Chicken respiratory infections

Protective roles of macrophages and cathelicidins

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Chicken respiratory infections

Protective roles of macrophages and cathelicidins

Respiratoire infecties in de kip

Beschermende rol van macrofagen en cathelicidines

(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. H.R.B.M Kummeling, ingevolge het besluit van het college voor promoties in het openbaar te verdedigen op dinsdag 7 juli 2020 des ochtends te 10.30 uur

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Chapter 1

General introduction

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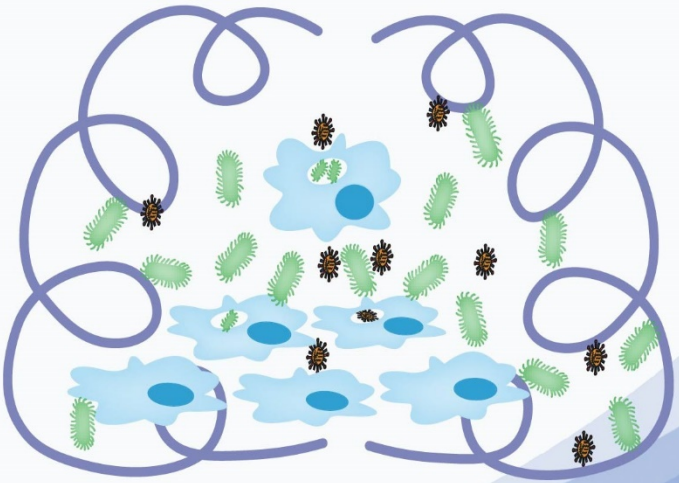

Avian respiratory diseases

Avian respiratory diseases are a major cause of mortality and morbidity in poultry, leading to a huge economic loss. These diseases are due to Gram-positive or Gram-negative bacteria and viruses (Table 1). Bacterial infections include for instance, *Pasteurella multocida* (Fowl cholera), *Haemophilus paragallinarum* (Infectious coryza), *Escherichia coli* (Colibacillosis), *Ornithobacterium rhinotracheale* and *Bordetella avium* (Bordetellosis) (1). Although there is variety of mycoplasmas affecting commercial poultry, the main ones are *Mycoplasma gallisepticum*, *M. synoviae* (chicken and turkey), *M. iowae* (mainly turkey), and *M. meleagridis* (turkey only) (2). Viral infections include infectious bronchitis virus (IBV), newcastle disease virus (NDV), influenza virus, infectious laryngotracheitis virus (ILV), avian leukemia virus (ALV) and pneumonia viruses (3). All of these microorganisms can cause respiratory disease alone, but often co-infections occur by multiple microorganisms, such as simultaneous infections by bacteria and viruses. Currently, the main strategy to prevent infections is vaccination to induce protective immunity against the pathogens. However, there are no (effective) vaccines available for all pathogens and efficacy of vaccines has decreased in some cases especially due to antigenic drift of viruses.

Besides the protection provided by the adaptive immune system by natural antibody production or specific T cells through vaccination or natural exposure to pathogens, at least an equally important protection mechanism is provided by the innate immune system. Innate immunity consists of specific immune cells and effector molecules that can act quickly against (inhaled) pathogens. When pathogens are inhaled into the airway, phagocytes (Table 2) such as heterophils, dendritic cells and macrophages, quickly act to neutralize these microorganisms. In addition, specific molecules can be quickly released by immune cells upon infection, such as host defense peptides (HDPs). Compared to mammals, the avian innate immune system is understudied. Therefore, avian innate immunity including specific immune cells and HDPs were studied in this thesis.

Table 1. Brief overview of commonly diagnosed infectious respiratory pathogens in chickens.

Group	Pathogens
Virus	Infectious bronchitis virus (IBV)
	Newcastle disease virus (NDV)
	Avian influenza virus (AIV)
	Infectious laryngotracheitis (ILT)
	Avian metapneumovirus (APV)
	Fowlpox virus (FPV)
Bacteria	*Escherichia coli*
	Mycoplasma gallisepticum
	Mycoplasma synoviae
	Avibacterium paragallinarum
	Pasteurella multocida
	Ornithobacterium rhinotracheale
Fungi	*Aspergillus fumigatus*
Parasites	*Syngamus trachea*

Avian phagocytic cells

Heterophils

Heterophils are granulocytic white blood cells, the counterparts to the mammalian neutrophils which are generated in the bone marrow and circulate in the blood and considered as (one of) the first responder cells against invading pathogens through phagocytosis and by the release of antimicrobial compounds (4). Besides direct antimicrobial effects, neutrophils release cytokines, proteases and other factors that provide a signal of tissue damage and also regulate the adaptive immune response, such as the activation of T cells and B cells (5, 6). It is likely that heterophils can fulfil similar roles in chickens.

Similar to neutrophils, avian heterophils are highly phagocytic (7). They are rapidly recruited to the infected site where they phagocytose and kill pathogens (8). Chicken heterophils can effectively phagocytose opsonized or non-opsonized pathogens including avian pathogenic *E. coli* (APEC) and *Salmonella* Enteritidis via different receptors, such as toll like receptors (TLR), complement receptors (CR) and Fc receptors (FcR) (9-11). However, it has been shown *in vitro* that APEC phagocytosis by heterophils was associated with bacterial virulence. The type 1 fimbriae expressed in APEC promoted phagocytosis in heterophils but protected phagocytosed bacteria from subsequent killing, although the mechanism for this is still not

clear (11). In addition, heterophils had higher phagocytic activity towards both serum- and IgG-opsonized *S.* Enteritidis compared with non-opsonized bacteria (via TLR) and *S.* Enteritidis-induced immune response as measured by cytokine gene expression was also different. Both non-opsonzied (via TLR) and serum-opsonized *S.* Enteritidis (via CR) induced higher gene expression of IL-1β compared to IgG-opsonized *S.* Enteritidis (via FcR), but lower gene expression of TGF-β4 compared to IgG-opsonized *S.* Enteritidis, whereas no difference was found for IL-6, IL-8 and IL-18 expression (9). Priming heterophils with recombinant chicken IL-2 increased the gene expression of IL-8 and IL-18, regardless whether bacteria were opsonized or not (10). These results suggests that different receptors, including TLR, CR and FcR, are involved in the immune response induced by opsonized and non-opsonized bacteria in heterophils.

Once phagocytosed, pathogens are entrappped in the phagosome which triggers the fusion of cytoplasmic granules with the phagosome. The entrapped pathogens are killed by the release of many antimicrobial compounds from the granules in the phagosome. Similar to the granule contents of neutrophils (12, 13), these granule substances of heterophils have been reported to contain β-defensins (Gal-1 and Gal-2), cathepsin, lysozyme, acid phosphatase, β-glucuronidase, and α-glucosidase (14). The most notable contrast to granules in neutrophils is the lack of myeloperoxidase in heterophil granules (15). In addition, the granules of avian heterophils are also lacking alkaline phosphatase and catalase (16). The lack of these enzymes in heterophils leads to a relatively weak oxidative response compared to the activity of mammalian neutrophils.

Host defense peptides, such as defensins and cathelicidins are important granule compounds released by heterophils and play an important role in killing of invading pathogens. Although at least 14 β-defensins have been described in chicken, only AvBD1 (Gal-1) and AvBD2 (Gal-2) were identified to be associacted with heterophils granules (17). A study from our group showed that the chicken antimicrobial peptide chicken cathelicidin-2 (CATH-2), which belongs to the cathelicidin family of HDPs and has potent broad spectrum antibacterial activitity, is released by heterophils upon *Salmonella* lipopolysaccharides (LPS) stimulation (18). Another study also showed two other chicken cathelicidins (CATH-1 and CATH-3) are produced in heterophils and have antimicrobial activity (19). These cathelicidins exert important functions in the host and are studied in this thesis (introduced further in the cathelicidins section).

Another feature of heterophils is that they can release heterophil extracellular traps (HETs) upon stimulation. HETs contain DNA, histones and likely many other nuclear molecules and are thought to have a similar function as mammalian neutrophil extracellular traps (NETs), namely, trapping and killing pathogens (15, 20). In mammals, this process of NETosis is

believed to be related to the production of an oxidative burst in response to stimulation (21). However, avian heterophils lack myeloperoxidase and therefore produce a weak oxidative burst compared to mammalian neutrophils (22), so formation of HETs might be regulated differently. Despite the weak oxidative response, avian heterophils can still kill pathogens by using antimicrobial proteins (16, 18, 23). Furthermore, chickens with highly functional heterophils are not susceptible to infections compared with chickens with less active heterophils (24). Excessive heterophils infiltration can create lesions in chickens (25), indicating that the heterophil activity needs to be tightly controlled, although only limited knowledge is present about the exact role of heterophils in inflammation.

Table 2. Characteristics of innate immune cells

Cell type	Source	Function	Cell surface marker expression	Specific features in comparison with mammalian cells
Heterophil	Bone marrow/blood	• Phagocytosis • Release of granular content (antimicrobial peptides) • Activation of T and B cells		Release heterophil extracellular traps (HETs) but lack myeloperoxidase
Dendritic cell (DC)	Bone marrow	• Recognition of pathogens • Antigen presentation	MHC-II, CD11c, CD40, CD86, CD83 and DEC205 (upon stimulation)	Chicken DCs exist in different tissues and they have special migration due to the lack of lymph nodes
Macrophage	Bone marrow/blood	• Phagocytosis • Production of ROS and NOS • Release of cytokines and chemokines	MRC1L-B, MHC-II, CSF-1	Polarization not (yet) described

Dendritic cells

Dendritic cells (DCs), members of the mononuclear phagocytic system, are professional antigen-presenting cells (APCs) (26-29). Once DCs recognize a pathogen, they increase the level of major histocompatibility molecules (MHC) and co-stimulatory molecules (CD80/86), migrate to the lymph nodes and present the antigen to the T cells.

Different subtypes of chicken DCs have been identified in different tissues. Chicken DCs were first described in the bursa of Fabricius and in the cecal tonsil's germinal center, named bursal secretory dendritic cells (BSDC) (30, 31). Recently, DCs have also been identified in the chicken thymus (32). In spite of the existence of chicken DCs in different tissues, still little is known about the exact function of chicken DCs. Especially about the migration and APC function of DCs, since chickens actually lack lymph nodes. Most studies on chicken DCs make use of chicken bone marrow-derived DCs (chBM-DCs) *in vitro*, which can be generated in the presence of granulocyte macrophage colony stimulating factor (GM-CSF) and IL-4 (33). These cultured cells show the typical morphology of DCs containing stretched cells with dendrites. Further characterization of the immature chBM-DCs showed high level expression of MHC class II and CD11c (a DC specific marker), low expression of CD1.1 and the co-stimulatory molecules such as CD40 and CD86, and no expression of CD83 or DEC-205 which is expressed in human mature DCs (34, 35). Also the immature DCs have the capacity to phagocytose and endocytose bacteria. Upon LPS stimulation chBM-DCs increase expression of CD40, CD1.1, CD86, CD83 and DEC-205, indicating maturation of DCs. However, the capacity of phagocytosis and endocytosis is lost (33).

Cultured cells *in vitro* have mainly been used to investigate the behaviour of DCs upon infection, but relatively little research is performed on the characterization of DCs in tissues due to limited number of DC-specific antibodies. CD83 monoclonal antibody has been used to identify both DC-like cells and follicular DCs in chicken spleen (36). Although CD83 is highly expressed on DCs, many other immune cells might also express CD83 (36). Indeed, the CD83 antibody actually also recognized a similar protein expressed by the chicken macrophage HD11 cell line (37). Another CD83 specific antibody (clone: IAH F890:GE8) strongly stained DC-like cells in the thymus, but did not stain DCs of bursal follicles and germinal centers (38). The DEC205 antibody recognizes a C-type lectin receptor on the surface of chicken DCs, but also specifically recognizes thymus cortical epithelial cells (32). These examples show the difficulty to specifically identify DCs in chicken tissues, especially with a single antibody. Some progress has been made by double labelling techniques. Recently, the 8F2 mAb was used to distinguish isolated splenic DC-like cells from KUL01 (a myeloid marker) macrophages (39) and double immunofluorescence staining with vimentin (type III intermediate filament (IF) protein that is expressed in mesenchymal cells)

and CSF1R provide a valuable tool for identification of chicken DCs (40). However, to further explore morphology and ontogeny of chicken DC subsets *in vitro* or *in vivo*, more reagents need to be developed.

Macrophages

Macrophages belong to the mononuclear phagocytic system and are considered as one of the first responders against pathogenic invasion. Macrophages are actively involved in the phagocytosis and killing of microorganisms. In addition, they are also key regulatory cells of the immune system by the production of an inflammatory or anti-inflammatory response upon stimulation (41, 42).

Development of macrophages

Macrophages originate from bone marrow stem cells (Fig. 1) and can be self sustaining in the tissues. They can also develop from monocytes in the bloodstream under the influence of colony stimulating factor (CSF) (Fig. 1). Macrophages are present in a variety of tissues, where they have specialized functions. Tissue macrophages can, for example, be found in the lung (alveolar macrophages). About 70% of alveolar macrophages are self-sustaining through division inside the lung, whereas 30% of alveolar macrophages are monocyte-derived. In the liver almost all macrophages (called Kupffer cells) are actually derived from blood monocytes. Macrophages are also present in the bone where they are called osteoblasts (43).

In mammals, macrophages have different phenotypes, such as M1, M2, M (Hb), Mox, and M4. However, they should be regarded more as cells with high plasticity that can easily switch from one phenotype to the other (and all intermediate phenotypes) where the designated types are at the extremes of the spectrum (44). The M1 and M2 phenotype have been mainly studied and they have very different immune functions (Fig. 1). M1 macrophages, differentiated using GM-CSF and interferon-γ, produce mainly pro-inflammatory cytokines such as IL-6 and TNF-α. On the other hand, M2 macrophages, differentiated using macrophage colony stimulating factor (M-CSF) and IL-4, produce mainly anti-inflammatory cytokines such as IL-10 (45, 46). Functionally, M1 macrophages play a role in killing intracellular pathogens while M2 macrophages are important for wound healing and tissue repair (Fig. 1). M1 macrophages have a “fried egg” appearance and can morphologically be distinguished from M2 macrophages which are more streched. Also the expression of surface markers and chemokine receptor ligands are different (47). For instance, M1 macrophages express CXCL9, CXCL10, and CXCL5 whereas CD163 is highly expressed on M2 macrophages (47-49).

Unlike that of mammalian macrophages, polarization and phenotypes of chicken macrophages are rarely studied. Monoclonal antibody KUL01 recognizes the chicken mannose receptor and was first used to characterize macrophages in different tissues, including spleen and gut (50). However, unlike humans, chicken has five paralogous genes of this receptor (MRC1L-A to MRC1L-E) of which KUL01 only recognises MRC1L-B (51).

To facilitate studies on chicken macrophages, two chicken macrophage-like cell lines, HD11 and MQ-NCSU, have been developed (52, 53) that are used most often in chicken macrophage studies. Cell lines provide convenience and great reproducability for experimental control, but one should be careful with extrapolating results using these cell lines to an *in vivo* situation. Therefore, several groups also have cultured primary chicken macrophages derived from monocytes in peripheral blood mononuclear cells (PBMCs) and bone marrow (54-57). So far, there is no standard method to culture these macrophages *in vitro*, but macrophage-like cells are obtained when cells were cultured with chicken GM-CSF. In addition, both chicken CSF-1 and IL-34 can promote proliferation of macrophages *in vitro* (58). These cultured macrophages are morphologically similar to macrophage M1 in mammals (59). In our study, after 3 days culture with GM-CSF, blood monocytes differentiated into M1 like macrophages with proinflammatory properties. A recent study showed that IL-4 induced gene expression associated with M2 markers in HD11 cells and PBMCs, indicating the possible existence of M1/M2 in chicken (60) but better characterizations of M1-like and M2-like macrophages are still needed.

Free avian respiratory macrophages

In the mammalian lung, respiratory macrophages provide a first line of defense against invading pathogens. These cells reside on the luminal surface of alveoli which enables them to internalize and kill pathogens before they can break the epithelial barrier and cause local and systemic infections (61). In birds, respiratory macrophages are known as free avian respiratory macrophages (FARM) (62, 63). FARM in the lung lavage are not only derived from the surface of lung but also from air sacs (64). Interestingly, FARM do not locate on the surface of air capillaries where gas exchange takes place, but are present on the surface of the atria and infundibulae (64, 65). Therefore, avian respiratory macrophages seem to locate at strategic places where fresh air is distributed into gas exchange areas, thereby entrapping and removing invading particles. However, the number of respiratory macrophages in chicken lung is almost 20 times lower than found in mammalian lungs (65, 66). One hypothesis is that this contributes to the relatively weak respiratory defense, which might partially explain the high mortality among birds upon invading pathogens.

Migration of macrophages to the lung in response to infection

Macrophages are present in all tissues, and can migrate to the site of infection and eliminate invading pathogens or to the site of injury to contribute in tissue repair. Macrophage migration is induced by pathogen-associated molecular patterns (PAMPs) released by invading pathogens or damage-associated molecular patterns (DAMPs) of damaged or dead cells. Macrophages in the inflamed or injured tissue release cytokines and chemokines which attract other immune cells among other macrophages (67, 68).

Similar to migration of mammalian macrophages, chicken macrophages can also migrate to sites of infection. This is especially useful in the lung where only low numbers of macrophages are located. For instance, intratracheal administration of live non-pathogenic *P. multocida* vaccine induced a three-fold macrophage increase in the lung and air sacs (69). Similar results were found for IBV and *E. coli* infection of the respiratory tract (70). Noticeably, macrophage migration to the lung is associated with respiratory inoculation of pathogens, because intravenous inoculation of bacteria only produced a weak induction of respiratory macrophages in the lung (66).

Macrophage function

Phagocytosis is one of the most classical functions of macrophages during evolution (Fig. 1). Similar to heterophils, phagocytic activity is also mainly mediated via specific receptors including TLRs, mannose receptors (MRs), CRs and FcRs present on the surface of macrophages. These receptors can recognize and bind to specific bacterial targets to induce phagocytosis, which is the key step to kill microbes. For instance, chicken macrophages took up a higher number of *S. enterica in vitro* when bacteria were opsonized with mannose-binding lectin (MBL) (71). Similarly, pre-incubation of *Campylobacter jejuni* with antiserum enabled chicken peritoneal macrophages to phagocytose more bacteria via FcRs than when bacteria were pre-incubated with PBS (72).

Chicken macrophages act differently in response to different bacterial strains, independent of the opsonization, and some bacteria have developed different protective systems to avoid antibacterial activity of macrophages. For example, the capsulated *P. multocida* adhered in macrophages, but were not internalized by macrophages. The degradation of capsule with hyaluronidase increased bacterial adhesion and subsequent internalization by macrophages (73).

Also in the chicken lung, macrophages have a high phagocytic activity. An *in vitro* comparative study showed that chicken respiratory macrophages had higher phagocytic capacity than rat alveolar macrophages (74). The chicken macrophage HD11 and MQ-NCSU cell lines have also shown high phagocytic activity and killing capacity against different

bacterial strains, such as *E. coli* and *Listeria monocytogenes* (75, 76), but actually failed to kill *Salmonella*. This is likely due to *Salmonella's* defense mechanisms against host cells that delay the phagolysosomal maturation and neutralize radical oxygen and nitrogen species (ROS and NOS) (77).

The production of ROS and NOS are important responses of macrophages against intracellular pathogens (Fig. 1). Mouse macrophages were shown to produce high levels of nitric oxide (NO) (78), which has been shown to mediate pathogen killing by macrophages *in vivo* (79). HD11 cells also produce high NO levels in response to infection or LPS stimulation (75, 80). In contrast, chicken monocytes produced a much lower amount of NO, which is more similar to human monocytes-derived macrophages that even failed to produce NO in response to stimulation (81, 82).

Besides NO production, macrophages also produce chemokines and cytokines (Fig. 1). Stimulation with TLR ligands and pathogenic infection *in vitro* induces pro-inflammatory cytokines such as IL-1β, IL-6, IL-8, interferons (IFNs) and IL-10 in both mammalian and chicken macrophages, which play a role in the initial inflammatory response of the host against infection (75, 83). Production of IL-1β has been found to promote antimicrobial immunity of macrophages against *Mycobacterium tuberculosis* by directly killling and upregulates further TNF-α secretion (84). *In vivo*, IBV infection increased expression of IL-1β produced by macrophages in the lung, indicating that IL-1β is an important component of chicken macrophages in response to infection (85). IL-6 can switch the differentiation of human monocytes from dendritic cells to macrophages (86), but it is also important in the modulation of the Th1/Th2 response defending invading pathogens in mice (87). Other cytokines such as IFNs, have been reported to play an important role in the regulation of chicken macrophage inflammatory response to baterial challenge (57). A recent study identified TNF-α, which was long considered to be absent in chicken (88) and mRNA expression of TNF-α was induced by LPS in chicken macrophages. The exact role is still not identified but it shares 45% homology with mammalian TNF-α indicating they may play similar roles in acute and chronic inflammation. So far, many functions as well as the cytokine response overlap between chicken and human macrophages and therefore it is likely that they play similar roles against pathogens.

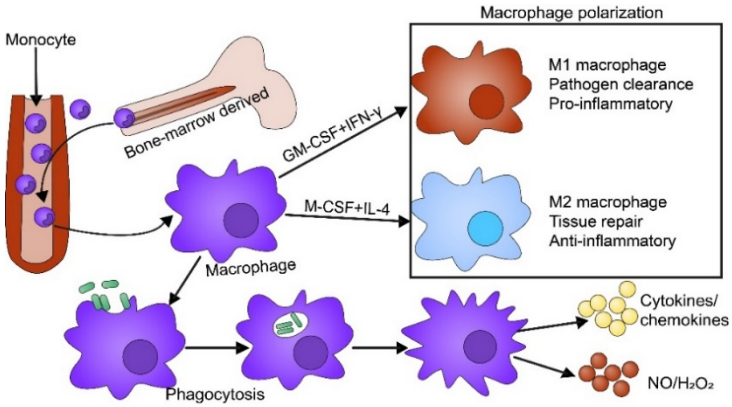

Fig 1. Summary of macrophage functions. Macrophages originate from bone marrow stem cells and also are developed from monocytes in the blood stream. Macrophages have different phenotypes. M1 and M2 phenotypes have been mainly studied and they have very different immune functions. M1 macrophages differentiated using GM-CSF and interferon-γ produce mainly pro-inflammatory cytokines and play a role in killing intracellular pathogens. M2 macrophages differentiated using M-CSF and IL-4 produce mainly anti-inflammatory cytokines and are important for wound healing and tissue repair. Macrophages can phagocytose bacteria and kill them; subsequently cytokines and chemokines as well as nitric oxide and peroxide are produced to activate macrophages.

The interaction of macrophages with viruses

The interaction between avian macrophages and viruses has been well studied. The source of macrophages is important, since peritoneal macrophages act differently from monocyte or bone marrow-derived macrophages (BMDM). Some but not all avian viruses can infect avian macrophages. For example, chicken BMDM have been shown to be resistant to Marek's disease virus (MDV), herpesvirus of turkeys (HVT-FC126), IBV and reticuloendotheliosis virus (REV) infection (89). On the other hand, adenovirus, infectious laryngotracheitis (ILT) virus, reovirus, infectious bursal disease virus (IBDV), myelocytomatosis virus, NDV, avian leukosis virus (ALV) and influenza A virus (IAV) infect and replicate in macrophages (89). In the end, all viral infections result in cell dead. The potential infectivity of avian viruses to macrophages *in vitro* is not correlated with pathogenity of viruses in chicken *in vivo*, although the *in vitro* viral infection provides useful information to investigate the role of chicken macrophages against viruses *in vivo*.

Macrophages respond differently to different viral strains. Intratracheal IBV infection increased the number of macrophages in the respiratory tract five days post-infection (85) while IBDV infection by eye-drop route led to decreased numbers of splenic macrophages three and five days post-infection (90). Both IBDV and IBV induce pro-inflammatory cytokine production by macrophages, like IL-1β, IL-6 and IFN-β (85, 90). Some viruses cause macrophage dysfunction. For instance, ALV, IBDV or IAV (H6N1) infection decreased macrophage phagocytosis and killing activity (91-93). In contrast, IAV (H5N1)

enhanced phagocytic activity of chicken macrophages (94). On the other hand, IAV has been shown to induce expression of pro-inflammatory cytokines (IL-6 and IL-1β) as well as the inflammatory chemokines (CXCLi1 and CXCLi2) in HD11 cells (95).

Chicken cathelicidins

Cathelicidins are HDPs, short cationic peptides that play an important role in innate immunity against pathogens. They have direct antimicrobial activity as well as immunomodulatory functions (Fig. 2) (96). Cathelicidins have been found in different species including mammals, birds, reptiles, amphibians and some fishes (97). Not all animals have the same number of cathelicidins. For instance, there is only one cathelicidin described in human, mouse and dog (98). So far, four cathelicidins have been characterized in chicken, named CATH-1, -2, -3 and -B1.

Structure and classification

All cathelicidins are composed of three parts: a signal peptide, a cathelin domain and the mature peptide. They are encoded by 4 exons. Exon 1 encodes the signal peptide of 29 or 30 amino acids (AA) and the cathelin domain of 99 to 114 AAs is encoded by exon 2 and exon 3. Exon 4 encodes the mature peptide of 12 to 100 AAs (99). The N-terminal signal peptide sequence and the cathelin-domain are highly conserved among species, contrary to the C-terminal domain sequence encoding the mature peptide, which is very diverse (100). This diversity leads to existence of mature cathelicidin peptides with differing length, charge density and structure. However, all the cathelicidins are positively charged due to cationic residues, and amphipathic due to spatial separation of the charged and hydrophobic residues.

The cationic and amphipatic nature enable cathelicidins to interact with negatively charged molecules such as LPS, lipoteichoic acid (LTA) and (phospho)lipids in bacterial membranes (101). Most mature peptides are mainly α-helical, such as LL-37 in human (102), SMAP-29 in sheep (103), and BMAP-27/28/34 in cow (104). Chicken CATH-1, -2 and -3 are also mainly α-helical segments of which CATH-2 contains 2 helices connected by a proline induced kink in the center. Such a helix-hinge-helix structure is common for more cathelicidins and similar configurations have been described in porcine PMAP-23 and sheep SMAP-29. Chicken cathelicidin (CATH-B1) is less studied compared to the other three chicken cathelicidins. The amino acid sequence of CATH-B1 has been predicted, but its molecular conformation has not been determined. Besides the helical peptides, some cathelicidins have other structural characteristics such as porcine protegrins that contain a β-hairpin, or peptides that are rich in specific amino acids (proline/arginine) like porcine PR-39 (105-107).

Expression

Cathelicidins are produced by leukocytes and epithelial cells. They are stored in granules as inactive precursors and then are released as mature peptide upon activation of cells after being cleaved by neutrophil elastase. On a protein level, expression of CATH-2 protein was determined using a specific anti-CATH-2 antibody. This showed that CATH-2 was exclusively expressed in heterophils. The human cathelicidin LL-37, which is produced by neutrophils, has a much broader expression spectrum (18, 108). It also indicates that it is well possible that the level of CATH-2 gene expression found in tissues is related to the number of heterophils present in that tissue at time of sampling. CATH-1 and CATH-3 protein were also identified in heterophils by liquid chromatography-mass spectrometry (LC–MS)/MS analysis (19). CATH-B1 is the second chicken cathelicidin for which an antibody is produced enabling the determination of its protein expression. CATH-B1 protein was only found to be expressed in secretory epithelial cells of the bursa surrounding M cells (109) which is a major entry point for pathogens in mucosal lymphoid tissues, but not many other tissues were tested for protein expression.

Gene expression of chicken cathelicidins is broader than actual (determined) protein expression. All four chicken cathelicidins mRNA are actually expressed in most tissues including the skin, the respiratory tract, gastrointestinal tract and lymphoid organs, except for breast muscle (110). CATH-1, -2 and -3 mRNA were highly expressed in bone marrow cells while CATH-B1 mRNA was highly expressed in bursa (109, 110). Interestingly, CATH-2 mRNA is highly expressed in the uropygial gland, which secretes preen oil containing antimicrobial factors that transferred to skin and provide protection against infections (111). Furthermore, these gene expression of four cathelicidins already has been detected during embryonic development (112, 113). In the early stage of life in chicken, increased gene expression of cathelicidins was observed in different tissues such as lung, bursa and intestine (110). The broader distribution of these cathelicidins *in vivo* plays an important role against invading pathogens.

Regulation of cathelicidin expression

Besides developmental regulated expression of cathelicidins, many external factors can also up or downregulate cathelicidin expression. Such factors include inflammatory and microbial stimuli but also vitamin D. Regulation of human cathelicidin expression by these factors and other factors are well studied, but less is known about chicken cathelicidins expression.

Vitamin D was discovered as an inducer of human cathlicidin gene expression in different cell types, such as keratinocytes, monocytes and neutrophils, and human cell lines (114). In chicken a similar effect was observed for vitamin D, gene expression of chicken cathelicidins

was induced in tissues upon vitamin D supplementation. Chickens fed vitamin D resulted in increased gene expression of CATH-1 and CATH-B1 in the spleen whereas the expression of CATH-3 was downregulated. These changes were tissue specific because the expression of cathelicidins was not affected in PBMCs (115).

Besides vitamin D, other stimuli such as short-chain fatty acids were found to regulate the expression of chicken cathelicidins. Butyrate is a short-chain fatty acid produced by bacterial fermentation of undigested dietary fiber. When HD11 cells and primary monocytes were incubated with butyrate for 24 h, upregulated CATH-B1 gene expression was detected but gene expression of the other three cathelidins was not affected by butyrate (116). Oral administration of butyrate also enhanced CATH-B1 gene expression in chicken jejunal and cecal explants (116). Furthermore, butyrate treatment enhanced antibacterial activity of primary monocytes against *S.* Enteritidis. These compounds may be good feed additives to increase the expression of cathelicidins and thereby potentially increase protection againt infection.

Microbial infection also regulates the expression of chicken cathelicidins, but whether they are up- or down-regulated depends on the bacterial strain. Abundant CATH-2 in heterophils has been observed in the jejunum of *S.* Eenteritidis-challenged broilers post 8 h infection (18). In contrast to *Salmonella* infection, *Campylobacter jejuni* infection in young chicks did not induce CATH-2 protein (heterophil) recruitment to the small intestine and CATH-2 mRNA expression was decreased 48 h post infection (117). CATH-1 and CATH-3 gene expressions were also downregulated by *Campylobacter jejuni*, but CATH-B1 gene expression was not affected (118). These differences indicate that possibly some bacteria evade the immune system partially by actively down-regulating cathelicidins.

Besides regulation of gene expression of cathelicidins, the functional regulation of cathelicidins might be on the level of release of the peptides. As mentioned cathelicidins are stored as inactive precursors in secretory granules and only upon secretion and subsequently proteolytic cleavage, active peptide is produced. For CATH-2, it was shown *in vitro* that LPS induces heterophils to release the mature form of peptide and this proteolytic process of proCATH-2 was mediated by serine proteases (18).

