Of Pigs and Peptides

Interactions between Host Defence Peptides, *Streptococcus suis* and Porcine Immune Cells

Roel M. van Harten

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Over Varkens en Peptiden – Interacties tussen Afweerpeptiden, *Streptococcus suis* en Immuuncellen van Varkens (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 maandag 13 maart 2023 des ochtends te 10.15 uur

door

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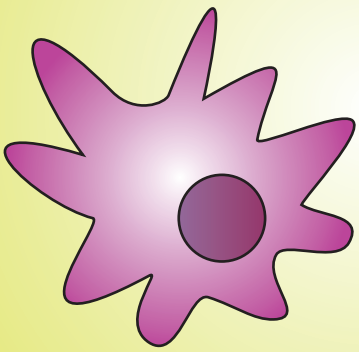

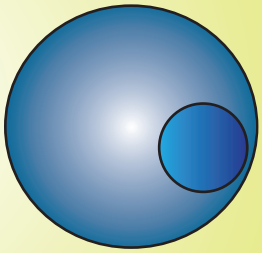

Chapter 1

General Introduction

Around 11.000 years ago, humans in the Near East first domesticated a strange, hoofed mammal with a large snout (Larson *et al.* 2005, Larson *et al.* 2007). Since then, the domestic pig (*Sus scrofa)* has become quite abundant worldwide. There were over 950 million pigs worldwide in 2020 (FAO 2022). This puts pigs in the top 5 of the most common large mammals, alongside sheep, cattle, goats, and humans. In 2018, the global gross production value of pigs in the meat industry was close to 230 billion US dollars (FAO 2022). Besides farming for meat, pigs were used to hunt for truffles, as the truffles produce a volatile compound that resembles male pig sex hormones, so female pigs are happy to dig them up. However, nowadays dogs are often preferred, as the pigs tend to eat the precious fungi while digging them up. Finally, some people keep pigs as pets, as they can be trained, are quite intelligent and can be perceived as affectionate and cute. However, pigs as pets do have quite complex needs in training, space and feed, and new owners often do not realize how large a pig can grow; even the smallest breeds can be 50-70 kg. So-called micro or teacup pigs are often sold as very young animals, and then grow up to normal, full-size pigs that no longer fit in a teacup.

Besides their economic impact, pigs have been playing an important role in health-care and health research. For example, the porcine pancreas was used for insulin production for treatment of diabetes until the 1980s, when recombinant insulin became commercially available (Bliss 1993, Landgraf and Sandow 2016). Due to their similar size to humans, pigs offer an advantageous model for cardiological research and xenotransplants, with pig heart valves being used in human hearts for decades (Manji *et al.* 2012). Recently, a genetically modified pig's heart was transplanted to a human being , although unfortunately the patient died two months later without a specific cause of death released (Brooks 2022, Wilson 2022). Besides the heart, other organs and the immune system share many characteristics to the human immune system, which makes pigs a relevant animal model for translational research (Meurens *et al.* 2012). In addition, since a tremendous number of (pre-)clinical trials fails (Rubin and Gilliland 2012), it is beneficial to use an animal model that can benefit from the obtained results. In addition, pigs are an attractive model for immune related research, due to the relatively similar size, genetics, physiology, and diet to humans. Furthermore, various molecular tools for pigs are available like antibodies, ELISAs, microarrays and a published genome, although these are limited compared

to human or mouse (Dawson 2011, Meurens *et al.* 2012, Käser 2021). Unfortunately, the use of pigs as experimental animals for translational immunological research has been inhibited by the cost of housing pigs, as well as their size and the time to mature.

The mammalian innate immune system

The first host defence mechanism that a pathogen encounters upon infection is the innate immune system. Innate immunity consists of cellular systems as well as epithelial barriers, production of acid, mucus and sweat, and cilia type movement, which form chemical or mechanical barriers to pathogen entry. Furthermore, there are protein components such as the complement system, which can lyse bacteria without additional cell mediation; aspecific antibodies, which are germline encoded and recognize conserved epitopes; circulating proteases like plasma kallikrein which can serve to start complement deposition; collectins like mannan-binding lectin (MBL) and surfactant proteins (SPs) - MBLs circulate with serum proteases in protein complexes called MASPs, which aggregate and neutralize pathogens but also start the complement cascades (Bergman 2011), while SPs like SP-A or SP-D are present in pulmonary surfactant and non-pulmonary epithelia and serve to opsonize pathogens through carbohydrate recognition domains (Geertsma *et al.* 1994, Kingma and Whitsett 2006, Sorensen 2018); and finally, host defence peptides (HDPs) like cathelicidins and defensins, which serve as antimicrobial, immunomodulatory and chemoattractant molecules. HDPs are discussed in detail in chapter 2.

