgements

We would like to thank Marloes Hoeksema, MSc, for technical support in ELISA experiments performed on RAW 264.7 cells.



Supplemental Figure 1: DNA-cathelicidin interaction. (A) NO production of HD11 cells after stimulation with ODN-2006 and 5 µM CATH-2 or LL-37. N = 3. Error bars = SEM. (B) Migration of ODN-2006 in presence of different concentrations of CATH-2 or LL-37 in a 1% agarose gel. Representative of 2 independent experiments. (C-E) Isothermal titration calorimetry with ODN-2006 and (C) CATH-2, (D) LL-37 or (E) C1-15*. Representative of 2 independent experiments.



Supplemental Figure 2: Processing of intracellular DNA/CATH-2-complexes. (A) ODN-2006-Alexa Fluor-488 (2.5 nM) internalization in HD11 cells at 4 °C or 37 °C in the presence or absence of CATH-2 (5 μM) analyzed by flow cytometry. N = 5-6. Error bars = SEM. (B) *IL1B* expression in HD11 cells after 4h stimulation with ODN-2006 (2.5 nM) with or without CATH-2 (5 μM), in presence or absence of chlorpromazine or EIPA. N = 3. Geometric mean with 95% confidence interval. (C) WST-1 assay on HD11 cells incubated with indicated endocytosis inhibitors for 5h. N = 6. Error bars = SEM. (D-E) NO production of HD11 cells after stimulation with ODN-2006 (2.5 nM) with or without CATH-2 (5 μM) in presence or absence of (D) NH_4Cl or (E) chloroquine. N = 3 or more. Error bars = SEM. (F-G) Isothermal titration calorimatry of ODN-2006 titrated with (F) CATH-2 at pH 4.4 or (G) D-CATH-2. Representative of 2 independent experiments. (H) *IL1B* expression in HD11 cells after stimulation with ODN-2006 (2.5 nM) and either 5 μM CATH-2 or D-CATH-2. N = 3. Geometric mean with 95% confidence interval.

Supplemental Table 1: Primer and probe sequences for qPCR

	Forward	Reverse	Probe
IFNA3	GACAGCCAACGCCAAAGC	GTCGCTGCTGTCCAAGCATT	TCCACCGCTACACCCAGCACCTCG
IFNB	CCTCCAACACCTCTTCAACATG	TGGCGTGTGCGGTCAAT	AGCAGCCCACACACTCCAAAACACT
IFNG	ACATCAAACACATATCTGAGGAG	AGCTTCTGTAAGATGCTGAAG	CGCTGGATTCTCAAGTCGTTCATCG
IL1B	GCTCTACATGTCGTGTGTGATGAG	TGTCGATGTCCCGCATGA	CCACACTGCAGCTGGAGGAAGCC
IL6	GTCGAGTCTCTGTGCTAC	GTCTGGGATGACCACTTC	ACGATCCGGCAGATGGTGA
CXCLi2	GCCCTCCTCCTGGTTTCA	CGCAGCTCATTCCCCATCT	TGCTCTGTCGCAAGGTAGGACGCTG
IL10	CATGCTGCTGGGCCTGAAG	ACGTCTCCTTGATCTGCTTGATG	CGCTGTCACCGCTTCTTCACCTGC
IL12B	TGGCTCGCACTGATAAATCTG	TGTAGTTCTCATATTCCACCTCATC	ACCTCCAGGAACATCTCAGTCGGCT
CCLi7	CTGCTGCTTCTCCTATGTTCAAC	TCTCTCCCAGGCAACGTAATTG	CTACTCCACTCCCATCCACCAGCATTG
GAPDH	GCCGTCCTCTCTGGCAAAG	TGTAAACCATGTAGTTCAGATCGATGA	AGTGGTGGCCATCAATGATCCC
28S	GGCGAAGCCAGAGGAAACT	GACGACCGATTTGCACGTC	AGGACCGCTACGGACCTCCACCA

HD11	Target	Exp.	Control 95% CI	p-value	Target	Exp.	CATH2 95% CI	p-value	Target	Exp.	LL37 95% CI	p-value
Control	***IFNA3***	**1.00**	1.00 - 1.00	-	***IFNA3***	**0.89**	0.83 - 0.96	-	***IFNA3***	**0.82**	0.56 - 1.22	-
	IFNB	**1.00**	1.00 - 1.00	-	***IFNB***	**0.89**	0.77 - 1.04	-	***IFNB***	**0.89**	0.68 - 1.17	-
	IFNG	**1.00**	1.00 - 1.00	-	***IFNG***	**0.99**	0.40 - 2.41	-	***IFNG***	**1.19**	0.57 - 2.48	-
	IL1B	**1.00**	1.00 - 1.00	-	***IL1B***	**1.19**	0.91 - 1.57	-	***IL1B***	**1.13**	0.89 - 1.43	-
	IL6	**1.00**	1.00 - 1.00	-	***IL6***	**0.91**	0.83 - 1.00	-	***IL6***	**0.89**	0.78 - 1.02	-
	CXCLi2	**1.00**	1.00 - 1.00	-	***CXCLi2***	**1.14**	0.76 - 1.72	-	***CXCLi2***	**1.16**	0.77 - 1.76	-
	IL10	**1.00**	1.00 - 1.00	-	***IL10***	**1.34**	0.29 - 6.18	-	***IL10***	**1.23**	0.32 - 4.67	-
	IL12B	**1.00**	1.00 - 1.00	-	***IL12B***	**0.94**	0.66 - 1.33	-	***IL12B***	**0.95**	0.73 - 1.23	-
	CCLi7	**1.00**	1.00 - 1.00	-	***CCLi7***	**1.16**	0.79 - 1.72	-	***CCLi7***	**1.09**	0.66 - 1.80	-
DNA	***IFNA3***	**0.85**	0.75 - 0.97	-	***IFNA3***	**0.80**	0.60 - 1.07	*0.71*	***IFNA3***	**0.87**	0.76 - 1.00	*0.97*
	IFNB	**0.90**	0.70 - 1.15	-	***IFNB***	**0.88**	0.77 - 0.99	*0.98*	***IFNB***	**0.92**	0.61 - 1.38	*0.98*
	IFNG	**1.96**	0.63 - 6.13	-	***IFNG***	**9.50**	3.09 - 29.20	*0.03* *	***IFNG***	**4.34**	1.13 - 16.61	*0.29*
	IL1B	**17.11**	9.43 - 31.04	-	***IL1B***	**47.81**	28.30 - 80.80	*0.01* *	***IL1B***	**31.28**	16.86 - 58.03	*0.08*
	IL6	**13.71**	4.07 - 46.12	-	***IL6***	**67.74**	35.94 - 127.68	*0.03* *	***IL6***	**21.55**	3.90 - 119.03	*0.65*
	CXCLi2	**8.89**	4.71 - 16.80	-	***CXCLi2***	**21.65**	16.43 - 28.52	*0.01* *	***CXCLi2***	**13.68**	7.24 - 25.85	*0.18*
	IL10	**14.63**	4.14 - 51.71	-	***IL10***	**46.08**	13.31 - 159.56	*0.18*	***IL10***	**24.93**	4.39 - 141.61	*0.63*
	IL12B	**1.40**	0.80 - 2.43	-	***IL12B***	**3.17**	1.65 - 6.09	*0.01* *	***IL12B***	**1.71**	1.22 - 2.40	*0.61*
	CCLi7	**1.63**	1.06 - 2.49	-	***CCLi7***	**2.07**	1.59 - 2.69	*0.28*	***CCLi7***	**1.99**	1.36 - 2.90	*0.40*

MQ-NCSU	Target	Exp.	Control 95% CI	p-value	Target	Exp.	CATH2 95% CI	p-value	Target	Exp.	LL37 95% CI	p-value
Control	***IFNA3***	**1.00**	1.00 - 1.00	-	***IFNA3***	**1.06**	0.60 - 1.89	-	***IFNA3***	**1.06**	0.78 - 1.44	-
	IFNB	**1.00**	1.00 - 1.00	-	***IFNB***	**1.03**	0.48 - 2.20	-	***IFNB***	**1.42**	1.15 - 1.75	-
	IFNG	**1.00**	1.00 - 1.00	-	***IFNG***	**1.02**	0.34 - 3.05	-	***IFNG***	**0.95**	0.32 - 2.84	-
	IL1B	**1.00**	1.00 - 1.00	-	***IL1B***	**1.03**	0.88 - 1.21	-	***IL1B***	**0.99**	0.81 - 1.22	-
	IL6	**1.00**	1.00 - 1.00	-	***IL6***	**0.92**	0.55 - 1.54	-	***IL6***	**0.81**	0.67 - 0.99	-
	CXCLi2	**1.00**	1.00 - 1.00	-	***CXCLi2***	**1.04**	0.85 - 1.27	-	***CXCLi2***	**1.06**	0.89 - 1.25	-
	IL10	**1.00**	1.00 - 1.00	-	***IL10***	**1.01**	0.71 - 1.43	-	***IL10***	**1.02**	0.67 - 1.55	-
	IL12B	**1.00**	1.00 - 1.00	-	***IL12B***	**1.17**	0.70 - 1.95	-	***IL12B***	**1.13**	0.72 - 1.79	-
	CCLi7	**1.00**	1.00 - 1.00	-	***CCLi7***	**1.02**	0.56 - 1.87	-	***CCLi7***	**1.04**	0.43 - 2.54	-
DNA	***IFNA3***	**1.02**	0.73 - 1.41	-	***IFNA3***	**0.74**	0.54 - 1.03	*0.04*	***IFNA3***	**0.91**	0.68 - 1.20	*0.48*
	IFNB	**0.91**	0.41 - 2.02	-	***IFNB***	**1.05**	0.49 - 2.26	*0.78*	***IFNB***	**1.37**	0.70 - 2.69	*0.24*
	IFNG	**0.92**	0.32 - 2.69	-	***IFNG***	**4.04**	0.58 - 28.05	*0.03* *	***IFNG***	**1.04**	0.37 - 2.95	*0.95*
	IL1B	**2.20**	1.68 - 2.87	-	***IL1B***	**10.81**	7.74 - 15.09	*0.00* *	***IL1B***	**1.93**	1.60 - 2.34	*0.32*
	IL6	**2.04**	1.09 - 3.81	-	***IL6***	**42.06**	18.68 - 94.72	*0.00* *	***IL6***	**1.59**	1.02 - 2.49	*0.45*
	CXCLi2	**1.64**	1.35 - 2.00	-	***CXCLi2***	**4.28**	3.36 - 5.46	*0.00* *	***CXCLi2***	**1.50**	1.29 - 1.74	*0.32*
	IL10	**1.37**	1.05 - 1.80	-	***IL10***	**3.78**	2.75 - 5.19	*0.00* *	***IL10***	**1.39**	0.87 - 2.24	*0.99*
	IL12B	**1.05**	0.69 - 1.61	-	***IL12B***	**10.18**	3.99 - 25.97	*0.00* *	***IL12B***	**1.36**	1.04 - 1.77	*0.40*
	CCLi7	**1.00**	0.37 - 2.71	-	***CCLi7***	**1.12**	0.26 - 4.84	*0.92*	***CCLi7***	**1.37**	0.77 - 2.46	*0.60*

Supplemental Table 2: HD11 or MQ-NCSU cells were stimulated with 2.5 nM ODN-2006 in the presence or absence of CATH-2 or LL-37 (5 μM). Data represents fold-increase in cytokine expression normalized to unstimulated cells. N = 3-4. Statistical analysis was performed by One-way ANOVA with Dunnett's Post-Hoc test comparing DNA-stimulated cells with DNA + CATH-2 and DNA + LL-37 stimulated cells.

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Chapter 5

The innate immune system uses cathelicidins to discriminate between viable and non-viable Gram-negatives and to inhibit TLR2 and TLR4 activation

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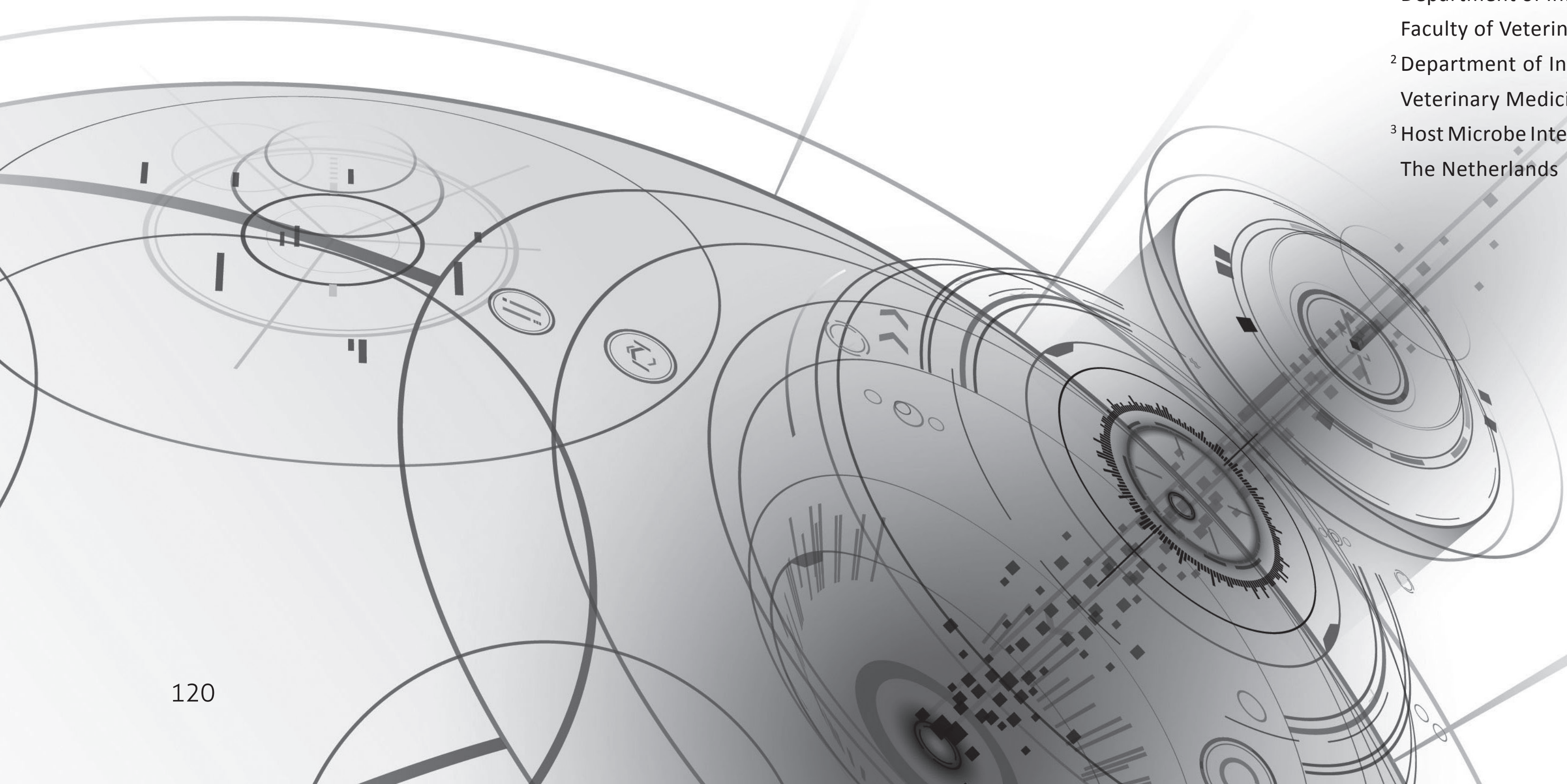


Abstract

Toll-like receptors (TLRs) are crucial in the induction of immune responses against bacterial infections. Activation of these receptors by microbial components at the site of infection causes the release of pro-inflammatory and antimicrobial mediators, which is crucial to combat the infection. Activation of TLRs can be influenced by host-derived effector molecules released at the site of infection. These effector molecules include cathelicidins, which have been described to have multiple functions, including direct antimicrobial activity as well as regulation of TLR activation. Nevertheless, the effects on TLR activation are mostly investigated in the context of single, purified or synthetic TLR ligands and very little is known about the role of cathelicidins during immune activation by intact and viable pathogens. In this study we show that cathelicidins are potent inhibitors of macrophage activation against Gram-negative bacteria. This inhibition is dependent on the loss of bacterial viability, either due to cathelicidin-mediated killing or other types of killing, and does not occur when bacterial viability remains intact. The inhibition results from direct interaction with lipoproteins and LPS derived from the bacterial membrane, which prevents activation of TLR2 and TLR4, respectively. These results provide a novel mechanism by which the immune system can discriminate between viable and non-viable Gram-negatives to respond accordingly and prevent excessive inflammation and sepsis against already neutralized pathogens.

Introduction

Toll-like receptors are important components of the innate immune system, with a crucial role in the protection against invading pathogens (1, 2). They are expressed on a wide variety of cell types, including leukocytes and epithelial cells, and can be activated by specific conserved microbial components, termed microbe-associated molecular patterns (MAMPs) (3, 4). A number of these MAMPs can be expressed on a single pathogen, which can lead to the simultaneous activation of multiple TLRs during infections. During *E. coli* infections, for instance, loss of TLR2 and TLR4, which are important in the recognition of lipoproteins and LPS, respectively, has been shown to limit cytokine production and increase the bacterial burden in mice (1, 5). In addition, *E. coli* expresses flagellin and contains DNA, which have been shown to activate TLR5 and TLR9, respectively (6-8). Once activated, TLRs promote the production and release of inflammatory cytokines, chemotactic factors, reactive oxygen intermediates (ROIs) and antimicrobial molecules (9-11), in order to recruit and activate leukocytes and counter the infection.

Activation of TLRs can be regulated by a variety of microenvironmental components, including cathelicidins. Cathelicidins are short cationic peptides that are released from leukocytes and epithelial cells upon activation of pattern recognition receptors (PRRs) (12) and have been shown to play an important role in the innate host defense system (13-15). Once released, cathelicidins are thought to exert a variety of functions, including direct antimicrobial activity against both Gram-positive and Gram-negative bacteria (16) as well as regulation of TLR activation (17). This regulation can be both positive and negative and includes the inhibition of TLR4 activation by LPS (17, 18), enhancement of flagellin-induced TLR5 activation (19, 20) and enhancement of DNA-induced TLR9 activation (21, 22). However, these effects have been demonstrated with single, purified or synthetic TLR ligands and it is unknown whether TLR regulation by cathelicidins also occurs in the context of viable and intact bacteria. In addition, since cathelicidins can both positively and negatively regulate TLR activation, it is unclear what the net-outcome of this TLR regulation will be when multiple TLRs are activated simultaneously by complete bacteria.

In this study, we demonstrate that cathelicidins inhibit *E. coli*-induced TLR2 and TLR4 activation, which is dependent on the loss of *E. coli* viability. Our results show that loss of bacterial viability, due to cathelicidin-mediated killing or other ways of killing, allows cathelicidins to inhibit TLR2 and TLR4 activation by directly neutralizing lipoproteins and LPS from the bacterial membrane. Together, these results provide a novel mechanism by which the immune system can discriminate between viable and non-viable pathogens

for a balanced immune response and prevent excessive inflammation against already neutralized Gram-negative bacteria.

Methods

Reagents

CATH-2 and LL-37 were synthesized by Fmoc-chemistry at CPC Scientific (Sunnyvale, CA, USA). All other peptides were synthesized by Fmoc-chemistry at the Academic Centre for Dentistry Amsterdam (Amsterdam, the Netherlands) (Table S1). Pam2CSK4, Pam3CSK4, LPS O111:B4, recombinant S. typhimurium Flagellin, Poly(I:C), CL264 and ODN-2006 were obtained from Invivogen (Toulouse, France). Human TNFα was obtained from Miltenyi Biotech (Bergisch Gladbach, Germany). Gentamicin was obtained from Sigma Aldrich (St. Louis, MO, USA).

Cell culture

J774.A1 cells (23) were a kind gift of Prof. Jos van Putten (Utrecht University, Utrecht, the Netherlands). J774.A1 cells were cultured in DMEM medium (Thermo Fisher Scientific, Waltham, MA, USA) supplemented with 10% FCS (Bodinco B.V., Alkmaar, the Netherlands) at 37 °C, 5.0% CO_2. HEK-Blue-hTLR reporter cell lines were obtained from Invivogen and cultured according to the manufacturer’s protocol. HEK-293 cells overexpressing hTLR5 and harboring a pNIFTY NF-κB luciferase reporter plasmid were cultured in DMEM supplemented with 10% FCS, 10 μg/ml blasticidin (Invivogen) and 250 μg/ml Zeocin (Invivogen). Chicken PBMCs were isolated and cultured as previously described (22). Bone-marrow cells were obtained by flushing the femur and tibia of C57BL/6j mice. All mice were kept under the guidelines and approval of the animal ethical comity of Utrecht University in the Netherlands and had free access to food and water. For differentiation of bone marrow-derived macrophages (BMDM), bone-marrow cells were cultured in RPMI 1640 supplemented with 10% FCS, 1% Pen/Strep and 20 ng/ml recombinant murine M-CSF (Peprotech, Rocky Hill, NJ, USA) for 3 days, after which they were washed with RPMI 1640 and incubated for another 3 days in RPMI 1640 supplemented with 10% FCS and 20 ng/ml M-CSF.