Cathelicidin functions

Antimicrobial activity

Cathelicidins have a broad spectrum of antimicrobial activity against Gram-positive and Gram-negative bacteria, fungi, protozoa and viruses (Fig. 2). This antimicrobial activity has been extensively investigated. CATH-1, -2 and -3 showed strong antibacterial activity against both Gram-positive (*L. monocytogenes, S. aureus*) as well as Gram-negative bacteria (*E. coli,*

S. typhimurium, K. pneumoniae, P. aeruginosa) including antibiotic-resistant strains (*S. aureus* MRSA) with minimum inhibitory concentrations (MIC) between 0.4-2.0 μM (119, 120). Antibacterial activity of HDPs is often negatively affected by the presence of salts and serum. However, antibacterial activity of CATH-1, -2 and -3 was not affected by the presence of physiological concentrations of salt (119, 120). Radial diffusion assays showed that the presence of serum had only a small effect on CATH-2's activity, but did not affect antimicrobial activity of CATH-1 and CATH-3. Whereas many antibiotics induce resistance, CATH-1, -2 and -3 did not induce resistance by multi-resistant bacteria such as *S. aureus* and *K. pneumoniae* (121). Besides their activity against planktonic bacteria, cathelicidins inhibited the formation of bacterial biofilms *in vitro.* In addition, CATH-2 has been shown to permeabilize the formed biofilm and directly kill the biofilm-associated bacteria (122, 123). Besides this antibacterial activity, CATH-2 also has been reported to rapidly kill fungi (*Candida albicans*) within 5 min by permeabilizing the fungal cell membrane (124). However, antimicrobial activity of another chicken cathelicidin CATH-B1 is less studied compared to the other three cathelicidins. CATH-B1 has been only tested against a limited number of bacterial strains including *E. coli*, *S. aureus*, and *P. aeruginosa* and the MIC value was in the range of 0.63-2.5 μM, however this was tested against a low amount of bacteria (2×10^3 CFU) (109). In another study the MIC value was increased to 12.8 μM against *E. coli* and *S. aureus* when higher bacterial concentrations (5×10^5 CFU) were used (125).

The antibacterial mechanism of cathelicidins has been extensively studied. Positively charged and amphipathic peptides can interact with negatively charged outer and/or inner membrane molecules of Gram-negative bacteria. LL-37 kills microorganisms by accumulating on the membrane to induce leakage of the membrane, finally disrupting membrane integrity (108, 126). In line with the mechanism of action of LL-37, CATH-2 directly binds to the bacterial membrane and permeabilizes the membranes to kill bacteria in 5 min (127, 128). Interestingly, at sub-MIC values, CATH-2 was also detected intracellularly in *E.coli* where it seemed to affect bacterial DNA and ribosome organization. In addition, CATH-2 also induced (outer) membrane vesicles release (128). These vesicles induced by CATH-2 might be interesting vaccine candidates.

Immunomodulation

In addition to direct microbial killing, many antimicrobial peptides including chicken cathelicidins have immunomodulatory effects on the host cells (Fig. 2). The immunomodulatory effect of human cathelicidin LL-37 has been extensively studied. LL-37 has many immunomodulatory properties including binding to LPS (108) and blocking LPS-induced production of cytokines and nitric oxide (129, 130), mediating migration of leukocytes (131, 132), modulating activation of TLRs (133-135) and regulating cell

differentiation (136, 137). Importantly, LL-37 can directly interact with cell receptors such as formyl-peptide receptor-like 1 (FPRL1) and P2X7 nucleotide receptor to contribute to innate and adaptive immunity against microbial invasion (131, 138). Chicken cathelicidins share some properties with LL-37 and some functions of chicken cathelicidins have been investigated.

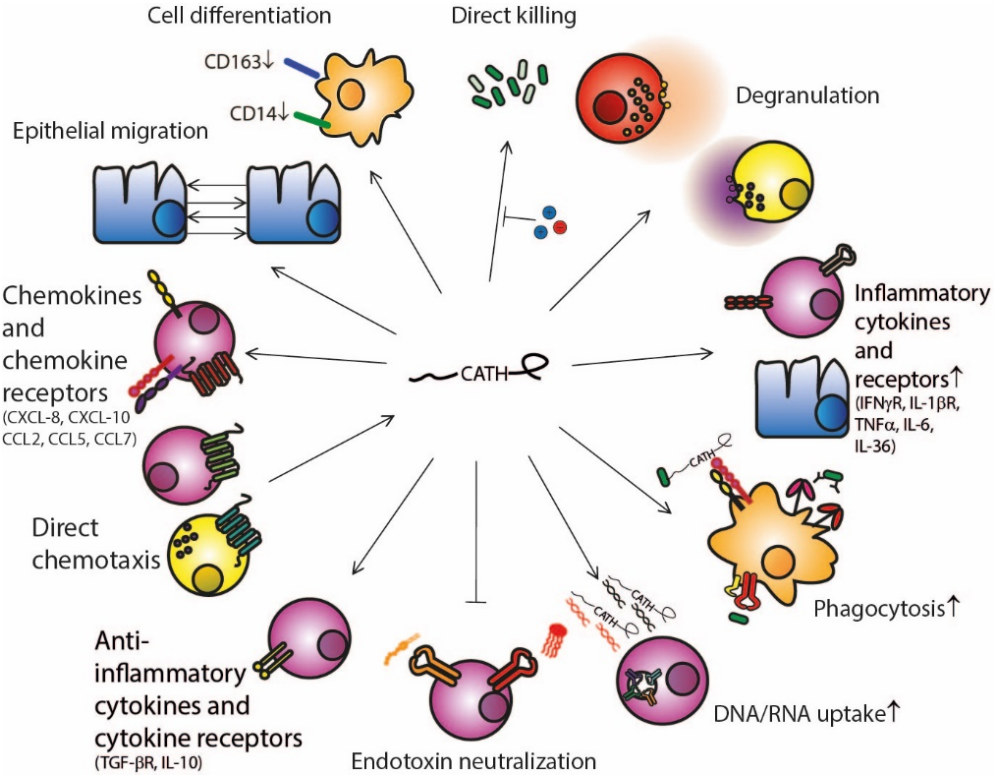

Fig 2. Summary of cathelicidin functions. Cathelicidins have direct killing activity against bacteria, viruses and fungi. They can induce degranulation of neutrophils, enhance phagocytosis, increase DNA/RNA uptake thereby boosting the activation of intracellular TLRs. Furthermore, they neutralize endotoxin via binding to LPS and LTA to inhibit LPS- and LTA-induced inflammatory response, thus directly inducing an anti-inflammatory response. They are directly chemotactic for mast cells and induce production of chemokines including CCL2, CCL5, CCL7, CXCL8 (IL-8) and CXCL10. They also have wound healing function via induction of epithelial migration. Finally, cathelicidins affect cell differentiation by polarizing macrophages to an inflammatory phenotype (M1). Figure was adapted from (96).

Similar to LL-37, chicken cathelicidins (CATH-1, -2, -3) can bind free LPS *in vitro* and inhibit LPS-induced nitric oxide production and cytokine expression such as monocyte chemotactic protein-1 (MCP-1), TNF-α, IL-1β, IL-8 and IL-6 (120, 139, 140). CATH-B1 has been shown to bind LPS (125), but it is still unknown whether CATH-B1 modulates LPS-induced immune response of the host cells. A CATH-1 analogue (6-26) directly induced

activation of macrophages *in vitro* resulting in cytokine expression and enhanced CD86 and MHC-II expression (141). In addition to blocking LPS-induced stimulation, CATH-2 can directly stimulate cells to produce MCP-1 (139). Unlike LL-37, which induces cytokine expression via the interaction with cell membrane receptors, so far chicken cathelicidins have not been shown to interact with any specific host receptors. However, CATH-2 has been shown to inhibit *E. coli*-induced TLR2 or TLR4 activation by direct killing of bacteria and to enhance DNA-induced TLR9 activation in macrophages (142, 143).

In conclusion, chicken cathelicidins have specific antimicrobial and immunomodulatory activities (Fig. 2). However, peptide aggregation, susceptibility to degradation and lack of knock-out chicken lines make it difficult to investigate the specific function of these chicken cathelicidins *in vivo*. At least, the D-enantiomer of chicken cathelicidin-2 has been shown to reduce chicken mortality and morbidity in an infection model (144). These results indicate that chicken cathelicidins, like other host defense peptides, can be potential therapeutics against microbial infections (145) and that upregulation of expression of chicken cathelicidins by feed additives can boost host immune system to promote chicken health.

An overview of the thesis

Avian respiratory diseases caused by pathogens including bacteria and viruses are important diseases leading to huge economic loss in poultry. Avian innate immunity including immune cells and HDPs provide important protection against pathogens, but it is rarely studied. Therefore, in this thesis, we aimed at exploring the role of avian innate immunity to increase our knowledge about the protective mechanisms of the host against pathogens.

In **chapter 2**, the interactions of chicken lung epithelial cells with APEC is described to understand the role of epithelial cells in APEC infections.

In **chapter 3**, the interactions of macrophage HD11 cells with APEC is described to understand the role of macrophages in controlling APEC.

In **chapter 4**, the establishment of a standardized culturing method of monocyte-derived macrophages is described and these cultured macrophages are characterized as pro-inflammatory macrophages with M1-like properties.

In **chapter 5**, the immunomodulatory functions of CATH-B1 are described increasing our understanding of the function of this cathelicidin.

In **chapter 6**, the anti-IAV activity of CATH-B1 is described exploring the possible use of cathelicidins in the development of anti-infective therapies.

In **chapter 7**, the main findings of this thesis are summarized and discussed.

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Chapter 2

Avian pathogenic *Escherichia coli* infection of a chicken lung epithelial cell line

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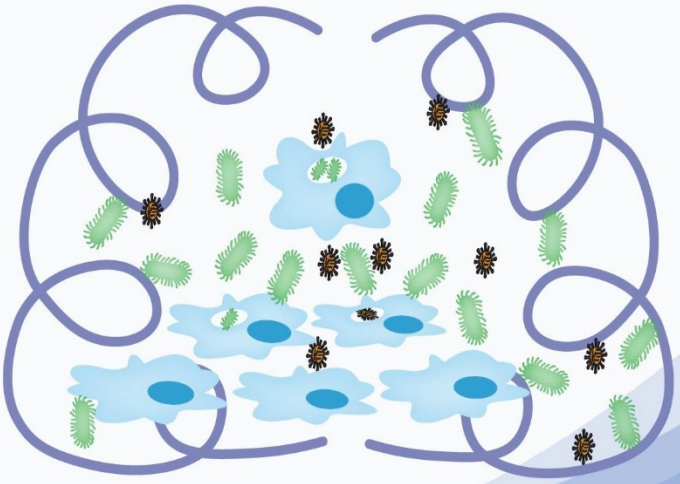

Abstract

Virulent strains of *Escherichia coli* (Avian Pathogenic *E. coli*: APEC) can cause initial infection of the respiratory tract in chickens potentially leading to systemic infection called colibacillosis, which remains a major cause of economic losses in the poultry industry. The role of epithelial lung cells as first targets of APEC and in initiating the innate immune response is unclear and was investigated in this study. APEC was able to adhere and subsequently invade cells from the chicken lung epithelial CLEC213 cell line exhibiting pneumocyte type II-like characteristics. Invasion was confirmed using confocal microscopy after infection with GFP-labelled APEC. Moreover, APEC infection resulted in a significant increase in IL-8 gene expression, a chemo-attractant of macrophages and heterophils. Gene expressions of interferon α and β (IFN- α and IFN- β) were not significantly upregulated and chicken surfactant protein A (SP-A), also did not show a significant upregulation on either gene or protein level. The immune response of CLEC213 cells towards APEC was shown to be similar to stimulation with *E. coli* LPS. These results establish CLEC213 cells as a novel model system for studying bacterial infection of the lung epithelium and show that these cells may play a role in the initial innate response towards bacterial pathogens.

Introduction

Escherichia coli (*E. coli*) is a natural inhabitant of the chicken's intestinal tract and to a lesser extent also of the trachea. However, virulent strains of *E. coli* (Avian Pathogenic *E. coli*: APEC) can cause initial infection of the respiratory tract potentially leading to systemic infection and disease in chicken (1). Colibacillosis is currently a major cause of economic losses in the poultry industry, due to decreased hatching rates, egg production, growth and increased mortality (2). There is no highly effective vaccine available to protect against APEC mainly due to the diversity of APEC strains in the field, and therefore often antibiotic-based treatment is required.

It is still unclear what makes an *E. coli* strain virulent in chickens. APEC strains from more than 6 serotypes have been identified often displaying multiple antibiotic resistance genes, but no clear systematic association with the APEC phenotype can be found (3). However the presence of 5 specific genes located on a the large colV virulence plasmid is found in approximately 70% of APEC strains (4). Nevertheless, a study comparing virulent and non-virulent *E. coli* could actually not find significant differences in lung histology of infected chickens nor in induction of apoptotic activity in lung cells, indicating again the lack of a thorough understanding of what makes certain *E.* coli strains pathogenic in chicken (5).

APEC pathogenesis has been studied mainly through the use of experimental infection models (6-8). Upon infection, heterophils and macrophages are attracted to the site of infection where they interact with bacteria. Heterophils are the fastest responders to an *E. coli* infection appearing within 6 h post infection contributing to bacterial clearance by degranulation, and the release of antibacterial compounds (9). Phagocytosis of *E. coli* by macrophages has been observed *in vivo* and some studies actually correlates virulence genes of *E. coli* to resistance towards phagocytosis (9, 10). In a recent study from our group it was shown that chicken macrophages (HD11 cells) *in vitro* are capable of providing an immune response towards APEC comparable to non-pathogenic *E.* coli (11). Besides macrophages and heterophils, other leukocytes such as NK cells could also contribute significantly to the innate response towards *E. coli*, but not many studies are present on this subject.

The exact role of lung epithelial cells as first line cellular target initiating innate immune responses towards invading respiratory APEC has not been studied in the chicken. A few studies have described adhesion characteristics of APEC to primary cell cultures of type II pneumocytes derived from 14-day-old chicken embryos (12, 13), but more extensive studies on immune responses are hampered by the relative difficulty to isolate epithelial cells from tissues. However, recently a new chicken lung epithelial cell line (CLEC213 cells) was described that showed many characteristics of type II pneumocytes, including the presence of cilia, alkaline phosphatase activity, and importantly the presence of pulmonary surfactant

protein A (SP-A) mRNA (a protein abundantly expressed by mammalian lung epithelial type II cells) (14). This cell line can be permissive to various chicken pathogens and is capable of developing a pro-inflammatory immune response, as was shown towards Influenza A viral infection and upon LPS stimulation (14, 15). In addition, CLEC213 cells were described as a novel chicken epithelial model system to study gametogony of *Eimeria Tenella* (16). However, except for a single study where the cell line was mainly used as a tool to determine the importance of the *Salmonella* T3SS secretion system (17), no bacterial infection studies with these cells have been performed. In this study we determined the interaction of APEC with these chicken epithelial cells. Invasion characteristics of APEC were determined, and the innate immune response of epithelial cells was measured and compared to stimulation of these cells by *E.* coli LPS.

Methods and materials

Bacterial strains

APEC 506 (O78, K80) isolated from chicken (18) was used in this study. Bacteria were cultured in Tryptic Soy Broth (TSB) and Tryptic Soy Agar (TSA) at 37 ℃. For preparation of the green fluorescent protein (GFP)-expression APEC strain, the plasmid pWM1007 was transformed into APEC 506 (O78, K80) by electroporation using an Electro Cell Manipulator according to the manufacturer's instructions. GFP-expression APEC was cultured in the same condition as APEC (11).

Chicken lung epithelial cells

The chicken lung epithelial cell line CLEC213 (14) was maintained in a humidified 41 ℃ incubator with 5% CO_2 and cultured in advanced DMEM supplemented with 4 % Fetal calf serum, glutamax (Thermo Fisher Scientific) and antibiotics (100 U penicillin/mL, 100 µg streptomycin/mL). Aliquots of cell suspension were seeded into 12 well plates at 2×10^5 cells/well and cultured overnight to reach 100% confluence at about 4×10^5 cells/well before being used for assays described below.

Bacterial adhesion and invasion assays

Before CLEC213 cells were incubated with APEC, culture medium was removed and cells were washed twice with PBS. APEC was grown to log-phase in 3 h. Bacteria were pelleted and resuspended in cell culture medium without antibiotics. Aliquots of 1 mL of bacterial suspension (10^6-10^8 CFU/mL) were added to each well. For association assays, the CLEC213 cells were incubated for 1, 2 and 3 h with APEC, washed three times with advanced DMEM medium (without supplements) and lysed in 1% Triton X-100 in PBS at room temperature (RT) for 5 min to release the associated bacteria. The suspensions were serially diluted and

100 µl of each dilution was plated on TSA (Oxoid Limited). From this, total cell-associated viable bacteria (both cell-adherent and intracellular) were calculated. For invasion assays, 1 mL of colistin at 250 µg/ml per well in advanced DMEM supplemented with Glutamax and 4% FCS was added to the CLEC213 cultures for 1 h to kill the remaining extracellular bacteria. Then the cells were washed, treated with Triton X-100 and plated out as described above to enumerate the number of invaded, intracellular bacteria. The number of adhered bacteria was calculated as: number of cell-associated - number of intracellular bacteria. Experiments were performed in at least three independent experiments in duplicate.

Metabolic activity

Metabolic activity of CLEC213 cells was determined by the WST-1 assay according to the manufacturer's instructions (Roche, Basel, Switzerland). Absorbance was measured after 30 min at 450 nm with a FLUOstar Omega microplate reader (BMG Labtech GmbH, Ortenberg, Germany) and was corrected for absorbance at 630 nm. Non-infected control cells were defined as 100% mitochondrial activity.

Gene expression

CLEC213 cells were infected with 1 mL of $1x10^7$ CFU/mL APEC (MOI = 25) at 41 ℃ for 3 h and subsequently treated with 250 µg/mL colistin as described above, or were stimulated with several doses of LPS ranging from 0.1 to 50 mg/mL (LPS EB: from *E. coli* O111:B4, Invivogen, Toulouse, France). After 4 and 24 h of culture total RNA was extracted by Trizol reagent (Ambion, Carlsbad, CA) according to manufacturer's instructions. RNA (500 ng) was reverse transcribed using the iScript cDNA synthesis kit according to the manufacturer's instructions. Quantitative real time PCR was performed on a CFX Connect qPCR with CFX Manager 3.0 (Bio-Rad). Reactions were performed as follows: 3 min at 95 °C; 40 cycles: 10 s at 95 °C, 30 s at 60 °C and 30 s at 72 °C. Relative gene expression levels were normalized against the expression levels of the house keeping genes GAPDH and 28S. Primer and probe sequences of the genes determined are depicted in Table 1.

Griess assay

To determine nitric oxide (NO) production, CLEC213 cells were incubated with APEC as described above for qPCR analysis. Subsequently nitrite, a stable metabolite of NO, was measured by the Griess assay in the cell culture supernatant as described before (11).

Table 1. Primer and probe sequences for qPCR.

Gene		5'→3'sequence
GAPDH	Forward	GCCGTCCTCTCTGGCAAAG
	Reverse	GTAAACCATGTAGTTCAGATCGATGA
	probe	AGTGGTGGCCATCAATGATCCC
IL-8	Forward	GCCCTCCTCCTGGTTTCA
	Reverse	CGCAGCTCATTCCCCATCT
	probe	TGCTCTGTCGCAAGGTAGGACGCTG
cSP-A	Forward	GGAATGACAGAAGGTGCAATCAG
	Reverse	GCAATGTTGAGTTTATTAGCTACAAATG
	probe	CCGGCTTGTTGTCTGCCAGTTTTAGTGG
IFN-α	Forward	GACAGCCAACGCCAAAGC
	Reverse	GTCGCTGCTGTCCAAGCATT
	probe	CCGGCTTGTTGTCTGCCAGTTTTAGTGG
IFN-β	Forward	CCTCCAACACCTCTTCAACATG
	Reverse	TGGCGTGTGCGGTCAAT
	probe	TCC-ACCGCTACACCCAGCACCTCG

Confocal microscopy

CLEC 213 cells were seeded on a 12 mm coverslip in 24-well plate and incubated overnight at 41°C to reach confluence. Cells were subsequently infected with GFP-APEC (MOI=25) for 3 h at 41 °C. After three wash steps with plain advanced DMEM, cells were fixed with 4% paraformaldehyde (PFA) in PBS for 30 min at RT. Subsequently, cells were incubated with 50 mM NH_4Cl in PBS for 10 min at RT and blocked with 5% normal goat serum in PBS for 1 h to block non-specific antibody staining. Then, cells were stained with *E. coli* antiserum (1:500) (19) for 1 h. After the wash steps, cells were incubated with Donkey anti-Rabbit Alexa 647 (1:100) (Jackson ImmunoResearch,West Grove, PA, USA) for 1 h. Finally, cells were washed with PBS or water and mounted in FluoroSave. Slides were observed on a Leica SPE-II DMI4000 microscope with LAS-AF software (Leica, Wetzlar, Germany) using a 63× HCX PLAN APO OIL CS objective.

Western blotting

The presence of cSP-A in the CLEC213 protein fraction was measured by western blot using mouse anti-cSP-A antibodies as described before (20). In short, CLEC213 cells were infected with APEC or stimulated with LPS as described above, after which CLEC213 cell proteins and secreted proteins in the supernatant, were dissolved in denaturing SDS sample buffer and separated on a 10% SDS-PAGE gel. Subsequently, proteins were blotted on nitrocellulose (Protran BA83, Whatman, Sigma-Aldrich). cSP-A was detected using monoclonal mouse anti-cSP-A antibodies as 1st antibody and horse radish peroxidase labelled Goat anti Mouse antibody (Sigma-Aldrich) as 2nd antibody.

Statistical analyses

Results are presented as the mean ± standard error of the mean (SEM) of at least three independent experiments. Statistical significance was assessed with one-way ANOVA followed by the Tukey Post-Hoc test in Prism software, version 6.02 (Graphpad, La Jolla, CA, USA). Differences were considered statistically significant at $P \leq 0.05$.

Results and discussion

Adhesion and invasion characteristics of APEC

Initial adherence studies were performed for 1 h with different densities of APEC (1 mL 10^6 -10^8 CFU/mL; corresponding to an MOI of 2.5, 25 and 250, respectively) as shown in Fig. 1A. Increased inoculum density resulted in an increased APEC adherence. If the adherence was expressed as percentage of the inoculum, the values correspond to approximately 7.5% for MOI 2.5 and 25, and 2.5 % for the highest bacterial density (MOI 250). Based on this, an inoculum density of 10^7 CFU/mL (MOI 25) was chosen for further studies.

The time-dependent adhesion and invasion of APEC to the CLEC213 cells is shown in Fig. 1B and 1C. Adherence of APEC at the initial MOI 25 significantly increased over time from 1 h to 3 h of incubation, roughly corresponding to 6 bacteria adhering to one CLEC213 cell after 3 h (2.5 x 10^6 CFU/ well vs 4 x 10^5 CLEC213 cells/well) (Fig. 1B). Invasion showed a similar trend towards higher number of invading bacteria with 4.5 x 10^4 CFU/well after 3 h (Fig. 1C). This indicates that after 3 h less than 2% of adhered bacteria was able to invade CLEC213 cells. In addition, after removal or killing of extracellular bacteria, the number of invaded bacteria was followed over time. As shown in Fig. 1D, the viability of invaded bacteria actually decreased over time, indicating that invasion does not lead to rapid multiplication of APEC intracellularly. It is unclear if this could be a bacterial strain specific effect or whether CLEC213 cells can potentiate an efficient intracellular immune response against a broader range of (invaded) bacteria.

Studies with isolated cultured chicken type II pneumocytes have shown similar high adherence of APEC, causing cell damage and the loss of microvilli (12, 21), but invasion into type II cells was not determined. APEC was also able to adhere to chicken breast and human colorectal adenocarcinoma cells (HCT-8) cells indicating that the adhesion is not specific for pneumocytes (22). With respect to invasion characteristics of APEC, one study tested this on a chicken hepatocyte cell line and found it to be relatively high (8% of the total adhered bacteria) but this number was much lower (0.2 %) when tested on human type II cells (23). Overall this is the first study quantitively determining the time and density dependency of APEC adhesion and invasion of chicken lung epithelial cells.

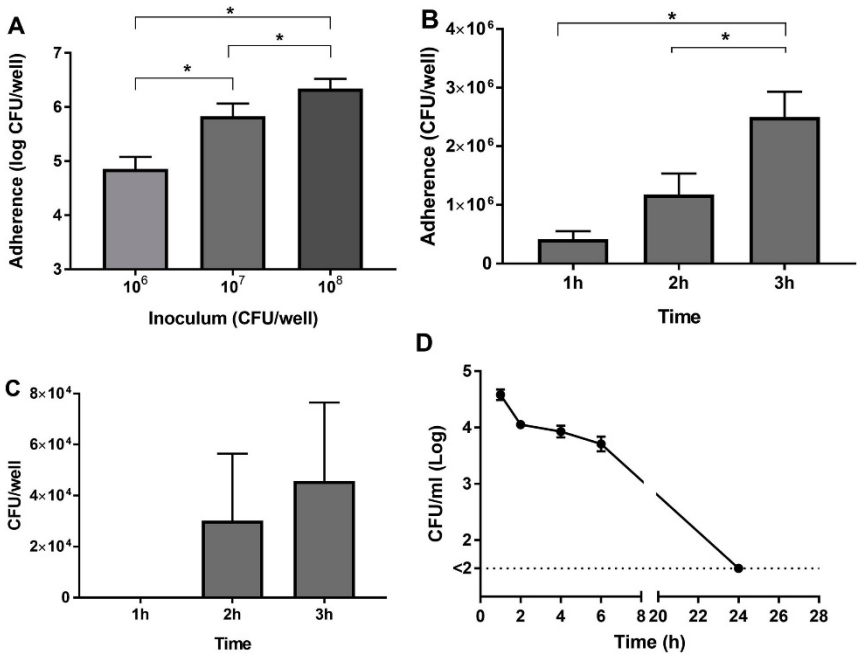

Fig 1. Adherence and invasion characteristics of APEC. (A) CLEC213 cells were incubated for 1 h with different densities of APEC. After washing of excess bacteria, associated bacteria were plated out and counted. (B) CLEC213 cells were incubated with 10^7 CFU/mL for 1-3 h, associated bacteria were determined as in A. (C) CLEC213 cells were incubated with 10^7 CFU/mL for 1-3 h, after which extracellular bacteria were killed with colistin. Intracellular bacteria were determined by colony counting. (D) CLEC213 cells were incubated with 10^7 CFU/mL for 3 h, after which extracellular bacteria were killed with colistin. Intracellular survival of APEC was followed in time, determined by colony counting. Data are shown as mean ± SEM of at least 3 independent experiments for each group (in duplicate). * indicates significant difference ($P \leq 0.05$).

Next, confocal microscopy was used to confirm the presence of intracellular bacteria after APEC infection in CLEC213 cells. GFP-producing APEC was used to infect CLEC213 cells for 3 h after which an antiserum against *E. coli* was used to detect the remaining adherent bacteria. Since the CLEC213 cells were not permeabilized, only extracellular APEC was detected by the antibody. As shown in Fig. 2, extracellular and intracellular bacteria can clearly be distinguished. Double labelled (yellow) are available for the anti-*E. coli* antibody and thus are extracellular, while intracellular APEC are shielded from the antibody and only show the green GFP signal. Control experiments to validate the model without infection or with permeabilized cells showed no staining or only double labelled bacteria, respectively (data not shown). These results confirm that APEC can invade CLEC213 cells and shows that the more indirect results obtained by the adhesion/invasion assay was not caused by, for example, incomplete killing of extracellular bacteria. In addition, a similar experiment was performed with APEC treated with gentamicin. No intracellular localization of these non-

viable bacteria was observed, showing that invasion is an active process. Our results correspond well to an earlier study where intracellular APEC in cultured chicken type II pneumocytes were detected using transmission electron microscopy (21), although this technique requires several fixation and staining steps, unlike our current confocal imaging set-up.

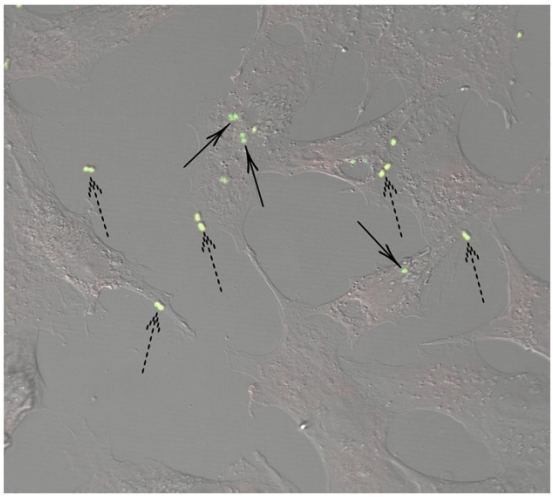

Fig 2. APEC is partially located intracellularly in CLEC213 cells. CLEC213 cells were incubated with GFP-APEC for 3 h. Subsequently, cells were washed and fixed, but not permeabilized. Extracellular APEC were labeled with rabbit anti-*E. coli* serum and Donkey anti-Rabbit Alexa 647 (red). CLEC213 cells appeared grey under the DIC channel. Intracellular bacteria appear green (solid arrows) while extracellular bacteria appear as red/yellow (dotted arrows).

Immune response upon APEC infection

CLEC213 cells were infected for 3 h with APEC after which extracellular bacteria were killed by colistin. At 4 and 24 h post infection (hpi), gene expression of several immune genes was determined. The largest effect was observed for IL-8 which showed a 6-fold increase in gene expression at 4 hpi. Interestingly, IL-8 is known for its chemotactic activity for macrophages and heterophils in chicken (24), indicating that the observed increase in macrophages in APEC infections *in vivo* could be partially explained by the initial epithelial response after the first interaction with the respiratory epithelia. SP-A and IFN-α showed a tendency towards upregulation at 4 hpi but this difference did not reach statistical significance (Fig. 3A). Gene expression was also determined after 24 h, but no significant upregulation could be observed anymore indicating a relatively short immune response for at least the genes studied. Metabolic activity (WST-1 assay) or viability (cell count, Trypan blue exclusion) of CLEC213 cells was not affected by bacterial infection after 4 and 24 h (data not shown).

LPS stimulation of CLEC 213 cells

In the next set of experiments, CLEC213 cells were stimulated with the potent immune stimulant LPS derived from *E. coli* (Fig. 3B). At the highest concentration of LPS a similar response as seen for APEC infection was observed and comparable to earlier studies using this cell line using LPS (14) or influenza virus infection (15). IL-8 was significantly upregulated, while cSP-A showed a non-significant tendency towards an increased expression. The large variation in cSP-A expression observed in both LPS and APEC stimulation/infection of CLEC213 cells could be partially explained by the low absolute level of cSP-A gene expression. The levels of cSP-A mRNA measured in these experiments were close to or just beyond the detection limit where a linear concentration-response correlation was observed, likely causing lower reproducibility of the data. However, since this protein is highly expressed in type II cells (in mammals) it was still valuable to show. Besides gene expression, the presence of cSP-A was also tested on a protein level by western blot using cSP-A specific antibodies. No cSP-A could be detected in either bacterially infected, LPS-stimulated or non-stimulated CLEC213 cells or their supernatants (data not shown). This apparent lack of detectable cSP-A in CLEC213 cells could indicate that they are a different cell type than surfactant producing cells in the chicken lung, or that these cells require a different stimulus *in vitro* to produce cSP-A. Expression of cSP-A was expected in CLEC213 cells, based on the presence of lamellar bodies which have been detected in long-term cultures (14), however the number remains much lower compared to what can be observed in the chicken lung by electron microscopy (25). In addition, lamellar bodies are not necessarily related to pulmonary surfactant synthesis, since they represent a general storage form of secretory lipids in multiple cell types (26).

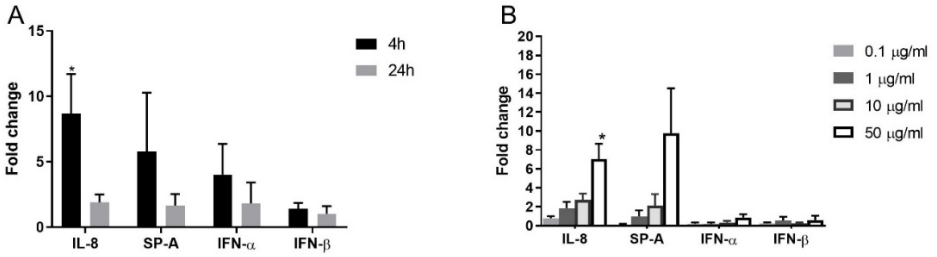

Fig 3. Immune response of CLEC213 cells. (A) CLEC213 cells were incubated with 10^7 CFU/mL for 3 h, after which non-associated bacteria were washed away. At 4 and 24 hpi mRNA was isolated and quantitative PCR was used to determine gene expression of selected genes. (B) CLEC213 cells were incubated with *E. coli* LPS at the indicated concentrations for 4 h. Gene expression was determined using qPCR. Data are shown ± SEM of at least three independent experiments for each group (in duplicate). * indicates significant difference ($P \leq 0.05$).