The innate immune system is characterized by its unbiased approach, recognizing either pathogens directly or pathogen induced damage through conserved molecular features. The innate immune system can recognize cell damage through damage associated molecular patterns (DAMPs). These DAMPs are only released when cellular integrity is compromised, and are therefore indicative of either pathogen induced damage, or other breaches of integrity which have an associated increased risk of infection. DAMP receptors typically recognize extracellular presence of molecules that are supposed to be intracellular. For instance, the P2X7 receptor detects extracellular ATP (Perregaux *et al.* 2002, Elssner *et al.* 2004). Besides DAMP receptors, many innate immune cells express pattern recognition receptors (PRRs), that recog-

nize conserved molecular structures of bacteria, fungi or viruses through pathogen associated molecular patterns (PAMPs) (Kumar *et al.* 2011). The desired effect of innate immune activation is to either clear the dangerous pathogen directly, or to provide a link to the adaptive immune system, which can set up a more targeted response. The most studied innate immune receptors are Toll-like receptors (TLRs), which can recognize intra- or extracellular ligands. The most important extracellular TLRs are TLR-4 (recognizing LPS of Gram-negative bacteria), TLR-2 (which hybridizes with TLR-1 or TLR-6 to recognize bacterial lipoproteins from both Gram-positives and Gram-negatives), and TLR-5 (recognizing flagellin), all of which are components that are strongly conserved and ubiquitously expressed by microbes. Intracellular TLRs located in endosomes recognize bacterial and viral nucleotides; TLR-3 recognizes ssRNA, TLR-7 and TLR-8 recognize dsRNA, and TLR-9 is specific for DNA with unmethylated CpG motifs. Other important innate receptors are the cytosolic NOD-like receptors (NLRs), which recognize many bacterial products including peptidoglycan and its precursors, as well as other bacterial toxins; cytosolic RIG-like receptors, recognizing viral RNA; and receptors like AIM2 and cGAS-STING, which bind to cytosolic DNA (Fernandes-Alnemri *et al.* 2009, Hornung *et al.* 2009, Hopfner and Hornung 2020). All these receptors function to detect pathogenic material and subsequently activate immune signalling cascades, many of which lead to production of cytokine and chemokine proteins, which in turn attract and activate more immune cells.

Innate immune receptors are expressed on many different epithelial and immune cells but are especially abundant on cells like dendritic cells and macrophages (Fig. 1). Macrophages are specialized, tissue resident phagocytic cells and very efficient at internalizing and breaking down both pathogens and cells debris. They are also involved in restoring homeostasis after a local infection is eliminated (Gordon and Martinez 2010). Macrophages are found in almost every different tissue type in the body, where they co-opt various tissue specific processes. For example, liver macrophages (Kupffer cells) are involved in haemoglobin breakdown, microglia (macrophage like cells in the brain) help regulate neuron synapses, while osteoclasts - which are multinucleated cells formed from fusion of multiple macrophages (Teitelbaum 2000) - produce bone degrading enzymes helping to remodel and repair bone tissue. Most tissue resident macrophages originate from monocyte-like cells t

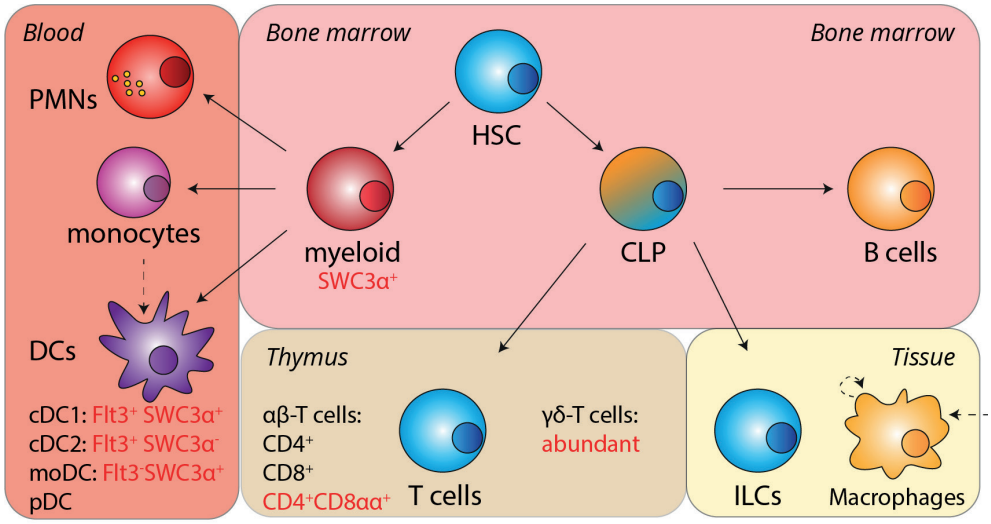

Figure 1: **Mammalian immune cell development starts with haematopoietic stem cells (HSC) in bone marrow.** There, the HSC can develop into common lymphoid progenitor cells (CLPs) or give rise to cells of a myeloid lineage. These myeloid cells can migrate into blood and become a variety of effector cells, being polymorphonuclear cells (PMNs), monocytes and dendritic cells (DCs). The CLPs further differentiate locally into B cells but can also migrate to the thymus and become T cells (either αβ T cells or γδ T cells) or migrate into tissues to become ILCs. In the tissues, macrophages are present that originate from a migration of monocyte-like cells during embryonic development. These macrophages self-renew but can be replenished by an influx of blood monocytes (not shown). In this figures, peculiarities of the porcine immune development are highlighted in red text.