Bacterial culture

E. coli O78 and *S. enteritidis* phage type 13a were obtained from Zoetis Animal Health (Kalamazoo, MI, USA). *E. coli* K12 MC4100 was a kind gift of Dr. Luirink (VU Amsterdam, Amsterdam, the Netherlands) and *E. coli* ATCC 22592 was obtained from the ATCC (Manassas, VA, USA). All bacteria were cultured in Luria Broth (BioTRADING Benelux B.V., Mijdrecht, the Netherlands). Prior to use, bacteria were grown to log-phase, centrifuged at 1200 x g, 10 min, 4 °C and diluted in either RPMI 1640 or DMEM to the correct OD_{620}. OD_{620} 0.1 = $3.3x10^7$ colony forming units (CFU)/ml for *E. coli* O78, *E. coli* K12 and *E. coli* 25922. OD 0.1 = 10^8 CFU/ml for *S. enteritidis.*

Stimulation set-up

$7.5x10^4$ J774.A1 cells/well were seeded in a 96-wells plate to adhere overnight. For stimulation, bacteria were diluted to the appropriate OD_{620} and either heat-killed (70 °C, 30 min), gentamicin-killed (37 °C, 30 min, 250 μg/ml gentamicin) or left untreated (4 °C, 30 min). Subsequently, bacteria were mixed with cathelicidins at indicated concentrations after which mixtures were used for cell stimulation and colony count assays. For macrophage stimulations longer than 2h, cells were washed twice after 2h and incubated for indicated times in culture medium containing 250 μg/ml gentamicin.

ELISA

ELISA Duoset kits for mouse TNFα, IL-1β, IL-6, IL-10, RANTES, IP-10 and IFNβ were obtained from R&D systems (Minneapolis, MN, USA). ELISAs were performed following the manufacturer’s protocol and samples were diluted in 1% BSA (Sigma Aldrich) in PBS, pH 7.4. Cytokine concentrations were determined after 2h for TNFα and 24h for all other cytokines. Samples were measured with a FLUOstar Omega microplate reader (BMG Labtech GmbH, Ortenberg, Germany) and analyzed with MARS data analysis software (BMG Labtech GmbH). OD_{450} measurements were corrected by subtracting OD_{570} measurements.

Colony count assay

Colony counts assays were performed by co-incubating bacteria with cathelicidins in 20 ml in DMEM or RPMI 1640 supplemented with 10% FCS at 37 °C for 2h. After incubation, samples were diluted with 180 ml PBS followed by spread-plating 10-fold dilutions in PBS on Tryptone Soy Agar plates (Oxoid Limited, Hampshire, United Kingdom). After overnight incubation at 37 °C, CFUs were counted (detection limit = 10^2 CFU/ml).

Quantitative PCR

For quantitative PCR (qPCR) experiments, chicken PBMCs were stimulated for 2h at 41 °C, 5.0% CO_2, and centrifuged at 400 x g, 8 min. Cell lysis of the obtained pellet and RNA isolation were performed with the High Pure RNA Tissue kit (Roche, Basel, Switzerland). RNA was converted to cDNA using the iScript cDNA synthesis kit (Bio-Rad, Veenendaal, the Netherlands). qPCR was performed using primers, probes (Table S2) and IQ supermix (Bio-Rad) in combination with the CFX Connect qPCR and CFX Manager 3.0 software (Bio-Rad). Quantification cycle (Cq) values were corrected for PCR efficiency

and housekeeping gene expression (GAPDH). When no signal was detected after 40 cycles, an arbitrary Cq value of 40 was given. Unstimulated samples were set to 1.

Transmission electron microscopy

For transmission electron microscopy (TEM), *E. coli* O78 was grown to log-phase and diluted to 10^8 *E. coli* O78/ml in DMEM. Bacteria were either untreated, incubated at 70 °C, or incubated with 40 μM CATH-2, 40 μM LL-37 or 250 μg/ml gentamicin at 37 °C for 0.5h or 2.5h. Mixtures were fixed with 2% glutaraldehyde (Polysciences, Eppelheim, Germany), 5 mM $CaCl_2$, 10 mM $MgCl_2$ (both Merck, Darmstadt, Germany) in 0.1 M sodium cacodylate buffer (Sigma-Aldrich) pH 7.4 overnight at 4 °C. After washing (3 x 10 min) in sodium cacodylate buffer, bacteria were embedded in 2% low-melting point agarose v/v (Sigma-Aldrich) and post-fixed with 4% osmium tetroxide (Electron Microscopy Sciences; EMS, Hatfield, USA) and 1.5% $K_4Fe(CN)_6$-$3H_2O$ (Merck) in distilled water for 2h at 4 °C. Bacteria were rinsed with distilled water (5 x 10 min) and incubated in 0.5% uranylacetate (EMS) for 1h at 4 °C. After washing (3 x 10 min) with distilled water, samples were embedded in Epon and ultrathin sections (50 nm) of each block were prepared on a Leica UCT ultramicrotome (Leica, Vienna, Austria). Finally, sections were stained with uranyl acetate and lead citrate using a Leica AC20 system (Leica). Electron microscopy was performed with a FEI Tecnai 12 electron microscope (FEI, Eindhoven, The Netherlands) at 80 kV.

HEK-TLR assay

Stimulation of HEK-TLR SEAP cells with $5x10^4$ CFU/ml heat-killed *E. coli* O78 was performed over 18h at 37 °C, 5.0% CO_2 in the presence or absence of 5 μM CATH-2 or LL-37. After incubation, NF-κB activity was determined with the Quantiblue assay (Invivogen). Stimulation of HEK-hTLR5-luciferase cells was performed with $5x10^4$ CFU/ml heat-killed *E. coli* O78 over 6h after which NF-kB activity was determined with the Bright-glo luciferase assay (Promega, Fitchburg, WI, USA).

Inner membrane permeabilization

Live, heat-killed or gentamicin-killed *E. coli* O78 were incubated with indicated concentrations CATH-2 or LL-37 for 30 min at 37 °C. After incubation, bacteria were centrifuged at 1200 x g, 10 min, 4 °C and washed with PBS. Subsequently, bacteria were resuspended in PBS with 2 μM Sytox Green Nucleic Acid stain (Thermo Fisher Scientific) and transferred to a black 96-wells plate. After 5 min, fluorescence was determined with a FLUOstar Omega microplate reader (BMG Labtech GmbH) and analyzed with MARS data analysis software (BMG Labtech GmbH).

Isothermal Titration Calorimetry

Isothermal titration calorimetry (ITC) was performed with the Low Volume NanoITC (TA Instruments-Waters LLC, New Castle, DE, USA). The 50 ml syringe was filled with 400 μM CATH-2 or LL-37 for titration into 190 ml 50 μM LPS O111:B4 or 165 μM Pam3CSK4. All components were diluted in PBS (6.04 mM Na_2HPO_4, 1.10 mM KH_2PO_4 103.45 mM NaCl, 2.0 mM KCl, 0.37 mM $MgCl_2$, 0.68 mM $CaCl_2$). Titrations were incremental with 2 ml injections at 300s intervals. Experiments were performed at 37 °C. Data was analyzed with the NanoAnalyze software (TA Instruments-Waters LLC).

Statistical analysis and graphics

Statistical analysis was performed using Prism 5 software (Graphpad, La Jolla, CA, USA) and IBM SPSS Statistics 20 (IBM, Armonk, NY, USA). * = $p < 0.05$; ** = $p < 0.01$; *** = $p < 0.001$. Graphics were designed using Prism 5 software (Graphpad) and Microsoft Excel 2010 (Microsoft, Redmond, WA, USA).

Results

CATH-2, but not LL-37, kills *E. coli* and inhibits *E. coli*-induced macrophage activation

To determine whether cathelicidins can regulate macrophage activation in the context of viable *E. coli*, J774.A1 macrophages were stimulated with *E. coli* O78 in the presence of either 5 μM chicken cathelicidin-2 (CATH-2) or human LL-37, which have strong and no antimicrobial activity against *E. coli* in DMEM + 10% FCS, respectively (Fig. 1A). Analysis of macrophage activation, as determined by TNFα (2h), IL-6 (24h) and IL-1β (24h) release, showed that CATH-2 strongly inhibits macrophage activation, while LL-37 only marginally inhibited TNFα release and did not affect IL-6 and IL-1β release (Fig. 1B). Use of other *E. coli* strains in combination with CATH-2 yielded similar results, with CATH-2 completely killing *E. coli* K12 (rough LPS) and *E. coli* ATCC 25922 (smooth LPS), as well as inhibiting the induction of macrophage activation by these bacterial strains (Fig. S1). To determine whether the observed inhibition by CATH-2 correlated with its antimicrobial activity, various ratios of CATH-2 and either *E. coli* O78 or *S. enteritidis* were used to both stimulate J774.A1 macrophages and to determine bacterial viability. Spearman correlation analysis on TNFα levels and bacterial viability showed a significant correlation for both *E. coli* ($r^2 = 0.720$) and *S. enteritidis* ($r^2 = 0.698$) (Fig. 1C). In addition, release of IL-6 and IL-1β also strongly correlated with *E. coli* viability (IL-6; $r^2 = 0.884$, and IL-1β; $r^2 = 0.794$), while a lower, but still significant correlation was observed for IL-6 and IL-1β release in relation to *S. enteritidis* viability (IL-6; $r^2 = 0.333$, and IL-1β; $r^2 = 0.306$) (Fig. S2). In contrast, no changes in IL-10 production were observed after *E. coli* stimulation in either presence or absence of CATH-2.

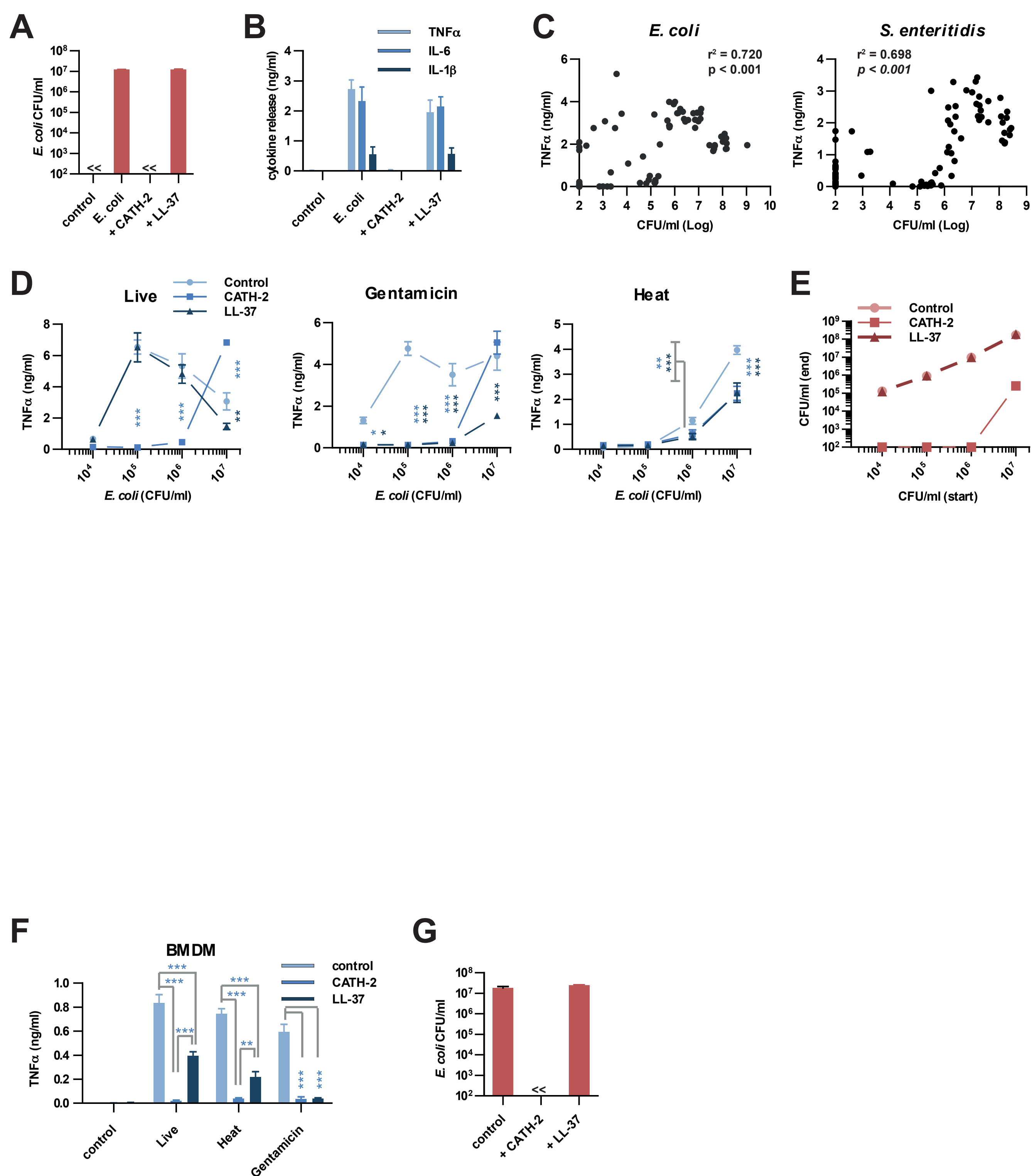


Figure 1: Cathelicidin-mediated inhibition of *E. coli*-induced macrophage activation depends on loss of *E. coli* viability. (A-B) 10^6 CFU/ml *E. coli* O78 was mixed with 5 µM LL-37 or CATH-2 in DMEM + 10% FCS, followed by (A) a colony count assay after 2h incubation and (B) stimulation of J774.A1 cells after which cytokine release was determined after 2h (TNFα) or 24h (IL-6 and IL-1β). (C) Different ratios of CATH-2 (0-10 µM) and either *E. coli* O78 (10^4-10^7 CFU/ml) or *S. enteritidis* ($3*10^4$-$3*10^7$ CFU/ml) were mixed in DMEM + 10% FCS and incubated for 2h for a colony count assay and were used for a 2h stimulation of J774.A1 cells after which TNFα concentrations in supernatant were determined. Spearman correlation analysis on bacterial viability and macrophage TNFα release in the context of *E. coli* (left panel) or *S. enteritidis* (right panel) is shown. (D-E) 10^4-10^6 CFU/ml *E. coli* O78 was either untreated, heat-killed or gentamicin-killed and mixed with 5 µM CATH-2 or LL-37 in DMEM + 10% FCS, followed by (D) stimulation of J774.A1 cells after which TNFα was determined after 2h and (E) a colony count assay after 2h incubation. Statistical analysis on TNFα release was performed by Two-way repeated measures ANOVA with Bonferroni post-hoc test. (F) TNFα release after stimulation of BMDMs with 10^6 CFU/ml untreated, heat-killed or gentamicin-killed *E. coli*, in combination with 5 µM CATH-2 or LL-37 in RPMI 1640 + 10% FCS. Statistical analysis on TNFα release was performed by Two-way repeated measures ANOVA with Bonferroni post-hoc test. (G) Colony counts assay of *E. coli* O78 in RPMI 1640 + 10 % FCS with 5 µM CATH-2 or LL-37. N = 3 or more ± SEM.

CATH-2 and LL-37 inhibit macrophage activation by non-viable *E. coli*

To determine whether the loss of bacterial viability is causing the inhibition of *E. coli*-induced macrophage activation observed with CATH-2 or is a prerequisite for this inhibition, J774.A1 cells were stimulated with either live, heat-killed or gentamicin-killed *E. coli* (Fig. 1D). In the absence of cathelicidins, both viable and non-viable *E. coli* induced TNFα release, although heat-killed *E. coli* is a less potent stimulus compared to live and gentamicin-killed *E. coli*. Release of IL-6 and IL-1β was strongly reduced after stimulation with non-viable *E. coli* compared to live *E. coli* (Fig. S3). Addition of CATH-2 to viable *E. coli* again inhibited TNFα release and completely killed *E. coli*, while LL-37 only showed limited inhibition at the highest *E. coli* concentration and did not affect *E. coli* viability (Fig. 1D-E). In contrast, both LL-37 and CATH-2 inhibited TNFα release by gentamicin-killed and heat-killed *E. coli*.

Similar to the effects on J774.A1 cells, CATH-2 also inhibited TNFα release in BMDMs when combined with viable, heat-killed or gentamicin-killed *E. coli* (Fig. 1F). Stimulation of BMDMs with live *E. coli* and LL-37 reduced TNFα release 2-fold, which was significantly less than the inhibition observed with CATH-2. Stimulation of BMDMs with heat-killed *E. coli* and LL-37 resulted in a stronger 3.5-fold inhibition, while stimulation with gentamicin-killed *E. coli* and LL-37 resulted in a 15-fold inhibition, which is similar to the inhibition observed with CATH-2. In addition, since BMDMs were cultured in RPMI 1640 medium, the antimicrobial activity of CATH-2 and the lack of LL-37-induced antimicrobial activity against *E. coli* were confirmed in this medium as well (Fig. 1G). Together, these results suggest that loss of bacterial viability is a prerequisite for the inhibition of TNFα release.

The cathelicidin inhibitory function is conserved between species

Although cathelicidin genes have been conserved in most vertebrate species, amino acid sequences of the mature peptides are highly variable (24). To determine whether the inhibition of macrophage activation by *E. coli* is conserved among cathelicidins, the inhibitory activity was assessed for 12 cathelicidins from 6 different species (Fig. 2A, Table S1). Interestingly, only CATH-2 and PMAP-36 were able to both kill *E. coli* and inhibit live *E. coli*-induced TNFα release (Fig. 2A-B). However, in combination with gentamicin-killed *E. coli*, LL-37, CRAMP and K9CATH showed similar inhibitory activity as CATH-2 and PMAP-36. Furthermore, in combination with heat-killed *E. coli,* significant inhibition was observed with chicken CATH-1, -2 and -3, LL-37, CRAMP, K9CATH, equine CATH-2 and PMAP-36, suggesting that the inhibition of macrophage activation by non-viable *E. coli* is mostly conserved between cathelicidins. In addition, the inhibitory effects of CATH-2 and LL-37 were not limited to murine macrophages,

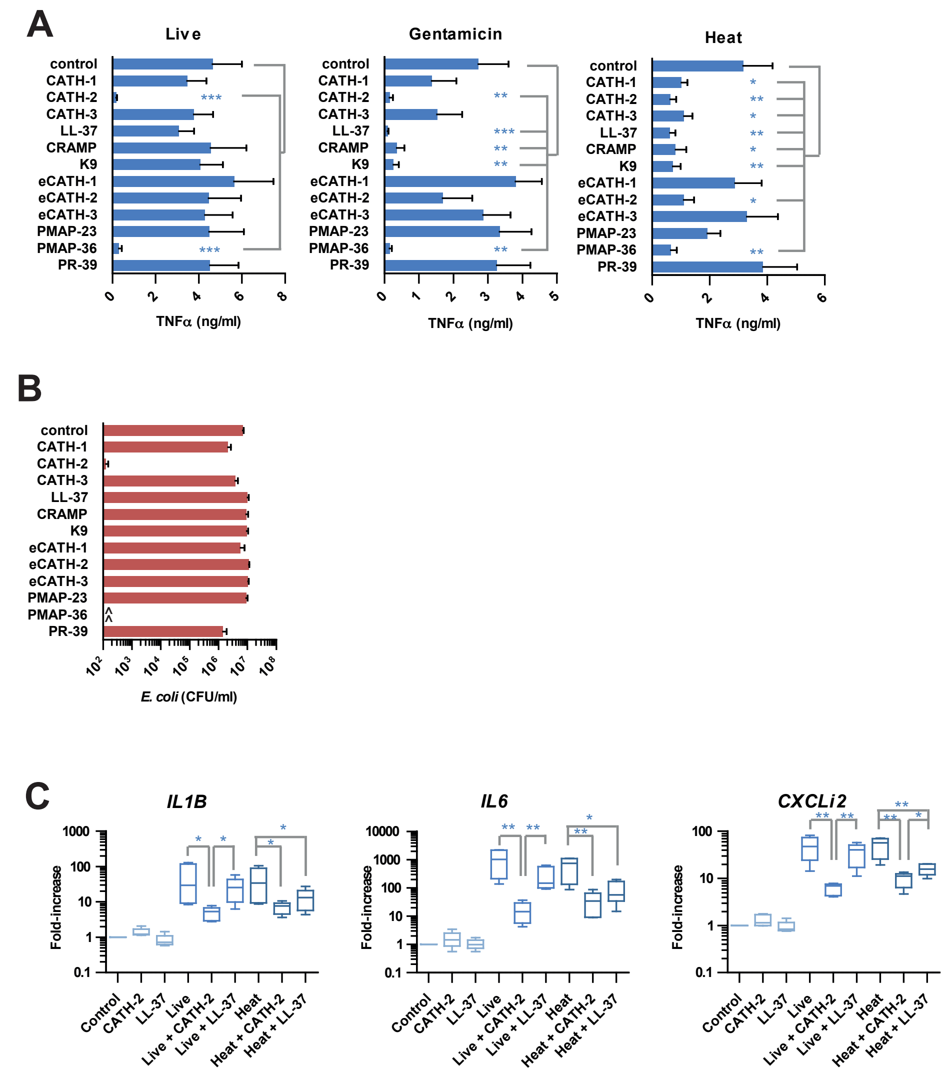


Figure 2: Cathelicidin effects are not species-specific. (A-B) 10^6 CFU/ml *E. coli* O78, untreated, heat-killed or gentamicin-killed, was mixed in DMEM + 10% FCS with 5 µM of the indicated cathelicidins, followed by (A) stimulation of J774.A1 cells after which TNFα release was determined after 2h and (B) colony count assay after 2h incubation. Statistical analysis on TNFα release was performed by One-way repeated measures ANOVA with Dunnett's post-hoc test. (C) chicken PBMCs were stimulated for 2h with 5×10^5 CFU/ml *E. coli* O78, untreated or heat-killed, in RPMI 1640 + 10% FCS with 5 µM of CATH-2 or LL-37, followed analysis of *IL1B, IL6* and *CXCLi2* gene expression by qPCR. Statistical analysis was performed by Two-way repeated measures ANOVA on Ln-transformed data, followed by Šídák post-hoc test. N = 3 or more ± SEM.

but were also observed with chicken PBMCs (Fig. 2C). In these cells, CATH-2 inhibits expression of *IL1B*, *IL6* and *CXCLi2* in combination with either live or heat-killed *E. coli*, while LL-37 only inhibits the expression induced by heat-killed *E. coli*.