In general, it is worthwhile to realize that in birds, there is not a clear distinction into only two types of alveolar epithelial cells as found in mammals. In the latter, alveoli contain elongated type I cells that are involved in gas exchange, while cuboidal type II cells contain secretory vesicles in which pulmonary surfactant is stored as surfactant-protein rich lamellar bodies. On the contrary, in birds besides granular secretory cells also squamous atrial and squamous respiratory cells (and squamous intermediate cells) are observed. Secretion of surfactant like material is not limited to the granular cells since also the squamous cells secrete a trilaminar substance that covers the epithelium (25, 27). Although immunohistochemistry has shown the presence of cSP-A in specific atrial cells (20), while also another antibody CVI-ChNL 74-3 was described to recognize secretory type II cells (28), it should possibly be concluded that the simple classification in epithelial type I and type II cells is convenient, but oversimplified when used for chicken lungs.

Finally, besides gene expression, also the NO production by CLEC213 cells after LPS stimulation was measured. Although NO production is a common feature for (stimulated) macrophages, the current lack of knowledge on the exact lung epithelial immune defense prompted us to check this. As expected, NO levels were low irrespective of LPS concentration (1-50 mg/mL) or duration of stimulation (4-24 h) (data not shown).

Overall, this study indicates that CLEC213 cells are a valuable tool to determine host pathogen interaction in the chicken lung, and can help in understanding the host response towards bacterial infections.

Acknowledgements

The authors would like to thank Richard Wubbolts of the Center for Cell Imaging (Faculty of Veterinary Medicine, Utrecht University) for his help with the confocal microscopy experiments. This work was supported by a personal fellowship from the China Scholarship Council (CSC) to Lianci Peng.

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Chapter 3

Avian pathogenic *Escherichia coli*-induced activation of chicken macrophage HD11 cells

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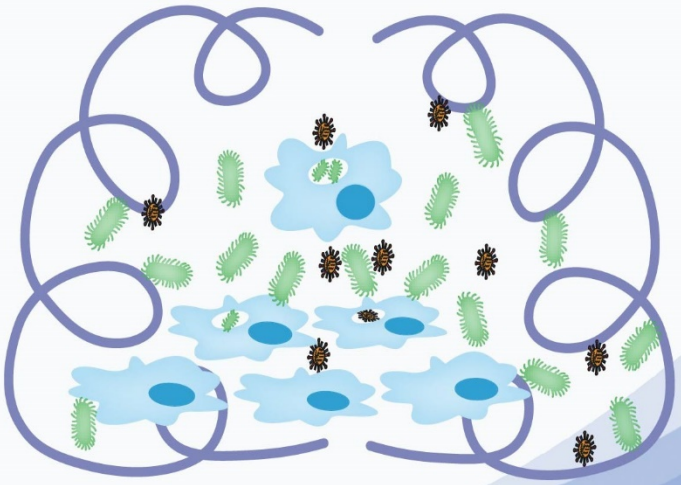

Abstract

Avian pathogenic *Escherichia coli* (APEC) can cause severe respiratory diseases in poultry. The initial interaction between APEC and chicken macrophages has not been characterized well and it is unclear how effective chicken macrophages are in neutralizing APEC. Therefore, the effect of APEC on activation of chicken macrophage HD11 cells was studied. Firstly, the effect of temperature (37 vs 41 ℃) on phagocytosis of APEC by HD11 cells was determined. The results showed that APEC was more susceptible to being phagocytosed by HD11 cells at 41 °C than 37 °C. Subsequently, it was shown that HD11 cells have the capacity to kill APEC. In addition, HD11 cells produced nitric oxide (NO) at 18 h post infection and a strong increase in the gene expression of IL-8, IL-6, IL-1β and IL-10 was detected, while IFN-β gene expression remained unaffected. Finally, it was shown that the response of HD11 was partially dependent on viability of APEC since stimulation of HD11 cells with heat-killed APEC resulted in a reduced gene expression level of these cytokines. In conclusion, APEC induces an effector response in chicken macrophages by enhanced NO production and cytokines gene expression.

Introduction

Avian pathogenic *Escherichia coli* (APEC) strains can cause severe infections in poultry, such as omphalitis, salpingitis, cellulitis and respiratory tract infections (1, 2). In all of these infections, the bacteria can enter the bloodstream and become systemic resulting in colibacillosis (2, 3). Nowadays, avian colibacillosis is one of main causes leading to mortality and morbidity in poultry resulting in huge economic losses in the poultry industry (4). So far, there is no highly effective vaccination to protect against APEC strains mainly due to the diversity of APEC strains in the field. Treatment of APEC infection mainly relies on antibiotics, but the increasing emergence of drug resistance makes treatment less successful.

An APEC infection originally starts in the respiratory tract, crosses to the blood stream and can subsequently infect internal organs causing septicemia (5). In the last few years, APEC pathogenesis to the host has been studied through the use of experimental infection models (6-8) and identification of virulence genes (9-12). Furthermore, APEC superinfection with infectious bronchitis virus infection leads to severe pathogenesis in the respiratory tracts as virus damages the respiratory mucosa and facilitates APEC colonization (2). Despite the importance of APEC pathogenesis, the knowledge about APEC invasion and interactions with host cells in chicken is limited and poorly understood.

In order to respond to a microbial infection, host innate immune cells are activated by interaction with (parts of) the pathogen. Activation of these immune cells subsequently leads to activation of intracellular signal pathways resulting in production of cytokines and microbial killing (13). Macrophages are one of the first responder innate cells upon a new infection, as seen in infection models where APEC infection causes a quick increase in the number of macrophages (14). They can phagocytose bacteria and subsequently produce multifunctional compounds including reactive oxygen species (ROS), nitric oxide (NO) and cytokines to kill the infectious microorganisms (15-17) and signal to other immune cells to establish an appropriate response to the infection.

A number of *in vitro* studies have shown phagocytosis and immune responses of chicken macrophages upon challenge with different bacterial strains, using primary macrophages, or chicken macrophage cell lines (18-21). Although most bacteria tested are phagocytosed by macrophages, some bacteria such as *Staphylococcus aureus*, can still escape from macrophages through the activation of caspase-3 followed by macrophage cell death (22). However, the role of macrophages in controlling APEC infection and the interaction between APEC and macrophages in chickens is less studied and remains mostly unclear.

In this study, we investigated the phagocytic capacity of HD11 macrophages towards APEC and the response towards infection as a first step towards understanding role of chicken macrophages in APEC infection.

Materials and methods

Bacterial strains

APEC strain (O78, K80) was isolated from chicken (23). *Salmonella* Enteritidis (strain, 13368) was cultured in Tryptic Soy Broth (TSB) and Tryptic Soy Agar (TSA) at 37 ℃. Heat-killed bacteria were prepared by incubating the bacterial suspension at 75 ℃ for 15 min; viability was checked by plating out heat treated bacteria on TSA plates. For preparation of the green fluorescent protein (GFP) – expressing APEC strain, the plasmid PWM1007 was transformed into APEC by electroporation using an Electro Cell Manipulator according to the manufacturer's instructions. GFP-expression APEC was cultured in the same condition with APEC.

Chicken macrophages, HD11 cells

The chicken macrophage-like cell line (24), HD11, was maintained in a humidified 41 ℃ incubator with 5% CO_2 and cultured in RPMI 1640-glutamax supplemented with 10% FCS and antibiotics (100 U penicillin/mL and 100 µg streptomycin/mL). Aliquots of cell suspension were seeded into each well at 2.5×10^5 cells/well for a 24-well plate and 5.0×10^5 cells/well for 12-well plates and cultured overnight before being used for assays described below.

Effects of temperature on the growth of APEC

Log-phase bacteria were diluted to $1x10^5$ CFU/mL in RPMI 1640-glutamax supplemented with 10% FCS. Aliquots of this bacterial suspension were added to 2 new tubes and incubated at 37 ℃ and 41 ℃, respectively. The OD_{620nm} was measured at 0-6 h to determine kinetics of bacterial growth.

Effects of temperature on HD11 phagocytic capacity and cell viability

Before HD11 cells were incubated with APEC, culture medium was removed and cells were washed once with RPMI 1640-glutamax. Aliquots of 1 mL of bacterial suspension (10^6 CFU/mL) were added to each well with four replicate wells for 24-well plates, at a multiplicity of infection (MOI) of 2. Cells were incubated for 1, 2, 3 and 4 h at 37 ℃ and 41 ℃. After incubation, the bacterial suspension was removed and HD11 cells were washed three times with RPMI 1640-glutamax. Then, RPMI 1640-glutamax containing 500 µg/mL gentamicin was added to each well in order to kill all extracellular, non-phagocytosed

bacteria and the plates were placed back at 37 ℃ and 41 ℃ for 1 h. At each appropriate time point (2, 3, 4 and 5 h, after gentamicin treatment), infected cells in three wells were washed three times with RPMI 1640-glutamax and lysed by 1 mL 0.5 % Triton X-100. After lysis, dilution series of cells were plated on TSA plates and incubated at 37 ℃ for 24 h to quantify viable bacteria. Cells in the fourth well received 0.1 mL 0.01 % trypsin-EDTA and were stained with trypan blue to quantify cell viability.

HD11 killing activity

Before incubation with APEC, HD11 cells were washed once with RPMI 1640-glutamax. Aliquots of 1 mL of bacterial suspensions ($1x10^6$ CFU/mL) were added to each well, with four replicate wells for 24-well plates at a MOI of 2 and incubated for 3 h at 41 ℃. At 3 h, the bacterial suspension was removed and cells were washed three times with RPMI 1640-glutamax and replaced with RPMI 1640-glutamax containing 500 µg/mL gentamicin for 1 h. After this high-gentamicin treatment, medium was replaced by cell medium containing 62.5 µg/mL gentamicin and cells were incubated back at 41 ℃. At each time point (4, 5, 6, 7 and 8 h), cells in three wells were washed three times with RPMI 1640-glutamax and lysed by 1 mL 0.5% Triton X-100. Then, suspensions were serially diluted, plated on TSA plates and incubated at 37 ℃ for 24 h to quantify viable intracellular bacteria. Cells in the fourth well received 0.1 mL 0.01% trypsin-EDTA and were stained with trypan blue to quantify cell viability.

Nitric oxide (NO) production assay

Nitrite, a stable metabolite of NO, produced by activated macrophages was measured by the Griess assay (25). HD11 cells were incubated with live or heat-killed bacteria at 41 ℃ for 3 h and treated with 500 µg/mL gentamicin for 15 h. After 18 h incubation, aliquots of 50 µL supernatant were transferred to the wells of a 96-well flat bottom plate. Fifty µL 1 % sulfanilamide (Merck, Darmstadt, Germany) was added in each well mixed with 50 µL 0.1 % N-(1-naphthyl) ethylenediamine dihydrochloride (VWR) at room temperature for 5 min. The nitrite concentration was determined by measuring optical density at 550 nm. Sodium nitrite (Sigma) was used as a standard to accurately determine the nitrite concentration in the cell supernatant.

Gene expression

HD11 cells were incubated with APEC at 41 ℃ for 3 h and subsequently treated with 500 µg/mL gentamicin for 1 h as described above. After 4 h incubation, total RNA was extracted by Trizol (Ambion, Carlsbad, CA) reagent according to manufacturer's instructions. RNA (500 ng) was reverse transcribed by the iScript cDNA synthesis kit (Bio-Rad, Veenendaal, the Netherlands) according to the manufacturer's instructions. Primers and probes were

designed and produced by Eurogentec (Seraing, Belgium) (Table 1). Quantitative real time PCR was performed on a CFX Connect qPCR with CFX Manager 3.0 (Bio-Rad). Reactions were performed as follows: 3 min at 95 °C; 40 cycles: 10 s at 95 °C, 30 s at 60 °C and 30 s at 72 °C. Relative gene expression levels were normalized against the expression levels of the house keeping genes GAPDH and 28S.

Table 1: Primer and probe sequences for qPCR

Gene		5'→3'sequence
GAPDH	Forward	GTCAACCATGTAGTTCAGATCGATGA
	Reverse	GCCGTCCTCTCTGGCAAAG
	Probe	AGTGGTGGCCATCAATGATCCC
28S	Forward	GACGACCGATTTGCACGTC
	Reverse	GGCGAAGCCAGAGGAAA
	Probe	AGGACCGCTACGGACCTCCACCA
IFN-β	Forward	CCTCCAACACCTCTTCAACACG
	Reverse	TGGCGTGTGCGGTCAAT
	Probe	AGCAGCCCACACACTCCAAAACACT
IL-1β	Forward	GCTCTACTAGTCGTGTGTGATGAG
	Reverse	TGTCGATGTCCCGCATGA
	Probe	CCACACTGCAGCTGGAGGAAGCC
IL-6	Forward	GTCGAGTCTCTGTGCTAC
	Reverse	GTCTGGGATGACCACTTC
	Probe	ACGATCCGGCAGATGGTGA
IL-8	Forward	GCCCTCCTCCTGGTTTCA
	Reverse	CGCAGCTCATTCCCCATCT
	Probe	TGCTCTGTCGCAAGGTAGGACGCTG
IL-10	Forward	CATGCTGCTGGGCCTGAA
	Reverse	CGTCTCCTTGATCTGCTTGATG
	Probe	CGACGATGCGGCGCTGTCA

Confocal microscopy

HD11 cells were seeded on a 12 mm coverslip in 24-well plate and incubated overnight. Cells were incubated with GFP-APEC for 3 h at 41 ℃ as described previously. After three wash steps with RPMI 1640-glutamax, cells were fixed with 4% paraformaldehyde (PFA) in PBS for 30 min at room temperature (RT). Subsequently, cells were incubated with 50 mM NH_4Cl in PBS for 10 min at RT and blocked with 5% normal goat serum in PBS for 1 h. Then, cells were stained with *E. coli* antiserum (23) (1:500) for 1 h. After the wash steps, cells were incubated with Donkey anti-Rabbit Alexa 647 (Jackson ImmunoResearch, West Grove, PA, USA) (1:100) for 1 h. Finally, cells were washed with PBS or water and mounted in FluoroSave. Slides were observed on a Leica SPE-II DMI4000 microscope with LAS-AF software (Leica, Wetzlar, Germany) using a 63× HCX PLAN APO OIL CS objective.

Statistical analysis

Data are represented as mean ± SEM of three independent experiments for each group (n=3) and were analyzed by a T-test for two groups or by one-way ANOVA with post-hoc t-test for more than two groups. $p \leq 0.05$ were considered significant. Bio-Rad CFX Manager 3.0 software was used for qPCR data analysis. All the graphs were made using GraphPad Prism® 5.0.

Results

Effect of temperature on the growth of *E. coli*

It is well known that the optimal culture temperature of some bacterial species is 37 ℃. However, enteric bacteria are specifically adapted to their hosts' body temperature. Since a chicken's body temperature is close to 41 ℃, we determined whether the temperature can affect the growth of APEC. For this, two *E. coli* strains (APEC and K88) were simultaneously cultured at 37 ℃ and 41 ℃. As shown in Fig. 1, both *E. coli* strains reached the logarithmic phase faster at 41 ℃ than 37 ℃, resulting in higher OD values at 2 h and 3 h. Interestingly, at 5 h and 6 h, the final OD of K88 was significantly lower at 41 ℃ compared to 37 ℃.

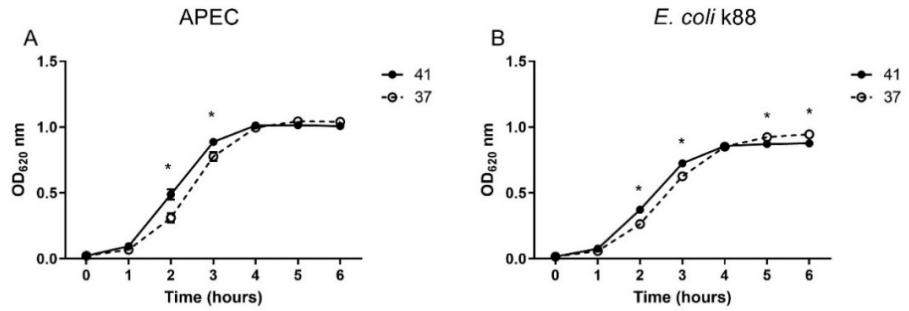

Fig 1. Temperature dependency of growth of *E. coli* strains. (A) APEC. (B) *E. coli* k88. Data are shown as mean ± SEM of three independent experiments (in triplicate per experiment) for each group (n=3). * indicates significant difference (P≤0.05) between 41 ℃ and 37 ℃ for a single time point using a student's T-test.

Effect of temperature on phagocytic capacity and viability of HD11 cells

The effect of temperature and incubation time on the phagocytic capacity of HD11 cells was tested. HD11 cells were incubated with APEC at 37 °C and 41 °C and the number of intracellular bacteria was determined after 1-5 h. As shown in Fig. 2A, the number of bacteria increased over time, and at all time points more bacteria were phagocytosed at 41 °C. In the same experiment, viability of HD11 cells after the infection was tested with trypan blue. No significant reduction in viability of HD11 was observed (Fig. 2B) at any time point. Based

on these results, an incubation time of 4 h at 41 °C was chosen as the optimal assay condition for subsequent experiments.

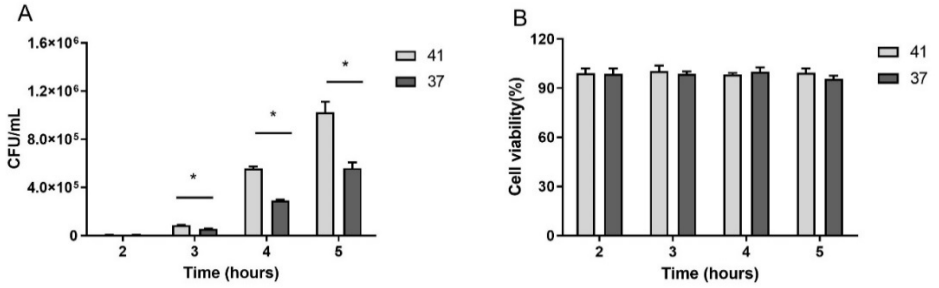

Fig 2. Temperature dependency of phagocytosis of APEC by HD11 cells. (A) Number of phagocytosed APEC in HD11 cells at 2-5 h post-incubation at 37 °C and 41 °C. (B) Viability of HD11 cells at 2-5 h post incubation. Data are shown as mean ± SEM of three independent experiments for each group (in triplicate per experiment). * indicates significant difference ($P \leq 0.05$) in bacterial number between 37 °C and 41 °C using a student's T-test.

Intracellular and extracellular APEC

In order to confirm the intracellular localization of APEC in our experimental set-up, confocal microscopy was performed. After HD11 incubation with GFP-APEC, the bacteria were stained with an anti-*E. coli* serum. Because cells were not permeabilized, only extracellular APEC was labelled with this antibody enabling a distinction between phagocytosed intracellular and adhered extracellular APEC. As shown in Fig. 3, intracellular bacteria, only GFP stained bacteria (green) are indeed present in HD11 cells, as well as extracellular bacteria (yellow/red), qualitatively confirming the results shown in Fig. 2.

Killing of APEC by HD11 macrophages

HD11 cells are capable of phagocytosing *S. typhimurium*, *S.* Enteritidis and *Listeria monocytogenes* (18, 20). Once phagocytosed, the number of viable intracellular bacteria decreased over the next 24 h (19). To determine whether macrophages are also capable of killing intracellular APEC, viability of APEC after phagocytosis was checked at several time points (Fig. 4). At 4 h, a lower number of APEC was present in the macrophage compared to *S.* Enteritidis (Fig. 4A), demonstrating a difference of HD11 uptake capacity towards different strains. At 6, 7 and 8 h, a significantly decreased number of APEC was observed (Fig. 4A), indicating that HD11 are able to kill APEC, contrary to *S.* Enteritidis. This observation indicated that *S. enteritidis* is more resistant to HD11 killing.

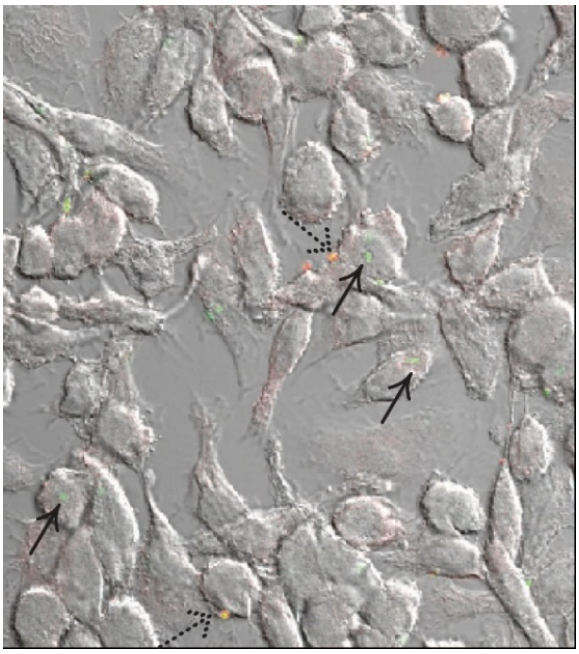

Fig 3. Localization of APEC in HD11 cells. Cells were incubated with GFP-APEC for 3 h. Subsequently, cells were washed and fixed, but not permeabilized. Extracellular APEC were labeled with rabbit anti-*E. coli* serum and Donkey anti-Rabbit Alexa 647 (red). HD11 cells appeared grey under the DIC channel. Intracellular bacteria appear green (black arrows), while extracellular bacteria appear as red/yellow (dashed arrows).

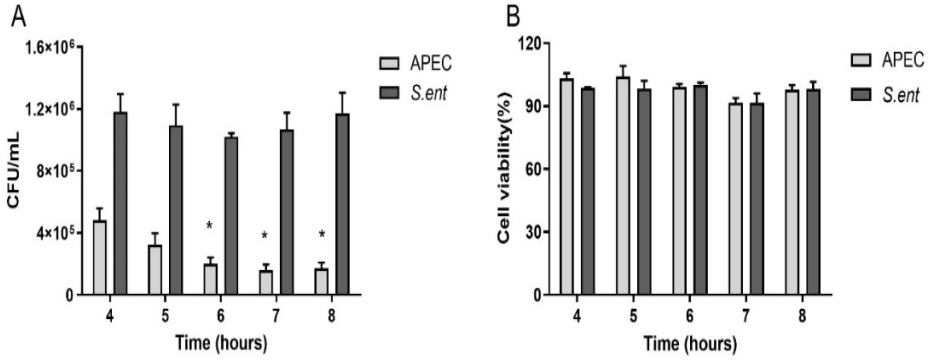

Fig 4. Survival of phagocytosed bacteria in HD11 cells. (A) Viable APEC and *S. enteritidis* in HD11 cells. (B) Viability of HD11 cells. Data are shown as mean ± SEM of three independent experiments for each group (in triplicate). * indicates significant difference ($P \leq 0.05$) in bacteria compared with 4h incubation using one-way ANOVA with post-hoc t-test.

NO production

Phagocytosis of APEC and *S.* Enteritidis induced a significant NO production in HD11 cells, with APEC giving higher levels of NO compared to *S.* Enteritidis (Fig. 5). In order to get a first indication whether bacteria had to be viable to activate cells, heat-killed APEC was also used in these experiments. Although the absolute amount of NO was lower for heat-killed APEC, they were still able to induce a significant amount of NO. These results indicate that activation of HD11 cells is partially depending on bacterial strain and viability but that bacterial products are responsible for most activation.

Cytokine expression

Besides NO production, activation of macrophages can lead to an increased expression of cytokines that further modulate the immune response in response to a bacterial infection. Quantitative real-time PCR (qRT-PCR) was used on selected genes (Table 1) to evaluate the effect of APEC on the immune response in HD11 cells. In addition, to evaluate the effect of viability of APEC on cytokines expression in HD11 cells, heated-killed APEC was also used in these experiments. At 4 h post-infection, APEC strongly up-regulated the expression of pro-inflammatory cytokines IL-1β and IL-6, inflammatory cytokine IL-8 and anti-inflammatory cytokine IL-10 in HD11 cells (Fig. 5), whereas IFN-β was not affected. Similar results were also found for *S.* Enteritidis stimulation in HD11, indicating that HD11 cells respond similarly towards both bacteria. Interestingly, addition of heat-killed APEC to HD11 cells resulted in a lower expression of IL-1β, IL-6 and IL-8, compared to viable APEC, but expression of IL-10 was not affected. Overall these results show that HD11 cells are capable of a strong cytokine production after phagocytosis of APEC, comparable to *S.* Enteritidis, and that viability of APEC affects most but not all cytokines indicating different signaling pathways could be involved for the production of these mRNAs.

Discussion

APEC can infect different kinds of birds including chickens, turkeys, and ducks, and causes systemic infections called avian colibacillosis due to immunosuppression and damage of the immune system (6). Despite of the identification of some virulent genes involved in bacterial adhesion and invasion that contribute to APEC pathogenesis (26), the pathogenic mechanism of APEC is still unknown as no specific virulence gene has been identified for the entire APEC pathogenesis in avian host. Most studies have focused on the role of virulence in APEC pathogenesis, but little research is performed on the interaction of APEC with host innate immune cells. Macrophages are quickly increased upon APEC infection response to APEC infection (14). Therefore, we studied the interaction between APEC and macrophages as an important step to determine the initial host response to APEC infection.

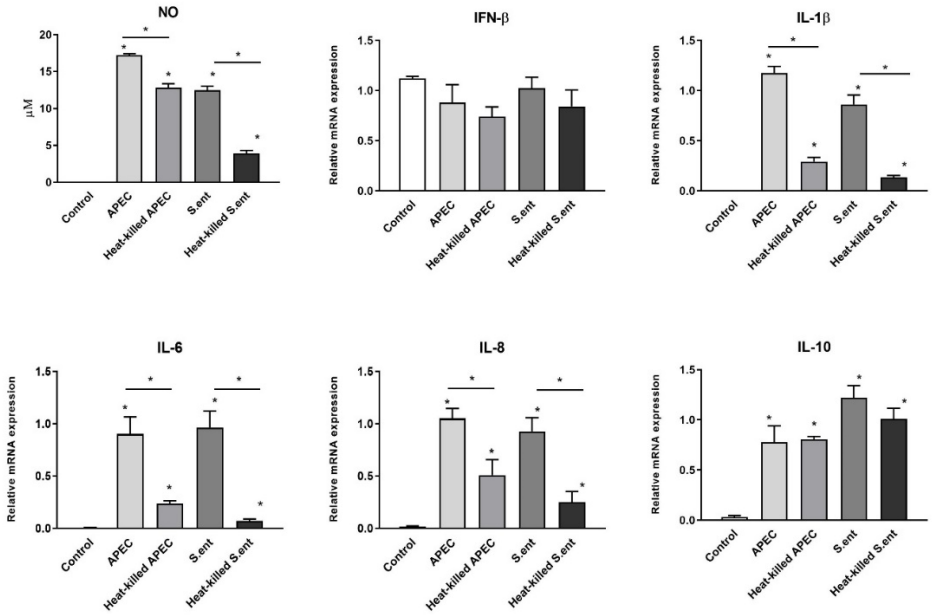

Fig 5. NO production and cytokine expression of HD11 cells after incubation with bacteria. HD11 cells were incubated with live or heat-killed APEC and *S.* Enteritidis for 18 h. Subsequently, nitrite (metabolite of produced NO) content in cell culture media was determined. For cytokines expression, HD11 cells were incubated with APEC and *Salmonella* for 4 h. Relative mRNA expression of cytokines in HD11 cells was determined using qPCR. Data are shown as mean ± SEM of three independent experiments (in triplicate) for each group. * indicates a significant difference compared to the control, or (indicated with horizontal bars) between specific treatment groups using one-way ANOVA with post-hoc t-test.

As a first step, the effect of temperature on the phagocytic activity of macrophages was determined. HD11 cells are often used at 37 °C (20, 27, 28) but the chicken body temperature is closer to 41 °C, which implies that the latter temperature would better resemble the *in vivo* situation. Indeed, some clear differences were observed at the higher temperature *E. coli* (APEC and non-APEC) grew faster at 41 °C (Fig. 1) indicating that the temperature was non-specific for the growth of APEC. On the other hand, HD11 cells phagocytized more *E. coli* at 41 °C (Fig. 2), demonstrating that HD11 cells have higher phagocytic activity at 41 °C. This is in line with available literature on the effect of temperature on phagocytic activity of immune cells in cold-blooded species. Phagocytic activity has been reported to decline in fish at low (<15 °C) or high (37 °C) temperature (29). Activity of other immune cells and antibody binding activity also reduced at decreased body temperature (30-32). In a mice model experiment, macrophages exposed to an acute cold environment (4 °C for 24 h) were suppressed in their activity (33). These studies imply that temperature plays an important role in remaining immune response and function of immune cells, which suggests that chicken macrophages should be used at 41 °C in order to resemble *in vivo* activity as much as possible.

The mononuclear phagocytic cells are part of the first line defense against invading pathogens. Activated macrophages can secrete a series of cytokines and chemokines and kill microorganisms by phagocytosis (34-37). For mammalian macrophages, many studies on phagocytic activity have been described, but the available data for chicken macrophages is limited. For chicken macrophage studies, phagocytosis of different bacteria has been investigated, such as *S. typhimurium* (18), *L. monocytogenes* (20) and *Mycoplasma synoviae* (28), but the research about APEC is lacking. Therefore, we studied phagocytosis of APEC in chicken macrophages (HD11). Our data showed that HD11 cells are relatively slow at uptake of APEC with relatively low numbers of APEC at 2 h post-infection after addition of the bacteria. However, longer incubation times significantly increased the number of phagocytosed APEC, although still much lower than *Salmonella* (Fig. 2). In initial studies to optimize *E. coli* uptake by HD11 cells, the effect of addition of chicken serum on phagocytosis of HD11 cells was tested but this had no effect (data not shown). Similarly, low numbers of phagocytosed (non-avian pathogenic) *E. coli* by HD11 macrophages were also observed by Wisner *et al* where *E. coli* DH5α was used as a control group for their *Salmonella* studies, although these authors suggested that this was due to fast killing of *E. coli* (18). These differences in phagocytosis indicate that macrophages have different phagocytic capacity to different bacterial strains. After all, phagocytosis is a complex process involving a diverse set of receptors that can stimulate phagocytosis. Different phagocytosed microbes have a different capacity to affect cells. In our study, it is visually shown for the first time that HD11 can indeed phagocytize APEC (Fig. 3) as intracellular APEC was clearly observed under the fluorescent microscopy (Fig. 3), indicating that a lack of uptake is not likely the cause of APEC virulence.

It is well known that macrophages can ingest and kill bacteria. Our results clearly indicated that APEC was killed by HD11 at 6 h post-infection while *S.* Enteritidis survived at all the time points. It has been reported that *Salmonella* can secrete different virulence factors in host cells to invade, survive and replicate within these host cells (38, 39), explaining the observed survival of *Salmonella*. In addition, *Salmonella* has been shown to delay the phagolysosomal maturation and neutralize radical oxygen and nitrogen species (ROS and NOS), as defense mechanisms against host cells (40-42). APEC apparently does not have such an evasion strategy although it must be said that after 6 h, the number of APEC did not further change.