that migrate to the tissues during embryonic development (Yona *et al.* 2013, Ginhoux and Guilliams 2016). In tissues, the macrophages can self-propagate without further influx of cells from bone marrow. However, upon infection, monocytes can migrate from blood and participate in phagocytosis and differentiate to the tissue resident macrophage phenotype. The original, non-monocytic population of tissue resident macrophages drives the resolution of inflammation and subsequently displaces the macrophages of monocytic origin (Davies *et al.* 2011). In the past, the macrophages that were inflammatory in nature were referred to as M1, and homeostatic or resolving macrophages were referred to as M2 to reflect the dichotomy found in T cell biology. Although macrophages are currently considered to be much more heterogenous, the M1/M2 dichotomy is still in use for *in vitro* cultured macrophages (Hume 2008, Geissmann *et al.* 2010a).

The key innate cells that provide instruction to the adaptive system are dendritic

cells (DCs). DCs principally present fragments of pathogens (antigen) on the major histocompatibility complex (MHC)-I or II receptor (Figure 2). MHC-I is present on almost all cells and presents fragments of intracellular proteins, which includes invading viral proteins. MHC-II is expressed by APCs exclusively and is loaded with fragments of extracellular proteins to be presented to $CD4^+$ T cells, although some APCs can cross-present extracellular protein fragments on MHC-I molecules (to induce a $CD8^+$ T cell response). Since these protein fragments or antigens are present in intact pathogens too, they are specifically targeted by this adaptive response mounted by the $CD4^+$ or $CD8^+$ T cells. The ability to present antigen and induce and regulate T cell function are therefore the main functions of dendritic cells. Plasmacytoid DCs (pDCs) circulate in blood and are more involved with producing IFN-α to modulate antiviral responses than with antigen presentation, although they do express both MHC receptors. Conventional DCs (cDCs) are the real professional APC and reside near barriers like skin, gut, and lungs where they sample the environment through dendrites and move to draining lymph nodes when receiving inflammatory signals, where they can interact with naïve T cells. The strength and type of immune response that is induced by the DCs is further regulated by the local cytokines present (among others, IFN-γ and IL-12 to induce a Th1 type response; IL-4 to induce a Th2 response, or IL-1β and IL-6 to induce a Th17 type response) and the interaction between co-stimulation receptor interactions between the DC and T cell, such as CD80/86 (present on DCs) interacting with either CD28 (which upregulates further activation) or CTLA-4 (which downregulates further activation) (Sharpe and Freeman 2002). cDCs can be further subdivided into cDC1 and cDC2. cDC1 are more specialized inflammatory stimulators and can interact with $CD4^+$ (and $CD8^+$ T cells through cross presentation), and often leads to a Th17 biased immune reaction. cDC2 are better suited to present endogenous and intracellular material to $CD8^+$ T cells and lead to a more Th1 phenotype. Both pDCs and cDCs originate from bone marrow precursor cells. Particularly cDCs acquire antigen by phagocytosing and degrading pathogens, while pDCs acquire antigen more through (Summerfield and McCullough 2009, Guilliams *et al.* 2014, Summerfield *et al.* 2015, Ginhoux and Guilliams 2016).