CATH-2 and LL-37 inhibit *E. coli*-induced TLR2 and TLR4 activation

To determine whether the inhibition of *E. coli*-induced activation involves the inhibition of TLR activation, HEK-TLR cells were stimulated with heat-killed *E. coli* in combination with CATH-2 after which NF-κB activation was determined (Fig. 3A-B). Control cells (TLR0), did not respond to heat-killed *E. coli* and activation of these cells by TNFα was not inhibited by CATH-2. Stimulation of the other HEK-TLR cells with heat-killed *E. coli* resulted in activation of TLR1/2/6, TLR4 and TLR5, but not TLR3, TLR7 or TLR9. Furthermore, the activation of TLR1/2/6 and TLR4, but not activation of TLR5, by heat-killed *E. coli*, was inhibited by CATH-2. In addition, CATH-2 inhibited activation of HEK-TLR cells and J774.A1 macrophages when stimulated with specific TLR ligands for TLR2 (Pam2CSK4 or Pam3CSK4) or TLR4 (LPS), but not TLR5 (flagellin) (Fig. 3A-C). Similarly, LL-37 also inhibited activation of HEK-TLR1/2/6 and HEK-TLR4 cells by heat-killed *E. coli*, but not the activation of HEK-TLR5 cells. Furthermore, LL-37 inhibited TLR2 and TLR4 activation of HEK-cells and J774.A1 cells by Pam3CSK4 and LPS, respectively (Fig. S4A-D). Together, these results indicate that both CATH-2 and LL-37 are able to inhibit the activation of TLR2 and TLR4 by non-viable *E. coli*.

To determine whether CATH-2 and LL-37 also inhibit NF-κB-independent TLR4 signaling, J774.A1 macrophages were stimulated with live or heat-killed *E. coli* in the presence of CATH-2 or LL-37, after which release of CXCL10, CCL5 and IFNβ was determined, since these cytokines have been linked to IRF3-dependent TLR4 signaling (25, 26) (Fig. S5A-C). Similar to the inhibition of TNFα, CATH-2 inhibited CXCL10 and CCL5 release by both live and heat-killed *E. coli*, while LL-37 only inhibited heat-killed *E. coli*. In addition, CATH-2 inhibited the release of IFNβ, although IFNβ release was also undetectable upon stimulation with heat-killed *E. coli*.

To investigate whether the inhibition of TLR2 and TLR4 activation is the result of direct interaction with lipoproteins and LPS, ITC analysis was performed. This demonstrated direct interaction of CATH-2 with LPS and Pam3CSK4, as well as direct interaction of LL-37 with LPS and Pam3CSK4 (Fig. 4A, Fig. S5D). Furthermore, to confirm that the direct interaction between *E. coli* and cathelicidins was required for inhibition of macrophage activation, J774.A1 macrophages were pre-incubated with CATH-2 or LL-37 before stimulation with live, heat-killed or gentamicin-killed *E. coli* (Fig. 4B). For all *E. coli* treatments, pre-incubation of macrophages with CATH-2 or LL-37 did not inhibit activation. In fact, pre-incubation with CATH-2 increased activation by live and

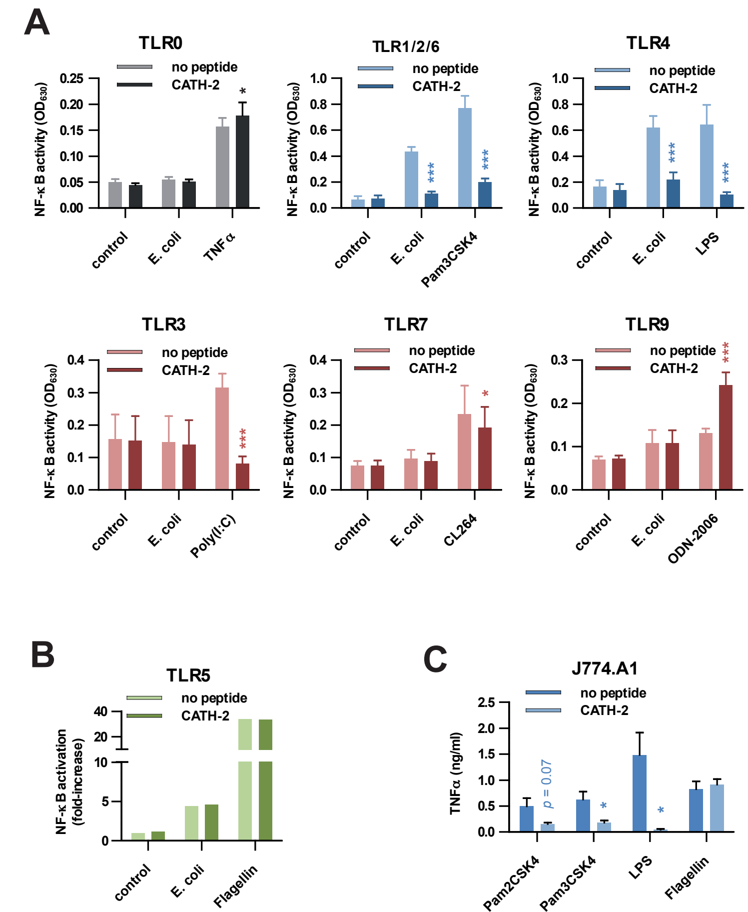


Figure 3: Inhibition of *E. coli*-induced TLR2 and TLR4 activation by CATH-2. (A) HEK-TLR cells overexpressing either no TLR (TLR0), TLR1,2 and 6 (TLR1/2/6), TLR4, TLR3, TLR7 or TLR9 as well as a SEAP reporter gene, were stimulated with $5x10^4$ CFU/ml heat-killed *E. coli* or TNFα (50 ng/ml), Pam3CSK4 (5 ng/ml), LPS (0.5 ng/ml), Poly(I:C) (250 ng/ml), CL264 (250 ng/ml) or ODN-2006 (50 nM) in the presence or absence of 5 μM CATH-2. After 24h, supernatant was used to determine NF-κB activation through Quantiblue analysis. Statistical analysis was performed by Two-way repeated measures ANOVA with Bonferroni post-hoc test. N = 3 or more ± SEM. (B) HEK-TLR5-luc cells were stimulated with $5x10^4$ CFU/ml heat-killed *E. coli* O78 or flagellin (10 ng/ml) in presence or absence of 5 μM CATH-2. NF-κB activation was determined after 6h by BrightGlo analysis. Representative result of N = 3. (C) J774.A1 cells were stimulated with Pam2CSK4 (10 pg/ml), Pam3CSK4 (10 ng/ml), LPS (10 ng/ml) or flagellin (1 μg/ml) in presence or absence of 5 μM CATH-2 for 2h after which TNFα release was determined by ELISA. Statistical analysis was performed by paired T-test. N = 3 or more ± SEM.

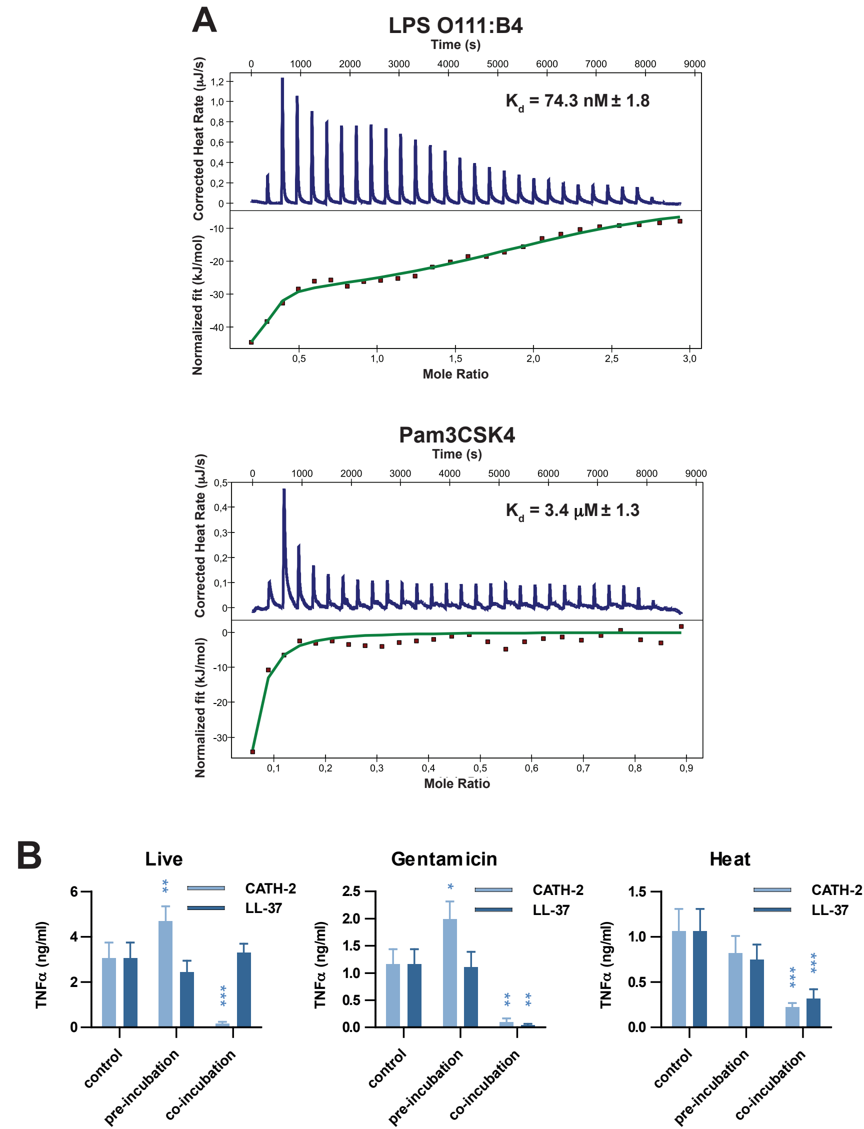


Figure 4: Inhibition of *E. coli*-induced macrophage activation depends on direct interaction between *E. coli* and cathelicidins. (A) ITC analysis of CATH-2 titration into LPS O111:B4 or Pam3CSK4 solution. Representative image of N = 2. Error = SEM. (B) J774.A1 pre-incubation for 2h with 5 µM CATH-2 or LL-37, after which cells were stimulated with 10^6 CFU/ml *E. coli* O78, either untreated, heat-killed or gentamicin-killed in the presence or absence of 5 µM CATH-2 or LL-37. TNFα release was determined after 2h. Statistical analysis was performed by Two-way repeated measures ANOVA with Bonferroni post-hoc test. N = 3 or more ± SEM.

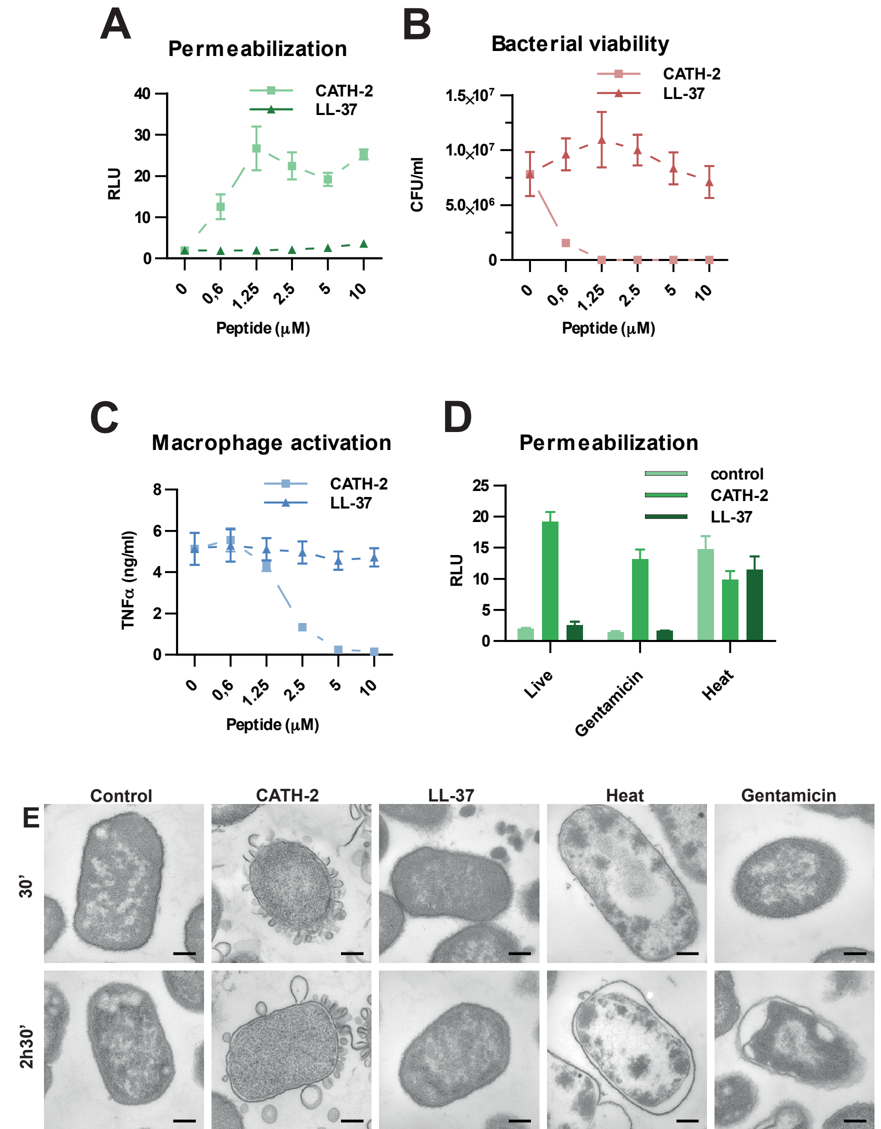


Figure 5: Dual activity of CATH-2 on bacterial inner and outer membrane. (A) 10^6 CFU/ml *E. coli* O78 was mixed with indicated concentrations of CATH-2 and LL-37 and used for (A) inner membrane permeability analysis with Sytox Green after 0.5h incubation, (B) stimulation of J774.A1 cells after which TNFα release was determined after 2h and (C) a colony count assay after 2h incubation. N = 3 or more ± SEM. (D) TEM images of 10^8 CFU/ml *E. coli* O78 in DMEM, either untreated, or treated with 40 µM CATH-2 or LL-37, heat (70 °C) or gentamicin (250 µg/ml) for 0.5 or 2.5h. Representative images of N = 2.

gentamicin-killed *E. coli*. Together, these results indicate that CATH-2 and LL-37 directly neutralize LPS and lipoproteins from the *E. coli* outer membrane upon loss of *E. coli* viability, leading to the inhibition of TLR2 and TLR4 activation and inhibition of both MyD88-dependent and –independent downstream signaling.

CATH-2 interacts with both *E. coli* inner and outer membrane

While most cathelicidins were unable to kill *E. coli* under cell culture conditions and only inhibited *E. coli*-induced macrophage activation when bacteria were killed prior to stimulation, CATH-2 showed dual activity, i.e. killing *E. coli* as well as inhibiting macrophage activation. To better understand how the interaction between CATH-2 and *E. coli* leads to the neutralization of LPS and lipoproteins, the antimicrobial activity of CATH-2 was investigated in more detail. Analysis of inner membrane (IM) integrity of *E. coli* showed that the IM was permeabilized by CATH-2 at 0.6-1.25 μM (Fig. 5A), which corresponds to the concentrations needed for *E. coli* killing (Fig. 5B). In contrast, inhibition of macrophage activation occurred at higher concentrations of 2.5-5 μM (Fig. 5C). Furthermore, LL-37 was unable to permeabilize the IM, even in the context of gentamicin-killed *E. coli* (Fig. 5D). This suggest that antimicrobial activity and neutralization of lipoproteins and LPS are two distinct processes. Finally, to visualize what happens to *E. coli* upon killing by CATH-2, electron microscopy was performed (Fig. 5E). This showed that killing by CATH-2 induces massive release of membrane fragments from *E. coli*. In contrast, LL-37 induces the release of a different type of bacterial components, which is most likely an active defense mechanism of *E. coli* to trap LL-37 and prevent killing. Images of heat- and gentamicin-killed *E. coli* showed that the membrane release observed upon CATH-2-mediated killing is not a general effect of the loss of *E. coli* viability. Together, these findings suggest that the antimicrobial activity of CATH-2 is dependent on IM permeabilzation, while inhibition of macrophage activation is the result of neutralization of LPS and lipoproteins released from the bacterial OM.

Discussion

While various studies have investigated the effects of cathelicidins on TLR activation by specific, purified or synthetic TLR ligands (19, 21, 27-29), this is, to our knowledge, the first study describing the regulation of TLR activation by cathelicidins in the presence of whole bacteria. In addition, this study shows for the first time a direct link between bacterial viability and cathelicidin-mediated regulation of TLR activation. Our results demonstrate that various cathelicidins can inhibit *E. coli*-induced macrophage activation, which is dependent on the loss of *E. coli* viability. Furthermore, this inhibition is caused by neutralization of LPS and lipoproteins from the bacterial outer membrane, thus preventing TLR2 and TLR4 activation (Fig. 6).

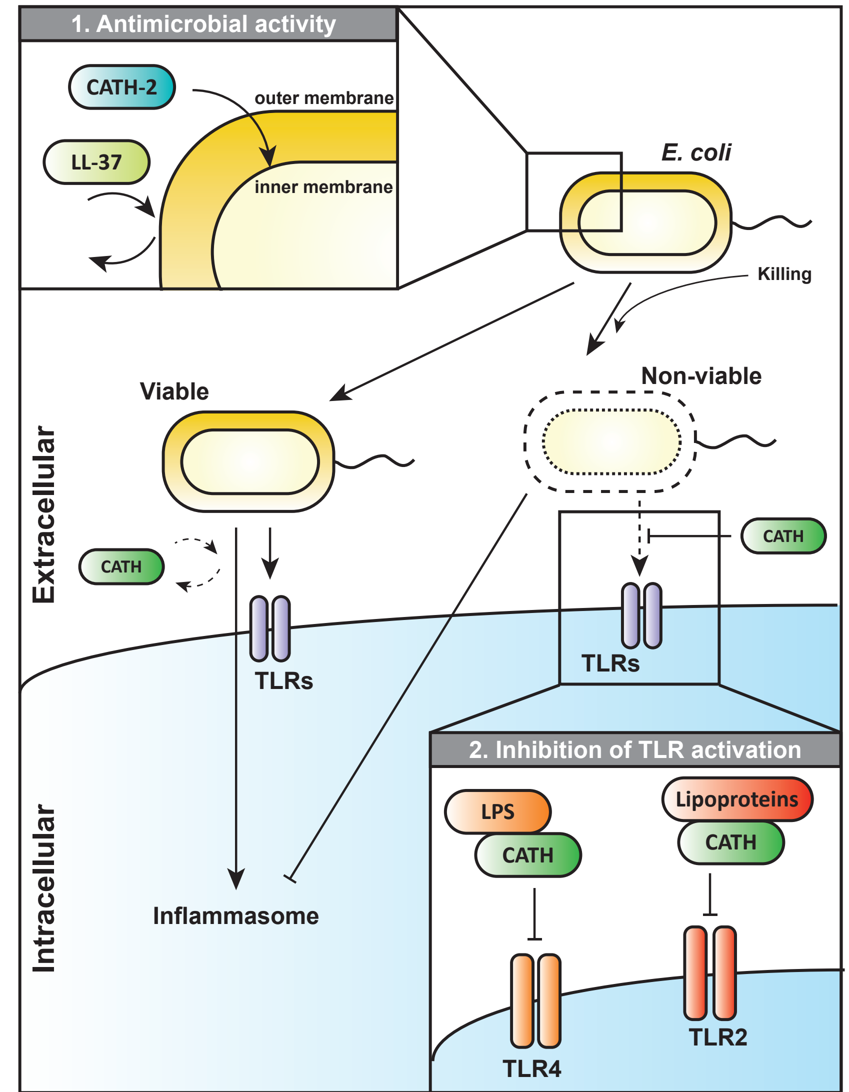


Figure 6: Model for effects of cathelicidins on *E. coli* viability and *E. coli*-induced macrophage activation. **(1)** Cathelicidins are attracted to the bacterial outer membrane due to ionic interaction between cationic residues on the cathelicidins and the anionic LPS. Depending on the cathelicidin, this is followed by either: bacterial killing due to translocation to and permeabilization of the inner membrane, or bacterial survival due to displacement of cathelicidins from the bacterial surface. **(2)** Upon bacterial killing by either cathelicidins or other antimicrobial mechanisms, cathelicidins can interact with the LPS and lipoproteins from the bacterial outer membrane to prevent activation of TLR4 and TLR2, respectively. In contrast, when bacterial viability remains intact, cathelicidins are unable to inhibit macrophage activation.

During infections, the immune system needs to induce a balanced immune response to prevent tissue damage and sepsis. This requires a correct assessment of the threat level of the infection, which includes discrimination between viable and non-viable bacteria, as immune activation can be dampened when bacterial viability is lost (30). Here we show how cathelicidins can be used by the immune system to specifically inhibit immune activation against non-viable Gram-negative bacteria. Thereby, cathelicidins can be used to prevent excessive inflammation when the bacterial threat is neutralized, while allowing inflammation when the antimicrobial host response has been insufficient to clear the infection.