NO plays an important role in the host defense against microbial infection (43). It can be produced by activated monocytes or macrophages, and act as effector molecules to kill invading pathogens. Previously, *S.* Enteritidis-induced NO production was described in chicken macrophages (16, 44). Similarly, our results showed that live and, to lesser extent, heat-killed bacteria induced NO production (Fig. 5), demonstrating that NO production might

partially depend on bacterial viability. Interestingly, even though HD11 cells took up lower numbers of APEC, compared to *S.* Enteritidis, NO production was higher, indicating that APEC is a relatively strong inducer of NO. One study actually showed that (viable) *S.* Enteritidis was able to inhibit production of NO in HD11 cells (19) probably as an evasion mechanism, but the exact mechanism was unknown. The discrepancy with our results could be related to strain dependency since the same study showed that other *Salmonella serovars* induced a strong NO response in HD11 cells. Overall, chicken macrophages seem to be able to produce a strong response towards APEC, comparable to, or even higher than *Salmonella*.

Besides intracellular killing of pathogens, professional phagocytes play an important role in modulating the immune response through expression of cytokines and chemokines. Therefore, we explored the initial immune response induced by APEC after 4 h incubation with chicken macrophages. Our results showed that both APEC and *S.* Enteritidis significantly induced the expression of IL-1β, IL-6 and IL-8 (Fig. 5). The induction of IL-6 and IL-1β in this study is consistent with other observations that APEC induced increased expression of IL-6 and IL-1β in chicken monocyte derived macrophages (28). In another study *S.* Enteritidis infection of chickens led to increased expression of IL-8 and IL-1β even through this was measured in the cecum (45). The surfaces of *E. coli* and *S.* Enteritidis have a variety of microbial associated molecular patterns (MAMPs), like LPS and flagellin, interacting with toll-like receptors (TLR) on the macrophage surface. The observation of induced pro-inflammatory cytokines IL-8 and IL-1β as well as inflammatory cytokine IL-6 is likely explained by activation of TLR signaling (46, 47). However, we observed that heat-killed APEC induced a lower amount of these three cytokines compared to live APEC, indicating that the level of cytokines expression induced by APEC is partially determined by bacteria and the presence of PAMPs. Interestingly, with respect to anti-inflammatory cytokine IL-10 expression, live APEC and heat-killed APEC induced almost the same level of cytokine expression, but *Salmonella* induced a higher expression, suggesting that this process might depend on amount and nature of PAMPs and is not necessarily bacterium-specific. Poly (I:C) and CpG-ODN have been reported to upregulate chicken IL-10 in chicken monocytes, indicating the occurrence of immune regulation to control excessive inflammation (48). Finally, there was not much difference for IFN-β expression in this study, which is in agreement with published studies on LPS and bacterial DNA stimulation of HD11 cells which, contrary to viral infection of HD11 cells, did also not have IFN-β production (48-50). These results suggest that APEC can induce a strong inflammatory response by the expression of some cytokines, but not for all the cytokines due to different signaling pathways.

In summary, we investigated the interaction of APEC with chicken macrophage HD11 cells. APEC was efficiently phagocytosed by HD11 cells at 41 °C and subsequently killed. Phagocytosis resulted in a clear pro-inflammatory immune response including production of

NO and pro-inflammatory cytokines, indicating that in principle chicken macrophages are capable of an appropriate immune response towards APEC infection. However, although the HD11 cell line is a well-established macrophage cell line, phagocytosis of APEC by chicken primary macrophages needs to be further explored to obtain extra confirmation of our current findings.

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Chapter 4

A method to differentiate chicken monocytes into macrophages with proinflammatory properties

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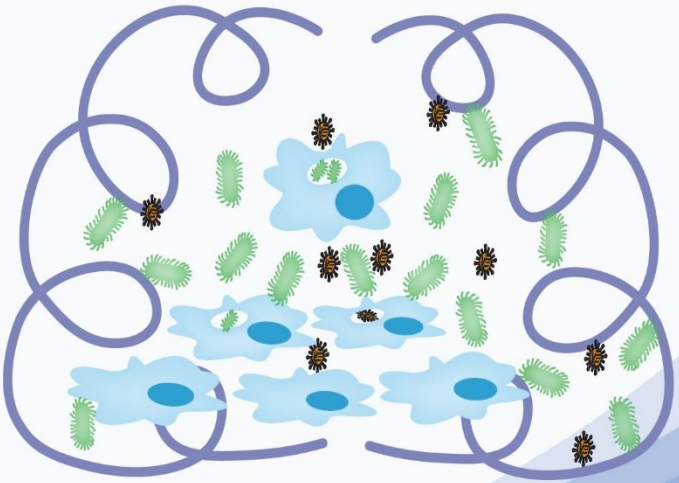

Abstract

Macrophages are part of the first line of defense against invading pathogens. In mammals, the *in vitro* culture of macrophages from blood monocytes or bone marrow cells is well established, including culturing conditions to differentiate them towards M1 or M2 macrophages. In chicken, monocyte-derived macrophages have been used in several studies, but there is no uniform protocol or actual characterization of these cells. Therefore, to generate chicken pro-inflammatory M1-like macrophages, in this study blood monocytes were differentiated using chicken GM-CSF for 4 days and characterized based on cell morphology, surface marker expression and cytokine expression response to toll-like receptors (TLRs) agonists stimulation at each (daily) time point. Cell morphology showed that one-day-cultured cells contained a mixture of cell populations, while on day 3 and day 4 the cells were a homogenous population of flat, 'fried-egg' like shaped cells, similar to human M1 macrophages. In addition, cell surface marker staining showed that 3- and 4-day-cultured cells expressed a high level of MRC1L-B (KUL01) and MHC-II. Furthermore, LPS stimulation of the cultured cells induced gene expression of the pro-inflammatory cytokines IL-1β, IL-6 and IL-8 after 3 days of culture. Finally, it was shown that 3-day-cultured macrophages were able to phagocytose avian pathogenic *E. coli* (APEC) and respond by nitric oxide production. Overall, our systematic characterization of the monocyte-derived macrophages from chicken blood showed that a 3-day culture was optimal to obtain pro-inflammatory M1-like macrophages, increasing our knowledge about chicken macrophage polarization and providing useful information for studies on chicken macrophage phenotypes.

Introduction

Macrophages play an important role in the innate immune system against invading pathogens. They are actively involved in phagocytosis and subsequent killing pathogenic microorganisms. In addition, they are key regulatory cells of the immune system by the production of a pro- or anti-inflammatory response upon stimulation. These immune responses are initiated by specialized pathogen recognition receptors, including the toll-like receptor (TLR) family. TLR4 and TLR7 are important to recognize components of bacteria (LPS) and viruses (RNA), respectively (1). In mammals, macrophages have different phenotypes, such as M1, M2, M (Hb), Mox, and M4. However, all these phenotypes are nowadays considered differentiated 'extreme-states' of a macrophage and depending on environmental factors macrophages can convert and cover the whole spectrum between these different states (2, 3).

In recent years, M1 and M2 macrophages have been studied the most and are linked to different macrophage functions. In a simplified view, M1 macrophages are usually considered pro-inflammatory macrophages that play a role in killing intracellular pathogens, while M2 macrophages are 'tolerant' anti-inflammatory macrophages important for wound healing and tissue repair (4). *In vitro*, M1 and M2 macrophages can be grown by applying different supplements (5, 6). Morphologically M1 macrophages differentiate into so-called "fried-egg" shaped cells that express inducible nitric oxide synthase (iNOS) and produce pro-inflammatory cytokines such as IL-6 and TNF-α in response to stimulation. M2 macrophages are stretched, spindle-like cells that express arginase and produce anti-inflammatory cytokines such as IL-10 in response to stimulation (7-9). Besides morphology, M1 macrophages can also be distinguished from M2 by their different expression of surface markers (5, 6). For instance, MHC-II is expressed by both subsets and high MRC1 is a characteristic of M2 macrophages. These different phenotypes of macrophages provide a useful tool for understanding the function and especially potential of macrophages *in vivo*.

Unlike *in vitro*-cultured mammalian macrophages that have well described distinct phenotypes under specific conditions, chicken macrophages have been poorly described or standardized. As a common initial step, chicken macrophages are cultured from monocytes purified from peripheral blood or bone marrow by adherence to glass or plastic. After that methodologies diverge and differentiation of monocytes to macrophages occurs without external stimulation, but usually in the presence of chicken serum, or is stimulated through addition of chicken (G)M-CSF (10-12). Interestingly, a recent manuscript used IL-4 to stimulate differentiation into a more M2-like phenotype (13). Besides differences in culturing conditions, there is also no consensus in literature on the duration of culture, which ranges from 1 to 6 days. This lack of culture standardization of monocyte-derived macrophages

affects reproducibility and comparability of different studies. Although the macrophage cell line (HD11) have been shown to take up and kill avian pathogenic *E. coli* (APEC) (14), the role of primary macrophages in controlling APEC is less studied due to the lack of standardization of culturing and characterization of macrophages.

In this study, a thorough characterization of chicken blood monocyte-derived macrophages was performed to optimize culture conditions, like culture duration and in the presence of chicken GM-CSF. The morphology, expression of cell surface markers and immune responses upon TLRs agonists stimulation during cell differentiation were determined. Finally, the optimized macrophage culture was functionally assessed for phagocytosis of APEC and nitric oxide (NO) production.

Methods and materials

Bacterial strains

APEC strain (O78, K80) was isolated from chicken (clinical isolate, Zoetis, USA) (15), stored as a 25% glycerol stock and cultured in Tryptic Soy Broth (TSB) (Oxoid) and Tryptic Soy Agar (TSA) (Oxoid) at 37 °C. Green fluorescent protein (GFP) labeling APEC strain was performed as described previously (14).

Cell isolation, culture and cell surface marker staining

Peripheral blood mononuclear cells (PBMCs) were isolated from 76-week-old healthy chickens blood using Ficoll gradient and cryopreserved in liquid nitrogen until use. PBMCs (1×10^7 cells) were seeded in a 24-well plate containing 1 mL RPMI 1640+glutamax medium (Gibco, UK) with 10% FCS (Bodinco, Alkmaar, The Netherlands) and penicillin (100 U/mL)/streptomycin (100 µg/mL) (P/S) (Life Technologies, Carlsbad, CA, USA) and incubated at 41 ˚C. After overnight incubation, all non-attached cells were removed and attached cells were maintained in RPMI 1640+glutamax medium with 10% FCS and P/S supplemented with chicken GM-CSF for another 3 days at 41 ˚C. Cell morphology was microscopically examined at each day. In addition, cells were harvested after 1, 2, 3, or 4 days using PBS containing 0.5 mM EDTA to detach the cells (hereafter referred to as day 1-day 4 cells). After centrifugation and washing steps, cells were stained for the chicken mannose receptor C-type 1-like-B (MRC1L-B) using the KUL01-FITC antibody (clone KUL01, isotype Southern Biotech, Birmingham, AL, USA) and MHC-II (MHCII-PE, ‘clone 2G11’; isotype Southern Biotech) in FACS buffer (0.5% BSA in PBS) at 4 ˚C for 30 min. Afterwards, cells were washed and analyzed using flow cytometry (FACSCanto-II, BD Biosciences, CA, USA) and FlowJo Software v. 10.5 (FlowJo LCC, Ashland, OR, USA).

LPS and R848 stimulation of monocyte derived macrophages

Cells were cultured as described above. After 1, 2, 3, or 4 days, ultrapure LPS *E. coli* O111:B4 (100 ng/mL) (InvivoGen, San Diego, CA, USA) or 10 µg/mL R848 (InvivoGen, San Diego, CA, USA) diluted in RPMI 1640-glutamax medium with 10 % FCS, was added to the cells for 4 h at 41 ˚C. Afterwards, cells were washed and lysed in Trizol (Ambion, Carlsbad, CA) stored in -20 ˚C for RNA isolation.

Quantitative real-time PCR (qPCR)

Total RNA was extracted by Trizol reagent according to the manufacturer's instructions. RNA (500 ng) was reverse transcribed using the iScript cDNA synthesis kit (Bio-Rad, Veenendaal, the Netherlands) according to the manufacturer's instructions. Primers and TaqMan probes were designed and produced by Eurogentec (Seraing, Belgium) (14). Quantitative real time PCR was performed on a CFX Connect qPCR with CFX Manager 3.0 (Bio-Rad). Reactions were performed as follows: 3 min at 95 °C; 40 cycles: 10 s at 95 °C, 30 s at 60 °C and 30 s at 72 °C. Relative gene expression levels were normalized against the expression levels of the housekeeping gene GAPDH.

APEC infection in monocyte derived macrophages

0.5 mL 1×10^6 CFU/mL APEC was added to day 3 macrophages with triplicate wells in a 24-well plate and incubated for 3 h at 41 ˚C. After 3 h, bacterial suspensions were removed and cells were washed three times with RPMI 1640-glutamax medium with 10% FCS. Then, RPMI 1640-glutamax containing 500 µg/mL gentamicin (Sigma-Aldrich) was added to cells in order to kill all extracellular, non-phagocytosed bacteria, followed by 1 h incubation at 41 ˚C. After that, cells were incubated at 41 °C for 0, 2, or 4 h. At each time point, infected cells were washed three times with RPMI 1640-glutamax and lysed by 0.5 mL 0.5% Triton X-100. After lysis, dilution series of cells were plated on TSA plates and incubated at 37 °C for 24 h to quantify viable bacteria.

Confocal microscopy

Day 3 cells were grown on a 12 mm glass coverslip in a 24-well plate and infected with GFP-APEC (1×10^6 CFU/mL) for 3 h at 41 °C as described above. After three wash steps with RPMI 1640-glutamax, cells were fixed with 4% paraformaldehyde (PFA) in PBS for 30 min at room temperature (RT). Subsequently, cells were incubated with 50 mM NH_4Cl (Merck) in PBS for 10 min at RT and blocked with 3% BSA (Sigma-Aldrich) in PBS for 30 min. Then, cells were stained with anti-*E. coli* rabbit serum (1:500) (15) for 1 h. After the wash steps with PBS, cells were incubated with Donkey anti-Rabbit Alexa 647 (Jackson ImmunoResearch, West Grove, PA, USA) (1:100) for 1 h. Finally, cells were washed with

PBS and water and mounted in FluoroSave (Merck Millipore, Billerica, MA). Slides were observed on a Leica SPE-II DMI4000 microscope with LAS-AF software (Leica, Wetzlar, Germany) using a 63 × HCX PLAN APO OIL CS objective.

NO production assay

Nitrite, a stable metabolite of NO, was measured by the Griess assay (16). PBMCs were seeded in a 24-well plate and incubated as described above for 3 days. Then, cells were incubated with bacteria at 41 °C for 3 h and treated with 500 μg/mL gentamicin for 15 h. After 18 h incubation, supernatants were collected and NO was determined as described previously (14).

Statistical analysis

Data are represented as mean ± SEM with three independent experiments for each group (n = 3) and were analyzed by a T-test for two groups or by one-way ANOVA with Tukey's multiple comparisons test for more than two groups. Bio-Rad CFX Manager 3.0 software (Bio-Rad) was used for qPCR data analysis. All the graphs were made using GraphPad Prism® 8.0.

Results and discussion

Alteration of cell morphology and surface marker expression during cell differentiation

Monocytes were isolated from the PBMC fraction of the blood from 76-week-old healthy chickens and cultured for 1, 2, 3, or 4 days in the presence of chicken GM-CSF to differentiate monocytes into macrophages. The cultures were inspected over time for changes in macrophage morphology and purity. The first day of culture, adherent cells were monocytes (Fig. 1A, black arrows). Non-adherent cells were removed. This was a mixed population of other cells including lymphocytes and heterophils and did not contain monocytes (FACS analysis, data not shown). Cells became flat after 2 days of culture (Fig. 1B, dashed arrow). At day 3 and 4, heterophils were lost from the culture and monocytes-derived macrophages remained, as indicated by the 'fried eggs-like' shape of the cells (Fig. 1C and D, dashed arrows), similar to classic mammalian M1 macrophages (9). Next, we used flow cytometry to characterize these cells. Macrophages were determined based on the forward scatter (FSC) (cell size) and side scatter (SSC) (granularity). Increasing amounts of these cells were detected up to 3 days and then leveled between day 3 and 4 (Fig 2A). These quantified macrophages on different days were MRC1L-B and MHC-II positive cells as shown in Fig. 2B. Then, the expression of MRC1L-B and MHC-II were quantified by the geometric mean fluorescence intensity (gMFI). High surface expression of MRC1L-B was detected at day 1 (Fig 2C), reflecting that monocytes in the blood have a high expression of MRC1L-B.

Subsequent monocyte differentiation and proliferation resulted in higher numbers of cells that were identified as macrophages by our FACS gating strategy, but with a tendency (although not statistically different from day 1) to lower average MRC1L-B expression at day 2. However, MRC1L-B expression increased at day 3 reflecting maturation of the newly differentiated macrophages. The expression of MHC-II increased from day 1 to 3 and then stabilized between day 3 and 4 (Fig 2C), indicating the differentiation and maturation of macrophages. In general, these results indicate that most of the differentiation of monocytes into macrophages was reached at 3 days post-incubation and then remained stable for at least 1 day.

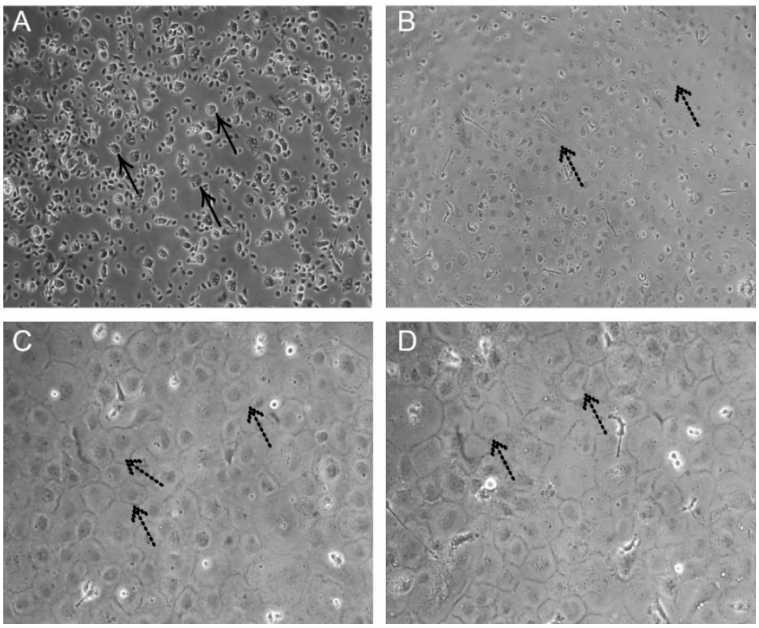

Fig 1. Morphology changes during cell differentiation. Representative microscopic images of monocyte-derived macrophages after (A) one-day-culture, (B) two-day-culture, (C) three-day-culture, and (D) four-day-culture. The black arrows show monocytes and dashed arrows show flat macrophages. All images are at 20 × magnification.

Based on presumed homology of chicken monocyte-derived macrophages with mammalian cells, expression of the cell surface markers (KUL01 and MHC-II) and morphology seem good indicators for chicken monocyte-to-macrophage differentiation. Although the expression of MRC1L-B and MHC-II were used to characterize macrophages, their expression is not limited to macrophages. MHC-II is highly expressed on chicken *in vitro* bone marrow-derived dendritic cells (DCs) (17) and also by B cells and DCs *in vivo* (18). Monoclonal antibody KUL01 recognizes a mannose receptor (also known as CD206 in mammals) and was first used to characterize macrophages by Mast *et al* in different tissues

including spleen and gut (19). A previous study has described that chicken macrophages actually have five paralogous genes of mannose receptor (MRC1L-A to MRC1L-E), contrary to mammals, and KUL01 only recognizes MRC1L-B (20). KUL01 does not exclusively bind to macrophages, since it also identified Langerhans cells in the chicken skin (19). Interestingly, in mammalian macrophages, MRC1 expression is considered to be connected to M2 macrophages. However, for MRC1L-B which is only one of the orthologs of MRC1 in chicken (20), it is unclear if mammalian data can be extrapolated.

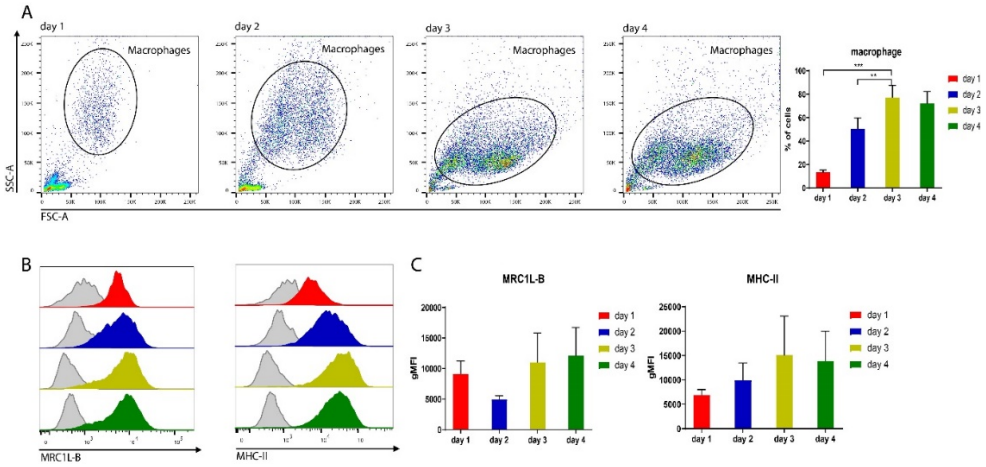

Fig 2. Expression of cell surface marker during cell differentiation. Chicken monocytes were cultured for 1-4 days in the presence of GM-CSF, after which cells were analyzed by flow cytometry. (A) Percentage of macrophages based on FSC (cell size) and SSC (granularity). (B) Representative histograms of MRC1L-B and MHC-II expression, with in grey the unstained controls. (C) The expression of MRC1L-B and MHC-II on the macrophages at different culture times were quantified by the geometric mean fluorescence intensity (gMFI). Data are represented as mean ± SEM of three independent experiments with three samples per experiment in the bar graphs. For data analysis, one-way ANOVA with Tukey's multiple comparisons test was used. *P≤0.05; **P≤0.01; ***P≤0.005.

LPS- or R848-induced cytokines during cell differentiation

The abilities of macrophages to induce an immune response upon stimulation with TLR4 agonist LPS and TLR7 agonist R848 was tested. R848 induced only low expression levels of IFN-β, IL-1β, IL-6, IL-8 and IL-10 (Fig. 3), and no significant effect of different culturing times on cytokine expression was observed. On the other hand, LPS induced a strong expression of these cytokines (Fig. 3). In addition, expression of all pro-inflammatory cytokines increased from day 1 to day 3 cultured cells, while IL-10 did not significantly change. Interestingly the gene expression of cytokines upon LPS stimulation decreased again at day 4 of culture indicating that there is not a lasting linear correlation between culture time and (pro)-inflammatory response.

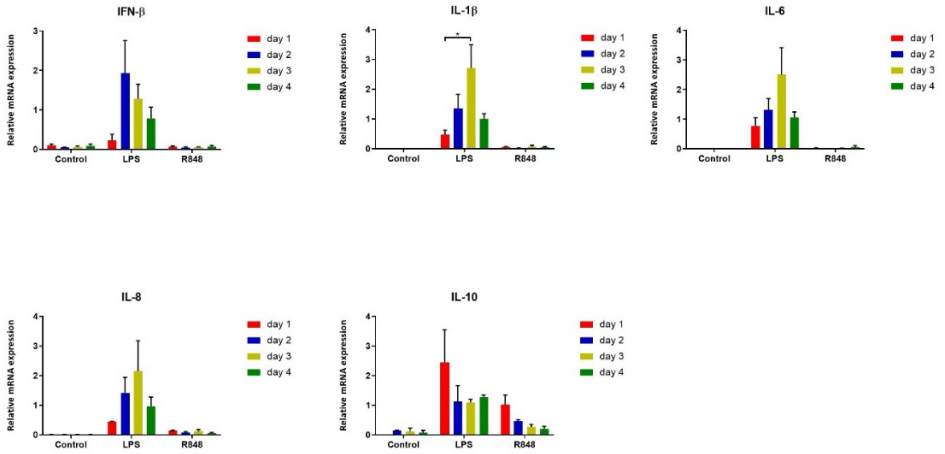

Fig 3. TLR ligands-induced cytokines expression during cell differentiation. Chicken monocytes were cultured for 1-4 days in the presence of GM-CSF, and subsequently stimulated with LPS or R848 for 4 h. Cells were lysed and mRNA was isolated. Finally, qPCR was used to detect gene expression of cytokines. Relative gene expression levels were normalized against the expression levels of the housekeeping gene GAPDH. Data is represented as mean ± SEM of three independent experiments with triplicate samples per experiment. For data analysis, one-way ANOVA with Tukey's multiple comparisons test was used. *P≤0.05;

The macrophage response upon TLR stimulation is related to their differentiation state. For example, in mammals, M1 macrophages produce pro-inflammatory cytokines, whereas M2 macrophages produce anti-inflammatory cytokines in response to TLR stimulation (8, 9). The production of these pro-inflammatory cytokines plays an important role in macrophages. IL-1β is an important pro-inflammatory cytokine for host defense against infection (21) and has been used as an immunoadjuvant to improve vaccination efficacy (22). IL-6 has multiple functions including the stimulating differentiation of monocytes to macrophages (23) and IL-8 is chemotactic for heterophils. This study showed that day 3 macrophages have a M1 pro-inflammatory differentiation state based on high expression of IL-1β, IL-6 and IL-8. Therefore, high expression of these cytokines upon stimulation indicate that macrophages were most responsive to LPS stimulation at day 3.

Unlike LPS stimulation, R848 did not induce any differences of the immune response in these macrophages. R848 has been reported to induce cytokines expression including TNF-α, IL-6 and IL-12 in mouse macrophages (24, 25). It also induced gene expression of IL-1β and IL-6 in chicken macrophage-like HD11 cell line containing TLR7 (26) although only low expression was detected. This is similar to our observation that low gene expression of IL-1β, IL-6 and IL-8 was induced upon R848 stimulation. A recent study also showed that low gene expression of IFN-β and IL-1β was induced upon R848 4 h stimulation in chicken

PBMCs (27). These results indicate that chicken macrophages might not be sensitively responsive to TLR7 agonist compared with mammalian macrophages.

APEC-induced activation of macrophages

To assess the function of day 3 macrophages, cells were incubated with APEC, one of the major bacterial pathogens for chicken. After 4 h, intracellular bacteria were detected (Fig. 4A) and after 6 h and 8 h, the number of bacteria in the cells was significantly decreased compared to 4 h initially intracellular bacteria (Fig. 4A), indicating that these macrophages are capable of phagocytosing and subsequent killing of APEC. To confirm that bacteria were taken up by macrophages, confocal microscopy was used to distinguish intracellular from extracellular bacteria. Macrophages were infected with GFP-APEC, after which bacteria were stained with anti-*E. coli* rabbit serum. Since the macrophages were not permeabilized in the procedure, only extracellular GFP-APEC were labeled with antibody and thus double-labelled (Fig. 4B, yellow bacteria), while intracellular bacteria were only positive for GFP fluorescence (Fig. 4B, green bacteria). A decreased number of bacteria over time and distinction between intra- and extra- cellular bacteria in macrophages are similar to observations that APEC were taken up by HD11 cells (14).

Phagocytosis is an important function of macrophages and in the current study it was shown that cultured primary macrophages phagocytosed and killed APEC. A number of *in vitro* studies have shown phagocytosis by chicken macrophage cell lines challenged with different bacterial strains (28-31). *In vivo*, increased numbers of macrophages have been detected in the lung and air sacs after chicken infection with APEC (32), indicating that macrophages play an important role in controlling APEC infection.

Finally, APEC-induced NO was determined. NO is an important mediator for host defense against microorganisms (33) and is mainly produced by activated pro-inflammatory M1 macrophages. APEC induced NO production in day 3 macrophages (Fig. 4C), although to a lower extent than previously observed for HD11 cells, which are actually known for producing high amounts of NO (14). Similarly, LPS has been shown to induce NO in other macrophage cell lines, such as chicken MQ-NCSU cells, and chicken monocytes (34-36). This shows that NO production is a substantial contribution to the TLR4 induced immune response.

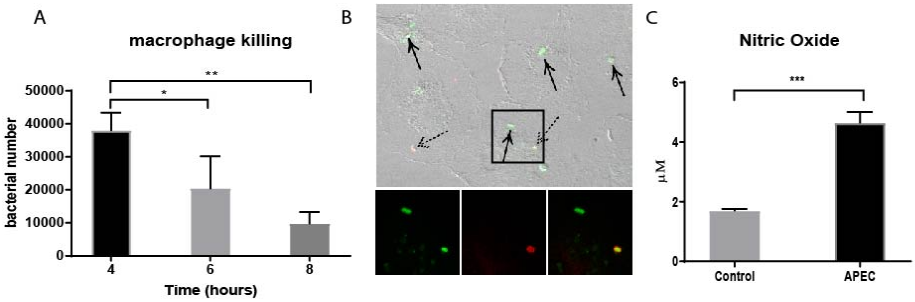

Fig 4. APEC-induced activation of macrophages. (A) Day 3 macrophages were infected with APEC for 3 h, then non-adherent bacteria were removed. Gentamicin was added to kill extracellular bacteria. Intracellular bacteria were quantified at each time point by plating out dilution series of cells on TSA plates. (B) Day 3 macrophages were infected with GFP-APEC for 3 h, after which they were fixed but not permeabilized. Extracellular APEC were stained with rabbit anti-*E. coli* rabbit serum and Donkey anti-Rabbit Alexa 647 (red). Macrophages were visualized with differential interference contrast (DIC) microscopy. Extracellular bacteria were yellow (double labelled green + red, dashed arrows) and intracellular bacterial were only labelled green (solid arrows). (C) Day 3 macrophages were infected with APEC for 3 h, then bacteria were removed. Extracellular bacteria were killed with gentamicin for 1 h and subsequently culturing of macrophages was continued 14 h. Nitric oxide was measured in the supernatant by the Griess assay. Data are represented as mean ± SEM of three independent experiments with triplicates per experiment. For data analysis, a T-test was used in two groups in figure C and one-way ANOVA with Tukey's multiple comparisons test was used in more than two groups in figure A. *P≤0.05; **P≤0.01; ***P≤0.005.

Conclusion

This study describes an *in vitro* chicken monocyte-derived macrophage culture in the presence of chicken GM-CSF over time. Our systematic characterization showed that a 3-day culture was optimal to obtain pro-inflammatory M1-like macrophages. This provides a tool for further studies on host-pathogens interactions on macrophages, in which plasticity and diversity of macrophage subsets are taken into account, in line with current studies on mammalian macrophages.