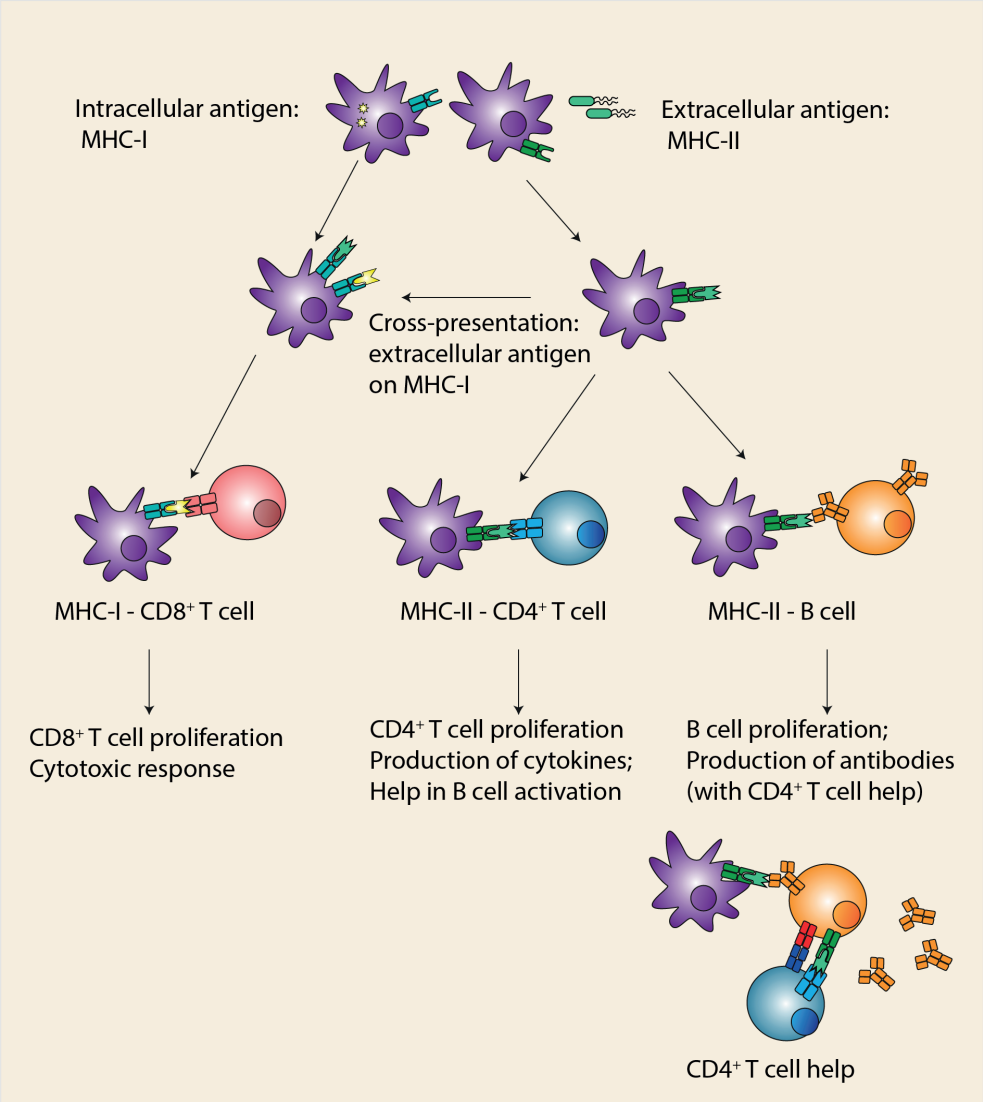

Figure 2: **Adaptive immunity is induced by antigen loaded on MHC receptors of DCs with T cells.** Intracellular antigen is loaded on MHC-I. Extracellular antigen is loaded on MHC-II but can also be presented by MHC-I through cross-presentation. $CD8^+$ T cells interact with MHC-I on DCs and can then proliferate and induce a cytotoxic response. $CD4^+$ T cells and B cells interact with MHC-II-antigen complexes, which leads to proliferation and a cellular or humoral response, respectively.

Besides macrophages and DCs, the innate immune system is comprised of many other haematopoietic cell types (Fig. 1). Natural killer cells are a type of type I innate lymphoid cell (ILC1) with cytotoxic abilities similar to $CD8^+$ T lymphocytes, but lack the T-cell receptor (TCR) and $CD3^+$ (Wu *et al.* 2020). Neutrophils are effector

granulocytes, carrying granules with large quantities of antimicrobial compounds and proteases. They are drawn to sites of infection by chemokines, produced locally in response to cellular damage by epithelial cells. The neutrophils then degranulate at the site of infection to either kill pathogens or trap them in neutrophil extracellular traps (NETs) (Amulic *et al.* 2012). Mast cells are granular myeloid cells activated by TLRs on the mast cells or specific IgE antibodies and release large quantities of cytokines and histamine, a compound that causes vasodilation, facilitating further influx of immune cells from the blood but also the main driver of allergy responses (Marshall 2004, Krystel-Whittemore *et al.* 2016). Eosinophils and basophils are less abundant than neutrophils but are also circulating granulocytes with similar granules full of immune regulating and antipathogenic molecules (Falcone *et al.* 2000, Ravin and Loy 2016). Blood platelets are technically not cells, as they lack a nucleus and are fragments of cytosol, but are filled with coagulation factors, cytokines and HDPs, which they release upon activation by DAMPs from damaged vessel walls (Jurk and Kehrel 2005). Monocytes are circulating phagocytic cells that can migrate into tissues in response to inflammation where they differentiate into dendritic cells or macrophages (Geissmann *et al.* 2010b). The large diversity of cells and functions results in the capacity of the body to deal with a large variety of pathogens, regardless of their molecular structure. Inflammatory dendritic cells and macrophages are essential effector cells of the innate immune system and are central to many chapters of the thesis.

Adaptive immunity

The adaptive immune response is slower than the innate immune response (7-15 days versus a few minutes to an hour)(Pennock *et al.* 2013), but can recognize and act against extremely specific parts of the pathogen, which can then be cleared with high efficiency and specificity. Due to the generation of memory cells, this response can be swiftly recalled in subsequent infections, which leads to a T cell response within a few hours, although proliferation starts a few days later (Whitmire *et al.* 2008). Central to the adaptive response are T and B lymphocytes (Fig. 2). T lymphocytes originate from common lymphoid progenitors (CLPs), which migrate from the bone marrow to the thymus during development. There, rearrangements in the TCR genes, followed by allelic exclusion, lead to an enormous diversity of TCRs