Previous studies have demonstrated the inhibitory effect of LL-37 and other cathelicidins on TLR4 activation by purified LPS (17, 18, 31, 32). This inhibition has been suggested to mainly depend on direct interaction between cathelicidins and LPS, although several studies have shown indirect effects of LL-37 on TLR4 activation as well (28, 31, 33). In addition, although less evident from literature, LL-37 has also been implicated in the inhibition of TLR2 by lipoproteins (20, 27, 31, 34). Our results show that CATH-2 and LL-37 are able to inhibit the activation of TLR2 and TLR4 in the context of non-viable *E. coli* by directly binding to LPS and lipoproteins. As TLR2 and TLR4 activation has previously been shown to be crucial in the *in vitro* and *in vivo* response against *E. coli*, as well as other Gram-negatives, the inhibitory effect of cathelicidins observed in this study could be important in the context of many Gram-negative bacterial infections (1, 5, 35-40).

Interestingly, a recent study demonstrated that LL-37 enhances LPS-induced activation in lung epithelial cells (41), an apparent result of increased LPS internalization and endosomal activation of TLR4. However, release of cytokines that have been linked to intracellular TLR4 activation, such as CXCL10, CCL5 and IFNβ (25, 26, 42, 43), are inhibited by CATH-2 and LL-37 upon stimulation with non-viable *E. coli*, suggesting that cathelicidins inhibit both extracellular and intracellular activation of TLR4 in macrophages in the context of complete *E. coli*.

While the interaction between cathelicidins and LPS is important for the inhibition of TLR4 activation, it is also thought that cathelicidins use LPS as an initial interaction point on the bacterial membrane to initiate their bactericidal activity (44). However, our findings suggest that cathelicidin-mediated antimicrobial activity and LPS neutralization are two distinct processes. First of all, under physiological conditions, CATH-2 is able to permeabilize the IM of *E. coli*, and kill *E. coli,* at concentrations that are lower than those needed for inhibition of macrophage activation. In addition, LL-37 is able to inhibit macrophage activation by non-viable *E. coli*, while not being able to kill *E. coli*. Furthermore, the antimicrobial activity of CATH-2 appears to depend on a threshold concentration (± 0.6 μM for over 90% killing), whereas higher CATH-2 concentrations are needed to inhibit macrophage activation at higher *E. coli* concentrations. This is most likely caused by the greater amount of available LPS and lipoproteins at higher *E. coli* concentrations, which demands more CATH-2 to directly neutralize the LPS and lipoproteins.

Although most peptides tested in this study were able to inhibit macrophage activation by non-viable *E. coli*, only CATH-2 and PMAP-36 showed strong antimicrobial activity under physiological cell culture conditions. This corresponds with previous findings that show that cathelicidin antimicrobial activity is limited under tissue culture conditions due to the presence serum components and mono- or divalent cations (45-47). This could mean that *in vivo*, other antimicrobial mechanisms are needed to kill the bacteria and that cathelicidins are used to sense the loss of bacterial viability and subsequently inhibit immune activation. On the other hand, cathelicidins have been shown to act in synergy with other host-derived antimicrobial components, such as lysozyme and lactoferrin, which are stored in the same neutrophil granules as cathelicidins (46, 48, 49). Thus, while most cathelicidins have limited antimicrobial activity under cell culture conditions, their bactericidal activity during infections *in vivo* might be greater by acting in concert with other host-derived antimicrobial components.

Nevertheless, regardless of possible synergism, chicken CATH-2 was observed to have strong antimicrobial activity by itself under physiological cell culture conditions in addition to its inhibitory effect on TLR2 and TLR4 activation. Because of this dual activity, CATH-2 can induce non-immunogenic, or "silent" killing, i.e. kill *E. coli* and subsequently inhibit *E. coli*-induced macrophage activation (50). This led to the initial hypothesis that CATH-2 might prevent the release of inflammatory components from *E. coli*, however, EM images showed massive release of membrane components upon CATH-2-mediated *E. coli* killing. This implies that CATH-2 actually induces the release of bacterial outer membrane components and subsequently neutralizes them through direct interaction. This is further supported by results from Schneider et al. (2016, submitted results), where immuno-EM images show that upon bacterial killing, CATH-2 is bound to the fragments released from the *E. coli*.

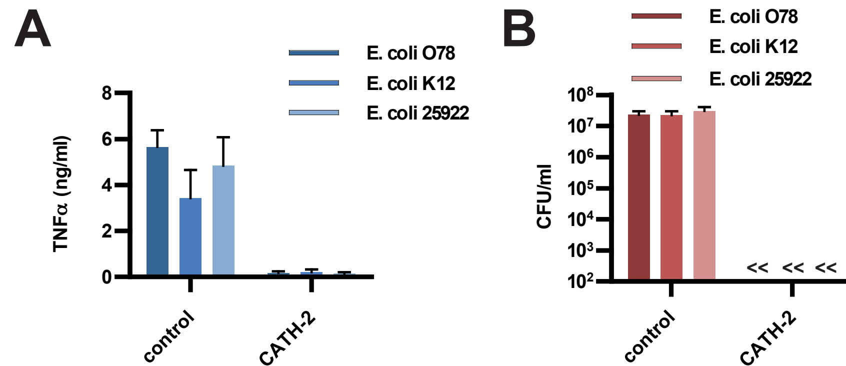
While these findings are interesting from a biological point of view, the dual activity of CATH-2 could be of great importance for the development of novel cathelicidin-based anti-infectives. Because of the development of antibiotic resistance in bacteria against many of the currently available antibiotics, host defense peptides, such as cathelicidins, have been suggested as novel alternatives for antibiotics (51-55). From a therapeutical point of view, the silent killing as observed for CATH-2, could be an additional benefit. A dual activity based drug could kill infectious bacteria as well as inhibit the inflammatory

response, but only when the antimicrobial activity is sufficient to clear the infection. These effects could help in the clearance of an infection as well as the prevention of sepsis in infected patients.

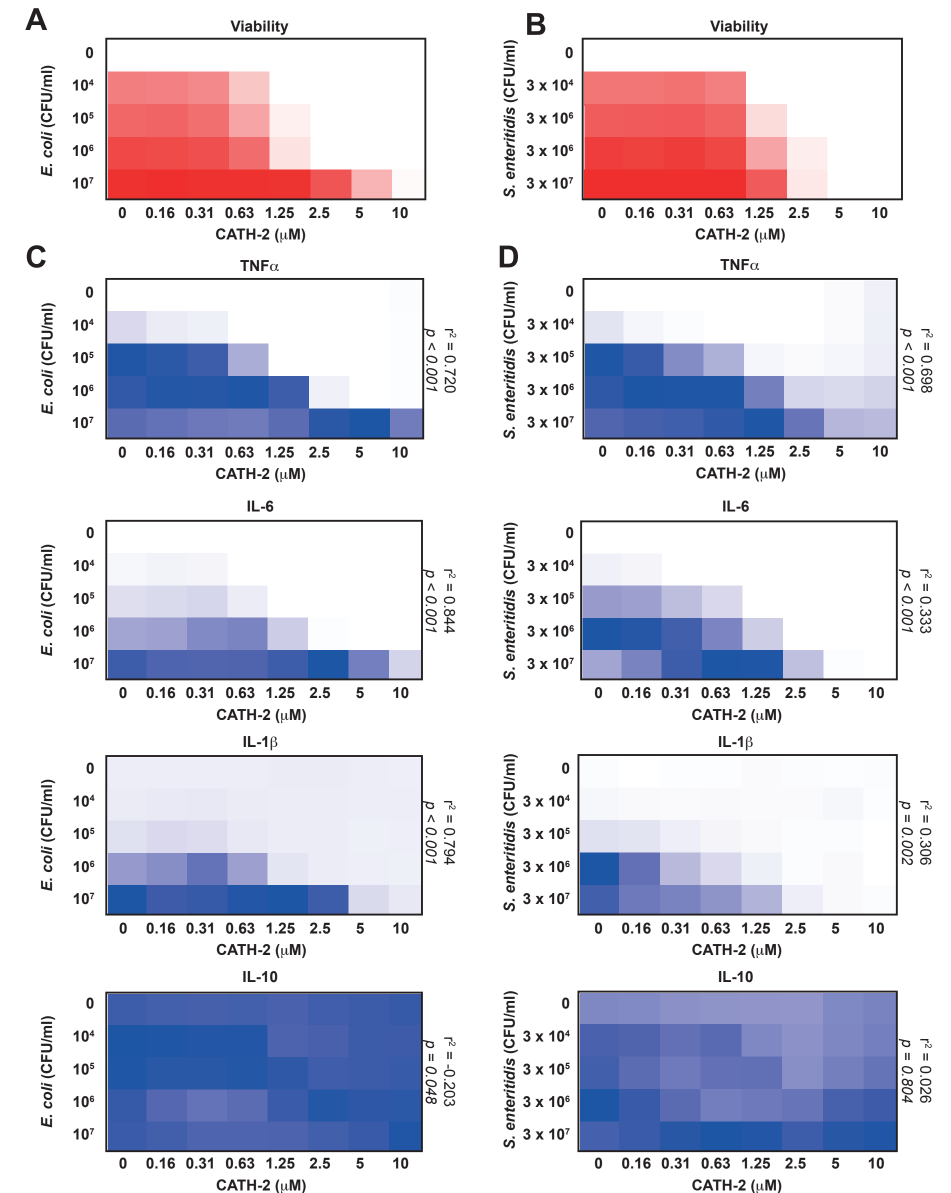
In conclusion, this study shows for the first time the regulatory effects of cathelicidins during macrophage activation by whole bacteria. Our results show how CATH-2 can silently kill Gram-negative bacteria by permeabilizing the bacterial inner membrane and subsequently neutralizing lipoproteins and LPS released from the bacterial outer membrane to prevent TLR2 and TLR4 activation, respectively. In addition, while most other cathelicidins have limited bactericidal activity under physiological conditions, they are able to inhibit macrophage activation by non-viable *E. coli*. These results describe a novel role for cathelicidins in the discrimination between viable from non-viable bacteria by the immune system and their function in inhibiting immune activation when a bacterial threat has been neutralized in order to prevent excessive inflammation and sepsis.

Acknowledgements

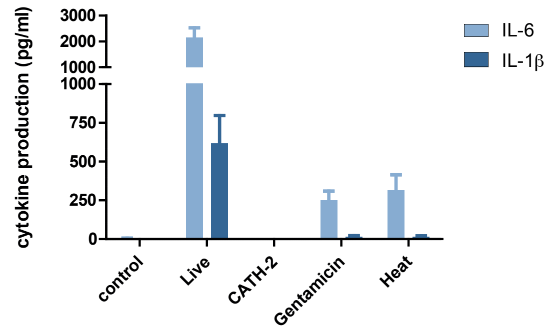
We would like to acknowledge George Posthuma and Renee Scriwanek for their assistance in the electron microscopy experiments and analysis.



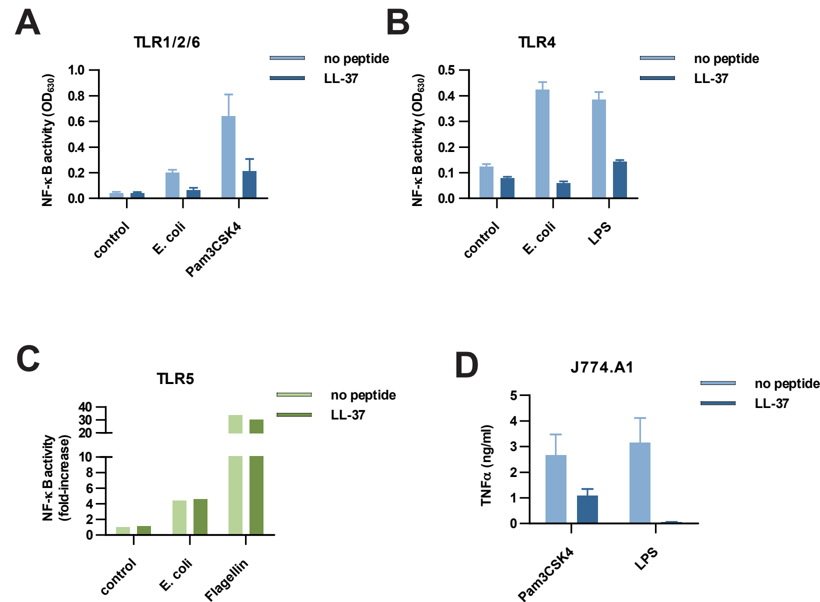
Supplemental Figure 1: CATH-2 inhibits macrophage activation against *E. coli* with smooth and rough LPS serotypes. (A) TNFα release after 2h stimulation of J774.A1 cells with 10^6 CFU/ml *E. coli* O78, *E. coli* K12 or *E. coli* ATCC 25922 in the presence or absence of 5 μM CATH-2. (B) *E. coli* viability after 2h incubation in DMEM + 10% FCS in the presence of 5 μM CATH-2. N = 3 or more ± SEM.



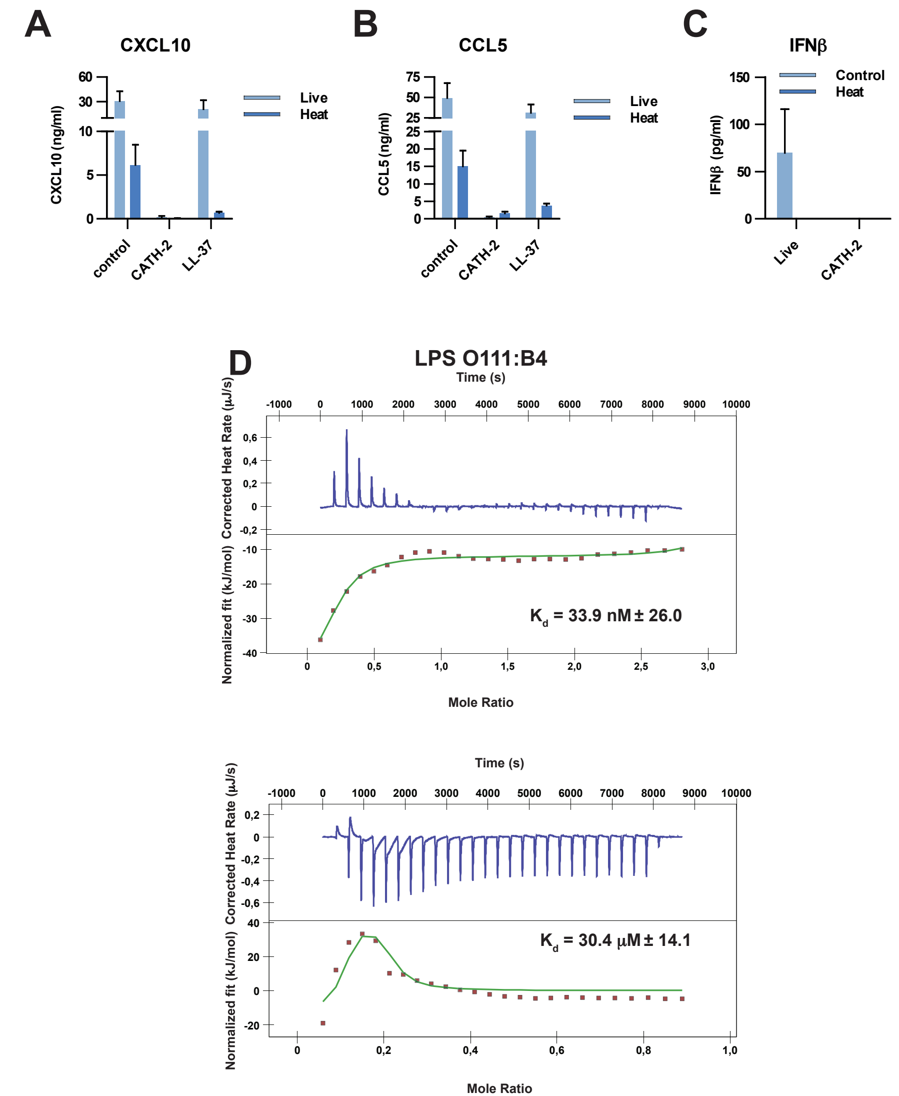
Supplemental Figure 2: CATH-2-mediated inhibition of *E. coli*-induced macrophage activation correlates with loss of *E. coli* viability. (A-B) Heat map of (A) *E. coli* O78 viability (Log) and (B) *S. enteritidis* viability (Log) after 2h incubation of indicated bacterial concentrations with indicated CATH-2 concentrations. (C-D) Heat maps of TNFα, IL-6, IL-1β and IL-10 release by J774.A1 cells after stimulation with (C) *E. coli* O78 and CATH-2 or (D) *S. enteritidis* and CATH-2 at various ratios with indicated correlation between cytokine release and bacterial viability (Spearman's correlation analysis). N = 3.



Supplemental Figure 3: Effect of bacterial viability on IL-6 and IL-1β release. IL-6 and IL-1β release by J774.A1 cells after 24h stimulation with 10^6/ml *E. coli* O78, which were either alive (Live), CATH-2-killed (CATH-2), gentamicin-killed (Gentamicin), or heat-killed (Heat). N = 3 ± SEM.



Supplemental Figure 4: Inhibition of *E. coli*-induced TLR2 and TLR4 activation by LL-37. (A) HEK-TLR2-SEAP and (B) HEK-TLR4-SEAP cells were stimulated with 5x10⁴ CFU/ml heat-killed *E. coli* O78, 10 ng/ml LPS or 10 ng/ml Pam3CSK4 in presence or absence of 5 µM LL-37 for 24h, after which NF-kB activity was determined by measuring Quantiblue OD at 630 nm. N = 2 ± SEM. (C) HEK-TLR5-luc cells were stimulated with 5x10⁴ CFU/ml heat-killed *E. coli* O78 or 10 ng/ml flagellin in presence or absence of 5 µM LL-37 for 6h, after which NF-kB activity was determined by measuring luciferase activity with BrightGlo. Representative of 2 independent experiments. (D) TNFα release after 2h stimulation of J774.A1 cells with 50 ng/ml Pam3CSK4 or 10 ng/ml LPS in the presence of 10 µM LL-37. N = 3 ± SEM.



Supplemental Figure 5: Inhibition of alternative TLR4 activation. (A) CXCL10, (B) CCL5 and (C) IFNβ release after 24h stimulation of J774.A1 cells with 10^6 CFU/ml live or heat-killed *E. coli* O78 in the presence or absence of 5 µM CATH-2 or LL-37. Cells were stimulated for 2h, followed by two wash steps and subsequent 22h incubation in culture media supplemented with 250 µg/ml gentamicin. N = 3 ± SEM. (D) Representative image of ITC experiments with LL-37 titration into LPS O111:B4 or Pam3CSK4 solution, N = 2. Error = SEM.