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Chapter 5

The immunomodulatory effect of cathelicidin-B1 on chicken macrophages

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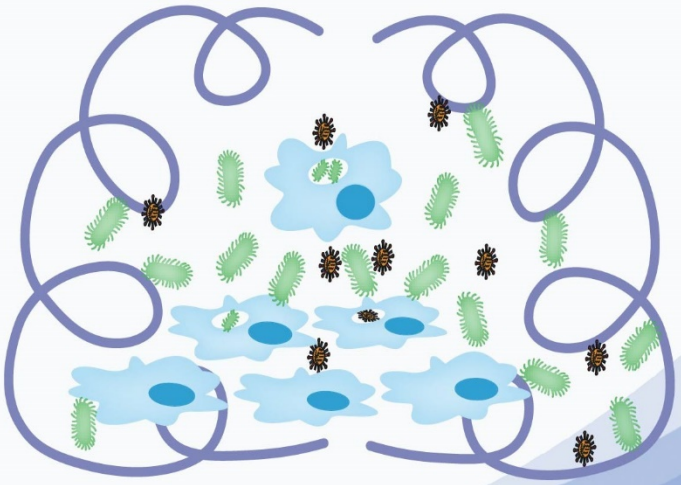

Abstract

Cathelicidins (CATHs) play an important role in the innate defense against microbial infections. Among the four chicken cathelicidins, CATH-B1 is studied the least. In this study, the effect of CATH-B1 on the macrophage response towards avian pathogenic *E. coli* (APEC) and bacterial ligands was investigated. Our results show that APEC induced CATH-B1 gene expression in both a chicken macrophage cell line (HD11 cells) and primary macrophages, while expression of the other three CATHs was virtually unaffected. While the antimicrobial activity of CATH-B1 is very low under cell culture conditions, it enhanced bacterial phagocytosis by macrophages. Interestingly, CATH-B1 downregulated APEC-induced gene expression of pro-inflammatory cytokines (IFN-β, IL-1β, IL-6 and IL-8) in primary macrophages. In addition, CATH-B1 pre-incubated macrophages showed a significantly higher gene expression of IL-10 after APEC challenge, indicating an overall anti-inflammatory profile for CATH-B1. Using isothermal titration calorimetry (ITC), CATH-B1 was shown to bind LPS. This suggests that CATH-B1 reduces toll like receptor (TLR) 4 dependent activation by APEC which may partly explain the decreased production of pro-inflammatory cytokines by macrophages. On the contrary, direct binding of CATH-B1 to ODN-2006 enhanced the TLR21 dependent activation of macrophages as measured by nitric oxide production. In conclusion, our results show for the first time that CATH-B1 has several immunomodulatory activities and thereby could be an important factor in the chicken immune response.

Introduction

Cathelicidins are host defense peptides (HDPs) with antimicrobial activity and immunomodulatory functions. They are produced as inactive precursors (prepropeptides), stored in granules, and upon cell activation released as mature peptides by proteolytic cleavage (1). Cathelicidins have been found in many different species, including mammals, reptiles, amphibians, fishes and birds (2, 3). Interestingly, the number of functional genes encoding cathelicidins in different species is highly variable (4). For example, only a single cathelicidin (LL-37) is present in human, while chicken has four cathelicidins with varying length and structure (CATH-1, -2, -3 and -B1) (5-8).

Of the four chicken cathelicidins, CATH-2 has been studied extensively. CATH-2 has broad antimicrobial activity and strong immunomodulatory effects, such as lipopolysaccharide (LPS) binding, neutralization of the immune response and enhanced DNA-induced activation of toll like receptor (TLR) 21 (9-12). In addition, *in ovo* administration of the all D-amino acid enantiomer of CATH-2 (D-CATH-2) at embryonic day 18 resulted in a protective effect against avian pathogenic *E. coli* (APEC) infection up to 7 days after hatch (13). However, less is known about the activities of CATH-B1, which means that it is challenging to properly compare functionalities and activities (14). When comparing expression patterns, one clear difference between CATH-1, -2, -3 and CATH-B1 is apparent: using immunostaining and mass spectrometry CATH-1, -2, -3 were detected in heterophils (15), while CATH-B1 was shown to be produced by epithelial cells in the bursa of Fabricius, although an extended description of CATH-B1 protein expression among cells/tissues was not described (6).

APEC is an important pathogen that causes severe respiratory diseases in chicken, leading to huge economic losses in poultry farming. APEC infection starts in the trachea and damages the respiratory mucosa. Subsequently, it crosses the epithelial layer and enters the blood stream spreading to other tissues (16). APEC can be phagocytosed by macrophages both in the lungs and in the blood stream, which leads to (partial) killing of the pathogen, but also induces an immune response that attracts other immune cells such as heterophils to infected sites.

Compared to mammalian lungs, the healthy chicken lung has a relatively low number of macrophages, but a large increase in number of macrophages occurs in the lung and air sacs after APEC infection (17-19). This suggests that macrophages play an important role in the host defence against microbial infection in the lung. Interestingly, inflammatory stimuli (butyrate) derived from bacteria induced the gene expression of CATH-B1 in chicken macrophages including HD11 cells and primary monocytes, whereas gene expression of the other three cathelicidins was very low in macrophages compared to heterophils (20). This

implies that CATH-B1 might play an important role in macrophages upon interaction with *E. coli*.

Therefore, in this study, our main aim was to investigate the effect of CATH-B1 on chicken macrophages and their response towards APEC and TLR agonists. We found that gene expression of CATH-B1 was induced by APEC in both HD11 cells and blood monocyte-derived macrophages. CATH-B1 enhanced phagocytosis of APEC by macrophages. Furthermore, CATH-B1 inhibited APEC- and LPS- induced immune response but enhanced DNA-induced nitric oxide (NO) in macrophages. Our study provides additional insights in the functions of CATH-B1, that are clearly different from those of the other chicken cathelicidins.

Methods and materials

Peptides

All peptides were synthesized by China Peptides (Shanghai, China) using Fmoc-chemistry and purified by reverse phase high-performance liquid chromatography to a purity of >95%.

Bacterial strains

The APEC strain (O78, K80) is a clinical isolate from chicken (13). Heat-killed bacteria were prepared by heating the bacterial suspension at 75 °C for 15 min. Viability was checked by plating out on Tryptic Soy Agar (TSA) (Oxoid) plates.

Antimicrobial activity assay

APEC was cultured in Tryptic Soy Broth (TSB) (Oxoid) at 37 °C and grown to mid-logarithmic growth phase before testing. Bacterial suspensions were pelleted by centrifugation, resuspended in TSB or cell culture medium (RPMI 1640- or DMEM-glutamax (Gibco, UK) with 10 % FCS (Corning)) and diluted to 2.0×10^6 CFU/mL. Twenty-five µL of peptides (0-80 µM) were mixed with an equal volume of bacterial suspension and incubated for 3 h at 37 °C. After incubation, dilution series of bacteria were plated out on TSA plates and incubated at 37 °C for 24 h to quantify viable bacteria.

Cell culture

Peripheral blood mononuclear cells (PBMCs) were isolated from blood of 76-week-old healthy chickens using Ficoll density gradient centrifugation and were frozen in liquid nitrogen until use. PBMCs (1×10^7 cells) were seeded in a 24-well plate and incubated at 41 °C. After overnight culture, all non-attached cells were removed and attached cells (monocytes) were maintained in RPMI 1640-glutamax medium with 10% FCS and 1% P/S (100 U penicillin/mL; 100 µg streptomycin/mL (Gibco, UK) supplemented with chicken

GM-CSF for another 2 days at 41 °C. These monocyte-derived macrophages were used for further analysis.

The chicken macrophage cell line HD11 was maintained in RPMI 1640-glutamax supplemented with 10% FCS and 1% P/S at 41 °C. HD11 cells were seeded in a 24-well plate (2.5×10^5 cells/well) or 96-well plate (0.5×10^5 cells/well) and cultured overnight to adhere before further analysis.

Mouse macrophages (RAW 264.7 cells) were maintained in DMEM-glutamax supplemented with 10 % FCS at 37 ℃. RAW cells were seeded in a 96-well plate (0.5×10^5 cells/well) and cultured overnight to adhere before further analysis.

Cell viability

Cell viability was determined using the WST-1 assay following the manufacturer's protocol. Briefly, primary macrophages were incubated with peptides for 3 h at 41 ℃. Subsequently, peptides were washed and cells were further incubated for 3 h at 41 ℃. Cell culture medium was removed and replaced with fresh culture medium containing 10% WST-1 reagent (Roche, Germany). After 20 min incubation, absorbance was measured at 450 nm with a FLUOstar Omega microplate reader and was corrected for absorbance at 630 nm.

APEC infection in chicken macrophages

Primary macrophages and HD11 cells were cultured as described above. Aliquots of 0.5 mL of bacterial suspensions (1×10^6 CFU/mL) were added to each well in the presence or absence of 5 μM CATH-B1 or CATH-2, with three replicate wells for a 24-well plate and incubated for 3 h at 41 ℃. In phagocytosis studies, bacteria were removed at 3 h post infection and cells were washed three times with RPMI 1640-glutamax medium with 10% FCS. Then, RPMI 1640-glutamax containing 500 μg/mL gentamicin (Sigma-Aldrich) was added to cells in order to kill all extracellular, non-phagocytosed bacteria and plates were put back at 41 ℃ for 1 h. Infected cells were washed three times with RPMI 1640-glutamax and lysed by 0.5 mL 0.5% Triton X-100. After lysis, dilution series of cells were plated on TSA plates and incubated at 37 °C for 24 h to quantify viable bacteria.

In pre-incubation studies, CATH-B1 was added to primary macrophages for 3 h, washed away with cell culture medium after which APEC were added for 3 h. In post-incubation studies, APEC were added to primary macrophages for 3 h, washed away and infected cells were treated with CATH-B1 and gentamicin for 3 h. After that, cells were treated with TriZol (Ambion, Carlsbad, CA) for RNA isolation.

LPS and ODN-2006 stimulation

Primary macrophages and RAW cells were cultured as described above. Ultrapure LPS *E. coli* O111:B4 (100 ng/mL) (InvivoGen, San Diego, CA, USA), was diluted in RPMI 1640-glutamax medium with 10% FCS, and added to cells in the presence or absence of 5 μM CATH-B1 or CATH-2 for 4 h. Afterwards, primary macrophages were washed and treated with TriZol for RNA isolation.

HD11 cells were prepared in a 96-well plate as described above. ODN-2006 (5 nM) (InvivoGen, France) was added to HD11 cells in the presence or absence of different concentrations (0-10 μM) of CATH-B1 and CATH-2 for 20 h. After this incubation, cell supernatants were collected to measure nitric oxide production (see below).

Quantitative real-time PCR (qPCR)

Primary macrophages were treated with APEC and LPS as described above. After incubation, total RNA was extracted by TriZol (Ambion, Carlsbad, CA) reagent according to the manufacturer's instructions. RNA (500 ng) was reverse transcribed by the iScript cDNA synthesis kit (Bio-Rad, Veenendaal, the Netherlands) according to the manufacturer's instructions. Primers and probes were designed and produced by Eurogentec (Seraing, Belgium) (Table 1). qPCR was performed on a CFX Connect qPCR with CFX Manager 3.0 (Bio-Rad). Reactions were performed as follows: 3 min at 95 °C; 40 cycles: 10 s at 95 °C, 30 s at 60 °C and 30 s at 72 °C. Relative gene expression levels were normalized against the expression levels of the house keeping gene GAPDH.

Griess assay

HD11 cells were stimulated with ODN-2006 as described above. Supernatant was collected to measure NO production. Briefly, 30 μL of sample were added to the well in a 96-well flat bottom plate. An equal volume of 1% sulfanilamide (Merck, Darmstadt, Germany) was added in each well, followed by 30 μL 0.1% N-(1-naphthyl) ethylenediamine dihydrochloride (VWR) at room temperature for 5 min. The nitrite concentration was determined by measuring optical density at 550 nm. Sodium nitrite (Sigma-Aldrich) was used as a standard to accurately determine the nitrite concentration in the cell supernatant.

Table 1: Primer and probe sequences for qPCR

Gene		5'→3'sequence
GAPDH	Forward	GTCAACCATGTAGTTCAGATCGATGA
	Reverse	GCCGTCCTCTCTGGCAAAG
	Probe	AGTGGTGGCCATCAATGATCCC
IFN-β	Forward	CCTCCAACACCTCTTCAACACG
	Reverse	TGGCGTGTGCGGTCAAT
	Probe	AGCAGCCCACACACTCCAAAACACT
IL-1β	Forward	GCTCTACTAGTCGTGTGTGATGAG
	Reverse	TGTCGATGTCCCGCATGA
	Probe	CCACACTGCAGCTGGAGGAAGCC
IL-6	Forward	GTCGAGTCTCTGTGCTAC
	Reverse	GTCTGGGATGACCACTTC
	Probe	ACGATCCGGCAGATGGTGA
IL-8	Forward	GCCCTCCTCCTGGTTTCA
	Reverse	CGCAGCTCATTCCCCATCT
	Probe	TGCTCTGTCGCAAGGTAGGACGCTG
IL-10	Forward	CATGCTGCTGGGCCTGAA
	Reverse	CGTCTCCTTGATCTGCTTGATG
	Probe	CGACGATGCGGCGCTGTCA

Isothermal titration calorimetry

Interaction between CATH-B1 and *E. coli* LPS O111:B4 or ODN-2006 was tested using isothermal titration calorimetry (ITC). All ITC experiments were performed on a Low Volume NANO ITC (TA instruments - Waters LLC, New Castle, USA). LPS was diluted in PBS to 0.5 mg/mL rigorously vortexed for 5 min and added to the cell chamber (167 µL). ODN-2006 was diluted to 25 nM in 75% PBS. The syringe was filled with a 50 µL solution of 200 µM CATH-B1 in 75% PBS. Titrations were incremental with 2 µL injections (for LPS) or 1 µL injections (for ODN-2006) at 300 seconds intervals. Experiments were performed at 37 °C and data were analyzed with the Nano Analyze software (TA instruments - Waters LLC).

ELISA

RAW 264.7 cells were prepared in a 96-well plate as described above. RAW cells were stimulated with *E. coli* LPS (100 ng/mL) in the presence or absence of 5 µM CATH-B1 or CATH-2 for 24 h. Cell supernatants were collected to measure cytokine expression. The mouse IL-6 ELISA kit (Minneapolis, MN) was used to determine the IL-6 concentration of samples. This assay was performed following the manufacturer's protocol.

Statistical analysis

Data are represented as mean ± SEM of three independent experiments for each group (n = 3) and were analyzed by a T-test for two groups or by one-way ANOVA with Tukey's multiple comparisons test for more than two groups. Bio-Rad CFX Manager 3.0 software was used for qPCR data analysis. All the graphs were made using GraphPad Prism® 8.0.

Results

APEC induced CATH-B1 gene expression in macrophages

CATH-B1 protein has so far only been detected in the bursa of Fabricius, but CATH-B1 mRNA is found in different tissues including the respiratory tract, gastrointestinal tract and lymphoid organs (21), indicating that a broader expression of CATH-B1 is likely. In our study, APEC significantly induced gene expression of CATH-B1 in both HD11 cells (Fig. 1A) and chicken primary macrophages (Fig. 1B). At 6 h post-infection, up to a 30-fold increase in CATH-B1 gene expression was detected in primary macrophages compared to non-infected cells (Fig. 1B). Gene expression of CATH-1, -2, -3 was not or only mildly induced by APEC in both cell types (Fig. 1A and 1B).

The effect of CATH-B1 on phagocytosis in macrophages

To determine the effect of CATH-B1 on the function of macrophages, peptide was added to primary macrophages or HD11 cells together with APEC. Bacterial phagocytosis by macrophages was significantly enhanced in HD11 cells (Fig. 2A) when CATH-B1 was present, and slightly enhanced in primary macrophages (Fig. 2B).

Antibacterial activity of chicken cathelicidins against APEC

The antimicrobial activity against APEC in various culture media was tested for the four chicken cathelicidins (CATH-1, -2, -3 and -B1) in order to determine the minimal bactericidal concentration (MBC). This showed that CATH-1, -2, -3 had similar antibacterial activity with MBC values between 5-10 μM. In contrast with the other three peptides, CATH-B1 showed a weaker anti-APEC activity at 5 μM but killed all bacteria at 10 μM (Fig. 3). In cell culture conditions (DMEM-glutamax with 10% FCS), CATH-2 still showed strong antibacterial activity, whereas the antibacterial activity of CATH-1 and CATH-3 was strongly reduced. CATH-B1 completely lost its antibacterial activity, showing no growth inhibition of APEC at the highest concentration tested (40 μM) (Supplementary Fig. 1A and 1B).

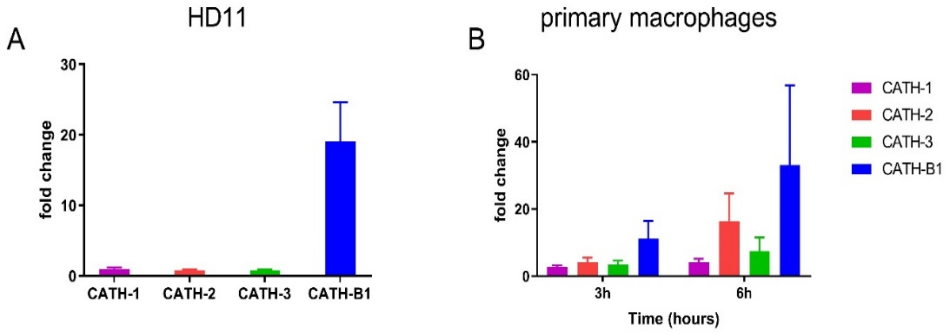

Fig 1. APEC-induced gene expression of chicken cathelicidins (CATHs). Cells were infected with APEC for 3 h and 6 h. After that, RNA was isolated to determine gene expression of CATHs by qPCR analysis in (A) HD11 cells and (B) primary macrophages. Relative gene expression levels were normalized against the expression levels of the house keeping gene GAPDH; fold change is presented compared to non-infected cells. Data are represented as mean ± SEM of three independent experiments of triplicate samples per experiment.

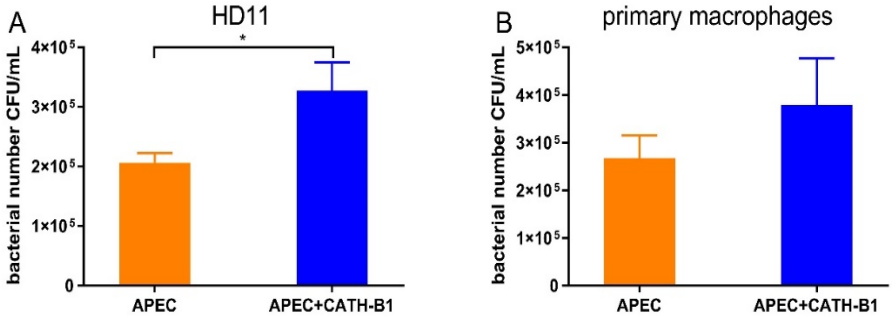

Fig 2. The effect of CATH-B1 on bacterial phagocytosis in chicken macrophages. Cells were infected with APEC for 3 h in the presence of 5 μM CATH-B1, then gentamicin was added to kill extracellular bacteria for 1 h. Finally, cells were lysed to quantify intracellular bacteria in (A) HD11 cells and (B) in primary macrophages. Data are represented as mean ± SEM of three independent experiments of triplicate samples per experiment. *P≤0.05.

Cytotoxicity of CATH-B1

To determine the toxic effect of CATH-B1 on host cells, the WST-1 assay was used to measure metabolic activity of primary macrophages. CATH-2 was used as a control in this study, which induced cell damage at 5 μM, at which a 40% reduction in metabolic activity was detected. CATH-B1 was less toxic than CATH-2 but reduced metabolic activity at concentrations of 10 μM or higher (Fig. 4).

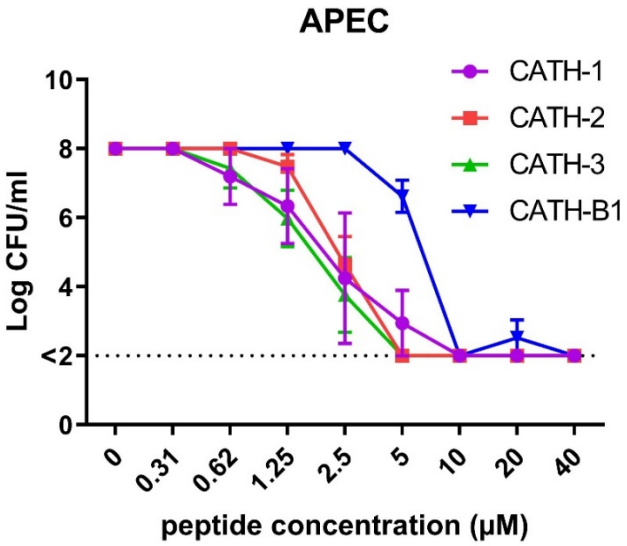

Fig 3. Antibacterial activity of chicken cathelicidins against APEC. Bacteria were incubated with different concentrations of cathelicidins for 3 h, serially diluted and spread plated on TSA plates to quantify viable bacteria. Data are represented as mean ± SEM of three independent experiments of triplicate samples per experiment.

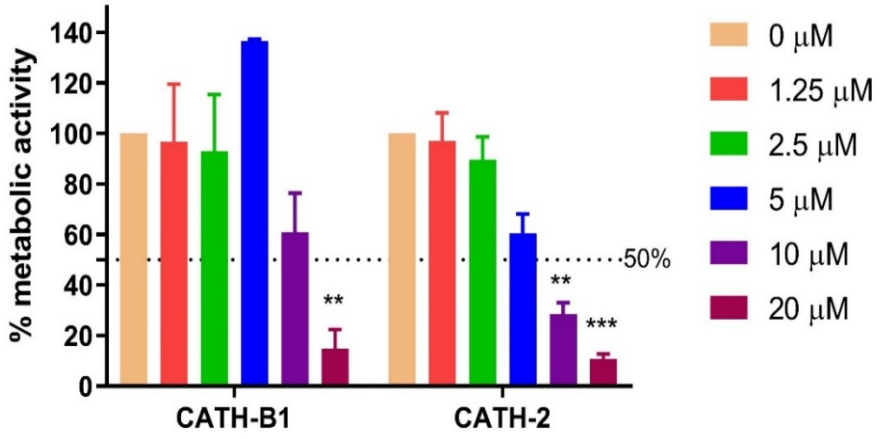

Fig 4. Cytotoxicity of chicken CATH-B1 and CATH-2. Primary macrophages were incubated with different concentrations (0-20 μM) of CATH-B1 and CATH-2 for 3 h. Peptides were removed and cells were maintained in new medium for another 3 h. Metabolic activity was tested using WST-1 reagent. Data are represented as mean ± SEM of three independent experiments of triplicate samples per experiment. *P≤0.05; **P≤0.01; ***P≤0.005; ****P≤0.001.

The effect of CATH-B1 on APEC-induced cytokine expression in macrophages

Activation of macrophages resulting in the release of cytokines is a key immune response against pathogens. However, overexpression of inflammatory cytokines can cause apoptosis of cells leading to tissue damage. Therefore, it is important to have a balanced response of the immune system with respect to release of these cytokines. To investigate whether CATH-B1 regulates APEC-induced activation of macrophages, APEC-induced cytokine expression in the presence or absence of CATH-B1 (and CATH-2 as control) was determined using qPCR. To separate immunomodulatory effects from antibacterial activity of CATH-B1, heat-killed APEC was also used in this experiment. At 3 h post infection, both viable and heat-killed APEC strongly up-regulated gene expression of pro-inflammatory cytokines IL-1β and IL-6, chemokine IL-8 and the anti-inflammatory cytokine IL-10 (Fig. 5). Both CATH-B1 and CATH-2 downregulated gene expression of these cytokines. Gene expression of IFN-β was also upregulated by APEC and both CATH-B1 and CATH-2 significantly inhibited IFN-β expression (Fig. 5). Interestingly, APEC-induced gene expression of IL-10 was increased by CATH-B1 and CATH-2 (Fig. 5) but the increase was not significant.

To investigate how CATH-B1 inhibited APEC-induced activation, primary macrophages were pre-incubated with peptides prior to or post APEC infection. APEC-induced gene expression after 3 and 6 h (depending on the setup of the experiment) was similar for IFN-β, IL-1β, IL-8 and IL-10, except for IL-6 gene expression, which was significantly higher after 6 h (Fig. 6). The inhibitory effect of CATH-B1 on cytokine gene expression, observed in co-incubation conditions, was lost in pre- and post-incubation conditions. Noticeably, there was one exception, macrophages pre-incubated with CATH-B1 expressed significantly more IL-10 compared to macrophages without CATH-B1. Overall, this indicates an anti-inflammatory effect of CATH-B1 on APEC-infected macrophages.

The effect of CATH-B1 on LPS-induced cytokines expression in macrophages

To further investigate the functional properties of CATH-B1, *E. coli* LPS-induced cytokine gene expression in the presence or absence of peptides was determined. Again, in these experiments CATH-2 was used as a positive control since CATH-2 has been described to neutralize LPS and that CATH-2-LPS binding was essential for this (12). LPS-induced gene expression of IFN-β, IL-1β, IL-6, IL-8 and IL-10 was significantly downregulated by both CATH-B1 and CATH-2 (Fig. 7A). To investigate whether the inhibitory effect of CATH-B1 is host cell specific, we also tested IL-6 protein production in LPS-stimulated mouse macrophages using ELISA. Also, in mouse macrophages, the cytokine production was inhibited by CATH-B1 (Supplementary Fig. 2), suggesting that the inhibition is due to the interaction of CATH-B1 and LPS. Finally, ITC analysis was used to determine the direct interaction of CATH-B1 and LPS (Fig. 7B). Peptide binding to LPS was detected with an

observed dissociation constant of Kd = 1.0 µM in a reaction driven by both enthalpy (ΔH = -19.6 kJ.mol^{-1}) and entropy ΔS = 51.3 J.mol^{-1}). This indicates that CATH-B1 inhibits LPS-induced cytokine expression in macrophages by binding to LPS and thereby neutralizing LPS, similar as has been described for CATH-2.

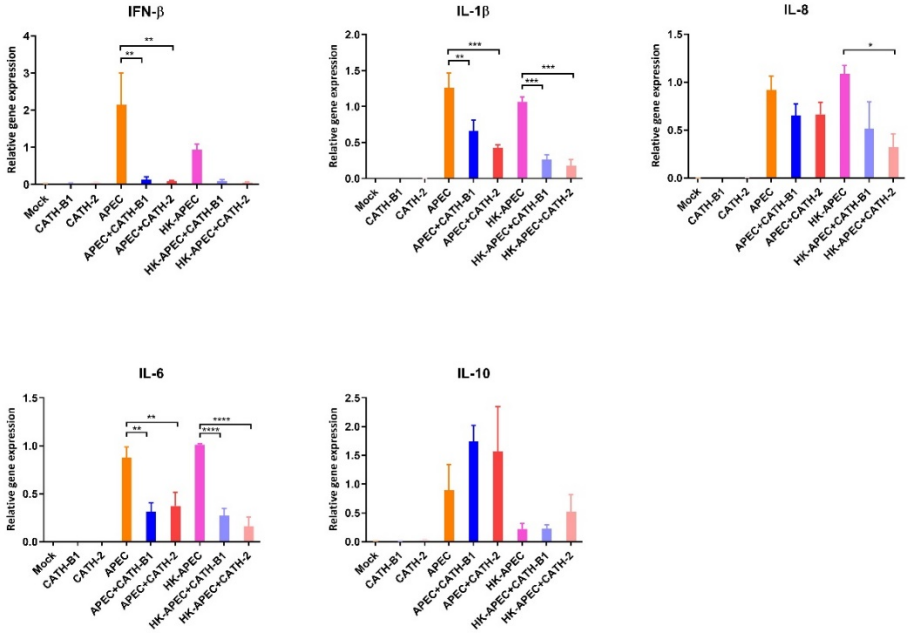

Fig 5. The effect of CATH-B1 on APEC-induced cytokine expression in primary macrophages. Gene expression of cytokines in primary macrophages were determined by qPCR at 3 h post infection in the presence or absence of 5 µM CATH-2 and CATH-B1. Relative gene expression levels were normalized against the expression level of the house keeping gene GAPDH. Data are represented as mean ± SEM of three independent experiments of triplicate samples per experiment. *P≤0.05; **P≤0.01; ***P≤0.005; ****P≤0.001.

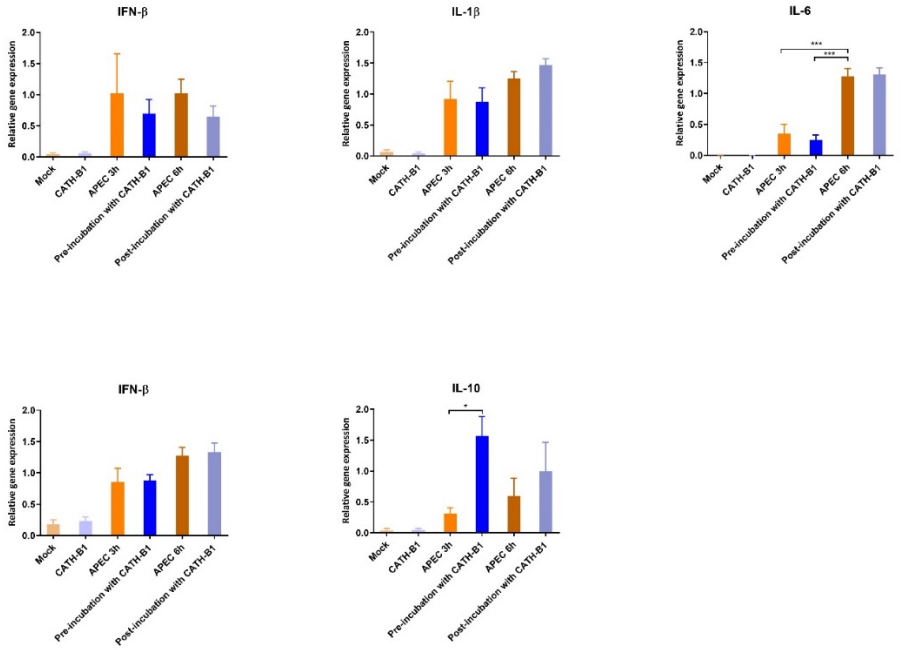

Fig 6. APEC-induced cytokine expression in primary macrophages upon pre- and post-incubation of CATH-B1. Primary macrophages were pre-incubated with 5 μM CATH-B1 for 3 h before APEC infection, or post-incubated with 5 μM CATH-B1 after APEC infection. Then, gene expression of cytokines was determined by qPCR. Gene expression levels were normalized against the expression levels of the house keeping gene GAPDH. Data are represented as mean ± SEM of three independent experiments of triplicate samples per experiment. *P≤0.05; **P≤0.01; ***P≤0.005; ****P≤0.001.

The effect of CATH-B1 on DNA-induced NO production in HD11 cells

Extracellular microbial DNA is an important signaling molecule in infection and inflammation. Bacterial DNA can be released from phagolysosomes after phagocytosis and bacterial degradation by macrophages, leading to activation of bystander macrophages (22). CATH-2 has been shown to increase uptake of extracellular DNA and boost subsequent TLR9 or TLR21 activation (11). To investigate whether CATH-B1 enhances DNA-induced macrophage activation, as shown before for CATH-2, HD11 cells were incubated with ODN-2006 in the presence or absence of CATH-B1 and CATH-2 as control. ODN-2006-induced NO production was determined by the Griess assay. HD11 cells did not produce NO without stimulation nor did peptides alone induce NO, whereas high concentration of ODN-2006 (40 nM) strongly increased the NO production (data not shown). The ODN-2006-induced NO production was clearly enhanced by the presence of CATH-2 and CATH-B1, although a higher concentration of 5 μM CATH-B1 was needed to enhance NO production compared to

CATH-2 (Fig. 8A). This shows that although the overall effect of CATH-B1 on stimulation of macrophages by APEC is inhibitory, the potential to increase stimulation by enhancing uptake of bacterial DNA is also present. For CATH-2, it was shown that the increased response of macrophages towards ODN-2006 was depended on direct binding of CATH-2 to ODN-2006. Using ITC, it was shown that CATH-B1 indeed also strongly binds ODN-2006 (Fig. 8B) with a Kd-value of 64 nM. This binding between CATH-B1 and DNA was enthalpy-driven (ΔH = -65.5 kJ.mol^{-1}) with a negative entropy value (ΔS = -73.5 J.mol-1).