being generated. The T cells that then carry TCRs that are not reactive to MHC are eliminated (positive selection), and T cells reactive to native epitopes are also eliminated (negative selection) to prevent auto-immunity (Robey and Fowlkes 1994). Both these selection processes together with the TCR genetic rearrangements lead to a T cell repertoire that can recognize MHC, is able to distinguish self from non-self, and carries such a large diversity of TCRs that almost any non-self-epitope can be recognized. The adaptive response to a pathogen is triggered by T helper (Th) cells interacting with antigen loaded on MHC-II of antigen presenting cells. After T cell activation, the cell proliferates, and daughter cells differentiate into different subsets. Specific cytotoxic $CD8^+$ T cells will seek out and eliminate cells carrying the antigen on MHC-I, making them good against intracellular pathogens like viruses. Depending on the inflammatory milieu, Th cells can differentiate into different subtypes, which should lead to a more tailored response. For instance, under influence of IFN-γ and IL-12, cells are driven more into Th1 subtype, which is biased towards cell mediated immunity. On the other hand, cytokines such as IL-4, IL-5 and IL-13 polarize towards a Th2 subtype, which is more associated with humoral (e.g., antibody) immunity. Some other subtypes exist, but another major type is the Th17 subtype, induced by IL-1β, IL-6, IL-23 and TGF-β, which produces cytokines and chemokines directed towards eliminating extracellular pathogens, and is therefore critical in immunity against bacteria. Tregs are a special subset induced by IL-2 and TGF-β, which act to suppress immune reaction and instead induce tolerance, which is essential to prevent auto-immune activation (Mills 2004). Many pathogens elicit a balance of different Th responses, and indeed produce compounds aimed to changing this balance or suppress this response entirely. After activation, the T helper cells proliferate and differentiate into different cell types. Besides effector T cells, part of the daughter cells become memory T cells, which are preserved and serve to recall the antigen response in several hours to a few days in subsequent infections. Central memory cells (Tcm) tend to stay in the lymph nodes, while effector memory cells (Tem) are found predominantly in circulation. Tissue resident T memory cells (Trm), which stay in tissues, are increasingly appreciated for their role in local defence.

The other important type of adaptive effector cells is the B cell. B cells carry a B cell receptor (BCR) which, much like the T cell receptor, is specific for an antigen. During

development, B cell precursors undergo recombination of receptor genes, leading to a large variety of B cell receptors. Cells with BCRs that already have been activated by this stage are eliminated, as they are apparently triggered by autoantigens. The cells then migrate from the bone marrow primarily to follicular zones in lymph nodes. During activation, B cells can be activated with the help from T follicular helper cells (Tfh), which are $CD4^+$ T cells that co-stimulate the B cell's interaction with APCs through CD40 and release of cytokines. Then, the B cell proliferates and becomes an effector cell. B cells produce antibodies, which are circulating Y-shaped proteins that bind to the antigen and thereby can neutralize it. Furthermore, the bound antibodies are used to flag the pathogen for destruction by complement or by cellular phagocytosis, as many phagocytes carry receptors like CD16 or CD32 specific for the antibody tail (Hume 2008, Geissmann *et al.* 2010a). B cells can also present antigen by internalizing the MHC-II from APCs, degrading the complex and then processing the antigen on newly synthesized MHC-II (Adler *et al.* 2017).

Other important types of lymphoid cells are innate lymphoid cells (ILCs), which often reside in tissues, roughly mirror the Th subtyping but through innate activation instead of antigenic activation, and are also thought to regulate $CD4^+$ T cell response (Rao *et al.* 2020) and γδ-T cells, which carry specialized γδ-TCRs. The epitope of many γδ-TCRs is often not known. Where characterized, they can recognize a great variety of (often non-proteinaceous) epitopes compared to the conventional αβ-TCR recognizing peptide-MHC-complexes. The best characterized groups of antigens are phosphorylated isoprenoid precursors, and lipid-linked antigens presented by CD1 isoforms CD1a, CD1b, CD1c or CD1d (Ferreira 2013).

Vaccines attempt to simulate infection to generate a memory response to a pathogen without causing the illness. As the memory response is then developed against the antigens specific for this pathogen, the host is protected against future 'real' infections. The word vaccine is derived from the first documented application in 1796, where needles inoculated with cowpox (then called *variolae* v*acciniae*) were successfully used for immunization against human smallpox by the British doctor Edward Jenner (Stewart and Devlin 2006). It should be noted that the Dutch farmer Geert Reinders was successfully immunizing cattle since the end of the 1770's and human immunization appeared to start in the 1790's, although Reinders did