Supplemental Table 1: Cathelicidin peptide sequences

Peptide	Sequence
LL-37	LLGDFFRKSKEKIGKEFKRIVQRIKDFLRNLVPRTES
CRAMP	GLLRKGGEKIGEKLKKIGQKIKNFFQKLVPQPEQ
K9CATH	RLKELITTGGQKIGEKIRRIGQRIKDFFKNLQPREEKS
chCATH-1	RVKRVWPLVIRTVIAGYNLYRAIKKK
chCATH-2	RFGRFLRKIRRFRPKVTITIQGSARF-NH_2
chCATH-3	RVKRFWPLVPVAINTVAAGINLYKAIRRK
eCATH-1	KRFGRLAKSFLRMRILLPRRKILLAS
eCATH-2	KRRHWFPLSFQEFLEQLRRFRDQLPFP
eCATH-3	KRFHSVGSLIQRHQQMIRDKSEATRHGIRIITRPKLLLAS
PMAP-23	RIIDLLWRVRRPQKPKFVTVWVR
PMAP-36	Ac-GRFRRLRKKTRKRLKKIGKVLKWIPPIVGSIPLGCG
PR-39	RRRPRPPYLPRPRPPPFFPPRLPPRIPPGFPPRFPPRFP

Supplemental Table 2: Primers and probes for qPCR on chicken samples

	Forward	Reverse	Probe
IL1B	GCTCTACATGTCGTGTGTGATGAG	TGTCGATGTCCCGCATGA	CCACACTGCAGCTGGAGGAAGCC
IL6	GTCGAGTCTCTGTGCTAC	GTCTGGGATGACCACTTC	ACGATCCGGCAGATGGTGA
CXCLi2	GCCCTCCTCCTGGTTTCA	CGCAGCTCATTCCCCATCT	TGCTCTGTCGCAAGGTAGGACGCTG
GAPDH	GCCGTCCTCTCTGGCAAAG	TGTAAACCATGTAGTTCAGATCGATGA	AGTGGTGGCCATCAATGATCCC

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Chapter 6

Chicken cathelicidin-2-mediated killing of *P. aeruginosa* prevents lung inflammation *in vivo*

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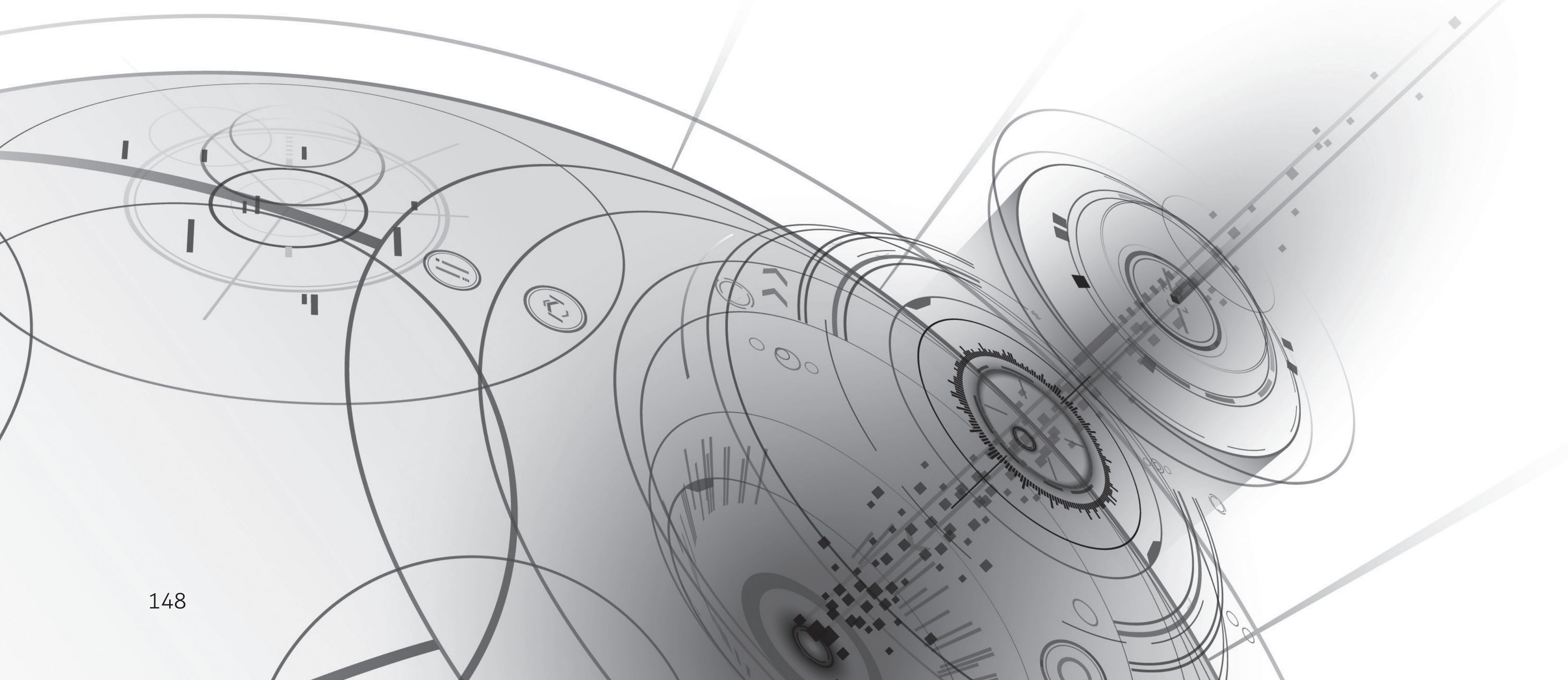


Abstract

Pseudomonas aeruginosa is an opportunistic pathogen that is commonly found to infect the lungs of cystic fibrosis (CF) patients. The chronic infection by this bacteria is a major cause of chronic inflammation, which leads to tissue damage and lung dysfunction in CF patients, and treatment is often difficult due to the development of antibiotic resistance. Novel treatment therapies that are aimed at clearing *P. aeruginosa* from the lung and at the same time preventing additional inflammation, could be very beneficial in the treatment of chronic infection in the lungs of CF patients. This study tested the potential of the chicken cathelicidin-2 (CATH-2) as a novel anti-infective against *P. aeruginosa* infections. Our results show that CATH-2 kills *P. aeruginosa* in an immunogenically silent manner, which limits the associated inflammation *in vitro*, as well as *in vivo* in a murine lung model. In this model, CATH-2 limited *P. aeruginosa*-induced neutrophil recruitment and reduced cytokine and chemokine production. Together, these results demonstrate the potential of CATH-2 as a dual-activity antibiotic in CF patients, which can both kill *P. aeruginosa* and prevent excessive inflammation.

Introduction

Pseudomonas aeruginosa is a Gram-negative bacterium which can cause opportunistic infections in the lungs of susceptible patients (1-3). Chronic *P. aeruginosa* infections are commonly associated with cystic fibrosis (CF) and chronic obstructive pulmonary disease (COPD) and effective treatment is difficult due to the development of multidrug resistance (MDR) by these bacteria (4-6). Adding to the complexity of the pathophysiology of the infected CF and COPD patients is the presence of chronic inflammation within the lung (7, 8). This chronic inflammation is characterized by high neutrophil numbers and release of pro-inflammatory mediators, which are insufficient to clear the infection. The tissue damage and lung dysfunction associated with the chronic infection, are ultimately the most common cause of death in CF and COPD patients (9-11).

Research into novel therapeutics for the treatment of *P. aeruginosa* infections has shown that cathelicidins are a promising alternative to conventional antibiotics (12-15). Cathelicidins are short cationic peptides with broad-spectrum antimicrobial activity against various pathogens, including Gram-positive and Gram-negative bacteria (16, 17). This broad-spectrum antimicrobial activity has also been observed for the chicken cathelicidin-2 (CATH-2) and includes activity against MDR *P. aeruginosa* strains. In addition, CATH-2 has been shown to retain antimicrobial activity under physiological conditions, in contrast to many other cathelicidins such as the human LL-37 and equine CATH-1 (15) (Chapter 3). Importantly, we showed that CATH-2 has a dual function, with regard to both killing gram-negative bacteria and subsequently inhibiting the inflammatory response against the killed microbe (Chapter 5). This "silent killing" was demonstrated against *E. coli* and *S. enteritidis*, where CATH-2 neutralizes LPS and lipoproteins released from the bacterial outer membrane, which prevents TLR2 and TLR4 activation on macrophages. However, it is unknown whether CATH-2 is able to silently kill other clinically relevant Gram-negatives, such as *P. aeruginosa*, and whether this reduced inflammation is also observed in an *in vivo* situation.

This study tests the hypothesis that CATH-2 mediates silent killing of *P. aeruginosa* both *in vitro* and *in vivo*. To test silent killing *in vitro*, TNFα and IL-6 release by murine macrophages was determined after stimulation with CATH-2-killed *P. aeruginosa* and was compared to stimulation with viable, heat-killed and gentamicin-killed *P. aeruginosa*. Subsequently, the *in vivo* effect of CATH-2-killed *P. aeruginosa* on leukocyte recruitment and release of cytokines in the bronchoalveolar lavage fluid (BALF) was determined after intratracheal instillation in mice. Overall, this study demonstrates CATH-2-mediated silent killing of *P. aeruginosa* in both an *in vitro* and *in vivo* setting and underlines the potential therapeutic value of CATH-2-based anti-infectives.

Materials and methods

Reagents

P. aeruginosa LPS was obtained from Sigma Aldrich (St. Louis, MO, USA) and *P. aeruginosa* flagellin was obtained from Invivogen (Toulouse, France). CATH-2 and LL-37 were synthesized by Fmoc-chemistry at China Peptides (CPC scientific, Sunnyvale, CA, USA) and eCATH-1 was synthesized by Fmoc-chemistry at the Academic Centre for Dentistry Amsterdam (Amsterdam, the Netherlands). Gentamicin solution was obtained from Sigma Aldrich.

Bacterial culture

For *in vitro* experiments, *P. aeruginosa* ATCC 27853 (ATCC, Manassas, VA, USA) was grown to log-phase in Luria Broth (BioTRADING Benelux B.V., Mijdrecht, the Netherlands). After measuring the optical density (OD), bacteria were centrifuged at 1200 x g, 10 min and diluted in DMEM medium (Thermo Fisher Scientific, Waltham, MA, USA). To prepare killed bacteria, bacteria were either incubated 1h at 90 °C (heat-killed), 1h with 1 mg/ml gentamicin at 37 °C (gentamicin-killed) or 1h with 20 μM CATH-2 at 37 °C (CATH-2-killed).

Cell culture

J774.A1 murine macrophages were a kind gift of Prof. Jos van Putten (Division of Infection Biology, Dept. of Infectious Diseases and Immunology, Utrecht University, The Netherlands). Cells were cultured in DMEM supplemented with 10% FCS (Bodinco B.V., Alkmaar, The Netherlands). Cells were seeded in 96-wells plates ($7.5x10^4$ cells/well) for adherence overnight. Cells were subsequently stimulated with live, heat-killed, gentamicin-killed or CATH-2-killed bacteria in the presence or absence of other cathelicidins. After 2h stimulation, supernatant was used to determine TNFα concentrations. Alternatively, cells were washed twice after the 2h incubation, followed by incubation for an additional 22h in DMEM + 10% FCS + 250 μg/ml gentamicin. After this incubation supernatant was harvested to determine IL-6 concentrations.

ELISA

ELISA Duosets for mouse TNFα and mouse IL-6 were obtained from R&D systems (Minneapolis, MN, USA). Samples were diluted in PBS with 1% BSA, pH 7.4 before analysis (5x for TNFα and 25x for IL-6). ELISAs were performed according to manufacturer's protocol. For ELISA plate analysis, absorbance was determined at OD_{450} and was corrected for absorbance at OD_{570}. Absorbance was determined with a FLUOstar Omega microplate reader (BMG Labtech GmbH, Ortenberg, Germany) and analyzed with MARS data analysis software (BMG Labtech GmbH).

Colony counting assay

Colony counting assays were performed after co-incubation of *P. aeruginosa* with cathelicidins in 20 ml DMEM + 10% FCS at 37 °C for 2h in round-bottom polypropylene 96-wells plates. After incubation, samples were diluted with 180 ml PBS followed by spread-plating 10-fold dilutions in PBS on Tryptone Soy Agar (TSA) plates (Oxoid Limited, Hampshire, United Kingdom). Plates were incubated overnight at 37 °C, after which CFUs were determined, with a detection limit of 10^2 CFU/ml.

Preparation of killed bacteria for *in vivo* analysis

An overnight culture of *P. aeruginosa* ATCC 27853 was diluted 10-fold in tryptic soy broth (TSB). The optical density was measured, and bacteria were further diluted in sterile saline to reach an initial concentration of approximately $2x10^6$ CFU/ml. Subsequently, the bacteria were killed by CATH-2, heat or gentamicin as described above. After intratracheal instillation, part of the bacterial solutions was plated via spot plating on TSA, and incubated overnight at 37 °C, to ensure complete bacterial killing.

Administration of killed bacteria *in vivo*

Male C57Bl/6 mice (Charles River, Sherbrooke, Qc, Canada), weighing 23-32 g, were used for this experiment. All animal procedures were approved by the Animal Use Subcommittee at the University of Western Ontario, and followed the approved guidelines described by the Canadian Council of Animal Care. Mice were anesthetized by intraperitoneal injection of ketamine (130 mg/kg body weight) and dexmedetomidine (0.5 mg/kg BW), and then intubated using a 20 G catheter, with the aid of a fiber-optic stylet (BioLite intubation system for small rodents, BioTex, Inc., Houston, Texas, USA). Once intubated, mice were instilled with 50 μL of heat-, gentamicin- or CATH-2-killed bacterial preparations (see above), or instilled with an air bolus (naïve controls). Mice were extubated following successful instillation and were subsequently injected with the reversal agent for dexmedetomidine, Antisedan, and allowed to breathe spontaneously for the following six hours. After six hours, the mice were euthanized by IP injection of sodium pentobarbital and dissection of the descending aorta. The animals were placed on a FlexiVent© in order to measure lung compliance and elastance. Following these measurements, whole lung lavage was collected by 3 x 1 ml aliquots of sterile saline. The whole lung lavage was immediately centrifuged at 150 x g at 4 °C, and the pellet was collected for cell analysis, while the supernatant was collected and used to measure protein content and cytokine concentrations. Differential cell analysis of the cells obtained in the lavage was done as previously described (18). Protein content of the lavage fluid was measured using a Micro BCA protein assay kit (Pierce, Rockford, Ill., USA), according to manufacturer's instructions. Levels of mouse cytokines were measured using multiplexed immunoassay kits according to manufacturers' instructions

(R&D Systems, Minneapolis, MN). A Bio-Plex 200 readout system was used (Bio-Rad), which utilizes Luminex® xMAP fluorescent bead-based technology (Luminex Corporation, Austin, TX). Cytokine levels (pg/mL) were automatically calculated from standard curves using Bio-Plex Manager software (v. 4.1.1, Bio-Rad).

Results

CATH-2 inhibits *P. aeruginosa*-induced macrophage activation

CATH-2 has been shown to inhibit macrophage activation against *E. coli* and *S. enteritidis* by silently killing the bacteria under cell culture conditions (Chapter 5). To determine whether CATH-2 also retained its antimicrobial activity against *P. aeruginosa* under these conditions, a colony counting assay was performed in DMEM + 10 % FCS (Fig. 1A). Activity of CATH-2 was compared to human LL-37 and equine CATH-1 (eCATH-1). 5 μM of CATH-2 completely killed $3x10^5$ – $3x10^6$ CFU/ml *P. aeruginosa* and decreased *P. aeruginosa* viability a 1000-fold at $3x10^7$ CFU/ml. In contrast, LL-37 and eCATH-1 did not show any antimicrobial activity, consistent with previous finding against *E. coli* (Chapter 3 and 5). To determine whether CATH-2-mediated killing also resulted in reduced macrophage activation by *P. aeruginosa*, J774.A1 murine macrophages were stimulated with viable *P. aeruginosa* in combination with 5 μM CATH-2, LL-37 or eCATH-1, after which TNFα production (Fig. 1B) and IL-6 production (Fig. 1C) were determined after 2h and 24h, respectively. CATH-2 significantly reduced *P. aeruginosa*-induced TNFα and IL-6 production, in contrast to LL-37 and eCATH-1, which did not affect cytokine production.

CATH-2 silently kills *P. aeruginosa*

To determine the effect of bacterial killing on macrophage activation, *P. aeruginosa* was either untreated, heat-killed, gentamicin-killed or CATH-2-killed (Fig. 2A). Subsequently, J774.A1 macrophages were stimulated for 2h after which TNFα release was determined (Fig. 2B). Live and gentamicin-killed bacteria induce similar TNFα release at $3x10^6$ CFU/ml, while live *P. aeruginosa* is more potent at $3x10^7$ CFU/ml compared to gentamicin-killed bacteria. Heat-killed *P. aeruginosa* did not induce TNFα release below $3x10^7$ CFU/ml, while CATH-2-mediated killing almost completely inhibited TNFα release at all bacterial concentrations, indicating that CATH-2-mediated killing of *P. aeruginosa* is immunologically silent. Because both CATH-2 and LL-37 were shown to inhibit activation of macrophages by non-viable *E. coli* (Chapter 5), gentamicin-treated bacteria (250 μg/ml) were combined with CATH-2, LL-37, or eCATH-1, after which TNFα production was measured after 2h (Fig. 2C). Both CATH-2 and LL-37 were able to inhibit macrophage activation by gentamicin-treated *P. aeruginosa*, while eCATH-1 did not affect activation. To determine the effect of CATH-2 on known *P. aeruginosa*-derived TLR ligands, J774.A1 macrophages were stimulated with either *P. aeruginosa*-derived

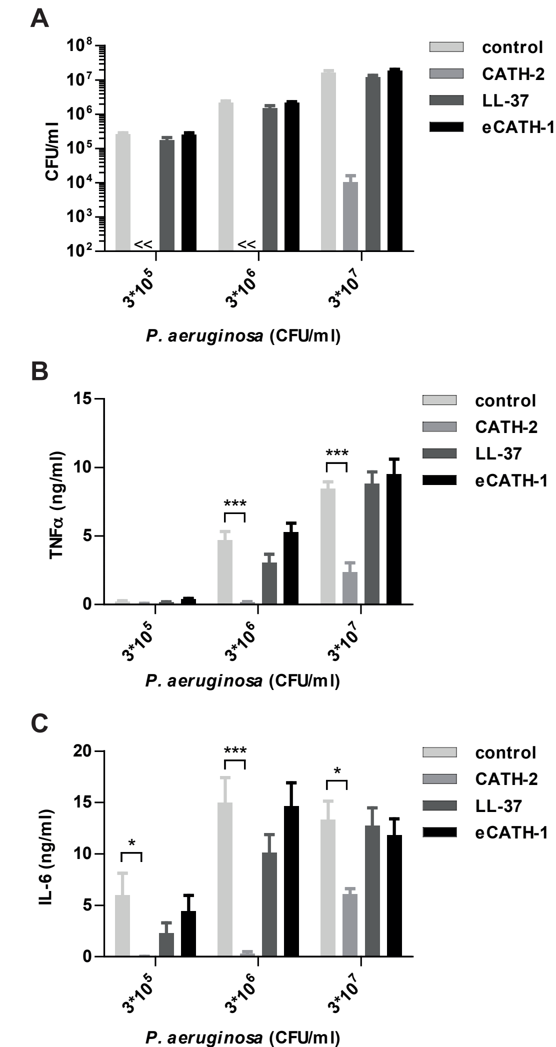


Figure 1: CATH-2 inhibits *P. aeruginosa*-induced macrophage activation. (A) Various concentrations of *P. aeruginosa* were incubated with 5 μM CATH-2, LL-37 or eCATH-1 in DMEM + 10% FCS for 2h at 37 °C, after which viability was assessed by colony counting assays. N = 4 ± SEM. (B-C) J774.A1 cells were stimulated for 2h with various concentrations of *P. aeruginosa* in combination with 5 μM CATH-2, LL-37 or eCATH-1 in DMEM + 10% FCS for 2h at 37 °C, followed by a double wash and incubation for an additional 22h in DMEM + 10% FCS + 250 μg/ml gentamicin. TNFα production (B) was determined after 2h, while IL-6 production (C) was determined after 24h. N = 3 or more ± SEM. Statistical differences are determined by Two-way ANOVA with Bonferroni's Post-Hoc test. * = $p < 0.05$; ** = $p < 0.01$; *** = $p < 0.001$.

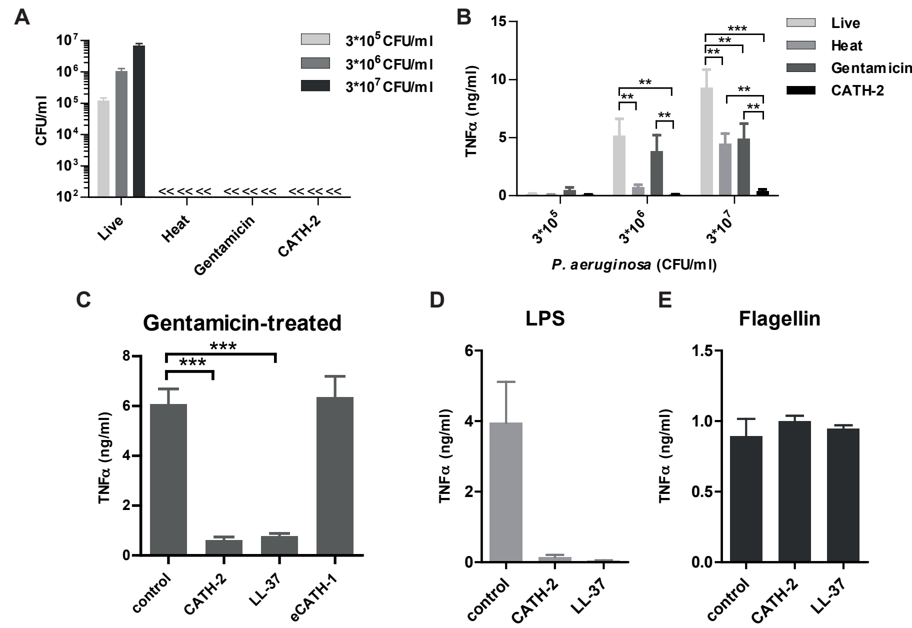


Figure 2: CATH-2 silently kills *P. aeruginosa* and inhibits LPS-induced macrophage activation. Various concentrations of *P. aeruginosa* were either untreated, CATH-2-killed, heat-killed or gentamicin-killed and used for (A) colony counting assays or (B) stimulation of J774.A1 cells for 2h, after which TNFα release was determined. Statistical differences were determined by Two-way ANOVA with Bonferroni's Post-Hoc test. N = 3 ± SEM. (C) *P. aeruginosa* (3x10^6/ml) was incubated for 0.5h with 250 μg/ml gentamicin, followed by addition of 5 μM CATH-2, LL-37 or eCATH-1. These mixtures were used for stimulation of J774.A1 cells for 2h, after which TNFα release was determined. N = 4 ± SEM (D-E) J774.A1 cells were stimulated with (D) *P. aeruginosa* LPS (100 ng/ml) or (E) *P. aeruginosa* flagellin (10 ng/ml) in combination with CATH-2 or LL-37, after which TNFα release was determined after 2h. N = 3 ± SEM. Statistical differences were determined by One-way ANOVA with Dunnett's Post-Hoc test. * = $p < 0.05$; ** = $p < 0.01$; *** = $p < 0.001$.

LPS (Fig. 2D) or flagellin (Fig. 2E) in the presence of 5 μM CATH-2 or LL-37. While LPS-induced TNFα production was potently inhibited by both CATH-2 and LL-37, flagellin-induced activation was unaffected by either peptide.