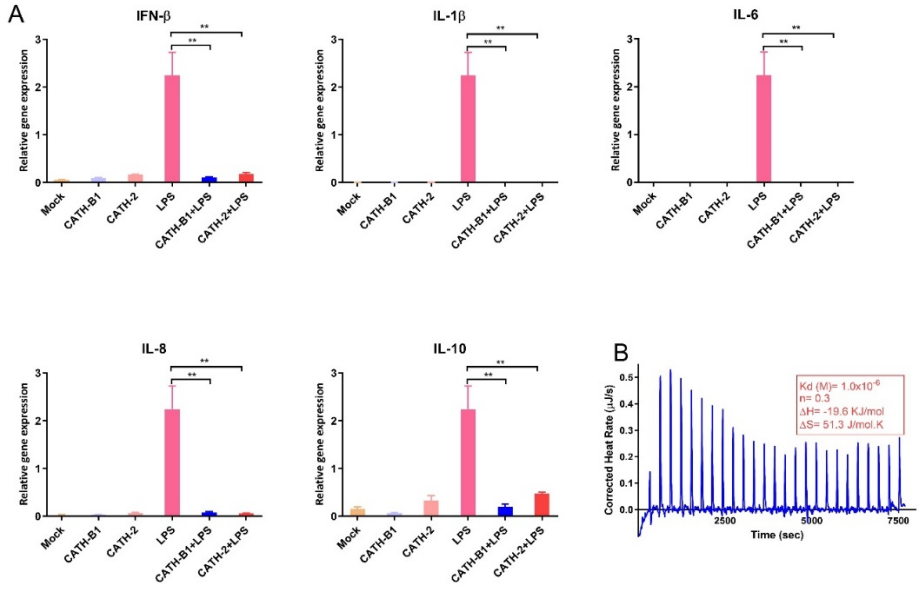

Fig 7. The effect of CATH-B1 on LPS-induced cytokine expression in primary macrophages. Primary macrophages were stimulated with LPS (100 ng/mL) for 4 h in the presence or absence of 5 μM CATH-2 and CATH-B1. (A) gene expression of cytokines. Relative gene expression levels were normalized against the expression levels of the house keeping gene GAPDH. Data are represented as mean ± SEM of three independent experiments of triplicate samples per experiment. *P≤0.05; **P≤0.01; ***P≤0.005; ****P≤0.001. (B) ITC thermogram of CATH-B1 binding to LPS *E. coli* O111:B4. ITC analysis data were calculated as $K_d = 1.0 \times 10^{-6}$ M, ΔH = -19.6 kJ mol^{-1} and ΔS = 51.3 J.mol^{-1}.

Discussion

So far, four cathelicidins have been characterized in chicken, CATH-1, -2, -3, and -B1. In this study, we showed that APEC upregulates gene expression of CATH-B1 in macrophages but not of CATH-1, -2, -3. Recently, it was found by our group that CATH-B1 has strong antiviral activity against influenza A viruses *in vitro* (14). In this study, the

immunomodulatory effect of CATH-B1 on APEC-, LPS- or ODN-2006-activated chicken macrophages was explored.

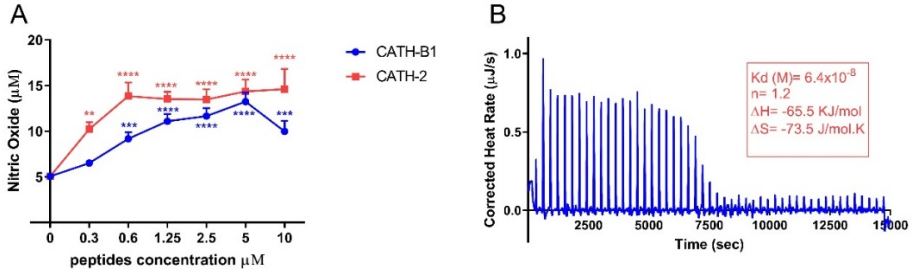

Fig 8. The effect of CATH-B1 on DNA-induced nitric oxide production in HD11 cells. HD11 cells were stimulated with 5 nM ODN-2006 for 20 h in the presence or absence of different concentrations of peptides. (A) The amount of NO in the cell supernatant were measured by Griess assay. Data are represented as mean ± SEM of three independent experiments of triplicate samples per experiment. *P≤0.05; **P≤0.01; ***P≤0.005; ****P≤0.001. (B) ITC thermogram of CATH-B1 binding ODN-2006. ITC analysis data were calculated as Kd= 6.4×10^{-8} M, ΔH = -65.5 kJ.mol^{-1}and ΔS = -73.5 J mol^{-1}.

The expression of cathelicidins is regulated by many factors including inflammatory and microbial stimuli. In our study, APEC infection enhanced gene expression of CATH-B1 but no or limited upregulation of the other three cathelicidins genes was detected in chicken macrophages. CATH-1, -2, -3, in contrast to CATH-B1, are mainly expressed in heterophils, indicating that CATH-B1 gene expression is regulated separately from CATH-1, -2, -3 and might play a non-redundant role in macrophages. Similarly, butyrate which has known immunostimulatory activity was previously shown to enhance gene expression of CATH-B1 (but not CATH-1, -2, -3) in chicken primary monocytes. Butyrate also enhanced antibacterial activity of primary monocytes against *S. enteritidis* (20). In contrast to APEC infection, CATH-B1 gene expression was actually downregulated in peripheral blood leukocytes from a *Salmonella typhimurium*-infected chicken (23), but its expression was not affected by *Campylobacter jejuni* infection, suggesting that regulation of CATH-B1 expression is dependent on the infecting bacterial species, and likely also on the cell type studied. However, since gene expression and protein production do not always correlate, it is still needed to determine the localization and amount of CATH-B1 in different tissues and cells in normal and stimulated conditions.

Antimicrobial activity is an important function of cathelicidins. CATH-1, -2, -3 showed good anti-APEC activity, which is consistent with the described broad antimicrobial activity of CATH-1, -2, -3 against a set of both Gram-positive and Gram-negative bacteria (24). Compared to the other three cathelicidins, the antimicrobial activity of CATH-B1 is less studied. CATH-B1 has only been tested against a limited number of bacterial strains

including *E. coli*, *S. aureus*, and *P. aeruginosa* with MIC values in the range of 0.63-2.5 μM when tested against a low number of bacteria (2×10^3 CFU) (6). However, in another study the MIC value of CATH-B1 was as high as 12.8 μM against *E. coli* and *S. aureus* using a higher number of bacteria (25), more closely resembling the results of this study. In addition, CATH-B1 had very weak anti-APEC activity in cell culture conditions, suggesting that direct killing of bacteria might not be the main activity of this cathelicidin.

Many studies have tried to correlate structure, charge and hydrophobicity to the antimicrobial activity of peptides. Chicken CATH-1, -2, -3 are largely unstructured in aqueous solution but can form an α-helical amphipathic conformation in membrane-mimicking environment (5, 26, 27). Proline residues often induce a kink in the helical structure of cathelicidins and this kink between the two helices is involved in antibacterial activity (26, 28). The structure of CATH-B1 has been predicted, but its conformation has not been determined. However, there are some clear differences between CATH-1, -2, -3 and CATH-B1. CATH-B1 is longer and contains a lower number of positively charged residues. These differences could at least partially explain the observed difference in antimicrobial activity of CATH-B1, but future structure-activity studies should be performed to determine which characteristics of CATH-B1 play a role in its antimicrobial activity.

In addition to direct microbial killing, cathelicidins can exert immunomodulatory effects on host cells. In previous studies, it has been shown that CATH-2 can strongly reduce activation of macrophages by neutralization of bacteria or bacterial products. In fact, it was hypothesized that CATH-2 has a dual role in first killing a pathogen and subsequently reducing an unwanted inflammatory reaction towards the dead bacterium (or its products) (12). Our results showed that CATH-B1 inhibited both viable- and heat-killed APEC-induced inflammatory responses in macrophages, although CATH-B1 did not actually kill bacteria. Subsequent studies on LPS binding and neutralization of LPS-induced immune responses suggest that CATH-B1 exerted similar anti-inflammatory properties to CATH-2 and several other host defense peptides (29, 30). Unlike the immunomodulatory functions of LL-37 that are described to be mediated by several receptors, such as FPR2/ALX (31), P2X7 (32) and EGFR (33), it is still unknow whether chicken cathelicidins regulate immune responses via interaction with specific cell receptors, or only act on bacterial ligands like LPS and DNA. Interestingly, when primary macrophages were preincubated with CATH-B1, upregulation of IL-10 expression was observed in response to APEC infection, suggesting that CATH-B1 might modulate inflammation via interaction with host factors.

Besides anti-inflammatory activity, host defense peptides also exert pro-inflammatory effects on host cells (34). In our previous study, CATH-2 has been shown to bind to DNA and enhance the DNA-induced TLR9/21 activation of macrophages (11). Similar to CATH-2, our

results showed that CATH-B1 also enhances DNA-induced NO production in macrophages, likely using a similar mechanism in which peptide binds to DNA and is taken up as a complex. ITC showed indeed that CATH-B1 strongly bound DNA, with similar entropy-driven binding characteristics as CATH-2. This enhanced response was also induced by other host defense peptides, such as human/porcine cathelicidins and defensins (35-37). The combination of anti-inflammatory and pro-inflammatory properties of cathelicidins provide insight for development of therapeutic immunomodulators to maintain a balanced immune system in the host against microbial infection (38).

In this study, we found that CATH-B1 has no antibacterial activity in cell culture medium. This corresponds with previous studies that many cathelicidins lose their antimicrobial activity in the presence of serum or physiological salt concentrations (39, 40). This means that *in vivo* other antimicrobial mechanisms are needed to kill bacteria. One such way could be that cathelicidins use their immunomodulatory properties to regulate the immune system. On the other hand, it has been shown that cathelicidins can have synergistic effects with other host-derived antimicrobial agents against invading pathogens, such as lysozyme and lactoferrin (41). Therefore, participating in bacterial killing in *in vivo* conditions might still be an important feature of cathelicidins.

In conclusion, these studies show the overall anti-inflammatory effect of CATH-B1 on APEC-infected or LPS-stimulated macrophages. This functional exploration of CATH-B1 provides a useful first set of information that justifies further investigations into the role of this less studied chicken cathelicidin *in vivo*.

Acknowledgement

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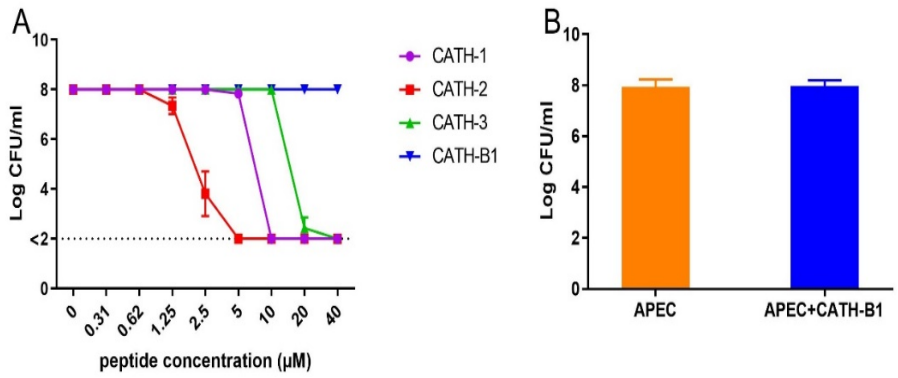
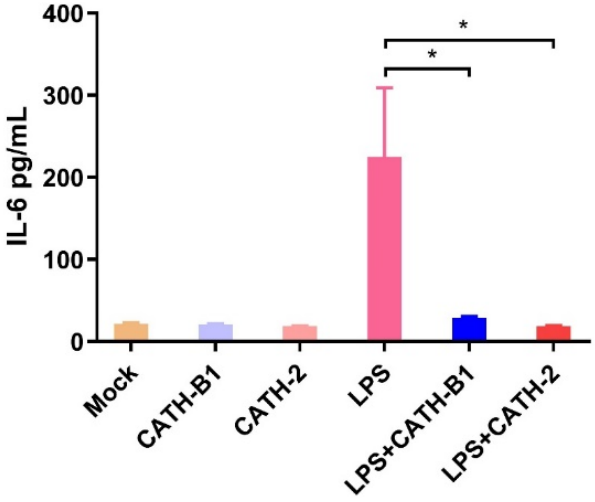


Supplementary fig 1. Antibacterial activity of chicken cathelicidins against APEC in cell culture medium. Bacteria were incubated with different concentrations of cathelicidins in DMEM or RPMI 1640-glutamax containing FCS for 3 h, serially diluted and spread plated on agar media to quantify viable bacteria. (A) Antibacterial activity of cathelicidins in DMEM-glutamax medium containing FCS. (B) Antibacterial activity of 5 μM CATH-B1 in RPMI 1640-glutamax medium containing 10% FCS. Data are represented as mean ± SEM of three independent experiments of triplicate samples per experiment.



Supplementary fig 2. The effect of CATH-B1 on LPS-induced IL-6 protein production in mouse macrophages. RAW cells were incubated with LPS (100 ng/mL) in the presence or absence of 5 μM CATH-2 and CATH-B1. Concentrations of IL-6 in the cell supernatant were determined by ELISA. Data are represented as mean ± SEM of three independent experiments of triplicate samples per experiment. *P≤0.05.

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Chapter 6

Antiviral activity of chicken cathelicidin B1 against influenza A virus

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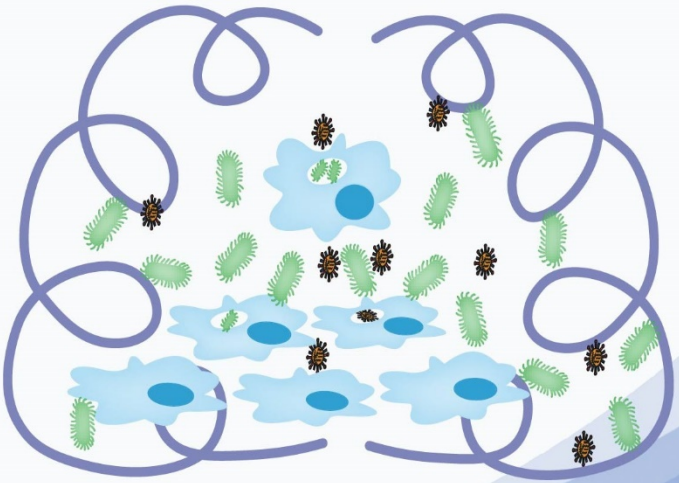

Abstract

Cathelicidins (CATHs) are host defense peptides (HDPs) that play an important role in the innate immune response against infections. Although multiple functions of cathelicidins have been described including direct antimicrobial activity and several immunomodulatory effects on the host, relatively little is known about their antiviral activity. Therefore, *in vitro* antiviral activity of chicken cathelicidins and the underlying mechanism was investigated in this study against different influenza A virus (IAVs) strains. Our results show that chicken CATH-B1 has broad anti-IAV activity compared to other cathelicidins (CATH-1, -2, -3, LL-37, PMAP-23 and K9CATH) with an inhibition of viral infection up to 80% against three tested IAV strains (H1N1, H3N1 and H5N1). In agreement herewith, CATH-B1 affected virus-induced inflammatory cytokines expression (IFN-β, IL-1β, IL-6 and IL-8). Incubation of cells with CATH-B1 prior to or after their inoculation with virus did not reduce viral infection indicating that direct interaction of virus with the peptide was required for CATH-B1's antiviral activity. Experiments using combined size exclusion and affinity-based separation of virus and peptide also indicated that CATH-B1 bound to viral particles. In addition, using electron microscopy, no morphological change of virus itself was seen upon incubation with CATH-B1 but large aggregates of CATH-B1 and viral particles were observed, indicating that aggregation might be the mechanism of action reducing IAV infectivity. Neuraminidase (NA) activity assays using monovalent or multivalent substrates, indicated that CATH-B1 did not affect NA activity *per se*, but negatively affected the ability of virus particles to interact with multivalent receptors, presumably by interfering with hemagglutinin activity. In conclusion, our results show CATH-B1 has good antiviral activity against IAV by binding to the viral particle and thereby blocking viral entry.

Introduction

Cathelicidins are short cationic peptides with an important role in the innate immune response against infections. They are mainly expressed by leukocytes and epithelial cells at infection sites in the host. Cathelicidins have been found in all vertebrates, including pigs, dogs, human and chicken, but with some diversity in number and structure. For example, only one 37 amino acid long cathelicidin (LL-37) is present in human while chicken has four cathelicidins (CATH-1, -2, -3 and -B1) with varying length. Cathelicidins have direct antimicrobial activity against a broad range of bacteria and also possess many immunomodulatory functions on host cells (1, 2). Out of the chicken cathelicidins, CATH-2 has been most studied. Besides having broad antibacterial activity, it can inhibit LPS-induced TLR4 activation and enhance DNA-induced TLR9 or TLR21 activation in macrophages (3, 4). Furthermore, CATH-2 treatment *in ovo* has been described to reduce mortality induced by avian pathogenic *E. coli* in chicken (5). Less information is known about the other chicken cathelicidins although some studies already focused on CATH-1 and CATH-3. CATH-1 and CATH-3 seem to share at least the antimicrobial potency and their localization with CATH-2 (6, 7). On the contrary, the function of CATH-B1 is hardly studied, but it is different from CATH-1, -2, -3 by its localization in the bursa of Fabricius in chicken (8). In addition, the antiviral activity for all chicken cathelicidins is still unknown.

Influenza A virus (IAV) is an important pathogen of human and animals. Infection with IAV causes acute respiratory diseases leading to serious morbidity and mortality in human and many animal species. In the past 100 years, influenza A viruses, such as H1N1 in 1918 and H3N2 in 1968, have caused severe pandemics in human (9, 10). Animal IAVs, such as highly pathogenic IAV H5N1, pose a constant threat of causing a new pandemic. This latter virus has been reported to infect humans with a mortality rate of 52.8% from 2003-2019 (source: WHO). Moreover, due to rapid genomic variation of IAVs, novel variants are emerging (such as H7N9 in 2013) that pose a new threat to human health (11). Currently, vaccination and anti-IAV drugs are being used to prevent and treat IAV infections. The efficacy of vaccination is, however, limited in part due to antigenic variation, while the use of anti-IAV drugs is limited by the development of resistance. Therefore, novel preventive and therapeutic options against IAV infection are needed.

In this study, we investigated the antiviral activity and mechanism of chicken cathelicidins against IAVs. The outcome of our study provides useful information for the development of therapies against IAV infection.

Materials and Methods

Peptides

All the peptides were synthesized by China Peptides (Shanghai, China) using Fmoc-chemistry. All peptides were purified by reverse phase high-performance liquid chromatography to a purity >95% (Table 1).

Table 1: Characteristics of peptides used in this study

Peptide	Amino acid sequence	length	charge
CATH-1	RVKRVWPLVIRTVIAGYNLYRAIKKK	26	+8
CATH-2	RFGRFLRKIRRFRPKVTITIQGSARF	26	+9
CATH-3	RVKRFWPLVPVAINTVAAGINLYKAIRRK	29	+7
CATH-B1	PIRNWWIRIWEWLNGIRKRLRQRSPFYVRGHLNVTSTPQP	40	+7
LL-37	LLGDFFRKSKEKIGKEFKRIVQRIKDFLRNLVPRTES	37	+6
PMAP-23	RIIDLLWRVRRPQKPKFVTVWVR	23	+6
K9	RLKELITTGGQKIGEKIRRIGORIKDFFKNLQPREEKS	38	+6

Cell lines and viruses

HD11 cells (a chicken macrophage cell line) and Madin-Darby Canine kidney (MDCK-II; ATCC) cells were cultured in RPMI 1640-glutamax and DMEM-glutamax (Gibco, UK), respectively, supplemented with 10% FCS and antibiotics (100 U/mL penicillin and 100 µg/mL streptomycin).

Influenza virus A/Puerto Rico/8/34/Mount Sinai (H1N1/PR8) and reassortant viruses were propagated in MDCK-II cells as described previously and stored aliquoted at -80°C until use. Generation of reassortant viruses H3N1 (containing the HA gene from A/Bilthoven/1761/76 (H3N2) in the genetic background of PR8) and H5N1 (containing the HA gene from A/duck/Hunan/795/2002 (H5N1) in the genetic background of PR8) was described previously (12, 13). The H3N1 virus was kindly provided by Ron Fouchier (Erasmus Medical Center, the Netherlands). Virus titers were determined for MDCK-II cells by calculating 50% tissue culture infectious dose per ml (TCID50/mL) as described before (14).

Viral infection

MDCK-II and HD11 cells, seeded in 96-well plate and grown to confluency, were infected with virus at a multiplicity of infection (MOI) of 0.1 in the presence or absence of cathelicidins (CATHs) for 1 h at 37 ℃. In pre-incubation studies CATHs were added to the cells for 1 h, washed away with PBS after which IAV was added for 1 h. Post incubation studies were performed similarly but with the order of peptide and virus addition reversed. All the initial infection and cathelicidin incubation steps were performed in the absence of

serum. After these incubations, unbound virus or unbound peptide were removed by washing the cells twice with PBS (supplemented with Ca^{2+} and Mg^{2+}). MDCK-II and HD11 cells were incubated for another 7 h with opti-MEM or RPMI 1640-glutamax supplemented with 2% FCS, respectively, at 37 ℃. Subsequently, cells were fixed with cold methanol at -20 ℃ for 5 min, after which cells were stained with primary mouse monoclonal antibody HB65 (1:1000) specific for the viral nucleoprotein and Alexa Fluor 488-labeled Donkey anti-Mouse IgG antibodies (Life technologies, Eugene, USA) (1:1000) as described previously (15). Cells were visualized using the nuclear stain DAPI (Thermo Fischer Scientific) according to the manufacturer's instructions. Three images per well were taken using an EVOS FL microscope (Thermo Fisher Scientific) and the infected cells were counted. The number of infected cells in inoculated, mock-treated wells was set at 100%.

To investigate whether a direct interaction of CATH-B1 with virus was present and possibly required for CATH-B1's activity, Capto Core 700 beads (GE Healthcare) were used to remove CATH-B1 not bound to virus. To this end, viruses were pre-incubated in opti-MEM medium with or without CATH-B1 for 30 min at 37 ℃, after which Capto Core 700 beads were added to the samples and samples were incubated for 20 min at 4 ℃ while rotating. Afterwards, beads were spun down and supernatants were collected. To control for the efficient removal of CATH-B1, samples containing CATH-B1 but no virus were subjected to the same procedure. Cells were inoculated with the supernatants (or combinations thereof) and processed to determine the number of infected cells as described above.

Cell Viability

Cell viability was determined using the WST-1 assay following the manufacturer's protocol. In short, cells were incubated with peptides for 1 h at 37 ℃, then peptides were washed away and cells were further incubated for either 7 h or 23 h at 37 ℃ (corresponding to the incubation times used for immunohistochemistry and detection of cytokine gene expression respectively). Cell culture medium was removed and replaced with fresh culture medium containing 10% WST-1 reagent. After 20 min incubation, absorbance was measured at 450 nm with a FLUOstar Omega microplate reader and was corrected for absorbance at 630 nm.

Electron microscopy

IAV (H3N1) was incubated in the presence or absence of CATH-B1 for 1 h at 37 ℃ and 10 µL sample was placed on a carbon-coated copper grid. Grids were washed three times with PBS and fixed with 1% glutaraldehyde (Sigma-Aldrich) in PBS for 10 minutes. Next, grids were washed two times with PBS and four times with MilliQ. Subsequently, grids were shortly rinsed with methylcellulose/uranyl acetate (pH 4) and incubated for 5 minutes with

methylcellulose/uranyl acetate (pH 4) on ice. Finally, grids were looped out of the solution and air-dried. Samples were imaged on a Tecnai-12 electron microscope (FEI).

MUNANA and ELLA assay

The activity of NA in the presence of CATH-B1 towards the synthetic monovalent substrate 2'-(4-methylumbelliferyl)-alpha-D-N-acetylneuraminic acid (MUNANA) (Sigma-Aldrich) was determined by using a fluorometric assay similarly to what was described previously (16). In short, IAV was incubated with CATH-B1 (0-40 μM) for 1 h at 37 ℃, followed by addition of MUNANA for another 1 h at 37 ℃. Next, the reaction was stopped, and fluorescence intensity was measured using a FLUOstar Omega microplate reader. The activity of NA toward the sialylated glycoprotein fetuin was analyzed in a solid phase cleavage assay using a previously described enzyme linked lectin assay (ELLA) (16, 17). Fetuin (2.5 ug/mL) was coated on Maxisorp Nunc 96-well plates (Thermo Fisher Scientific). Plates were incubated with IAV PR8 (1.78×10^8 PFU/mL) in the presence or absence of 5 μM CATH-B1 (in 50 mM Tris-HCl with 4 mM $CaCl_2$, pH =6) for 2 h at 37 °C. Subsequently, the plates were washed three times with PBS/ 0.05 % Tween 20 after which terminal galactose moieties were quantified using biotin-conjugated peanut agglutinin E. Cristagalli (ECA) lectin (Vector laboratories) (1.5 μg/mL) in combination with streptavidin-HRP (Thermo Fisher Scientific) (1:1000). After washing, TMB was added and plates were incubated for 1-4 minutes at room temperature. Sulfide acid (25%) was used to stop the reaction. Finally, the plate was read at OD450 nm using the FLUOstar Omega microplate reader. Final OD450 nm values are presented as OD450 nm_{sample}-OD450 $nm_{backgroud}$.

Quantitative real-time PCR (qPCR)

HD11 cells were infected with virus for 1 h at MOI of 1 in the presence or absence of CATH-B1 as described above. After 8 or 24 h incubation, total RNA was extracted by Trizol (Ambion, Carlsbad, CA) reagent according to the manufacturer's instructions. RNA (500 ng) was reverse transcribed by the iScript cDNA synthesis kit (Bio-Rad, Veenendaal, the Netherlands) according to the manufacturer's instructions. Primers and probes were designed and produced by Eurogentec (Seraing, Belgium) (Table 2). Quantitative real time PCR was performed on a CFX Connect qPCR with CFX Manager 3.0 (Bio-Rad). Reactions were performed as follows: 3 min at 95 °C; 40 cycles: 10 s at 95 °C, 30 s at 60 °C and 30 s at 72 °C. Relative gene expression levels were normalized against the expression levels of the house keeping gene GAPDH.

Table 2: Primer and probe sequences for qPCR

Gene		5'→3'sequence
GAPDH	Forward	GTCAACCATGTAGTTCAGATCGATGA
	Reverse	GCCGTCCTCTCTGGCAAAG
	Probe	AGTGGTGGCCATCAATGATCCC
IFN-a	Forward	GACAGCCAACGCCAAAGC
	Reverse	GTCGCTGCTGTCCAAGCATT
	Probe	TCCACCGCTACACCCAGCAGCACCTCG
IFN-β	Forward	CCTCCAACACCTCTTCAACACG
	Reverse	TGGCGTGTGCGGTCAAT
	Probe	AGCAGCCCACACACTCCAAAACACT
IL-1β	Forward	GCTCTACTAGTCGTGTGTGATGAG
	Reverse	TGTCGATGTCCCGCATGA
	Probe	CCACACTGCAGCTGGAGGAAGCC
IL-6	Forward	GTCGAGTCTCTGTGCTAC
	Reverse	GTCTGGGATGACCACTTC
	Probe	ACGATCCGGCAGATGGTGA
IL-8	Forward	GCCCTCCTCCTGGTTTCA
	Reverse	CGCAGCTCATTCCCCATCT
	Probe	TGCTCTGTCGCAAGGTAGGACGCTG

Statistical analysis

Data are represented as mean ± SEM of three independent experiments for each group (n = 3) and were analyzed by a T-test for two groups or by one-way ANOVA with Tukey's multiple comparisons test for more than two groups. Bio-Rad CFX Manager 3.0 software was used for qPCR data analysis. All graphs were made using GraphPad Prism® 8.0.

Results

Cytotoxicity and anti-IAVs activity of cathelicidins

To investigate the anti-IAVs activity of cathelicidins, three IAV strains (H1N1/PR8, H3N1 and H5N1) were used in this study. Both HD11 and MDCK cells were inoculated with IAVs in the presence or absence of 5 μM cathelicidins for 1 h. After 1 h, viruses and peptides were removed, and cells were incubated for another 7 h. At 8 hours post infection (hpi), the number of infected cells was quantified by immunofluorescent labeling of the influenza nuclear protein. As shown in Fig. 1, the cathelicidins displayed different antiviral activities, which for some of them depended to some extent on the viral strain and the cell line used. PMAP-23 and K9 did not significantly inhibit infection. Interestingly, LL-37 only showed activity against H3N1 and to a lower extent H1N1 but not against H5N1, while the chicken

cathelicidins were active against all three influenza strains with inhibition of infectivity of 40-70%. However, regardless of the cell line or viral strain used, CATH-B1 clearly displayed the strongest antiviral activity, inhibiting infection up to 80-90%.

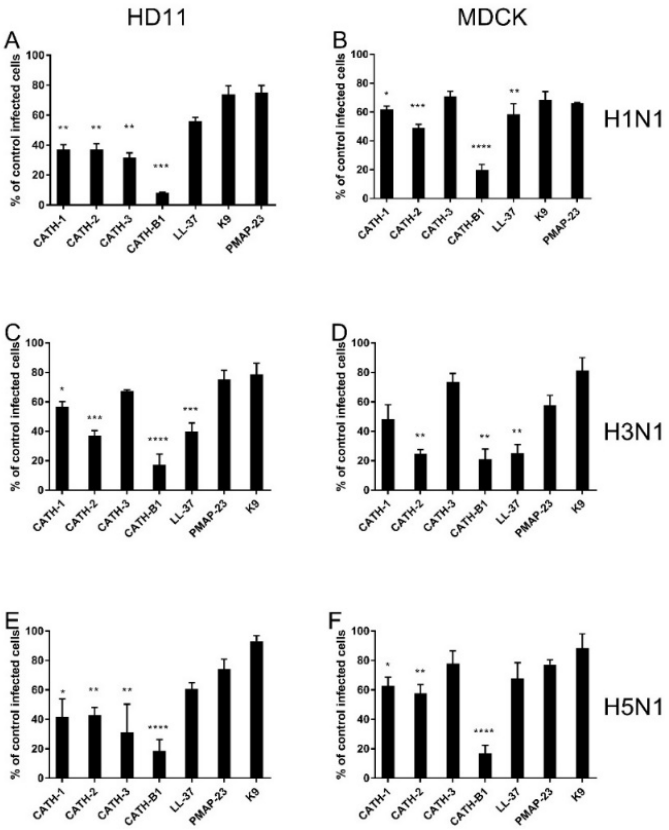

Fig 1. The antiviral effect of cathelicidins against 3 IAV strains (H1N1/PR8, H3N1 and H5N1). Cathelicidins were mixed with virus strains before addition to either HD11 or MDCK cells. H1N1/PR8 infection in the presence of cathelicidins of HD11 (A) and MDCK (B) cells. H3N1 infection in the presence of cathelicidins of HD11 (C) and MDCK (D) cells. H5N1 infection in the presence of cathelicidins of HD11 (E) and MDCK (F) cells. Viral infection was determined by immunofluorescent detection of IAV nuclear protein. Three images per well were taken using an EVOS FL microscope (Thermo Fisher Scientific) and the infected cells were counted. The infection rate in the presence of cathelicidins was normalized against only virus-treated wells. Data are represented as mean ± SEM of three independent experiments of triplicate samples per experiment. *p≤0.05; **p≤0.01; ***p≤0.005; ****p≤0.001.

The inhibitory infectivity of CATH-B1 was dose-dependent (Fig. 2), with an almost complete inhibition of viral infectivity of the H1N1 and H3N1 strains, while inhibition of H5N1 reached 85% (Fig. 2). The observed reduction in infected cells was not due to toxicity of cathelicidins towards the mammalian cell lines as shown by the WST assay (Fig. 3).

Nevertheless, cytotoxicity was observed for CATH-1 and CATH-3 at 10 µM to HD11 cells (Fig. 3A).