not publish his results until after 1800 (Huygelen 1997, Braggaar 2021). Since then, vaccination has been celebrated as one of the greatest healthcare technologies of the modern era and has led to eradication of smallpox in humans and rinderpest in cattle, and near-eradication of polio in humans. Many other diseases are successfully repressed due to vaccination in large parts of the world, like hepatitis A, hepatitis B, diphtheria, and tetanus (Das *et al.* 2019, Mosser *et al.* 2019, Andani *et al.* 2022). Vaccine antigens can come in different forms; whole pathogens (live but attenuated or killed) contain the whole breadth of antigens and all other innate stimuli needed. Subunit vaccines contain only the proteins that are essential for a successful memory response. However, as the proteins only are often not enough to elicit the chain of cellular activations necessary for immune memory, these subunit vaccines often require additional stimulation of the innate system in the form of adjuvants (Coffman *et al.* 2010). Other vaccination techniques are toxoid vaccines - where only the (inactivated) compounds causing illness are injected instead of the pathogenic organism, viral vectors - where a safe virus is used to shuttle in pathogenic material, leading to an immune reaction against only the target protein(s), and nucleic acid vaccinations - where DNA or RNA is injected, which leads to local production of pathogenic material (Kindrachuk *et al.* 2009, Vemula *et al.* 2013, Corbett *et al.* 2020, Polack *et al.* 2020). Like a conventional infectious immune response, vaccines must also recruit the 'correct' Th response to achieve protective immunity. Especially for non-whole cell vaccines this can be challenging but can sometimes be nudged in the right direction by choice of adjuvant or other immunomodulators (Coffman *et al.* 2010, Nanishi *et al.* 2020).

The porcine immune system

The porcine immune system is characterized by several similarities as well as differences in comparison to the human immune system. Anatomical differences compared to humans are inverted lymph nodes (which results in afferent lymph fluid diffusing from the centre to the periphery in pigs, which is the inverse of humans and mice) and a continuous Peyer's patch in the ileum (Rothkötter 2009). Passive immunity is principally transferred from sow to piglet through colostrum and not through the placenta, as is predominantly the case with human maternal immunity (Salmon *et al.* 2009, Dawson 2011, Meurens *et al.* 2012). Especially when looking at

specific breeds of mini pig, the size of organs is quite like humans, making them an attractive model organism for translational research.

Besides anatomy, pigs have a high degree of similarity with humans on the molecular and cellular level of the immune system. Porcine PRRs have a high degree of similarity with humans on both sequence level and expression patterns, making porcine innate cells good tools for *in vitro* translational research (Mair *et al.* 2014). Like human macrophages, porcine macrophages do not produce iNOS in response to TLR signalling, but instead produce IDO, which leads to a cytokine signalling cascade instead of an antimicrobial effector response in the form of NO radicals (Fairbairn *et al.* 2011, Kapetanovic *et al.* 2012, Mair *et al.* 2014). In swine, macrophages and monocytes express CD172α (in swine also known as SWC3α) and CD163, which is also the target receptor for the widespread porcine respiratory and reproductive virus (PRRSV) (Fairbairn *et al.* 2011, Singleton *et al.* 2016).

There are some clear differences between the porcine and human immune system as well. In contrast to humans, pigs have a relatively high abundance of γδ-T cells in the blood and lymphoid tissues which can reach up to 85% of total peripheral lymphocytes (Takamatsu *et al.* 2006, Sedlak *et al.* 2014). Pigs have an unusual population of circulating $CD4^+$ T cells co-expressing CD8αα homodimers, which are mutually exclusive in any other species. These $CD4^+CD8^+$ double positive cells are considered to correspond to activated and memory $CD4^+$ T cells. CD27 expression can further split these cells into CD27- Tcm and CD27+ Tem (Gerner *et al.* 2015). Not much is known about Trm in pigs. Th1, Th2, Th17 and Tregs have all been identified in pigs, and while they do have some minor differences in marker expression, the functions are conserved (Kiros *et al.* 2011, Käser *et al.* 2012, Stepanova *et al.* 2012, Sedlak *et al.* 2014, Gerner *et al.* 2015).

The downsides of having pigs: zoonotic diseases

Like any other animal, pigs can be infected with a large variety of potential pathogens. Classical swine fever is a viral disease that causes fever and death after 1-2 weeks after the start of acute disease and is strictly monitored by veterinary agencies around the world (Moennig 2000, Madera *et al.* 2016). African swine fever (ASF) has had a devastating epidemic starting in 2018 that infected millions of pigs

in Asia and led to extremely large number of preventive culling (Li and Tian 2018, Mason-D'Croz *et al.* 2020). ASF causes fever and death, and infection mortality can reach up to 100%, without an effective vaccine available. Another virus, PRRSV, is also particularly economically damaging, as infected sows do not reproduce (Ruedas-Torres *et al.* 2021). Bacteria also pose threats to porcine health. Common bacterial diseases threatening swine cause respiratory diseases (like *Mycoplasma hyopneumoniae* and *Actinobacillus pleuropneumoniae*) (Gottschalk and Taylor 2006, Maes *et al.* 2008), intestinal infections (like *Escherichia coli* and *Lawsonia intracellularis*) (Wu *et al.* 2013, Vannucci and Gebhart 2014), and systemic disease (like *Streptococcus suis*) (Staats *et al.* 1997, Gottschalk *et al.* 2010, Dong *et al.* 2021, Maes *et al.* 2021).