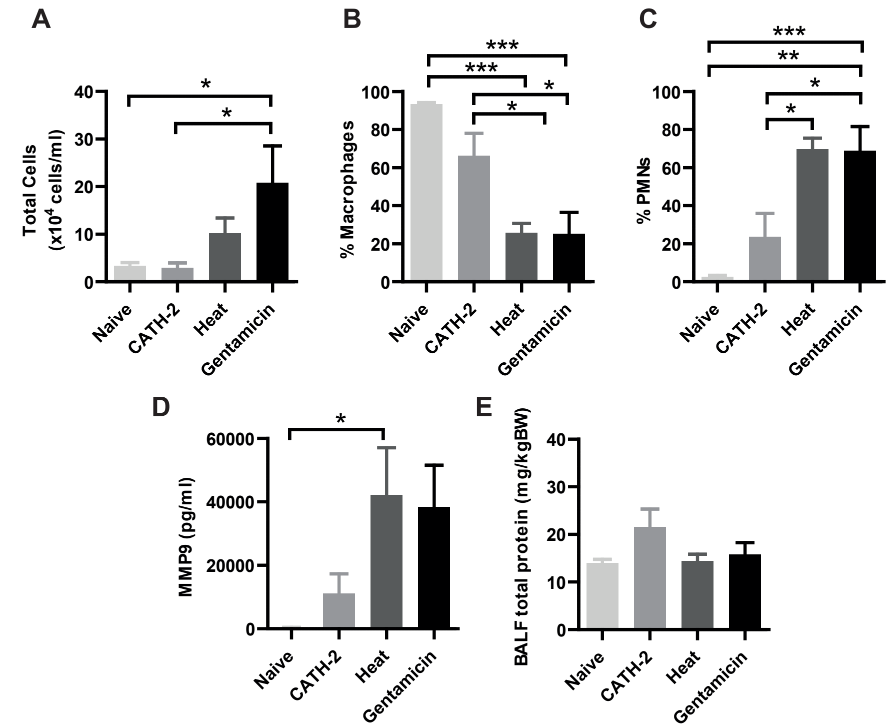


Figure 3: CATH-2-mediated killing prevents *in vivo* lung inflammation. Male C57Bl/6 mice were intratracheally instilled with 50 μl 2x10^6 CFU/ml *P. aeruginosa*, which was either CATH-2-killed (20 μM), gentamicin-killed (1 mg/ml) or heat-killed (90 °C, 1h). Alternatively, control mice were instilled with an air bolus. After 6h, (A) total cell counts in BALF, as well as (B) macrophage and (C) PMN percentages were determined by flow cytometry. In addition, (D) MMP-9 concentrations in BALF and (E) total protein concentration in BALF were determined. N = 5 or more ± SEM. Statistical differences were determined by One-way ANOVA with Bonferroni's Post-Hoc test. * = $p < 0.05$; ** = $p < 0.01$; *** = $p < 0.001$.

CATH-2 inhibits *P. aeruginosa*-induced *in vivo* inflammation

While the results described above show that CATH-2 is able to inhibit *in vitro* macrophage activation against *P. aeruginosa*, it is unknown whether this inhibitory effect is maintained in an *in vivo* setting. To determine whether this is the case, heat-killed, gentamicin-killed or CATH-2-killed *P. aeruginosa* (2x10^6 CFU/ml) was instilled in mouse lungs for 6h, after which lung function was assessed and leukocyte numbers,

cytokine/chemokine release and total protein content were determined in BALF. Lung compliance and elastance were determined to assess whether the different experimental conditions would affect lung function, however, no significant changes were observed (Fig. S1A-B). Analysis of total cell numbers in the BALF showed that killing of *P. aeruginosa* by gentamicin resulted in the highest cell count (Fig. 3A). Treatment with heat-killed bacteria also increased cell numbers in the BALF, albeit not significantly, and no change in total cell numbers was observed after treatment with CATH-2-killed *P. aeruginosa*, as compared to naïve animals. In both the heat-killed and gentamicin-killed treatment group, polymorphonuclear cells (PMNs) were the main cell type in BALF, while macrophages remain the largest portion of cells in the naïve mice and mice treated with CATH-2-killed bacteria (Fig. 3B-C), although the mice treated with CATH-2-killed bacteria did show a non-significant increase in PMNs compared to the naïve mice. The number of PMNs in the treatment groups correlated with the higher matrix metallopeptidase 9 levels measured in the BALF (Fig. 3D), which has previously been linked to PMN recruitment (19). Furthermore, no changes in the BALF protein content were detected after treatment with CATH-2-killed, heat-killed or gentamicin-killed bacteria, although there was a tendency of higher protein levels in the group that received CATH-2-killed bacteria (Fig. 3E).

To further examine the extent of inflammation in the lung, multiplex analysis was performed on various pro- and anti-inflammatory cytokines, as well as various chemokines. Both heat-killed and gentamicin-killed bacteria induced the release of pro-inflammatory cytokines TNFα (Fig. 4A) and IL-6 (Fig. 4B), while gentamicin-killed bacteria also significantly induced IL-23p19 (Fig. 4C) and IL-12p70 release (Fig. 4D) into the BALF. Treatment with CATH-2-killed bacteria resulted in significantly lower concentrations of TNFα, IL-6, IL-23p19 and IL-12p70 compared to treatment with gentamicin-killed bacteria and did not induce a significant increase of these cytokines compared to naïve mice (Fig. 4A-D). Similar induction patterns were observed for G-CSF (Fig. 4E), KC (Fig. 4F) and MIP-2 (Fig. 4G), with gentamicin-killed *P. aeruginosa* being the strongest inducer of cytokine release, followed by heat-killed *P. aeruginosa*. CATH-2 killed *P. aeruginosa* values were close to naïve mice and significantly lower than cytokine release induced by gentamicin-killed *P. aeruginosa*. Furthermore, IL-33 was only significantly increased in the gentamicin-killed treatment group (Fig. 4H). Other cytokines levels, including IL-1β, IL-4, IL-10 and MCP-1 levels (Fig. S2A-D), remained low and did not show any significant changes.

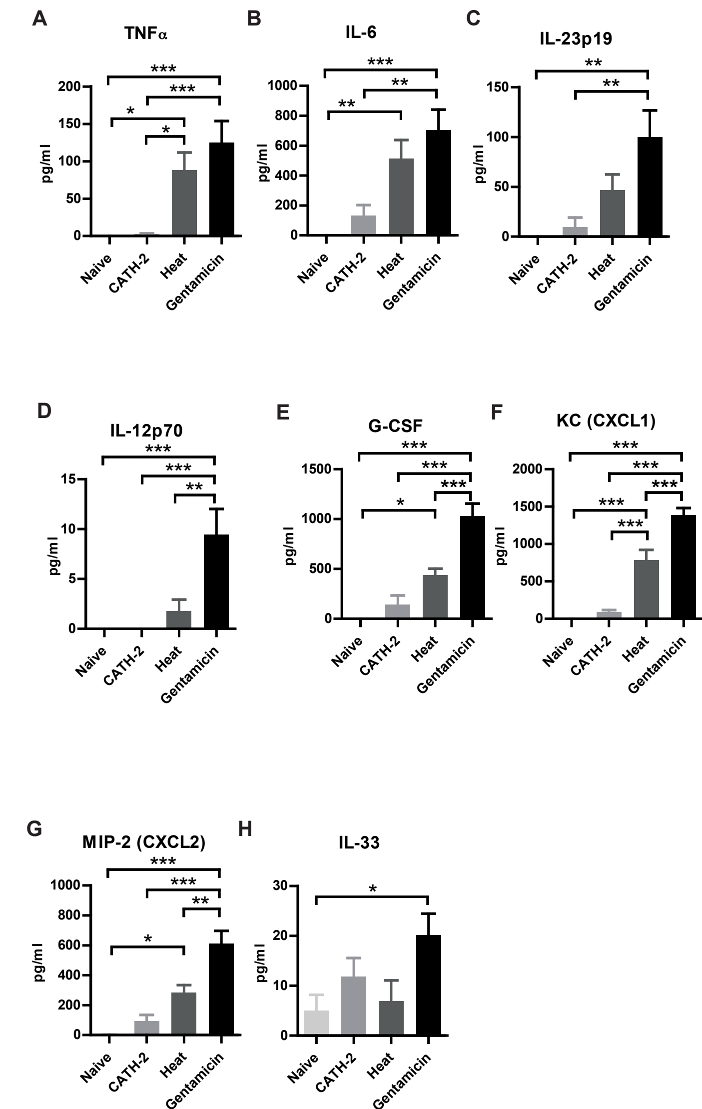


Figure 4: Effect of CATH-2-mediated killing on cytokine/chemokine release *in vivo*. Male C57Bl/6 mice were instilled with 50 µl $2x10^6$ CFU/ml *P. aeruginosa*, which was either CATH-2-killed (20 µM), gentamicin-killed (1 mg/ml) or heat-killed (90 °C, 1h). Alternatively, control mice were instilled with an air bolus. After 6h, (A) TNFα, (B) IL-6, (C) IL23p19, (D) IL-12p70, (E) G-CSF, (F) KC, (G) MIP-2 and (H) IL-33 were determined by Luminex. N = 5 or more ± SEM. Statistical differences were determined by One-way ANOVA with Bonferroni's Post-Hoc test. * = $p < 0.05$; ** = $p < 0.01$; *** = $p < 0.001$.

Discussion

CATH-2 has previously been shown to cause "silent killing" of *E. coli* and *S. enteritidis*, i.e. kill bacteria in a non-immunogenic manner (Chapter 5). This study provides evidence that silent killing *in vitro* by CATH-2 is not restricted to *E. coli* and also occurs against *P. aeruginosa*, a clinically relevant lung pathogen. In addition, our results provide evidence that CATH-2-mediated killing of *P. aeruginosa* also inhibits pulmonary inflammation in a mouse lung model by reducing PMN recruitment and preventing the release of pro-inflammatory cytokines and chemokines. Based on this data it is concluded that CATH-2 kills Gram-negative bacteria in an immunogenically silent manner, limiting inflammation both *in vitro* and *in vivo*.

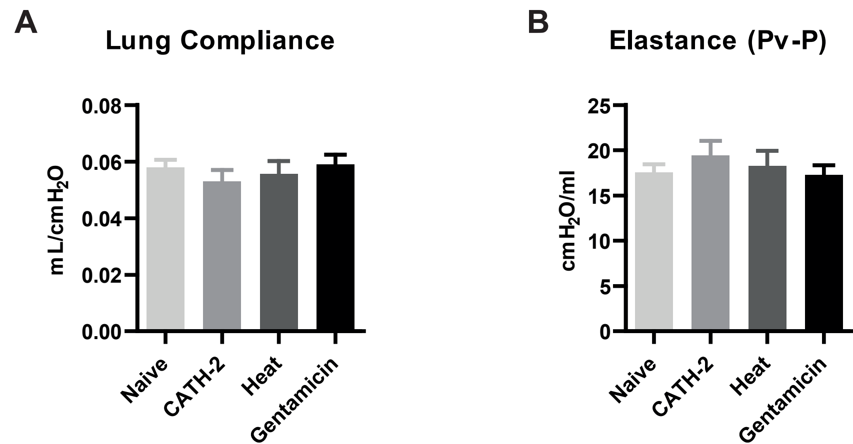
While CATH-2 potently inhibits *P. aeruginosa*-induced macrophage activation, it also strongly inhibits TLR4 activation by *P. aeruginosa* LPS. This is in line with our previous study, which shows that silent killing of *E. coli* by CATH-2 is a two-step process, in which CATH-2 first kills *E. coli* and subsequently neutralizes LPS from the bacterial outer membrane to inhibit TLR4 activation (Chapter 5). In addition, our results show that while LL-37 is unable to kill *P. aeruginosa* under cell culture conditions, it is able to inhibit macrophage activation by gentamicin-treated *P. aeruginosa* and also inhibits TLR4 activation by *P. aeruginosa* LPS. This corresponds to the previously reported lack of antimicrobial activity of LL-37 against *E. coli* under cell culture conditions and the inhibition of macrophage activation by LL-37 in the context of non-viable *E. coli* only (20) (Chapter 5). Together, this strongly suggests that both CATH-2 and LL-37 inhibit *P. aeruginosa*- and *E. coli*-induced macrophage activation through a similar mechanism, but that only CATH-2 has the dual function of both killing Gram-negatives and subsequently inhibiting macrophage activation.

The anti-inflammatory effect of CATH-2 on TLR4 activation is a strong potential benefit for the development of cathelicidin-based anti-infective therapies for CF patients. In CF patients, TLR4-mediated immune activation plays an important role in inflammation during *P. aeruginosa* infections. This is partially caused by adaptations of *P. aeruginosa* to the environment of CF patient's lungs. It has been shown that *P. aeruginosa* modifies its LPS from a penta- to a hexa-acylated form, which is more potent in the activation of TLR4 (4, 21-23). In addition, regulation of immune activation, including TLR4 activation in alveolar macrophages and epithelial cells, appears to be dysregulated in CF patients, in part due to the lack of a functional cystic fibrosis transmembrane conductance regulator (CFTR) (24-26). This dysregulation includes the lack of proper TLR4 degradation in lysosomal compartments (27), as well as a lack of negative feedback upon TLR4 activation (28-30). Together, this ultimately causes a higher inflammatory response in the lungs of CF patients. Since CATH-2 has a dual function of both killing *P. aeruginosa* as well as inhibiting TLR4 activation, treatment of *P. aeruginosa* infections in CF patients with CATH-2 (or CATH-2-derived compounds) can potentially reduce both bacterial numbers as well as limit inflammation in the lung.

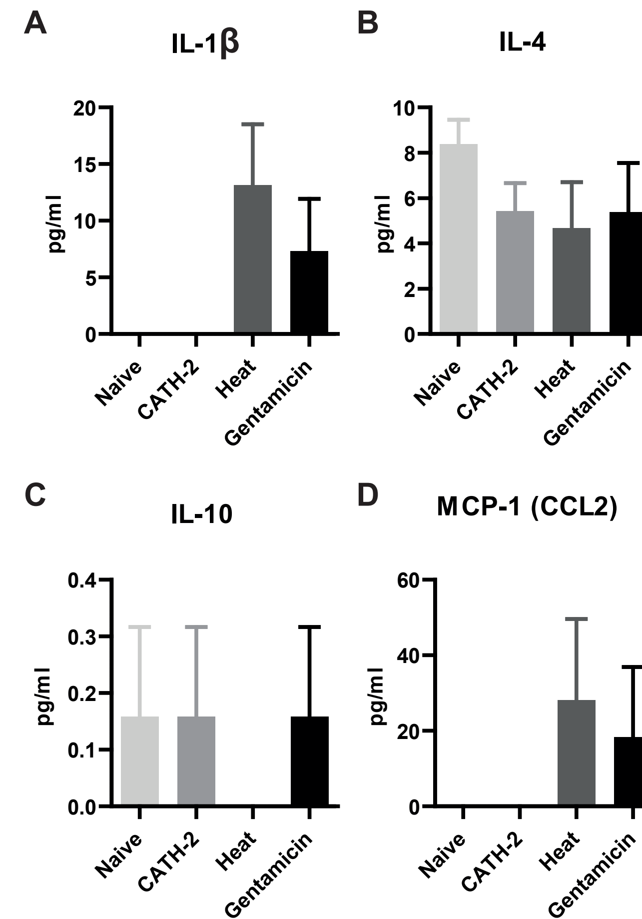
Another important characteristic of CATH-2 for anti-infective drug development is its antimicrobial activity under complex conditions, which includes solutions containing salt and serum components or bovine lipid extract surfactant (31) (Chapter 3). Furthermore, CATH-2 has broad-spectrum antimicrobial activity, which includes activity against MDR *P. aeruginosa* (15), as well as activity against *S. aureus*, which is another common infectious pathogen in CF patients (32). Similar to the killing of *E. coli* and *P. aeruginosa*, *S. aureus* is also killed by CATH-2 in complex environments (31) (Chapter 3). While no proof of silent killing of Gram-positives, such as *S. aureus*, is available yet, CATH-2 has been shown to inhibit macrophage TLR2 activation by *S. aureus*-derived LTA, suggesting silent killing might not be restricted to Gram-negatives (Chapter 3).

While our study has focused on silent killing, other studies have demonstrated additional cathelicidin effects that could be beneficial in the treatment of lung infections. For instance, while LL-37 is unable to directly kill *P. aeruginosa* under physiological conditions, a recent report showed that LL-37 can lower *P. aeruginosa* bacterial loads in a murine lung model, suggesting that indirect effects can also play an important role in bacterial clearance from the lung (33). Furthermore, CATH-2-derived peptides, as well as other cathelicidins, have anti-biofilm activity, which could be important because of the biofilm formation during chronic infections in CF patients (34-36). However, further research is needed to determine which of these functions will actually be beneficial for the treatment of infections and additional studies are required to understand the silent killing and potential other effects of CATH-2 in the context of an *in vivo P. aeruginosa* infection under CF conditions.

Overall, our results provide evidence for silent killing of a relevant lung pathogen by CATH-2. While silent killing by CATH-2 has been observed against *E. coli* and *S. enteritidis in vitro*, this is the first study that shows that CATH-2-mediated killing of *P. aeruginosa* leads to inhibition of inflammation *in vitro* as well as *in vivo*. Together with previous reports, these results underline the potential for CATH-2 as a template for the development of an anti-infective therapy, for instance for CF patients, with both antimicrobial and anti-inflammatory functions.



Supplemental Figure 1: Effect of CATH-2-treatment on lung function. Male C57Bl/6 mice were instilled with 50 µl 2x10^6 CFU/ml *P. aeruginosa*, which was either CATH-2-killed (20 µM), gentamicin-killed (1 mg/ml) or heat-killed (90 °C, 1h). Alternatively, control mice were instilled with an air bolus. After 6h, (A) lung compliance was determined and (B) lung elastance was calculated. N = 3 or more ± SEM. Statistical differences were determined by One-way ANOVA with Bonferroni's Post-Hoc test. * = $p < 0.05$; ** = $p < 0.01$; *** = $p < 0.001$.



Supplemental Figure 2: Effect CATH-2-mediated killing on cytokine/chemokine release *in vivo*. Male C57Bl/6 mice were instilled with 50 µl 2x10^6 CFU/ml *P. aeruginosa*, which was either CATH-2-killed (20 µM), gentamicin-killed (1 mg/ml) or heat-killed (90 °C, 1h). Alternatively, control mice were instilled with an air bolus. After 6h, (A) IL-1β, (B) IL-4, (C) IL-10 (D) and MCP-1 concentrations were determined by Luminex. N = 5 or more ± SEM.

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Chapter 7

General Discussion

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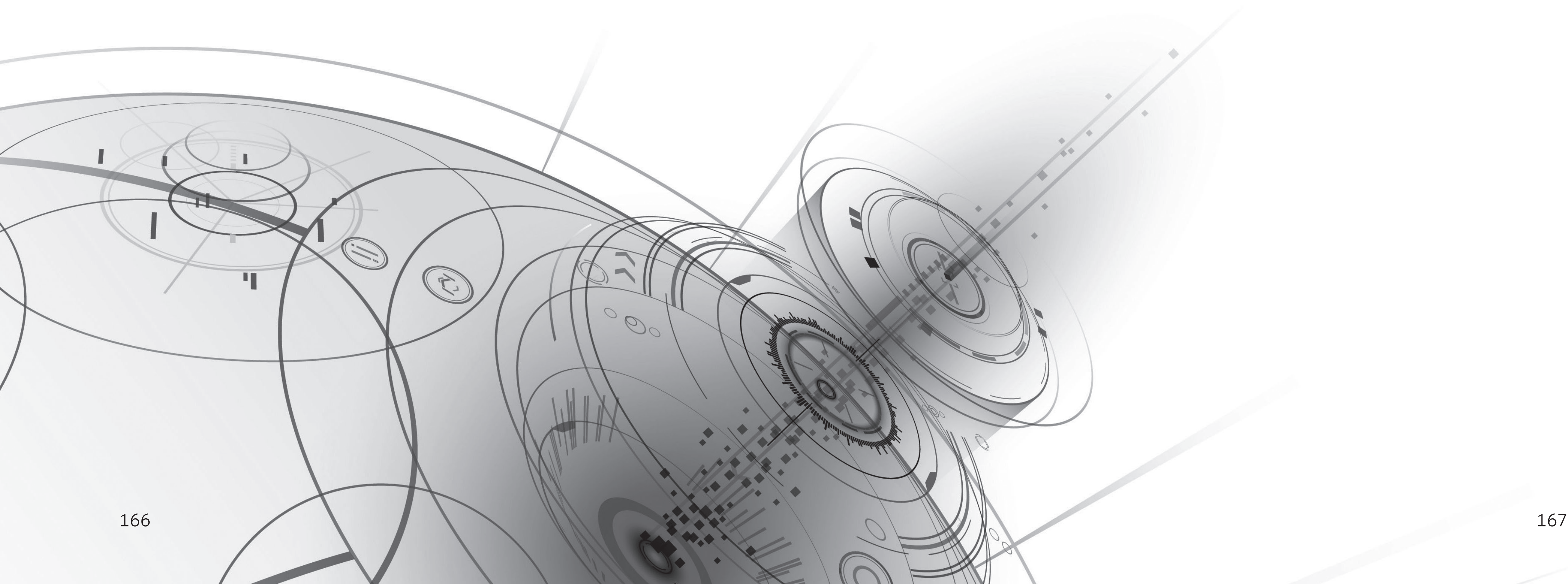

Cathelicidins are commonly accepted as important components of the innate host defense system in vertebrates and are considered crucial in the protection against pathogens in a wide variety of species (1). While these short cationic peptides were first identified as antimicrobial peptides, their functional repertoire has quickly broadened with various functions in the regulation of immune responses (2). One of the immunomodulatory effects of cathelicidins is the modulation of TLR activation. While first described in the context of TLR4, further research identified other TLRs of which the activation can be altered by cathelicidins, including mammalian TLR1 to 9 (3-9). Nevertheless, much still remains unclear about this TLR regulation by cathelicidins, especially in the context of complete bacteria. To this end, a macrophage stimulation setup was developed, as described in **Chapter 5**, where macrophages were incubated for 2h with viable *E. coli* in the presence of cathelicidins after which TNFα release was determined. The use of this setup had several advantages: 1) Complete and viable bacteria simultaneously present multiple MAMPs in their proper context, in contrast to single, purified or synthetic TLR ligands. 2) Because TNFα release by macrophages can be measured after 2h, activation can be determined without overgrowth of bacteria in the cell culture samples and without the influence of washing steps and post-incubation steps in gentamicin-containing media. 3) The system enables bacteria to defend themselves against the presence of cathelicidins. 4) By determining the bacterial viability for each sample, it is possible to correlate the effects of cathelicidins on bacterial viability and macrophage activation. 5) The antimicrobial activity is assessed under physiological cell culture conditions. With this setup, we demonstrated that cathelicidins inhibit *E. coli*-induced macrophage activation and that the inhibition is dependent on loss of *E. coli* viability. The following sections will expound on the various effects of cathelicidins on bacterial viability and macrophage activation and how these effects can play a role during infections.