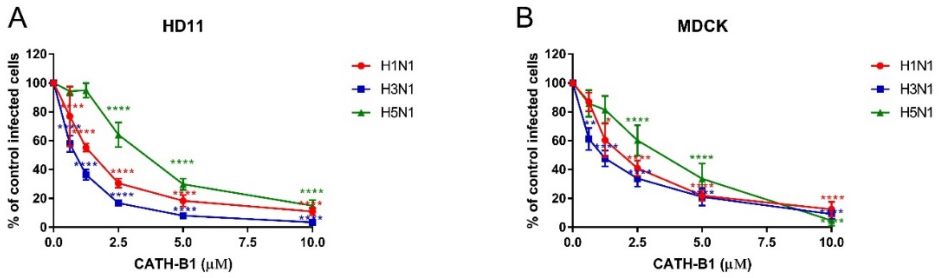

Fig 2. Dose-dependent antiviral activity of CATH-B1 against IAV strains (H1N1/PR8, H3N1 and H5N1). (A) Viral infection with CATH-B1 in HD11 cells. (B) Viral infection with CATH-B1 in MDCK cells. Viral infection was determined by immunofluorescent detection of IAV nuclear protein. Three images per well were taken using an EVOS FL microscope (Thermo Fisher Scientific) and the infected cells were counted. The infection rate in the presence of cathelicidins was normalized against only virus-treated wells. Data are represented as mean ± SEM of three independent experiments of triplicate samples per experiment. *p≤0.05; **p≤0.01; ***p≤0.005; ****p≤0.001.

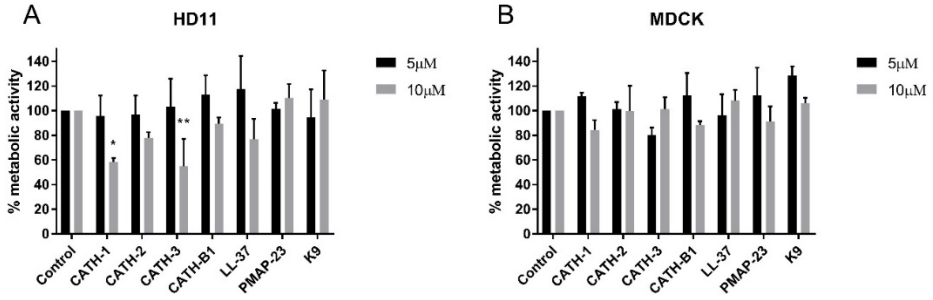

Fig 3. Cytotoxicity of cathelicidins. HD11 and MDCK cells were incubated with cathelicidins, and metabolic activity was tested using WST-reagent. (A) Metabolic activity of HD11 cells incubated for 24 h with cathelicidins. (B) Metabolic activity of MDCK cells incubated for 8 h with cathelicidins. Data are represented as mean ± SEM of three independent experiments of triplicate samples per experiment. *p≤0.05; **p≤0.01.

When cathelicidins and virus were sequentially added to cells (either pre- or post-incubation of peptide relative to virus inoculation), the inhibitory effect was mostly lost (Supplementary fig. 1). This indicates that the antiviral effect of the peptides was not achieved through interaction with the HD11 or MDCK cells or by an inhibitory effect on viral replication after the viruses entered the cells, but that CATH-B1 likely blocked viral entry to the cells by direct interaction with the virus.

The effect of CATH-B1 on IAV-induced gene expression of cytokines in HD11 cells

Activation of macrophages is important for viral clearance during IAV infection, but an excessive inflammatory response might cause morbidity and mortality (18-20). As several cathelicidins have been reported to affect innate immune responses (3, 4), we analyzed to what extent the presence of CATH-B1 affected these responses induced by infection of cells with IAV. To this end, virus-induced gene expression of cytokines in HD11 macrophages was determined by qPCR in the presence or absence of CATH-B1.

Virus infection resulted in induced gene expression of IFN-β, IL-1β, IL-6, and IL-8, but surprisingly not IFN-α. However, whether this lack of IFN-α gene expression was IAV strain specific was not further investigated. CATH-B1 downregulated PR8-induced gene expression of IFN-β, IL-1β, IL-6, and IL-8, but the relative mRNA level of IFN-α was unaffected (Fig. 4A and supplementary fig. 2). CATH-2 and LL-37 also showed similar effects on gene expression upon virus infection, but the inhibition was not as pronounced as observed for CATH-B1, correlating with the effect of the peptides on virus infection shown in Fig. 1. The effect of CATH-B1 on virus-induced gene expression was diminished when the cells were incubated with CATH-B1 prior to, or immediately after virus infection (Fig. 4B), indicating that the reduction of the response results from the ability of the peptide to inhibit infection.

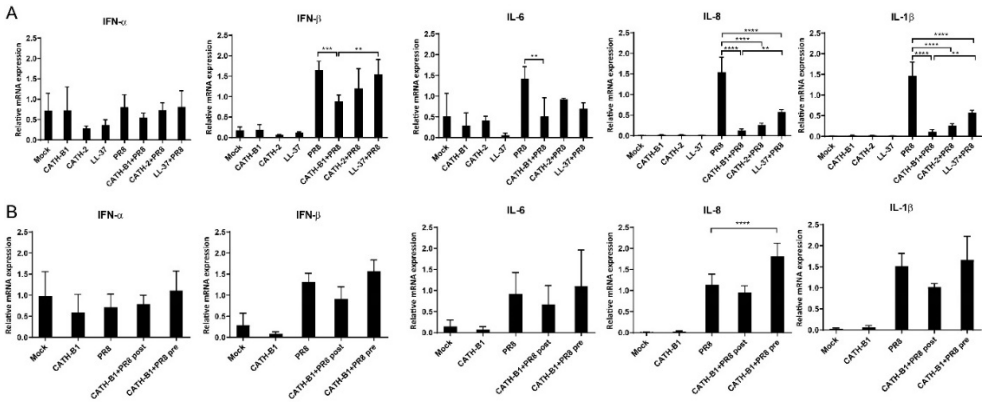

Fig 4. The effect of CATH-B1 on PR8-induced immune response in HD11 cells. (A) Cytokine expression in HD11 cells at 24 hpi in the presence or absence of peptides. (B) Cytokine expression in HD11 cells for pre- or post-incubation with CATH-B1. Relative gene expression levels were normalized against the expression levels of the house keeping gene GAPDH. Data are represented as mean ± SEM of two or three independent experiments of triplicate samples per experiment. *P≤0.05; **P≤0.01; ***P≤0.005; ****P≤0.001.

The interaction of CATH-B1 with IAV

The inhibitory effect of CATH-B1 on virus infection and induction of cytokine responses is only observed when the peptide is present during inoculation of cells with virus, but not when cells are exposed to the peptides prior to or immediately after virus infection. This suggests that a direct interaction of the peptide with the virus is required for its antiviral effect. To further investigate the antiviral mechanism of CATH-B1, a series of experiments was performed using H1N1. Firstly, a crucial role for a direct interaction of CATH-B1 with virus particles was analyzed by removal of unbound CATH-B1 using Capto Core 700 beads. As controls, incubation of virus itself with the beads did not affect virus infectivity (Fig. 5, red bar), while addition of CATH-B1 solution to virus preparations again resulted in 80% reduction of virus infectivity on HD11 cells (Fig. 5, dark green bar). Incubation of CATH-B1 with beads prior to virus addition resulted in very little antiviral effect (Fig. 5, orange bar), indicating that CATH-B1 was efficiently removed from solution by the beads. However, when CATH-B1 and virus were mixed prior to their treatment with Capto Core 700 beads, the antiviral activity of CATH-B1 was maintained indicating that CATH-B1 is directly associated with the virus and not captured by the beads (light green bar, Fig. 5). The proposed binding of CATH-B1 to virus is almost instantaneous because in the absence of the 30 min incubation time upon mixing of virus and CATH-B1 prior to addition of the beads, a similar antiviral activity of CATH-B1 was observed (data not shown). Similar results were obtained using MDCK cells (Supplementary fig. 3).

The effect of CATH-B1 on morphology of virus

Some host defense peptides, such as human neutrophil defensins, have been shown to induce viral aggregation, which might contribute to their antiviral activity (21, 22). Other peptides such as LL-37 have been found to directly disrupt the viral membrane (23). To study the effect of CATH-B1 on viral morphology, H3N1 was used in this study as an example. As shown in Fig. 6, there is no clear alteration of the viral structure for CATH-B1, CATH-2 or LL-37 (Fig. 6 A-D). However, large aggregates were observed that contained viral particles and electron dense material at high concentration (20 μM) of CATH-B1 (Fig. 6E), while some smaller aggregates were observed at this concentration for CATH-2 and LL-37 (Fig. 6F and 6G). At 5 μM CATH-B1 smaller aggregates were observed (Fig. 6H-J). These results indicate that binding and aggregation of virus particles is likely involved in the antiviral mechanism of CATH-B1.

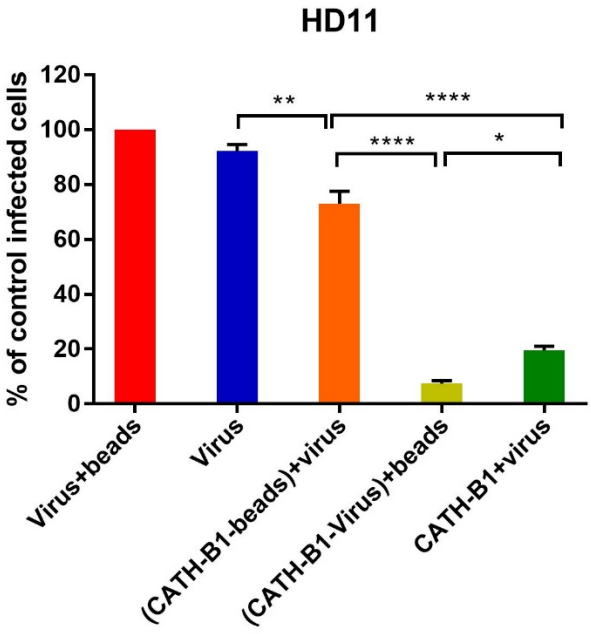

Fig 5. Binding of CATH-B1 to PR8 virus. CATH-B1 was pre-incubated with H1N1 virus after which peptide and virus were separated using Capto beads. (Virus containing) supernatant was then used to infect HD11 cells. Viral infection was determined by immunofluorescent detection of IAV nuclear protein. Three images per well were taken using an EVOS FL microscope (Thermo Fisher Scientific) and the infected cells were counted. The infection rate in the presence of CATH-B1 was normalized against only virus-treated wells. Data are represented as mean ± SEM of three independent experiments of triplicate samples per experiment. *p≤0.05; **p≤0.01; ***p≤0.005; ****p≤0.001.

The effect of CATH-B1 on hemagglutinin and neuraminidase activity

Hemagglutinin (HA) and neuraminidase (NA) are important functional proteins on the surface of IAVs. During viral infection, the function of HA is binding to sialic acid receptors on host cells and subsequent membrane fusion, while release of newly assembled virus particles requires the sialidase activity of NA. First, we analyzed the ability of CATH-B1 to interfere with the receptor-binding properties of HA by performing a hemagglutination inhibition assay. Unfortunately, CATH-B1 to some extent, induced lysis of erythrocytes, which precluded further analysis of the hemagglutination inhibition assay. Next, we analyzed the ability of CATH-B1 to interfere with NA activity using the substrate MUNANA. Clearly, even at the highest CATH-B1 concentrations, no inhibition of NA activity was observed (Fig. 7A). Next we performed a solid phase cleavage (ELLA) assay using the glycoprotein fetuin. Cleavage of fetuin by NA in this assay depends on the activity of NA, but also on the activity of HA, as receptor-binding by HA contributes significantly to NA cleavage (24, 25), at least when multivalent receptors are used. CATH-B1 inhibited cleavage of sialic acids on fetuin

(Fig. 7B). As CATH-B1 did not affect NA activity *per se* as demonstrated with the MUNANA assay, we conclude that the inhibitory effect in the ELLA assay results from the ability of CATH-B1 to interfere with virus-receptor binding.

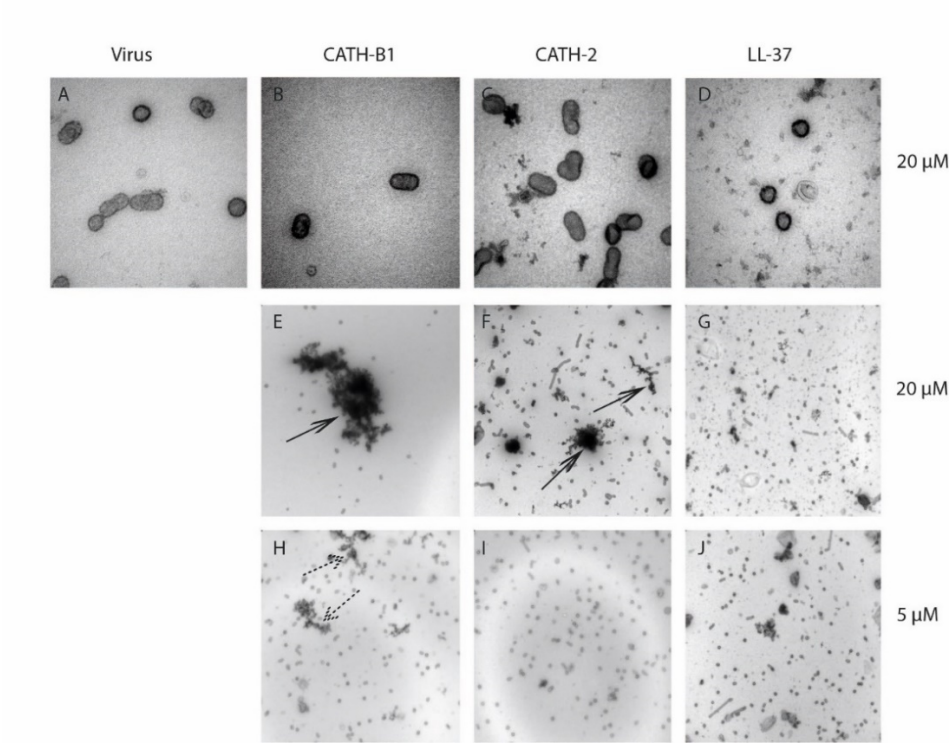

Fig 6. The effect of CATH-B1 on viral morphology. Representative electron microscopic images of H3N1 IAV alone (A) versus IAV pretreated with 20 µM (B-G) and 5 µM (H-J) of peptides. Large peptides aggregates (black arrows) containing viruses and small aggregates (dashed arrows) were visible at high concentration and low concentration of peptides, respectively. Representative images of 60,000 × magnification (A-D). Representative images of 16,500 × magnification (E-J).

Discussion

Cathelicidins are important peptides of the innate immune system that protect against invading pathogens. Although cathelicidins have mostly been studied with respect to their antibacterial activity, more recently, studies show potential antiviral activity of these peptides. For example, the human cathelicidin LL-37 has been found to have antiviral activity against IAV, adenovirus, respiratory syncytial virus and HIV (26-28). In addition, defensins including α- and β-defensins have also been found to exhibit antiviral activity against IAV (22).

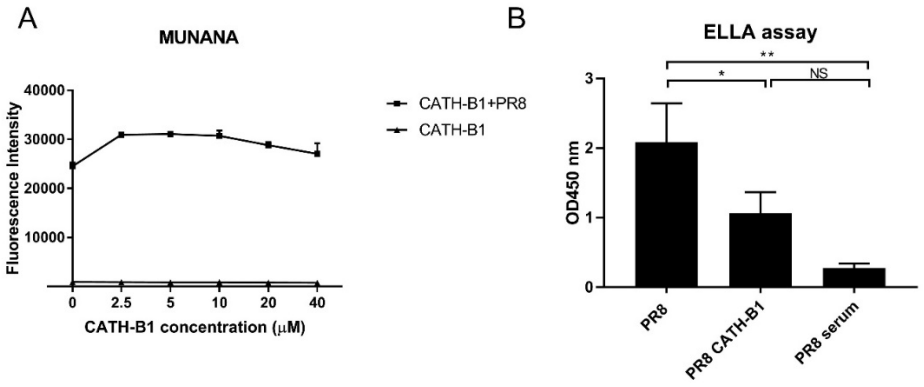

Fig 7. The effect of CATH-B1 on HA and NA activity. (A) NA activity of H1N1/PR8 was directly measured using MUNANA substrate in presence or absence of CATH-B1. (B) Desialylated N-glycans of fetuin were detected using HRP-conjugated ECA lectins, after incubation with H1N1/PR8 in presence or absence of CATH-B1. Data are represented as mean ± SEM of two independent experiments of triplicate samples per experiment. *p≤0.05; **p≤0.01.

Chicken cathelicidins have been studied quite extensively and they have shown to possess many different activities. Besides broad spectrum antibacterial activity, they enhance phagocytosis, neutralize LPS-induced immune responses and enhance DNA-induced TLR21 activation (3, 4, 6). However, antiviral activity of chicken cathelicidins was never described. Therefore, we investigated the anti-IAV activity and mechanism of inhibition of chicken cathelicidins, since IAV is an important pathogen causing disease in chicken and also in humans.

Four chicken cathelicidins (CATH-1, -2, -3, -B1) were used in this study together with a porcine (PMAP-23), canine (K9CATH) and human cathelicidin (LL-37) for comparison. Our results showed that CATH-B1 has the strongest anti-IAV activity against all three tested virus strains in this study. Comparison of the peptides does not give a clear indication what the main determinant for antiviral activity might be. All peptides have a (predicted) helical structure, are highly cationic and amphipathic. However, the sequence homology itself is quite low between peptides (except for CATH-1 and CATH-3 that also seem to have comparable activity). CATH-B1 is slightly longer than the other cathelicidins tested but it is unclear if that contributes to antiviral activity. Only for LL-37 some structure-antiviral activity studies have been performed which indicated that the central 20 amino acid fragment of LL-37 played a critical role in inhibiting the infection IAV (29). Future mutational studies on CATH-B1 could indicate which domains or residues are important for its observed activity against IAV.

CATH-B1 is different from the other three chicken cathelicidins in several ways. Besides some structural and sequence differences (Table. 1), it was reported to be exclusively present in the bursa of Fabricius. The peptide is expressed by secretory epithelial cells but is located after secretion surrounding bursal M-cells (8). In contrast, CATH-1, -2, and -3 are mostly expressed in the bone marrow and at least for CATH-2 it was shown that it is present in specific granules in heterophils (7, 30) where the peptide is released upon infection (30). In order to determine if CATH-B1 is important *in vivo* against viral infections, more detailed studies on its expression and localization are needed. If CATH-B1 is indeed only present in the bursa, only a limited antiviral role against for example infectious bursal disease virus can be envisioned, but not really against IAV or other repiratory or intestinal viruses. However, CATH-B1 gene expression seems not restricted to the bursa of Fabricius. Although at much lower levels than found in the bursa, CATH-B1 mRNA was present in several tissues, including spleen and multiple segments of the respiratory and gastrointestinal tract of chicken (31, 32). CATH-B1 gene expression was also observed in chicken HD11 macrophages and primary monocytes (33). Finally, some studies have described induced gene expression upon LPS and LTA stimulation *in vitro*, indicating that higher CATH-B1 levels in multiple tissues upon viral infection could be obtained.

HDPs as antiviral therapeutics are gaining interest with the increasing knowledge on their antiviral potential. However, the antiviral mechanism of action can be quite different from one HDP to another, and is also depended on viruses. Human cathelicidin LL-37 has been found to directly interact with the IAV virion thereby limiting viral replication and virus-induced inflammation *in vivo* (26). *In vitro*, LL-37 was described to directly induce disruption of the IAV viral membrane (23), although we did not observe this in our current study, possibly related to differences in the viral strains used. Human neutrophil peptides (HNPs) have been shown to induce viral aggregation and inhibit infectivity mainly through direct interactions with virus without any inhibition of HA activity of IAV (21). Another group of HDPs, defensins, also showed antiviral activity against IAV and HIV-1 but mainly through immunomodulatory effects during viral infection (34, 35). The current study showed that CATH-B1 binds to viral particles but this was not accompanied by any obvious disruption of the viral membrane. Instead, peptide-virus aggregates were observed using electron microscopy, indicating that CATH-B1 might exert this mechanism of aggregating pathogens to block infection for viral invasion.

The viral membrane of IAV is characterized by the two key proteins on the surface of the virus, hemagglutinin (HA) and neuraminidase (NA), both of which are important for IAV infection and could potentially be affected by binding of CATH-B1 to the viral surface. HA functions are as a receptor binding and fusion protein, while the NA protein is involed in release of (nascent) virus particles from decoy receptors or the cell surface (36, 37). Recently,

it has been reported that NA activity of IAV is influenced by virus-receptor binding (17, 24, 25). NA activity is altered based on enhanced or a reduced HA-receptor binding property. Our results suggest that CATH-B1 did not affect NA activity but rather inhibited virus-receptor binding activity, in agreement with CATH-B1 only affecting virus infection when present during virus inoculation. Presumably the inhibiting effect on virus-receptor interaction is related to CATH-B1 induced aggregation of virus. Whether this phenomenom results from a direct interaction of CATH-B1 with HA remains to be established, and other or additional antiviral mechanisms, such as the interaction of CATH-B1 with the viral membrane should be explored further. This antiviral mechanism of CATH-B1 appears to differ from that of LL-37. LL-37 bound to virus but did not inhibit HA-receptor binding and failed to inhibit virus binding to and uptake into cells (23, 29). Moreoever, the inhibitory activity of LL-37 depends on the IAV strain used which is consistent with our observation that LL-37 showed much more antiviral activity against H3N1 than against H1N1 and H5N1. Of note, CATH-B1 showed broad antiviral activity against IAVs carrying different HA proteins.

In conclusion, this study showed the potential of CATH-B1 to bind and inhibit the infectivity of IAV, likely by interfering with HA-mediated virus-receptor binding and thereby blocking viral entry. This new activity is important to understand the *in vivo* role of this cathelicidin, but might also have important implications for the future development of new antivirals based on cathelicidins in general.

Acknowledgements

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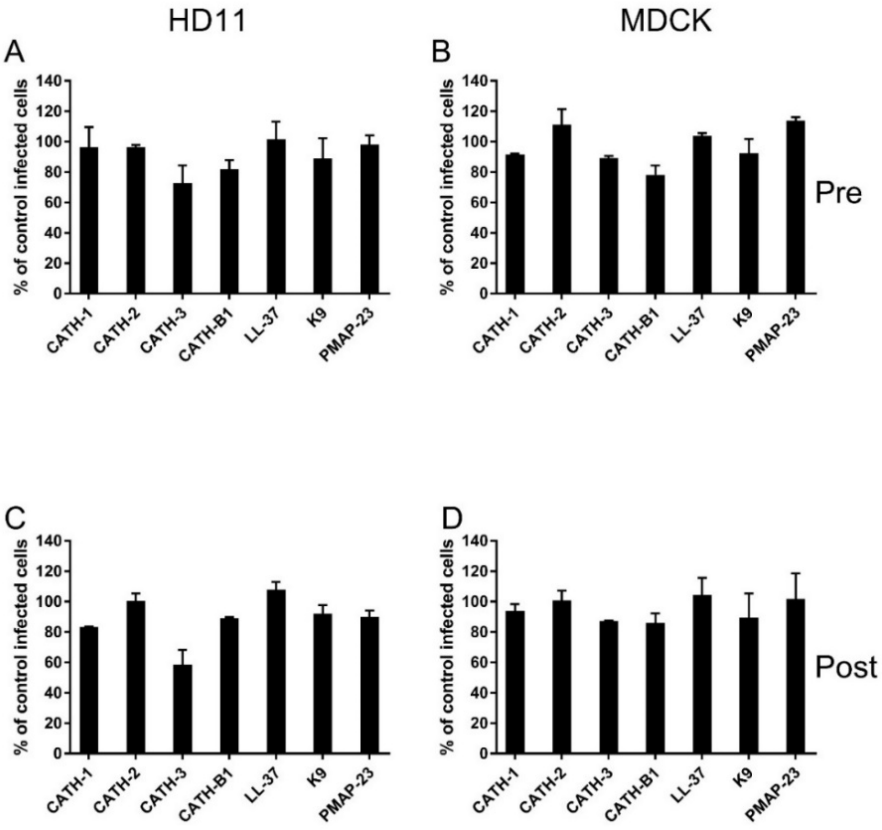
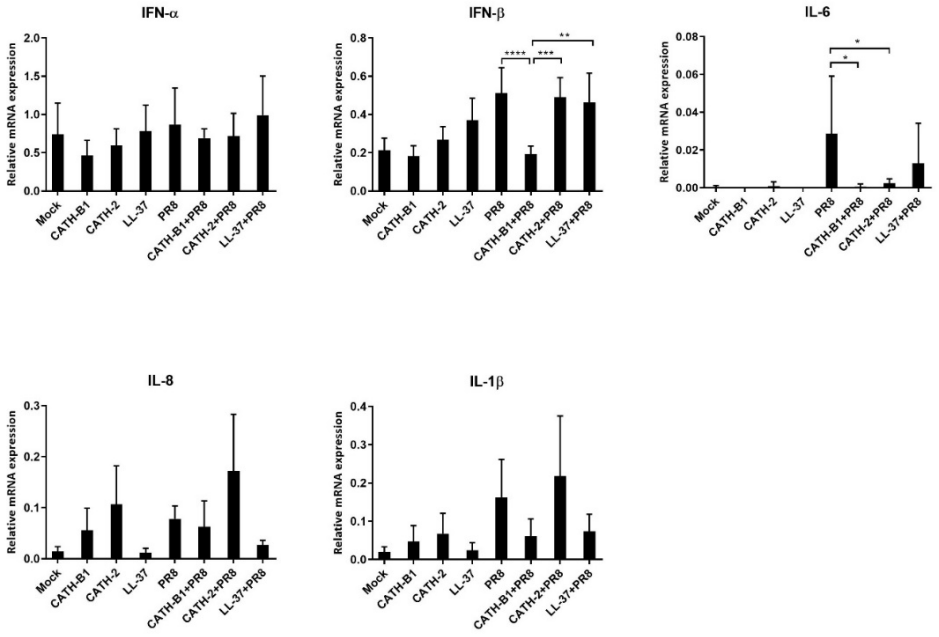
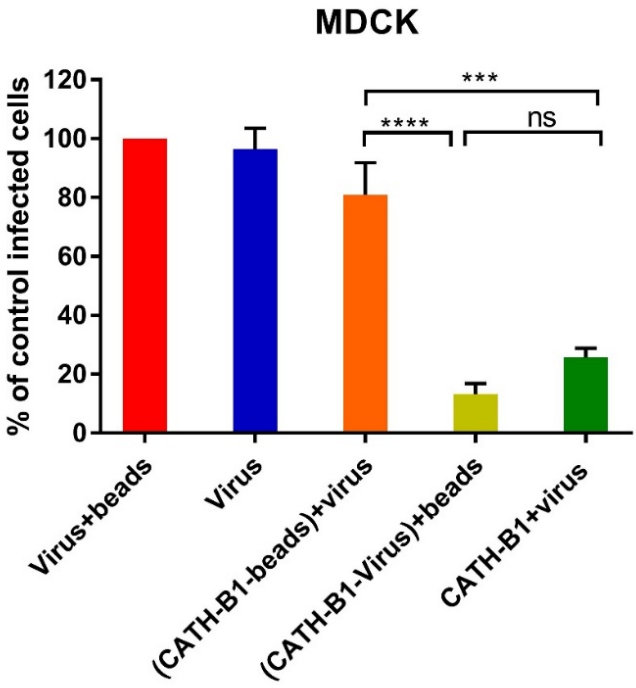


Supplementary fig 1. The effect of pre-incubation or post-incubation of cathelicidins on viral replication of H1N1/PR8 strain. PR8 infection in HD11 cells (A) or MDCK cells (B) for pre-incubation with cathelicidins. PR8 infection in HD11 cells (C) or MDCK cells (D) for post-incubation with cathelicidins. Viral infection was determined by immunofluorescent detection of IAV nuclear protein. Three images per well were taken using an EVOS FL microscope (Thermo Fisher Scientific) and the infected cells were counted. The infection rate in the presence of cathelicidins was normalized against only virus-treated wells. Data are represented as mean ± SEM of two independent experiments of triplicate samples per experiment.



Supplementary fig 2. The effect of CATH-B1 on PR8-induced immune response in HD11 cells. Cytokine expression in HD11 cells at 8 hpi in the presence or absence of peptides. Relative gene expression levels were normalized against the expression levels of the house keeping gene GAPDH. Data are represented as mean ± SEM of three independent experiments of triplicate samples per experiment. *p≤0.05; **p≤0.01; ***p≤0.005; ****p≤0.001.



Supplementary fig 3. Binding of CATH-B1 to PR8 virus. CATH-B1 was pre-incubated with H1N1 virus after which peptide and virus were separated using Capto beads. (Virus containing) Supernatant was then used to infect MDCK cells. Viral infection was determined by immunofluorescent detection of IAV nuclear protein. Three images per well were taken using an EVOS FL microscope (Thermo Fisher Scientific) and the infected cells were counted. The infection rate in the presence of cathelicidins was normalized against only virus-treated wells. Data are represented as mean ± SEM of three independent experiments of triplicate samples per experiment *p≤0.05; **p≤0.01; ***p≤0.005; ****p≤0.001.

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Chapter 7

General discussion

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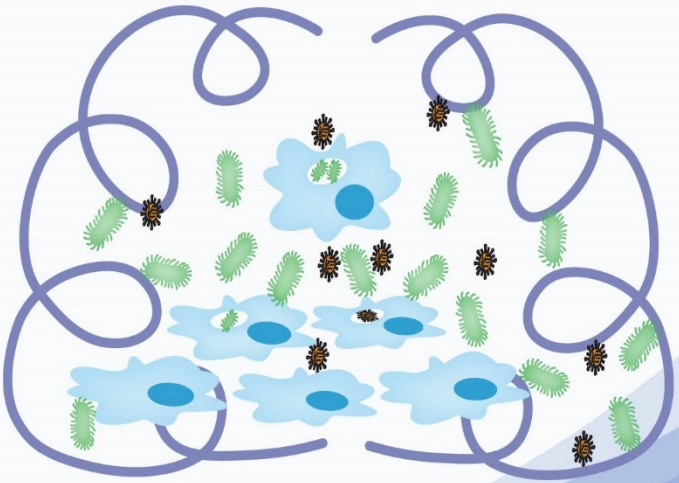

The lung is a major target organ for pathogens causing respiratory diseases in poultry. Avian respiratory diseases (ARD) lead to serious economic losses for most poultry industries (1). ARD are mainly caused by mycoplasmas, bacteria and viruses. For bacterial infection, avian pathogenic *E. coli* (APEC) is one of most prevalent pathogens causing systemic diseases called avian colibacillosis (2). APEC infection starts in the trachea and results in damaged respiratory mucosa; the bacteria subsequently cross the epithelium and enter the blood stream, finally spreading to other tissues (2). Furthermore, damaged epithelial cells facilitate colonization of other pathogens, such as viruses. During this process of infection, epithelial cells are the first cellular defense line against APEC infection and will produce cytokines and chemokines to initiate the immune response. Similarly, macrophages and heterophils will be quickly recruited to the site of infection to fight against the infection in the respiratory tract. Nevertheless, the exact role of these cells in controlling APEC infection is still unclear and therefore investigated in the studies described in this thesis.

So far, the treatment of an APEC infection in chicken mainly depends on antibiotics, but increasing antibiotic resistance makes this less effective (3). Therefore, new anti-infectives are needed and host defense peptides (HDPs) such as cathelicidins are considered as a promising alternative to antibiotics. They are mainly expressed by leukocytes and epithelial cells at infection sites in the host (4). They have broad antimicrobial activity and immunomodulatory effects on the host cells. These functions are important features for the development of anti-infectives.

In chicken, four cathelicidins (CATH-1, -2, -3 and -B1) have been described of which CATH-2 is the best studied. CATH-2 has strong antibacterial and antifungal activities (5, 6). In addition, CATH-2 can bind to LPS and inhibits LPS-induced production of inflammatory cytokines (7), inhibits *E. coli*-induced TLR2 and TLR4 activation (8) and enhances DNA-induced TLR21 activation in chicken macrophages (9). Some of these activities have also been described for CATH-1 and CATH-3 (10, 11), but especially for CATH-B1 knowledge on its role is lacking. Contrary to the localization of CATH-1, -2, -3 in heterophils, the CATH-B1 peptide is only described to be produced by epithelial cells in the bursa of Fabricius, while its function is speculated to protect this organ, but this is not backed up with any functional assays (12).