Porcine pathogens pose risks that are unfortunately not limited to pigs. In general, pathogens are restricted to one or a few host species. This restriction can be due to specific conditions in the host that are required for infection, such as organ structure, specific animal behaviour or habitat restriction, or expression of a receptor that the pathogen requires. In some cases, these conditions overlap between multiple related species, so the pathogen can then also infect those, although not always equally effectively. This process is facilitated when conditions are similar, such as when a virus uses a receptor that is genetically conserved in two or more animal species. Random mutations can also cause a previously incompatible pathogen to acquire new compatible hosts, and exchange of genetic material can also allow new host specificity.

When an animal-specific pathogen infects a human, this is called zoonosis. In some cases, the pathogen can infect humans, but can not spread or spreads poorly from human to human. For instance, in the case of MERS-CoV spread from dromedary camels to humans, viral outbreaks could be quickly contained with disease control measures (Raj *et al.* 2014). For these diseases, each human infection or cluster of infections requires a new and separate animal-to-human transmission incident. In other cases, the pathogen can be easily transmitted between humans, and new diseases can enter the population. This process of zoonosis is most recently dramatically illustrated by the COVID19 pandemic, which started from a virus circulating in bats (MacLean *et al.* 2021), possibly through an intermediate host in a wet market

in Wuhan, China (Xiao *et al.* 2021), which led to contact with humans and a subsequent global pandemic. Interestingly, the African swine fever epidemic causing enormous deaths among pigs in China might have caused such severe shortages of pork, that demand for other meats increased, leading to an increased risk of disease crossover between different animal species present in these wet markets (Mason-D'Croz *et al.* 2020). Perhaps the most known zoonotic epidemic directly originating from swine was the 2009 swine flu epidemic. The H1N1 influenza virus likely reassorted in central Mexico from multiple influenza A viruses introduced by importing live Eurasian pigs (Mena *et al.* 2016).

S. suis infections in humans and pigs are a risk for antimicrobial resistance development

S. suis is a Gram-positive pathogen and zoonotic bacterium that can infect both humans and pigs (Yongkiettrakul *et al.* 2019, Segura *et al.* 2020, Dong *et al.* 2021, Maes *et al.* 2021). In its natural host, pigs, it colonizes the tonsil and upper respiratory tract, and is transferred to new-borns through vertical transmission (Maes *et al.* 2021). Although many virulence factors have been discovered, it remains unclear under what circumstances an *S. suis* outbreak starts (Segura *et al.* 2017). Acute infections can start when bacteria cross the epithelial barrier in the respiratory tract or gut and enter the bloodstream. There, they can cause sepsis and sudden death syndrome (in other words, the infection is not noticed until it is too late), but also cross into other organs, most notably joints (causing arthritic inflammation) and the cerebrospinal fluid, causing meningitis (Reams *et al.* 1994). In humans, *S. suis* can cross into the bloodstream through cuts in the skin when handling infected meat, or by consumption of poorly cooked pork products (Dutkiewicz *et al.* 2017). There is no evidence of human-to-human transmission(Haas and Grenier 2018). Infections lead most notably to sepsis and meningitis, with a surprisingly high occurrence of co-morbidity of deafness in one or both ears (Yen *et al.* 1994, Tan *et al.* 2010, Rayanakorn *et al.* 2019). Death rate in humans are around 10%, while death rates in pigs are harder to estimate (Gottschalk M. 2010).

Treatment of *S. suis* infections is certainly possible in both humans and pigs by application of beta-lactams and/or fluoroquinolones (Seitz *et al.* 2016). However, there is the problem of antibiotic resistance (Hernandez-Garcia *et al.* 2017, Haas and Gre-

nier 2018, Murray *et al.* 2022). Antimicrobial resistance (AMR) is a global problem, with an estimated 5 million deaths related to AMR in 2019 and more than 1.25 million directly attributable to AMR in the same year (Murray *et al.* 2022). In *S. suis* specifically, resistance has been demonstrated against multiple antibiotics, including tetracycline, doxycycline, fluoroquinolones, linezolid, and vancomycin (Holden *et al.* 2009, Wang *et al.* 2013, Gurung *et al.* 2015, Seitz *et al.* 2016, Hernandez-Garcia *et al.* 2017, Du *et al.* 2019). Treatment of resistant bacteria is obviously more challenging, and results in risks of introducing further resistance genes into the population due to selective pressure. Additionally, S. *suis* could become a reservoir or source of antimicrobial resistance genes that could transfer to more widespread and directly pathogenic bacterial species through mobile genetic elements (Munk *et al.* 2018). Antimicrobial resistance is directly induced by selective pressure exerted by use of antimicrobials. Indeed, the prevalence of resistance in pigs of various European countries is correlated with the use of antimicrobials in those countries (Munk *et al.* 2018). Therefore, antimicrobial use in veterinary care poses a risk for human associated AMR development, and should be restricted where possible.