Antimicrobial activity

Antimicrobial activity of cathelicidins under physiological conditions

It is notable that although CATH-2 and PMAP-36 retain their antimicrobial activity, most cathelicidins tested in **Chapter 3 and Chapter 5** showed a reduced antimicrobial activity against *E. coli* under physiological cell culture conditions in comparison to MHB. This is most likely caused by mono- and divalent cations and serum components present in the cell culture media, which are known to have a detrimental effect on the antimicrobial activity of cathelicidins. Ions, such as Na^+ and K^+, are thought to hamper the interaction between the negatively charged bacterial membrane and cationic peptides (10, 11), while cations, such as Ca^{2+} and Mg^{2+}, stabilize the bacterial outer membrane due to interaction with the negatively charged phosphate-groups in the core-region of the LPS molecules (12, 13). Furthermore, cathelicidins have been shown to interact with serum components, such as lipoproteins, which can also inhibit their antimicrobial activity (14, 15). On the other hand, carbonate, which is also present in cell culture media, has been shown to promote the bactericidal activity of antimicrobial peptides, including cathelicidins (16). Nevertheless, the balance of beneficial and detrimental factors for cathelicidin-mediated killing of *E. coli* appears to cause a general lower antimicrobial activity in cell culture media.

Even though antimicrobial activity against *E. coli* was reduced under cell culture conditions, this does not exclude a potential role for cathelicidin-mediated antimicrobial activity *in vivo*. Other host-derived antimicrobial components, such as lysozyme and lactoferrin, which utilize different antimicrobial mechanisms than cathelicidins, have been shown to act synergistically with LL-37 in bacterial killing (17). It will be interesting to see whether other bactericidal components from the innate immune system, such as reactive oxygen species (ROS) or complement components, have a similar potential to synergize with LL-37. In addition, the antimicrobial activity of cathelicidins against *S. aureus* was enhanced in cell culture medium, suggesting that these conditions can also be beneficial for killing of certain bacterial strains. Of note, it has been shown that treatment of antibiotic resistant bacteria with a combination of cathelicidins, or with host defense peptides and antibiotics, improves bactericidal activity, which opens new doors for the development of cathelicidin-based anti-infective therapies (18-20).

Bacterial viability and inflammasome activation

The antimicrobial activity of cathelicidins, but also of other bactericidal components, can influence immune activation by bacteria in multiple ways. For instance, the results in **Chapter 5** demonstrate that loss of bacterial viability prevents the release of IL-1β. This could be caused by a lack of inflammasome activation against non-viable bacteria. Inflammasome activation induces the activation of caspase-1, which is important for the cleavage of pro-IL-1β into mature secretable IL-1β (21). The inflammasome can be activated by several cytosolic receptors, including various receptors for nucleic acids (22), NAIP5 and NLRC4 for the detection of cytosolic flagellin (23-26) and NAIP2 for the detection of Type III secretion system-derived rod proteins (27). Because these receptors are located in the cytosol, their activation depends on the active delivery of these components to the cytosol. This can be done, for instance, through Type III and Type IV secretion systems or via delivery by outer membrane vesicles (23, 28-31). The loss of these active delivery systems in non-viable bacteria might prevent inflammasome activation by killed *E. coli* in **Chapter 5**. In addition, previous studies have shown that loss of viability in other bacteria also hampers IL-1β release, which could have a similar underlying cause (23, 32).

Regulation of TLR2 and TLR4 activation

Loss of *E. coli* viability not only prevents IL-1β release, but is also important for cathelicidin-mediated inhibition of TNFα and IL-6 release. In **Chapter 5**, it was demonstrated that this inhibition is the result of LPS and lipoprotein neutralization, which prevents TLR2 and TLR4 activation. In contrast, activation of TLR5 by *E. coli* was unaffected by cathelicidins.

Cathelicidins interact with LPS and lipoproteins

Inhibition of TLR2 and TLR4 activation has previously been described for LL-37 in the context of purified LPS and LTA (3, 9)**.** Inhibition of TLR4 activation has further been demonstrated in the context of different LPS sources, including *P. aeruginosa, E. coli*, *P. gingivalis* and *S. typhimurium* (5, 9, 33-36)**.** In **Chapter 3**, the inhibitory activity of LL-37 on TLR2 and TLR4 activation was confirmed and extended with the demonstration of LPS and LTA neutralization by various other cathelicidins from different species, including canine K9CATH and porcine PMAP-36. However, to our knowledge, the TLR2 and TLR4 inhibition observed in **Chapter 5** is the first description of inhibition of these receptors in the context of complete bacteria. This inhibition was caused by direct interaction between the cathelicidins and LPS and lipoproteins, as macrophage pre-incubations with cathelicidins were ineffective in inhibiting TNFα release upon stimulation with *E. coli*. In addition, by using isothermal titration calorimetry, CATH-2 and LL-37 were shown to directly interact with *E. coli* LPS and Pam3CSK4. These results are in line with a previous study demonstrating the binding of LL-37 and CATH-2 to *S. minnesota* LPS in a Polymyxin displacement assay (36) and a study demonstrating the interaction between LL-37 and lipoproteins in serum (15). Other cathelicidins, including canine K9CATH, guinea pig CAP11, chicken CATH-1 and sheep SMAP-29 have also been shown to interact with LPS either by NMR (37), LBP-competition assays (38) or the LAL assay (39-41).

Cathelicidin-*E. coli*-interaction

As shown in **Chapter 5**, the cathelicidin-mediated neutralization of LPS and lipoproteins depends on the loss of bacterial viability. This suggests that these components are only accessible to cathelicidins once bacteria are killed. However, LPS has been suggested as an important component on the surface of Gram-negatives for the initial interaction with cathelicidins and induction of bacterial killing. Our results in **Chapter 5** show that the killing of *E. coli* by CATH-2 correlated with the loss of inner membrane integrity. In addition, inhibition of macrophage activation only occurred at higher concentrations of CATH-2, suggesting that CATH-2 passes the outer membrane and permeabilizes the inner membrane before it can actually neutralize the OM LPS and lipoproteins. Furthermore, EM images showed that upon killing, CATH-2 induces the release of outer membrane fragments, which suggests that LPS and lipoproteins need to be released from the bacteria, before they can be neutralized by cathelicidins. This is further supported by recent findings by Schneider et al. (2016, submitted results), which show by immuno-EM that CATH-2 co-localizes with the released outer membrane fragments of *E. coli* upon killing by CATH-2.

In contrast to CATH-2, LL-37 was unable to kill *E. coli* under cell culture conditions. However, it was able to inhibit TLR2 and TLR4 activation when incubated with killed bacteria and direct interaction of LL-37 with LPS and lipoproteins was observed in **Chapter 5**. Nevertheless, even when bacteria are killed by gentamicin, which disrupts the bacterial OM (42, 43), LL-37 was unable to permeabilize the bacterial inner membrane, even though the OM LPS and lipoproteins are neutralized. Interestingly, a recent report on the antimicrobial activity of LL-37 has shown interaction between LL-37 and the bacterial outer membrane as well as the inner membrane (44). These experiments, however, are conducted under different conditions and at bactericidal concentrations of LL-37. This lack of activity of LL-37 on the IM could explain why LL-37 is unable to kill *E. coli* under cell culture conditions.

While LL-37 lacks IM permeabilizing activity, other bacterial defense mechanisms can also play a role in the inhibition of antimicrobial activity of LL-37. For instance, Gram-

negative bacteria can release outer membrane vesicles and capsule polysaccharides from the bacterial surface, which is a common mechanism to trap antimicrobial peptides (45-48). This could also explain the released components observed in the EM experiments after treatment of *E. coli* with LL-37. In addition, bacteria can use other active inhibitory mechanisms to prevent sustained interaction between antimicrobial peptides and the bacterial membrane. This includes degradation of peptides by proteinases (49) as well as modification of the LPS structure (50, 51). Furthermore, it has been suggested that binding of other host-derived components, such as C-reactive protein and LBP, is more efficient on dead bacteria than live bacteria (52, 53), suggesting these components are also actively removed from the surface of viable bacteria.

Overall, it appears that CATH-2 has the ability to penetrate the outer membrane of *E. coli* under physiological conditions and permeabilize the inner membrane to kill the *E. coli*. In contrast, while LL-37 might interact with the bacterial outer membrane, it lacks antimicrobial and inner membrane permeabilizing activity under physiological conditions, which appears to give the bacteria the opportunity to respond and remove LL-37 from the bacterial surface.

Silent killing of *P. aeruginosa* by CATH-2

Most of the results shown in this thesis describe effects of cathelicidins on macrophage TLR activation *in vitro*. To address the effects of CATH-2 on TLR activation *in vivo*, we made use of a *P. aeruginosa* murine lung inflammation model **(Chapter 6)**. Similar to the *in vitro* results on *E. coli* and *S. enteritidis*, CATH-2 was also able to silently kill *P. aeruginosa in vivo*, resulting in reduced neutrophil recruitment and cytokine/chemokine production compared to *in vivo* stimulation with heat-killed or gentamicin-killed bacteria. This suggests that silent killing and the inhibition of TLR4 activation also prevents inflammation in the context of the various cell types and environmental factors involved in the inflammatory response of the lung *in vivo*.

Silent killing of Gram-positives?

Since silent killing by CATH-2 appears to be a rather general phenomenon for Gram-negative bacteria, especially when TLR4 activation is important for their immunogenicity, it will be interesting to determine whether silent killing also applies to Gram-positive bacteria. Although we did not fully examine this within this thesis, **Chapter 3** does present some initial observations that suggest that silent killing by CATH-2 against Gram-positive bacteria could occur as well. First of all, CATH-2, as well as the other chicken cathelicidins, has very strong antimicrobial activity against *S. aureus* under cell culture conditions. Although salts and serum might still have an inhibitory effect on the killing of *S. aureus*, the presence of factors such as carbonate, and perhaps synergy with serum components, provides a better environment for killing of *S. aureus* compared to MHB conditions (10, 11, 14-16). In addition, CATH-2 was shown to inhibit *S. aureus* LTA-induced TLR2 activation. Although the role of LTA in Gram-positive infections is less clear than the role of LPS in Gram-negative infections, this compound is thought to be important for inflammation during Gram-positive infections (54, 55). Nevertheless, future studies will need to confirm whether CATH-2 also induces silent killing of Gram-positives.

Intracellular TLRs

While cathelicidin interaction with LPS and lipoproteins plays an important role in TLR2 and TLR4 activation, our results presented in **Chapter 3**, suggest that some cathelicidins also influence macrophage internalization processes. In our phagocytosis assay, six out of twelve cathelicidins were able to inhibit the phagocytosis of latex beads by RAW264.7 cells. In addition, four cathelicidins were able to significantly enhance activation of RAW264.7 cells by DNA, which activates the intracellular TLR9, and even more cathelicidins were able to enhance DNA-induced NO production in chicken HD11 macrophages, as described in **Chapter 4**. This suggests that cathelicidins have different effects on the internalization of different compounds by macrophages.

Intracellular cathelicidin degradation

Interestingly, while interaction between cathelicidins and extracellular TLR ligands appears to inhibit TLR activation, interaction between cathelicidins and intracellular TLR ligands appears to increase ligand internalization and thereby TLR activation. Similar to the cathelicidin-mediated increase in DNA-induced activation observed in **Chapter 3 and 4,** other studies have shown that LL-37 can enhance the intracellular activation of TLR3, TLR4, TLR7, TLR8 and TLR9. This has been linked to an increased uptake of LPS, RNA and DNA, due to direct interaction with LL-37 (4, 7, 8, 56, 57). The results presented in **Chapter 4** could explain why interaction between cathelicidins and intracellular TLR ligands does not hamper TLR activation, in contrast to the activation of extracellular TLRs. In **Chapter 4**, it is shown that CATH-2 directly interacts with extracellular DNA and promotes the uptake of the DNA in endosomal compartments. This is followed by endosomal acidification, which causes the degradation of CATH-2 by proteases and results in the release of DNA from the DNA/CATH-2-complex. This subsequently allows DNA to bind to TLR21 and induce cytokine gene expression and NO production. This process could also be important for the release of RNA or LPS in endosomal compartments to allow TLR activation. Of note, while our results in **Chapter 4** show that a lower pH does not strongly affect DNA-CATH-2 interaction, this has been suggested to play a role in the interaction between cathelicidins and RNA and subsequent activation of TLR3 activation (58).

Increasing TLR9 affinity for DNA

While cathelicidin degradation appears important for intracellular TLR activation, a recent report demonstrated that sustained interaction between cationic peptides and DNA does not necessarily lead to inhibition of TLR9 activation (59). In this report, it was shown that the structure and spacing between the DNA molecules upon complex formation with cationic peptides is crucial for the activation of TLR9. It appears that due to this specific spacing, affinity between the TLR9 and DNA/cationic peptide-complex is increased due to additional TLR-ligand interaction regions. In this model, LL-37 appears to be an excellent molecule for optimal spacing, which suggests that degradation of cathelicidins is not essential to increase TLR9 activation. However, this model was only applied to TLR9-induced IFNα production in pDCs, and sustained interaction between cathelicidins and DNA might only favor activation of this specific IFNα pathway. It has been suggested that signaling through TLR9 from early endosomes induces IFNα production, while signaling from late endosomes induces NF-κB activation (60). This could explain why macrophages, which induce faster endosomal acidification compared to DCs, increase NF-κB activation upon stimulation with DNA and cathelicidins, while slower acidification in pDCs might favor IFNα production (61-63).

Indirect TLR regulation

While interaction between cathelicidins and microbe-asssociated molecular patterns (MAMPs) plays an important role in regulation of TLR activation, as observed in **Chapter 4 and 5**, cathelicidins have also been suggested to indirectly affect TLR activation. Activation of TLR5 by flagellin has been shown to be enhanced in bronchial epithelial cells and keratinocytes by LL-37, resulting in higher IL-6 and CXCL8 production (6, 64, 65). This increase in TLR5 activation is caused by activation of two separate pathways by flagellin and LL-37, which has a synergistic effect on cytokine production. However, this effect appears to be cell type specific, as activation of TLR5 on monocytic cell types in our studies **(Chapter 5 and Chapter 6)**, as well as other studies, is not altered when cells are stimulated with flagellin in the presence of either CATH-2 or LL-37 (5, 66, 67). In addition, we were unable to detect any change in HEK-TLR5 activation by heat-killed *E. coli* in the presence of CATH-2 or LL-37.

Extrapolation to *in vivo* infections

While we have obtained much knowledge on cathelicidin functions *in vitro*, it is important to try to extrapolate these findings to *in vivo* scenarios in order to obtain a better understanding of their possible importance with respect to the physiological functions of cathelicidins, as well as to the potential effect of cathelicidin-based anti-infective therapies during infections. In the following section we discuss some of the discovered properties of cathelicidins with regard to the host response against a bacterial infection *in vivo*.

Starting an immune response

During infections, initial activation of the immune system occurs through the activation of pattern-recognition receptors (PRRs) (68), which are expressed on epithelial cells (69) and tissue resident macrophages (70). Activation of PRRs causes the release of pro-inflammatory cytokines, chemokines for neutrophil and monocyte recruitment, as well as cathelicidins and other potential antimicrobial components (71, 72). In addition to the antimicrobial function of cathelicidins, which can help to decrease the bacterial load at the site of infection and to prevent further bacterial spreading, cathelicidins can promote the recruitment of neutrophils (73). This is suggested to be the result of either direct binding to chemotactic receptors on neutrophils (74) or the local induction of chemokine expression (9). While both mechanisms could be of importance *in vivo*, our *in vitro* results in **Chapter 3** only show a limited increase of chemokine release from RAW264.7 cells, at relatively high peptide concentrations compared to the much higher quantities of chemokines released upon bacterial or LPS stimulation. Although this could be specific for the cell type tested in this study, other studies have shown similar results using other cell lines and cell types, including primary PBMCs (75-77). The other pathway for leukocyte recruitment involves direct binding to the formyl-peptide receptors on neutrophils (74). In a recent study, however, neutrophil recruitment to the lung after instillation with LL-37 in the absence of an infection was limited. Nevertheless, when LL-37 treatment was combined with a *P. aeruginosa* infection, higher neutrophil recruitment is observed within 6h compared to the infection group without increasing any of the measured chemokine levels (73). This suggests that induced neutrophil recruitment by LL-37 is more efficient in the context of an infection. In line with this observation, it has been shown that expression of the formyl-peptide-receptor on neutrophils can be increased upon stimulation with LPS (78). Since this is the receptor that has been implicated in the direct chemotactic activity of LL-37 for neutrophils (74), the upregulation of this receptor by pro-inflammatory molecules derived from pathogens or the host might increase the susceptibility to LL-37-mediated recruitment of neutrophils to the site of infection.

Bacterial clearance

With the recruitment of neutrophils, a new wave of antimicrobial factors arrives at the site of infection. These include ROS, RNS, pro-inflammatory cytokines and granular components, such as lysozyme, lactoferrin and cathelicidins, which promote the killing of extracellular and phagocytosed bacteria (79, 80). Although all these components and

antimicrobial mechanisms are aimed at killing the microbe, this response at the same time endangers the host by causing tissue damage if no adequate anti-inflammatory response is started (71, 79). At this stage, the inhibitory effects of cathelicidins against neutralized bacteria described in **Chapter 5 and 6** can be useful to prevent any additional and unnecessary inflammation against already killed pathogens. This allows for a balanced inflammatory response and can prevent much of the potential tissue damage due to excessive inflammation.

Resolution of inflammation

While the cathelicidins can function as part of a negative feedback mechanism during the inflammatory response, they can also play a role in the subsequent recruitment of monocytes to the site of infection to start the clearance of microbial and cellular debris (74, 81). Furthermore, it has been shown that CRAMP and LL-37 can promote macrophage differentiation, which can be important in the resolution of inflammation (82-84). Interestingly, LL-37 has also been shown to indirectly promote phagocytosis of opsonized bacteria, which could also promote the resolution of inflammation and return to a steady state (85). In contrast, our results in **Chapter 3** on the internalization of latex beads mainly showed inhibitory effects by several cathelicidins, which demonstrates the complexity of phagocytosis of different components due to the many receptors that can play a role in bacterial uptake (86).

Overall, the results presented in this thesis, together with previously described effects, further clarify the functions of cathelicidins during infections, where cathelicidins appear to play a role throughout the entire inflammatory process, from recruitment of leukocytes to the resolution of inflammation.

Therapeutic potential

While the biological functions of cathelicidins are very interesting, the observed mechanisms of inhibition of bacteria-induced inflammation can also be useful from a therapeutic point of view. Especially for CATH-2, which retains much of its antimicrobial activity under physiological conditions, its dual mode-of-action can help in both clearing infections as well as preventing excessive inflammation.

Cathelicidins as dual-mode-of-action anti-infectives

During Gram-negative infections, LPS is an important trigger for inflammation (87). However, while the infection-related inflammation is necessary for the clearance of the infection, excessive inflammation can result in sepsis and can cause tissue damage, organ dysfunction and death (88). Even when patients survive the initial phase of sepsis, there is the risk of Compensatory Anti-Inflammatory Syndrome (CARS) development, which is characterized by a systemic anti-inflammatory phenotype (89) that increases the patient's susceptibility to infections (90). Development of CATH-2-based anti-infectives might prove useful to both kill the infectious microbes and at the same time prevent excessive inflammation due to the inhibition of immune activation by the neutralized bacteria, in contrast to several other antibiotics, which are thought to increase inflammation due to the induction of LPS release from the bacterial membrane upon killing (91).

A specific case where CATH-2-based anti-infectives could be useful is in the treatment of infections in cystic fibrosis (CF) patients. In these patients, treatment of recurrent *P. aeruginosa* infections with antibiotics promotes antibiotic resistance and leads to chronic *P. aeruginosa* infections. These infections result in chronic inflammation, an important cause of tissue damage, lung dysfunction and ultimately death in CF patients (92-95). Treatment of these infections with CATH-2-based anti-infectives could prove beneficial. First of all, CATH-2 is able to kill *P. aeruginosa* under complex and physiological conditions **(Chapter 6)** (96). Secondly, CATH-2 can limit inflammation induced by *P. aeruginosa* LPS, which has been implicated to induce a strong inflammatory response in CF patients (97-100). Finally, while *P. aeruginosa* is adapting to more of the currently used antibiotics, the low induction of resistance formation against CATH-2 by Gram-negative bacteria, could be another useful characteristic of CATH-2-based anti-infective therapy (101). However, it should be noted that resistance development should be carefully assessed as LL-37 has been shown to potentially cause mutagenesis in *P. aeruginosa* (102).

Cathelicidins in vaccination therapies

In addition to the usefulness as an anti-infective, cathelicidins might prove useful as adjuvants in the development of vaccination therapies. Other host defense peptides have already been shown to increase vaccination efficiency in combination with CpG-DNA (103-107). Although no data is available on the effect of these specific peptides on DNA uptake or DNA-induced inflammation, part of the enhanced vaccination efficiency could come from enhancing DNA-induced immune activation, similar to the effect observed for CATH-2 in **Chapter 4**. This is supported by a study on the human β-defensin-3, which increased pro-inflammatory cytokine production *in vivo* when combined with CpG-DNA (108). In addition to the enhancement of DNA-induced immune activation, other cathelicidin effects such as leukocyte recruitment or regulation of leukocyte differentiation could be an additional benefit in reaching and activating the right cell types to increase vaccination efficiency (109, 110).