In this thesis, we investigated the interaction of chicken macrophages and epithelial cells with APEC to establish an appropriate APEC infection model *in vitro*. Then, we investigated the immunomodulatory effect of CATH-B1 on the chicken macrophages. Furthermore, antiviral activity of chicken cathelicidins against influenza A virus was studied.

The role of chicken lung-associated non-specific cellular defense against microbial infection

Lung epithelial cells act as a physical barrier, and are the first cells to produce an immune response upon infection (13). Due to the lack of a standard method of primary cultures, the chicken lung epithelial cell line (CLEC213) which exhibits pneumocyte type II-like characteristics is often used to investigate the interaction between lung epithelial cells and invading pathogens (14). As shown in **chapter 2**, APEC adhered to CLEC213 cells and subsequently invaded cells leading to a significant increase of IL-8 gene expression. Similarly, adherence of APEC was also observed in cultured chicken type II pneumocytes (15). Besides bacterial infection, viruses and parasites have also been shown to be able to infect CLEC213 cells (14, 16). These results show that CLEC213 cells can be used as infection model for multiple microorganisms. However, as shown in **chapter 2**, the immune response of CLEC213 cells was relatively low upon LPS stimulation since only IL-8 gene expression was highly induced, which is not completely in line with a previous study that showed a strong immune response upon LPS stimulation with high gene expression of IFN-α, IFN-β and IL-8 (14) using the same cell line. It is unclear what caused this discrepancy but these results indicate that this cell line might have a different behavior under slightly changed conditions. Therefore, one should be careful to use this cell line as a standardized system to study the chicken lung epithelial immune system. To further investigate the immune defense of lung epithelium against pathogens, a stabilized lung epithelial cell line or the culture of chicken primary epithelial cells need to be developed in the future.

The healthy avian lung has a low number of free respiratory macrophages compared to mammalian lung, but they can be quickly recruited from underneath the respiratory epithelia or from the blood upon infection (17-20), suggesting that macrophages indeed play an important role in the immune response towards microbes. To facilitate chicken macrophage studies, two chicken macrophage-like cell lines including HD11 and MQ-NCSU have been developed that have often been used by several groups (21, 22). In **chapter 3,** we report studies on the interactions of macrophages with APEC in HD11 cells. Besides phagocytosis of APEC, HD11 cells also showed phagocytic activity towards other bacteria that infect chicken, such as *Salmonella typhimurium* (23), *Listeria monocytogenes* (24) and *Mycoplasma synoviae* (25).

Besides the functional study of HD11 cells, primary macrophages are also a very useful tool for understanding the function of macrophages *in vivo*, expected to be resembling natural macrophages better than cell lines. However, there is no uniform protocol or characterization of chicken primary macrophages. Therefore, we established a standardized culturing system of monocyte-derived macrophages from chicken blood and characterized these cells by

morphology, cell surface marker expression and cytokine expression in response to TLR ligands stimulation. As shown in **chapter 4**, after 3 days of culturing monocytes with chicken GM-CSF, macrophages with a pro-inflammatory property were obtained. These macrophages showed high expression of IL-1β, IL-6 and IL-8 upon LPS stimulation, which is similar to classic mammalian M1-like macrophages (26). In addition, these cells had a "fried-egg" like shape and the three-day-cultured macrophages had a high expression of MRC1L-B and MHC-II, indicating the maturation of macrophages. In addition, these macrophages showed phagocytic activity towards APEC. Besides phagocytosis of APEC, monocyte-derived macrophages have been shown to take up fungi such as *Cryptococcus neoformans* (27). These results suggest that macrophages as non-specific defense cells have phagocytic activity against a broad array of microbial infections.

Macrophages have different killing capacities towards various bacterial strains. Our results in **chapter 3** and **chapter 4** showed that APEC were killed by HD11 cells and primary macrophages but *Salmonella* Enteritidis survived in HD11 cells. *Salmonella* can secrete different virulence factors to invade, survive and replicate within host cells (28, 29), explaining the observed survival of *Salmonella*. Recently, it has been shown that the interaction between *Salmonella* and macrophages was associated with host specificity (30). Avian host-specific *Salmonella* had a high survival rate while non-avian strains induced high level of cell death in chicken primary macrophages. Our *in vitro* infection model showed that APEC did not have such an evasion strategy since the number of viable intracellular APEC reduced with longer incubation time in both HD11 cells and primary macrophages.

When APEC is taken up by macrophages it also induces an immune response through the expression of cytokines and chemokines. As shown in (parts of) **chapter 3, 4 and 5** APEC did indeed induce nitric oxide (NO) production by macrophages as well as expression of several cytokines including IL-1β, IL-6, IL-8 and IL-10 in both HD11 cells and primary macrophages. Induction of this immune response suggests that activation of macrophages by APEC leads to a strong pro-inflammatory immune response, which is likely similar to the response of macrophages against APEC *in vivo*.

The host immune response to respiratory infections

Our results using the macrophage cell line and the monocyte-derived macrophages have clearly contributed to a better understanding of the defense provided by macrophages towards bacterial infections, but there are also some limitations to our studies. In our experiments we used the respiratory pathogen APEC (*E. coli* 506, O78:K80) as an example *in vitro*. This O78 serotype APEC strain is in most countries one of the most common pathogens causing avian colibacillosis (31). This APEC strain has been shown to cause purulent necrosis of parabronchi in chicken lung (32). Moreover, in a chicken lung infection model, *E. coli* 506

induced increased number of macrophages in the blood and spleen but the role of macrophages during infection is still understudied (19).

However, it needs to be pointed out that our infection studies are limited using only this APEC strain, while APEC is a general name for the large family of pathogenic *E. coli* with different genetic backgrounds. For example a pathogenic isolate (MT78, O2:K1), has also been reported to induce high numbers of heterophils and macrophages in the lung (33, 34). These macrophages have been observed to phagocytose this APEC *in vivo* and also showed high phagocytic activity towards these bacteria *in vitro* (34). It would therefore be worthwhile to extend our studies to determine if the results can be extrapolated to other *E. coli* strains. In addition, besides APEC, respiratory infections, caused by *Mycoplasma*, *Pasteurella multocida* and *Ornithobacterium rhinotracheale*, have also been shown to induce activation of macrophages resulting in increased phagocytosis and release of inflammatory cytokines (35-37). These results suggest that macrophages play a non-specific defense role against respiratory infections and it would be interesting to include these bacteria in our future studies.

The immunomodulatory effect of chicken cathelicidins on host cells

In addition to direct microbial killing, cathelicidins can exert immunomodulatory effects on the host cells. The immunomodulatory effect of human cathelicidin LL-37 has been extensively studied. LL-37 has many immunomodulatory properties including binding to LPS and blocking LPS-induced production of cytokines by macrophages (38, 39), mediating leukocytes migration (40), modulating activation of TLRs (41-43) and regulating cell differentiation (44, 45). The chicken cathelicidin CATH-2 shares many of these properties with LL-37, but other peptides such as CATH-B1, had not been investigated.

As shown in **chapter 5**, both CATH-2 and CATH-B1 bind to LPS and inhibit LPS-induced inflammatory cytokine expression in macrophages. These results are in line with previous studies that showed the binding of CATH-2 and CATH-B1 to *E. coli* LPS by ITC analysis and a chromogenic limulus amoebocyte lysate (LAL) assay, respectively (8, 46).

Besides LPS binding, both CATH-2 and CATH-B1 inhibited the APEC-induced inflammatory response in primary macrophages. CATH-2 has been shown to permeabilize the inner membrane of *E. coli* and thereby kills the bacterium. Subsequently, activation of TLR2 and TLR4 was inhibited by CATH-2, leading to inhibition of activation of macrophages (8). CATH-B1 did not kill bacteria and slightly enhanced bacterial phagocytosis by chicken macrophages, but interestingly it reduced APEC-induced activation of macrophages, indicating a different mechanism than CATH-2 which only reduces non-viable APEC-induced activation of macrophages. In addition, macrophages pre-incubated with CATH-B1 produced significantly higher expression of IL-10 after challenge with APEC.

These results suggest that CATH-B1 plays a role in the anti-inflammatory response, but the exact mechanism is still not clear. In earlier studies, CATH-2 was described to induce MCP-3 gene expression in macrophages (7), suggesting chicken cathelicidins may be involved in signaling pathways to regulate immune responses, but it is still unknown whether chicken cathelicidins regulate the immune response via interaction with other specific cell receptors or only act on bacterial ligands such as LPS and DNA. To further find out which host factors are affected by chicken cathelicidins, RNA-seq technology could be used to screen differences in host gene expression upon incubation with cathelicidins.

The role of chicken cathelicidins against viral infections

Viruses are important pathogens causing diseases, leading to morbidity and mortality in chickens. Development of drug resistance is the main problem that makes treatment less effective. Cathelicidins as endogenous proteins in the host can be an option to develop novel antiviral therapeutics. Recently, the human cathelicidin LL-37 was found to have antiviral activity against different viruses, including influenza A virus (IAV), adenovirus, respiratory syncytial virus and HIV (47-49). As shown in **chapter 6**, we described, for the first time, antiviral activity of chicken cathelicidins against IAV *in vitro*. The four chicken cathelicidins displayed different antiviral activities and CATH-B1 showed the strongest antiviral activity.

So far, it has been described that possible antiviral mechanisms of cathelicidins are due to a direct interaction with viral particles or to modulation of host cell responses upon viral infection (50). LL-37 was found to directly interact with the IAV virion and to disrupt the viral membrane thereby limiting viral replication and virus-induced inflammation *in vivo* (47, 51). In our studies, CATH-B1 did not induce an (obvious) disruption of viral particles, but CATH-B1 bound and aggregated viruses thereby blocking viral entry. These results suggest antiviral activity of cathelicidins at the early stage of viral infection. This activity could have important implications for the future development of new antivirals based on cathelicidins in general (52). Furthermore, this functional exploration of antiviral activity of CATH-B1 is an important indication that CATH-B1 might have local functions in the bursa against other pathogens, such as IBDV. It will be interesting to investigate anti-IBDV activity of CATH-B1 on the host cells, such as DT-40 cells, or perform *in vivo* experiments to further understand the exact role of CATH-B1.

The role of CATH-B1 against microbial infection of host cells

CATH-B1 is different from the other three chicken cathelicidins in several ways. First, CATH-B1 protein expression was only observed in the secretory epithelial cells of the bursa (12). Second, CATH-B1 antimicrobial activity differs from that of the other cathelicidins. CATH-B1 has limited antibacterial activity compared to the other three chicken cathelicidins,

but CATH-B1 actually showed the strongest antiviral activity against IAV. Many investigators tried to link the antimicrobial mechanism of cathelicidins to the structure of the peptide. The central kink of CATH-2 produced by a proline residue plays an important role in the antibacterial activity (53) and the central fragment of LL-37 is important to inhibit viral infection (54, 55). Our results show that CATH-1 and CATH-3 exerted similar antimicrobial activities against IAV and APEC, which is not surprising since they are 70% homologous, while CATH-B1 has no apparent homology with the other three chicken cathelicidins. These results indicate that some structural and sequence differences might lead to different antimicrobial activities of cathelicidins. However, with the limited data available for CATH-B1 and the lack of homology with other peptides, it is hard to deduce any structure-function relationships. A separate study using truncated and mutated CATH-B1 variants would be necessary to obtain some more information on this.

CATH-1, -2, and -3 have been reported to be expressed in bone marrow and their proteins were detected in heterophils using immunostaining and mass spectrometry (56, 57), but CATH-B1 protein is expressed by epithelial cells in bursa. However, gene expression of CATH-B1, in contrast to protein expression is not limited to the Bursa since CATH-B1 mRNA was also found to be present in several other tissues. Our results in **chapter 5** showed that CATH-B1 gene expression was induced by APEC in HD11 cells and in primary macrophages. This seemed like a specific upregulation because gene expression of the other three cathelicidins was stable or only very mildly induced, although it should be pointed out that mRNA levels of these cathelicidins was relatively low. In addition, CATH-B1 showed immunomodulatory activity towards the macrophages (**chapter 5**). These results could suggest that CATH-B1 may actually be produced upon infection in macrophages and subsequently may play an active role against microbial infections. However, it is essential for this to determine if CATH-B1 translation actually occurs because gene expression does not always correlate with protein expression. This could potentially be done, for example, with a CATH-B1 antibody or with mass spectrometry.

The clinical potential application of HDPs for anti-infective therapies

HDPs play an important role in the host's response to infection and inflammation. Initial researches focused on antimicrobial activity of HDPs was to develop alternatives to antibiotics against microorganisms. Over the past decades, some studies demonstrated that HDPs not only have antimicrobial activity since many HDPs lose their antimicrobial activity under physiological conditions, whereas their immunomodulatory activities are presented both in tissue cultures and *in vivo* (58).

As shown in **chapter 5**, CATH-B1 lost its antibacterial activity but exerted its anti-inflammatory activity under cell culture condition. Other synthetic innate defense regulator

peptides which lacked antimicrobial activity have been shown to protect mice from infection *in vivo* (59-61). Similarly, the human α-defensin HNP-1 also protected mice from infection by leukocyte accumulation, despite of its weak antimicrobial activity (62). These results indicate that anti-infective properties of HDPs are exerted by their immunomodulatory activity. Therefore, HDP-based therapies are developed not only as antimicrobials but also as immunomodulators. Recently, exogenous administration of HDPs have been used in many animal infection models to provide protection against microbial infection. For instance, LL-37 application protected animals from infection with *Pseudomonas aeruginosa*, influenza viruses and respiratory syncytial virus *in vivo* (52) in a neutrophil-dependent manner. Another study showed that *in ovo* administration of CATH-2 provided protection in chicken against respiratory *E. coli* infection (63). However, so far, most studies focused on potential application of HDPs are still limited in the preclinical stage. Only a few studies in humans were successful to use HDPs in clinical trials, such as LL-37 application in venous leg ulcers and protegrin 1 application in pneumonia (clinical trials have been reviewed in 52). Notably, the application of HDPs in the field of veterinary medicine is a promising approach to develop anti-infective therapies. One major problem of this approach is related to costs, since the production of peptides is relatively expensive, making treatment or prevention challenging using exogenous host defense peptides. Another approach would be to stimulate the endogenous production of HDP to increase the immune status of chicken. In several studies, this hypothesis was tested and it was shown that indeed HDP production (in chickens and mammals) can be increased by short chain fatty acids such as butyrate (64), and but also for example by vitamin D. Feed additives based on these compounds could therefore have great beneficial effect at relatively low costs. Our studies on CATH-B1 in this thesis add to the existing activity profile for chicken cathelicidins and contains new valuable information for the potential development of anti-infectives in later clinical studies.

Concluding remarks

In the work described in this thesis, we aimed to investigate chicken lung-associated cellular defense against microbial infections and the anti-infective role of CATH-B1 *in vitro* during infection. Studies on the interaction of APEC with epithelial cells and macrophages increase our knowledge about the chicken lung-associated immune system upon infection. The functional exploration on the immunomodulation and antiviral activities provide insight in the possible use of cathelicidins in the development of anti-infective therapies.

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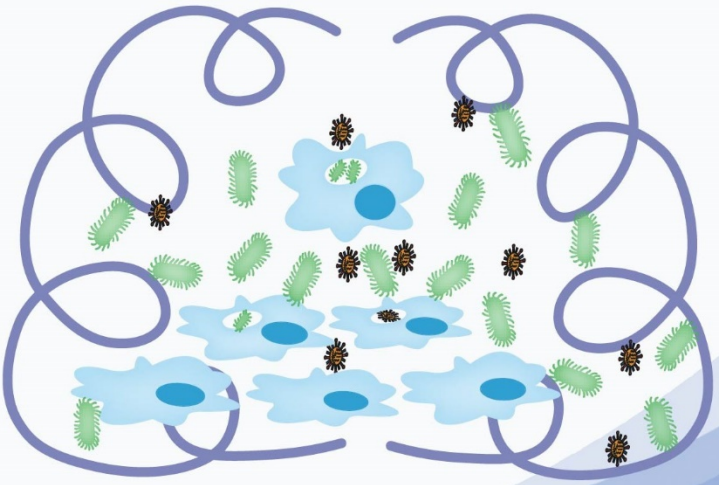
Appendices



Nederlandse samenvatting

Infecties van het respiratoire systeem zijn een belangrijk probleem in de pluimveesector. Ze veroorzaken veel uitval van vogels en zorgen daarbij voor een enorme economische schade. Longinfecties kunnen veroorzaakt worden door verschillende micro-organismen zoals virussen en bacteriën (inclusief mycoplasma). 'Avian Pathogenic *Escherichia coli*' (APEC) is één van de belangrijkste bacteriële ziekteverwekkers in de sector die leidt tot colibacillose, een systemische infectie waarbij meerdere organen geïnfecteerd worden. De initiële APEC-infectie start in de trachea waar de slijmvliezen van de luchtwegen beschadigd kunnen worden. Daarna kan de bacterie de longepitheelcellen infecteren en uiteindelijk ook in de bloedbaan komen waardoor het andere organen kan bereiken en infecteren. Daarnaast zorgt de mucosale beschadiging er ook voor dat andere pathogenen, zoals bijvoorbeeld virussen makkelijker de long kunnen infecteren, zogenaamde co-infectie.

Wanneer een infectie optreedt zal de gastheer een immuunreactie initiëren. Epitheelcellen produceren dan onder andere cytokines en chemokines, en cellen van het aangeboren immuunsysteem, zoals heterofielen en macrofagen worden snel naar de infectiebron gerecruteerd om de infectie te bestrijden. Echter, het exacte mechanisme van deze cellulaire en moleculaire afweerreactie bij de kip is nog niet bekend, mede door een gebrek aan goede modelsystemen waarmee dit onderzocht kan worden.

Een APEC-infectie wordt nog steeds behandeld met antibiotica maar de toenemende bacteriële resistentie tegen antibiotica zorgt ervoor dat de behandeling steeds minder effectief wordt. Hierdoor zijn nieuwe antimicrobiële middelen nodig om infecties te bestrijden en zogenaamde 'Host Defence Peptiden' (HDP) worden gezien als een potentieel nieuw antimicrobieel middel. Deze HDP zijn onderdeel van het aangeboren immuunsysteem van de gastheer en worden voornamelijk aangemaakt door witte bloedcellen en epitheelcellen. Vaak wordt de aanmaak ervan ook opgereguleerd op de plaats waar de infectie plaatsvindt. Deze HDPs hebben 2 verschillende functies, ze kunnen micro-organismen doden of neutraliseren, en ze kunnen ook het immuunsysteem sturen (immuunmodulatie). Beide eigenschappen zijn belangrijk voor de eventuele ontwikkeling van een alternatief voor antibiotica gebaseerd op deze HDP.

Binnen de familie van HDP zijn er verschillende subklassen te onderscheiden, waaronder defensines en cathelicidines; deze laatste groep wordt in dit proefschrift bestudeerd. De kip heeft 4 cathelicidines, CATH-1, -2, -3 en CATH-B1, en hiervan is CATH-2 veruit het beste bestudeerd in de literatuur. CATH-2 heeft een sterke antimicrobiële activiteit tegen een breed-spectrum aan bacteriën, maar het is bijvoorbeeld ook actief tegen bepaalde schimmels. Daarnaast is beschreven dat CATH-2: 1) aan Lipopolysaccharide (LPS, onderdeel van Gram negatieve bacteriemembraan) kan binden, waardoor het LPS geïnduceerde productie van cytokines vermindert, 2) *E. coli* geïnduceerde activatie van Toll- Like Receptor 2 (TLR2) en

TLR4 kan verminderen, en 3) DNA-geinduceerde activatie van TLR21 kan stimuleren. Sommige van deze aktiviteiten zijn (in mindere mate) ook voor CATH-1 en CATH-3 beschreven maar vooral voor CATH-B1 is weinig bekend over welke antimicrobiële en/of immuunmodulerende eigenschappen het bezit. Een ander verschil is dat CATH-1 t/m -3 in heterofielen worden geproduceerd terwijl gerapporteerd is dat CATH-B1 vooral in epitheelcellen van de Bursa van Fabricius gemaakt zou worden. Een hypothese is dat CATH-B1 specifiek dit orgaan moet beschermen maar er zijn nog geen goede functionele tests beschreven die deze hypothese onderbouwen.

In de experimenten beschreven in dit proefschrift werd de interactie van kippenmacrofagen en kippenepitheelcellen met APEC onderzocht, met als doel een *in vitro* APEC-infectiemodel op te zetten dat gebruikt kan worden voor respiratoire infectiestudies bij kippen. Daarnaast werd het immuunmodulerend effect van CATH-B1 op macrofagen onderzocht en ook werd de antivirale activiteit van CATH-B1 bepaald tegen het influenza A Virus.

Rol van epitheelcellen in de luchtwegen bij *E. coli* infectie.

Het longepitheel vormt niet alleen een fysieke barrière maar kan ook een specifieke immunologische reactie initiëren als het worden geïnfecteerd. Het onderzoek naar deze immuunreactie in kippen is beperkt omdat er geen goede en makkelijke methode is om primaire epitheelcelculturen te kweken waarmee geëxperimenteerd kan worden. Wat wel beschikbaar is, en dus voor onderzoek gebruikt wordt, is de kippen-epitheel cellijn CLEC213, die volgens de literatuur karakteristieken heeft die lijken op epitheliale type-II-cellen uit de long. In de experimenten beschreven in **hoofdstuk 2** werd de interactie van APEC met deze CLEC213 cellen bestudeerd om een goed beeld te krijgen in hoeverre deze cellen als een goed model kunnen dienen voor bacteriële longinfecties in de kip. Uit de experimenten bleek dat APEC goed aan deze epitheelcellen konden aanhechten en dat ze deze vervolgens konden binnendringen, vergelijkbaar met de *in vivo* situatie. Invasie van CLEC213 cellen leidde o.a. tot een verhoogde IL-8 gen-expressie, hetgeen vergelijkbaar is met observaties in een eerdere studie met geïsoleerde kippen type-II-pneumocyten. Andere beschreven studies hebben aangetoond dat virussen en parasieten de CLEC213 cellen kunnen infecteren wat aangeeft dat deze cellijn ook een goed representatief model kan zijn voor dit soort studies. Echter, uit de studies in hoofdstuk 2 bleek dat op IL-8 inductie na, de inductie van andere cytokinen in CLEC213 cellen, zoals bijvoorbeeld IFN-α en IFN-β productie door LPS stimulatie, vrijwel niet detecteerbaar was terwijl dit wel eerder beschreven was. Een verklaring hiervoor zou kunnen zijn dat relatief kleine veranderingen in de opzet van een experiment een groot effect op de immuunreactie van de cel kunnen hebben. Mede hierdoor werd geconcludeerd dat men voorzichtig moet zijn met de interpretatie van de resultaten bij het gebruik van deze cellijn. Voor toekomstige immunologische studies naar infecties van het respiratoire longepitheel is

er een noodzaak voor een beter modelsysteem. Een gestandaardiseerde cultuur van primaire epitheelcellen, dan wel een meer geschikte cellijn met betere epitheelspecifieke immuunkarakteristieken zou de voorkeur hebben.

De rol van de macrofaag in bacteriële infecties

In een gezonde kippenlong zijn relatief weinig macrofagen aanwezig in vergelijking met een zoogdierlong zoals bijvoorbeeld in de muis of de mens. Echter, als bij kippen een infectie in de long optreedt kunnen er zeer snel en veel macrofagen gerecruteerd worden vanuit het bloed of het weefsel onder de geïnfecteerde epitheellaag. Dit geeft aan dat macrofagen een belangrijke rol spelen in de bestrijding van infecties. Er zijn momenteel twee kippenmacrofaag cellijnen beschikbaar, HD11 en MQ-NCSU, die veel gebruikt worden voor studies naar de rol van kippenmacrofagen. In **hoofdstuk 3** zijn experimenten beschreven waarin HD11 cellen gebruikt werden om de interacties van macrofagen met APEC te bepalen. Met behulp van microbiologische technieken als ook met elektronenmicroscopie kon worden bepaald dat HD11 cellen APEC opnemen (fagocytose), zoals al was beschreven voor een select aantal andere bacteriën (*Salmonella typhimurium*, *Listeria monocytogenes* and *Mycoplasma synoviae).* Fagocytose van APEC leidde tot gedeeltelijke doding van de bacterie en een verhoogde productie van stikstofmonoxide en cytokinen, waaronder IL-8. Deze functionele bepalingen geven aan dat HD11 cellen in principe een goed werkend en makkelijk model zijn om de basisfuncties van macrofagen te bestuderen.

Naast het gebruik van een macrofaag cellijn kunnen primaire celculturen van macrofagen ook gebruikt worden om de immuunrespons van deze cellen te bepalen. Deze primaire culturen komen in het algemeen beter overeen met de *in vivo* situatie van macrofagen in vergelijking met (geïmmortaliseerde) cellijnen zoals HD11. Echter, hoewel meerdere groepen het gebruik van primaire macrofagen, gekweekt uit bloed of beenmerg hadden beschreven was er geen duidelijk uniform protocol beschikbaar voor de kweek van kippenmacrofagen. In **hoofdstuk 4** is daarom een standaard methode om primaire macrofagen te kweken uit monocyten beschreven. Deze macrofagen werden gekarakteriseerd aan de hand van belangrijke eigenschappen zoals morfologie, oppervlakte (celmembraan) -markers en cytokinerespons bij TLR-stimulatie. Uit deze studies bleek dat om de best reproduceerbare pro-inflammatoire macrofagen te krijgen, bloed-monocyten 3 dagen lang in cultuur gehouden moesten worden in de aanwezigheid van GM-CSF. Na 3 dagen hadden deze macrofagen: 1) een ‘gebakken ei’ vorm, karakteristiek voor zoogdier pro-inflammatoire macrofagen, 2) een hoge IL-1β, IL-6 and IL-8 expressie na LPS-stimulatie en 3) een hoge expressie van de specifieke celoppervlak eiwitten MHC-II en MRC1L-B. Deze macrofagen konden tevens APEC fagocyteren, wat een goede indicatie is dat deze manier van macrofagen

kweken een goede benadering kan geven van de natuurlijke *in vivo* functie van kippenmacrofagen.

Uit de resultaten van **hoofdstuk 3 en 4** kon ook worden afgeleid dat kippenmacrofagen (zowel HD11 als primaire macrofagen) APEC kunnen doden nadat deze bacteriën gefagocyteerd waren. Dit is echter wel bacterie-afhankelijk want bijvoorbeeld *Salmonella* Enteritidis kon wel worden gefagocyteerd maar werd niet gedood, omdat deze bacterie specifieke virulentiefactoren kan uitscheiden waardoor deze bacteriestam in macrofagen kan overleven en zich zelfs kan vermenigvuldigen. Dit fenomeen is mogelijk uniek voor *Salmonella* Enteritidis stammen die specifiek voorkomen bij vogels aangezien *Salmonella* soorten geïsoleerd uit zoogdieren niet konden overleven in kippenmacrofagen.

De immuunmodulerende werking van CATH-B1 op macrofagen

Cathelicidines spelen ook een grote rol tijdens een bacteriële infectie. Naast hun directe antimicrobiële rol kunnen ze ook het immuunsysteem moduleren. In hoofdstuk 5 is onderzoek beschreven naar de mechanismen waarop kippencathelicidines de immuunrespons van macrofagen kunnen beïnvloeden. Zowel CATH-2 als CATH-B1 konden de LPS-geïnduceerde cytokineproductie van primaire macrofagen verlagen. Uit zogenoemde 'isothermal titration calorimetry' metingen bleek dat directe binding van de cathelicidines aan LPS hieraan ten grondslag lag. CATH-B1 en CATH-2 verlaagden ook de immuunrespons van macrofagen bij infectie met APEC. Echter, CATH-2 deed dat door de APEC te doden, terwijl CATH-B1 de immuunrespons verlaagde zonder directe antimicrobiële werking tegen de bacterie. Daarnaast stimuleerde CATH-B1 lichtelijk de fagocytose van APEC, hetgeen niet het geval was met CATH-2. Pre-incubatie van macrofagen met CATH-B1 verhoogde ook de productie van het anti-inflammatoire cytokine IL-10. Het anti-inflammatoire werkingsmechanisme van beide cathelicidines is dus duidelijk verschillend, maar er is nog steeds veel onderzoek nodig om de precieze werking van de cathelicidines te ontrafelen.

Antivirale werking van CATH-B-1

Naast bacteriële infecties zijn ook virale infecties van de luchtwegen een belangrijke oorzaak van uitval bij kippen en economische schade in de sector. De kennis over antivirale activiteit van cathelicidines is erg beperkt maar van de humane cathelicidine LL-37 is beschreven dat het o.a. het Influenza A virus (IAV) kan neutraliseren. In dit hoofdstuk werd voor de eerste keer bepaald of ook kippencathelicidines antivirale activiteit tegen IAV bezitten. Gebruik makend van *in vitro* infectiemodellen bleek dat van de 4 geteste kippencathelicidines CATH-B1 de sterkste neutraliserende activiteit had tegen 3 verschillende IAV stammen. De neutraliserende werking trad alleen op als virus en peptide tegelijk werden toegevoegd na

een korte incubatieperiode, wat erop duidt dat de werking van het peptide gebaseerd is op interactie met het virus en niet door immuunmodulatie van de gastheercel. Met behulp van elektronenmicroscopie werd gevonden dat CATH-B1 niet het virus zelf kapot maakte maar dat het wel grote aggregraten vormde van virus en peptide. Hieruit werd de hypothese gevormd dat het peptide het virus neutraliseert door het te aggregeren en daarbij de eerste fase van de virusinfectie, namelijk de aanhechting van het virion aan de gastheel cel, kan verstoren. Toekomstige studies zouden kunnen uitwijzen of CATH-B1 dezelfde effectiviteit heeft tegen andere virusinfecties waaronder bijvoorbeeld het 'Infectious Bursal Disease Virus' dat zich vermenigvuldigt in de bursa van Fabricius, het orgaan met een hoge expressie van CATH-B1.

Conclusie

De studies beschreven in dit proefschrift laten zien dat zowel cellen, met name macrofagen, als moleculen zoals cathelicidines een belangrijke rol kunnen spelen in de bescherming tegen bacteriële en virale infecties van de kippenlong. Tevens dragen de studies bij aan de ontwikkeling van goede modelsystemen om longinfecties te kunnen bestuderen. Toekomstige studies kunnen op deze informatie verder bouwen om nog meer inzicht in het aangeboren immuunsysteem te krijgen en eventuele therapeutische of preventieve toepassingen te ontwikkelen tegen luchtweginfecties bij de kip.

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Curriculum Vitae

彭练慈（Lianci Peng） was born on 4th December 1990 in Chongqing, China. After finishing high school, she started her bachelor studies in the College of Veterinary Medicine at Sichuan Agriculture University in September 2009. After three years she obtained a bachelor degree, then she did her master studies in the same college under the supervision of Prof. Zhongqiong Yin. She mainly focused on the antibacterial activities and mechanisms of action of Chinese Traditional Medicines. She obtained her Master degree in June 2015. In September 2015, she was awarded financial support by the China Scholarship Council (CSC) for doing her PhD in the Netherlands. In November 2016, she started her PhD project at Utrecht University under the supervision of Prof. Dr. Henk Haagsman and Dr. Edwin Veldhuizen. Her PhD project focused on chicken innate immunity and exploring chicken lung-associated cellular defense against microbial infection and the anti-infective role of chicken cathelicidins. During her PhD study, she was actively attending international conferences and finished the required PhD courses with success. In July 2020, she finished her PhD dissertation in the group of Molecular Host Defence resulting in this thesis.

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