Besides the effects of CATH-2 on DNA-induced activation, CATH-2 could also affect the activity of other adjuvants. If, for instance, a lipid-containing TLR ligand is used as adjuvant, such as monophosphoryl lipid A (MPLA), immune activation could be inhibited instead of enhanced in the presence of cathelicidins. On the other hand, in situations where LPS toxicity might be a concern, for instance in the context of vaccinations with outer membrane vesicles, this inhibitory effect might be beneficial (111, 112). Furthermore, specific cathelicidin modifications can alter the effects on TLR activation, which can be useful in the development of novel therapeutics. For instance, truncations of CATH-2 or amino-acid substitutions can prevent LPS-neutralization (36) or DNA-induced immune activation, while use of D-amino acids cathelicidins can completely inhibit DNA-induced responses **(Chapter 4)**. As many cathelicidins appear to have a distinct functional repertoire, as shown in **Chapter 3**, screening of these properties can be used for the selection of templates for the development of novel anti-infective therapies or vaccination adjuvants that have specific functions desirable for the specific therapy.

Concluding remarks

Although first described as antimicrobial peptides, cathelicidins have been shown to play a crucial role in various inflammatory processes and are indispensable for optimal protection against infections (113-115). Increasing our knowledge about their mechanisms of action and structure-function relationships will help to further understand the complex role of these intriguing peptides in the defense against infections.

With the work presented in this thesis, we aimed to identify the mechanisms behind the cathelicidin-mediated regulation of TLR activation to better understand the physiological role of cathelicidins during infections as well as to obtain insight in the possible use of cathelicidins for the development of anti-infective therapies.

Abbreviations

CARS, compensatory anti-inflammatory syndrome; **CF**, cystic fibrosis; **CpG**, cytosine-guanine; **DC**, dendritic cell; **EM**, electron microscopy; **IM**, inner membrane; **LAL**, limulus amebocyte lysate; **LBP**, LPS-binding protein; **LPS**, lipopolysaccharide; **LTA**, lipoteichoic acid; **MAMP**, microbe-associated molecular pattern; **MHB**, Mueller Hinton broth; **MPLA**, monophosphoryl lipid A; **NAIP**, NLR family, apoptosis inhibitory protein; **NLRC4**, NLR family, CARD-containing 4; **NO**, nitric oxide; **OM**, outer membrane; **PBMC**, peripheral blood mononuclear cell; **PRR**, pattern-recognition receptor; **RNS**, reactive nitrogen species; **ROS**, reactive oxygen species; **TLR**, Toll-like receptor.

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Appendices

Nederlandse samenvatting

Cathelicidines zijn kleine eiwitten, oftewel peptiden, die een belangrijke rol spelen in het afweermechanisme van een groot aantal verschillende gewervelde dieren. Cathelicidines worden gemaakt door immuuncellen en epitheelcellen op de plaats van infectie, waar ze helpen in de immuunreactie tegen o.a. bacteriële infecties. De eerste beschrijvingen van cathelicidines gingen met name over de antibacteriële activiteit van deze peptiden. In deze eerste studies werd aangetoond dat cathelicidines sterke antibacteriële activiteit hebben tegen Gram-positieve en Gram-negatieve bacteriën. Latere studies hebben echter aangetoond dat cathelicidines ook een belangrijke rol kunnen spelen in het reguleren van de immuunreactie tijdens infecties. Dit houdt onder andere in dat ze kunnen zorgen voor migratie van immuuncellen naar de plek van infectie en dat ze de activatie van deze immuuncellen kunnen reguleren.

Immuuncellen kunnen worden geactiveerd door de aanwezigheid van microbe-gerelateerde moleculaire patronen (MAMPs). Dit zijn componenten die op of in bacteriën, schimmels of virussen voorkomen en kunnen worden herkend door zogenaamde Toll-like receptoren (TLRs). TLRs zijn receptoren die op het oppervlak of in intracellulaire compartimenten van cellen zitten en specifieke MAMPs kunnen binden. Elke TLR heeft eigen MAMPs die worden herkend, zoals TLR4, die lipopolysacchariden (LPS) kan herkennen die in het buitenmembraan van Gram-negatieve bacteriën zitten, of TLR9, welke DNA kan herkennen dat in Gram-positieve en Gram-negatieve bacteriën zit. Als een MAMP wordt herkend door een TLR zorgt dit voor een reeks aan intracellulaire reacties die uiteindelijk leiden tot de productie van pro-inflammatoire cytokinen en antibacteriële componenten. Verschillende studies hebben aangetoond dat de activatie van deze TLRs door hun specifieke MAMPs kan worden beinvloed door de aanwezigheid van cathelicidines. In dit proefschrift is het effect van cathelicidines op de activatie van verschillende TLRs door verschillende MAMPs verder beschreven.

Hoewel er al veel bekend is over de verschillende functies van cathelicidines, zijn de beschrijvingen vaak beperkt tot de functies van het humane LL-37 en in mindere mate het muizen CRAMP. De functies die wel zijn onderzocht voor andere cathelicidines worden vaak maar bekeken in de context van één of twee cathelicidines en het is onduidelijk hoe sterk of zwak deze functies zijn in vergelijking met andere cathelicidines. Daarnaast worden vaak verschillende condities gebruikt bij het bepalen van veel functies, zoals verschillende groeimedia voor bacteriën, verschillende soorten LPS om naar de effecten op TLR activatie te kijken en verschillende cathelicidine concentraties. Om een beter overzicht te krijgen welke functies daadwerkelijk goed bewaard zijn gebleven tussen cathelicidines van verschillende dieren, hebben we in **Hoofdstuk 3** de activiteit van twaalf verschillende cathelicidines uit zes verschillende diersoorten getest voor een aantal veel beschreven functies. Uit deze experimenten blijkt dat vooral antibacteriële activiteit en de remming van TLR4 activatie door LPS functies zijn die voorkomen bij veel verschillende cathelicidines. Daarnaast blijkt ook dat, hoewel eerder beschreven is dat fysiologische omstandigheden vaak een negatief effect hebben op de antibacteriële activiteit van cathelicidines, dit niet het geval is voor de Gram-positieve *S. aureus*. Deze bacterie wordt juist beter gedood onder fysiologische omstandigheden dan onder de standaard bacteriekweek condities. Daarnaast blijkt ook dat de productie van chemokinen in macrofagen in ons model niet sterk wordt beïnvloed, hoewel dit eerder wel beschreven is. Een interessante en belangrijke observatie is ook dat alle cathelicidines een andere set aan functies lijken te hebben. Zo bleek K9CATH (hondenpeptide) bijvoorbeeld geen antibacteriële activiteit tegen *S. aureus* of *E. coli* te hebben en kon eCATH-1 (paardenpeptide) de activatie van TLR2 door lipoproteïnen of activatie van TLR4 door LPS niet voorkomen. Samen geven deze resultaten weer dat men voorzichtig moet zijn met het generaliseren van cathelicidine functies, omdat deze sterk van elkaar kunnen verschillen. Dit houd ook in dat men voorzichtig moet zijn met het extrapoleren van de effecten die zijn verkregen met het gebruik van muizen die geen cathelicidine tot expressie brengen, omdat deze effecten niet representatief hoeven te zijn voor de functie van andere cathelicidines.

Bij het vergelijken van de verschillende functies van cathelicidines in **Hoofdstuk 3**, blijkt dat CATH-2 activatie van TLR9 door DNA kan versterken. Deze invloed op TLR9 activatie kan van belang zijn tijdens immuunreacties tegen infecties, wanneer DNA kan vrijkomen tijdens het doden van bacteriën of door schade aan cellen van de gastheer. Daarnaast wordt DNA ook gebruikt als adjuvans tijdens vaccinaties om een betere response te krijgen tegen het antigeen waarmee wordt gevaccineerd. Om meer inzicht te krijgen in de rol van CATH-2 in DNA-geïnduceerde activatie van kippenmacrofagen, is in **Hoofdstuk 4** onderzocht wat het mechanisme achter deze versterkte activatie is. De eerste resultaten in dit hoofdstuk tonen aan dat het effect onafhankelijk lijkt van het soort DNA, aangezien het optreedt bij zowel enkelstrengs als dubbelstrengs DNA. Verder blijkt dat CATH-2 direct aan het DNA kan binden, wat er voor lijkt te zorgen dat er meer DNA wordt opgenomen in de macrofagen. Het DNA dat door de macrofaag wordt opgenomen komt terecht in kleine compartimenten in de macrofaag die endosomen worden genoemd. In deze endosomen is ook TLR21 te vinden, de TLR in kippen die DNA herkent. Als het DNA aan TLR21 bindt zorgt dit voor activatie van de macrofaag en meer pro-inflammatoire cytokineproductie. Een interessante observatie in dit proces is dat activatie van macrofagen door DNA wordt geremd door een D-aminozuur analoog van CATH-2, terwijl de DNA opname in deze macrofagen wel versterkt wordt. Dit kan worden verklaard omdat D-aminozuur peptiden erg stabiel zijn en niet worden afgebroken door proteases die aanwezig zijn in endosomen. Dit zou ervoor kunnen zorgen dat de DNA/D-CATH-2-complexen niet worden afgebroken, waardoor het DNA niet vrijkomt en er geen TLR21 activatie plaats kan vinden. Om dit aan te kunnen tonen werd gebruik gemaakt van DNA gelabeld met een fluorescent molecuul en CATH-2 gelabeld met een molecuul dat deze fluorescentie kan doven. Op het moment dat het DNA dan aan CATH-2 is gebonden, wordt de fluorescentie van het DNA gedoofd, terwijl dit niet het geval is als er geen interactie is tussen het DNA en CATH-2. Door het gebruik van deze techniek kon inderdaad worden aangetoond dat in de aanwezigheid van het natuurlijke CATH-2, fluorescentie kan worden waargenomen in de cel (CATH-2 is dus afgebroken en is niet meer gebonden aan het DNA). Daarnaast kon ook worden aagetoond dat deze fluorescentie niet meer zichtbaar is in aanwezigheid van het D-aminozuur CATH-2. Samen tonen deze resultaten aan dat het proces van DNA opname en vervolgens afbraak van CATH-2 in endosomen cruciaal is voor de versterkte activatie van DNA-geïnduceerde macrofaag activatie.

In **Hoofdstuk 3 en 4** wordt beschreven hoe cathelicidines de activatie van TLRs kunnen beïnvloeden. Dit kan zowel negatief zijn, zoals bij TLR2 activatie door lipoproteïnen en TLR4 activatie door LPS, maar ook positief, zoals bij TLR9 en TLR21 activatie door DNA. Echter, tijdens een infectie zijn meerdere TLR liganden tegelijk aanwezig en tot op heden was het onbekend wat de netto uitkomst is van de regulatie van TLRs door cathelicidines in de aanwezigheid van een complete bacterie. In **Hoofdstuk 5** wordt beschreven wat de invloed is van cathelicidines op immuunactivatie in de aanwezigheid van *E. coli*, een Gram-negatieve bacterie. Om dit te testen werd *E. coli* gebruikt om marofagen te stimuleren in de aanwezigheid van CATH-2, waarna de activatie van de macrofagen werd gemeten door de productie van cytokinen te meten. Tegelijkertijd werd ook de levensvatbaarheid van *E. coli* onderzocht, aangezien CATH-2 sterke antibacteriële activiteit heeft, zoals aangetoond in **Hoofdstuk 3**. Hierdoor kon worden vastgesteld dat er een sterke correlatie is tussen het doden van de *E. coli* door CATH-2 en remming van de activatie van de macrofagen. Daarnaast blijkt dat andere cathelicidines, waaronder het humane LL-37, *E. coli* niet konden doden onder de fysiologische celkweekcondities en ook activatie van de macrofagen niet konden voorkomen. Daarentegen bleek dat veel van deze cathelicidines wel de activatie van macrofagen remden als de *E. coli* was gedood door hitte of een antibioticum, voordat het werd gemengd met de cathelicidines en bij de macrofagen werd gevoegd. Hieruit kan worden opgemaakt dat veel cathelicidines wel de capaciteit hebben om macrofaag activatie door *E. coli* te voorkomen, maar dat dit afhankelijk is van de levensvatbaarheid van de *E. coli*.

De volgende stap is om te onderzoeken of inderdaad regulatie van TLR activatie een rol speelt in de geobserveerde effecten. Om dit te bekijken zijn speciale cellen gebruikt. Deze cellen, genaamd HEK-cellen, zijn zo gemodificeerd dat ze enkel de specifiek gewenste TLR of TLRs op het celoppervlak tot expressie brengen. Op deze manier kan worden nagegaan welke TLRs worden geactiveerd door *E. coli* en activatie van welke TLRs vervolgens ook door CATH-2 kan worden geremd. Door gebruik te maken van deze cellen, bleek dat *E. coli* TLR2, TLR4 en TLR5 kan activeren. Vervolgens kon ook worden vastgesteld dat CATH-2 en LL-37 de activatie van TLR2 en TLR4 kunnen remmen. Om te kijken of deze remming veroorzaakt werd door de directe binding van CATH-2 en LL-37 aan lipoproteïnen and LPS, welke respectievelijk TLR2 en TLR4 kunnen activeren, werd gebruikt gemaakt van isothermale titratie calorimetrie. Met deze techniek kan de warmte die vrijkomt tijdens de interactie tussen twee componenten worden gemeten met een uitzonderlijk gevoelige thermometer. Uitvoering van deze experimenten liet inderdaad zien dat CATH-2 en LL-37 aan lipoproteïnen en LPS kunnen binden.

De resultaten die hier zijn beschreven tonen aan dat cathelicidines een immuunmodulerende rol hebben tijdens infecties met Gram-negatieve bacteriën, zoals *E. coli*. Tijdens een *E. coli* infectie wordt het immuunsysteem geactiveerd door activatie van TLR2 en TLR4. Dit zorgt voor de productie van cathelicidines en andere antibacteriële componenten die de bacteriën moeten doden, maar tegelijkertijd ook enigzins schadelijk zijn voor de gastheer. De resultaten hierboven beschreven geven aan

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dat op het moment dat de bacteriën dood zijn, cathelicidines kunnen voorkomen dat er verdere activatie van TLR2 en TLR4 plaatsvindt. Dit zorgt ervoor dat op het moment dat de infectie onder controle is, ook de immuunreactie wordt geremd om schade aan gastheercellen te voorkomen. Aan de andere kant, als de bacteriën niet gedood kunnen worden door de gastheer, zullen cathelicidines de immuunreactie ook niet remmen, om te voorkomen dat de respons wordt gestopt voordat de infectie is overwonnen.

Deze bevindingen kunnen belangrijk zijn om de rol van cathelicidines tijdens de immuunreactie van de gastheer beter te begrijpen. Echter, deze resultaten zijn verkregen in celkweek modellen die gebruik maken van een beperkt aantal celtypen en plaatsvinden in enigzins artificiële omstandigheden. In **Hoofdstuk 6** zijn de effecten uit **Hoofdstuk 5** in een levend organisme dat een compleet en functioneel immuunsysteem heeft beschreven. In deze experimenten werden Gram-negatieve *P. aeruginosa* bacteriën gedood door hitte, antibiotica of CATH-2 en vervolgens geïnjecteerd in de longen van een muis. Daarna werd gemeten hoeveel immuuncellen (in dit geval monocyten en neutrofielen) na 6 uur in de long aanwezig waren en tevens werd gekeken naar de concentraties van pro-inflammatoire cytokinen in de long. Analyse van deze parameters liet zien dat er nauwelijks een respons plaatsvond wanneer CATH-2 *P. aeruginosa* had gedood, in tegenstelling tot *P. aeruginosa* die was gedood door hitte of antibiotica, welke zorgden voor veel neutrofielen in de long en ook voor een hoge productie van cytokinen. Samen met de bevindingen uit **Hoofdstuk 5** kan worden gesteld dat CATH-2 een "sluipmoordenaar" is, wat inhoudt dat CATH-2 bacteriën kan doden en tegelijkertijd kan voorkomen dat componenten van de bacterie het immuunsysteem kunnen stimuleren. Dit kan onnodige ontsteking en mogelijke weefselschade voorkomen. Deze resultaten zijn niet alleen informatief om immuunactivatie tijdens infecties beter te begrijpen, maar kunnen ook nuttig zijn voor de ontwikkeling van nieuwe cathelicidine-gebaseerde antibiotica die én antibacterieel zijn én kunnen voorkomen dat er buitensporige immuunactivatie optreedt.

Dankwoord

Hierbij wil ik ook graag een aantal mensen bedanken voor hun hulp tijdens mijn promotie onderzoek. Ten eerste wil ik Henk bedanken voor zijn vertrouwen om mij aan dit project te laten beginnen en zijn begeleiding gedurende de afgelopen jaren. Daarnaast wil ik ook graag Albert en Edwin bedanken voor hun begeleiding en hulp tijdens mijn promotieperiode en Edwin in het bijzonder voor zijn slechte humor…

Verder wil ik ook alle andere mensen uit de Molecular Host Defence groep heel erg bedanken voor hun hulp tijdens experimenten, de hulpvolle bijdragen tijdens werkbesprekingen, de gezellige uitjes en de vrije en open sfeer op de afdeling, waardoor ik een hele fijne werkplek heb gehad de afgelopen jaren.

Daarnaast wil ik ook de hele Infectiebiologie groep bedanken voor de gezellige lunches, labuitjes en opbouwende kritiek tijdens de WIP meetings.

Ook wil ik graag de mensen van de andere onderzoeksgroepen bedanken die bij hebben gedragen aan het onderzoek in deze thesis. Floris, Jerry, Marjolein, Marit, Brandon en Ruud, bedankt voor alle hulp en de fijne samenwerking. Daarnaast wil ik ook iedereen van Pathobiologie waarmee ik heb samengewerkt bedanken. Hélène, Steven, Erik, Geert, Alinda en Iresha, bedankt voor de hulp en alle input de afgelopen jaren.

Als laatste wil ik graag Vicky heel erg bedanken voor haar geduld en begrip de afgelopen jaren en zeker de afgelopen maanden en ik kijk er heel erg naar uit om ons nieuwe avontuur in Zweden tegemoet te gaan!

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Curriculum Vitae

Maarten Coorens werd geboren op 10 maart 1988 te Sittard. In 2006 behaalde hij zijn VWO diploma aan SG Groenewald en startte met de Bachelor Moleculaire Levenswetenschappen aan de Universiteit van Maastricht, wat werd vervolgd met de Master Oncology and Developmental Biology. Tijdens zijn studie heeft hij stage gelopen bij de afdeling Molecular Cell Biology aan de Universiteit van Maastricht, onder begeleiding van Jos Broers. Hier deed hij onderzoek naar intracellulaire membraan stabiliteit in cellen van laminopathie patiënten. Daarna heeft hij stage gelopen bij het MAASTRO lab, waar hij, onder begeleiding van Ludwig Dubois, onderzoek heeft gedaan naar tumor acidificatie. Zijn afsluitende stage heeft hij gelopen bij de afdeling Tumor Immunologie aan de Universiteit Maastricht. Onder begeleiding van Ans Houben en Joris Vanderlocht deed hij onderzoek naar het effect van tumor bestraling op immuunactivatie en uitgroei van metastasen. In 2011 haalde hij zijn diploma en startte als AIO bij de afdeling Molecular Host Defence aan de Universiteit Utrecht, om onderzoek te doen onder leiding van Henk Haagsman. De resultaten van dit onderzoek staan beschreven in dit proefschrift.

List of publications

Cuperus, T.*, **Coorens, M.***, A. van Dijk, and H. P. Haagsman. 2013. Avian host defense peptides. *Dev. Comp. Immunol*. 41: 352-369.

Coorens, M., A. van Dijk, F. Bikker, E. J. A. Veldhuizen, and H. P. Haagsman. 2015. Importance of Endosomal Cathelicidin Degradation To Enhance DNA-Induced Chicken Macrophage Activation. *J. Immunol.* 195: 3970-3977.

Coorens, M.*, M. R. Scheenstra*, E. J. A. Veldhuizen, and H. P. Haagsman. 2016. Interspecies cathelicidin comparison reveals divergence in antimicrobial activity, TLR modulation, chemokine induction and regulation of phagocytosis. Submitted.

Coorens, M., V. A. F. Schneider, A. M. de Groot, A. van Dijk, M. Meijerink, J. M. Wells, M. R. Scheenstra, E. J. A. Veldhuizen, and H. P. Haagsman. 2016. The innate immune system uses cathelicidins to discriminate between viable and non-viable Gram-negatives and to inhibit TLR2 and TLR4 activation. Submitted.

Coorens, M.*, B. J. H. Banaschewski*, E. J. A. Veldhuizen, R. A. W. Veldhuizen, H. P. Haagsman. 2016. Chicken cathelicidin-2-mediated killing of *P. aeruginosa* prevents lung inflammation in vivo. In preparation.

Coorens, M.*, V. A. F. Schneider*, J. L. M. Tjeerdsma-van Bokhoven, G. Posthuma, A. van Dijk, E. J. A. Veldhuizen, H. P. Haagsman. 2016. Visualization of the anti-staphylococci activity of chicken cathelicidin-2. In preparation.

Coorens, M., S. J. van Beurden, G. de Vrieze, S. R. Ordonez, E. Weerts, A. Berends, H. P. Haagsman, E. J. A. Veldhuizen, M. H. Verheije. 2016. Differences in anti-viral activity of human and avian cathelicidins against avian infectious bronchitis virus. In preparation.

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