There are multiple strategies to prevent and combat infection with resistant bacteria. One important method is the reduction of use of antimicrobials in livestock industry, via good stewardship. Reduction of antibiotics use, particularly in veterinary practices, also reduces the risk of resistance development and spread. In many parts of Europe, reduction of antibiotics use has also led to a decrease of the bacterial resistome (Munk *et al.* 2018). Besides stewardship, phage therapy might be a useful technique. Bacteriophages are naturally occurring bacterial viruses that are lytic for bacteria and can be extremely specific in their target. The enormous biodiversity present in phages suggests that targeted therapy is an option given further research (Kortright *et al.* 2019). Phage therapy nowadays remains an experimental thought, with many people still preferring classical antibiotics. Finally, vaccination of pigs would certainly be a great alternative to antibiotics, as it could prevent *S. suis* infections and sudden death. Unfortunately, although autogenous vaccines do sometimes provide protection at a single farm level, vaccination against *S. suis* has thus far not proven broadly successful (Lapointe *et al.* 2002, Baums and Valentin-Weigand 2009, Büttner *et al.* 2012, Haas and Grenier 2018).

One possible factor that could contribute to solving these problems is treatment with host defence peptides (HDPs). HDPs are small peptides from the immune system of vertebrates and are both constitutively expressed at low levels by epithelial cells in the gut, lungs and skin as well as released in large quantities upon activation of innate immune cells, particularly neutrophils and mast cells. The function of HDPs is to kill microbes directly and modulate the subsequent immune reaction. The exact nature and properties of these peptides are described in detail in Chapter 2.

The scope of this thesis is to explore the use of HDPs as immunomodulators in swine, by examining effects of selected HDPs on *in vitro* cultured DCs and macrophages and explore the interaction of those most promising with *S. suis* and in a mouse model of *S. suis* infection. A better understanding the immunomodulatory activity of HDPs can contribute to more effective vaccines. In Chapter 3, the immunomodulatory effects of various HDPs are shown when applied to porcine macrophages, with a strong anti-inflammatory effect of D-CATH-2. In Chapter 4, effects of peptides on porcine dendritic cells are examined, particularly in their interaction with *S. suis,* revealing a strong anti-inflammatory role for D-CATH-2. In Chapter 5, the interaction of *S. suis* with HDPs is shown in the context of antimicrobial killing, showing an *S. suis* related evasion of HDP killing. In Chapter 6, the *in vivo* effects of cathelicidin peptide CATH-2 are shown in a mouse infection model of *S. suis*., which led to a slight decrease of morbidity after prophylactic administration. Finally, in Chapter 7, the results are discussed and put in a general perspective for their application in pigs.

Abbreviations

AMR – antimicrobial resistance
APC – antigen presenting cell
ASF – African swine fever
ATP – adenosine triphosphate
BCR – B cell receptor
CD – cluster of differentiation
CLP – common lymphoid progenitor
DAMP – damage associated molecular pattern
DC – dendritic cell
DNA – desoxyribonucleic acid

HDP – host defence peptide
IFN – interferon
Ig – immunoglobulin
IL – interleukin
ILC – innate lymphoid cell
MBL – mannan binding lectin
MERS-CoV – Middle Eastern respiratory syndrome coronavirus
MHC – major histocompatibility complex
NET – neutrophil extracellular trap
NLR – NOD like receptor
PAMP – pathogen associated molecular pattern
PRR – pattern recognition receptor
PRRSV – porcine reproductive and respiratory syndrome virus
RLR – RIG like receptor
SP – surfactant protein
(ss/ds) RNA – (single/double strand) ribonucleic acid
SWC – swine workshop cluster
Tcm – central memory T cell
TCR – T cell receptor
Tem – effector memory T cell
Th – T helper
TLR – Toll like receptor
Trm – (tissue) resident memory T cell

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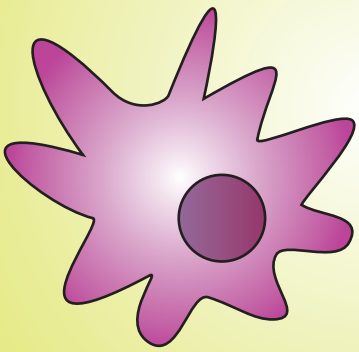

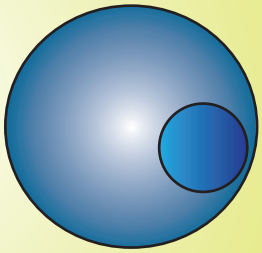

Chapter 2

Cathelicidins: Immunomodulatory Antimicrobials

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Abstract:

Cathelicidins are host defence peptides with antimicrobial and immunomodulatory functions. These effector molecules of the innate immune system of many vertebrates are diverse in their amino acid sequence but share physicochemical characteristics like positive charge and amphipathicity. Besides being antimicrobial, cathelicidins have a wide variety in immunomodulatory functions, both boosting and inhibiting inflammation, directing chemotaxis, and effecting cell differentiation, primarily towards type 1 immune responses. In this review, we will examine the biology and various functions of cathelicidins, focusing on putting in vitro results in the context of in vivo situations. The pro-inflammatory and anti-inflammatory functions are highlighted, as well both direct and indirect effects on chemotaxis and cell differentiation. Additionally, we will discuss the potential and limitations of using cathelicidins as immunomodulatory or antimicrobial drugs.

Introduction

The rise of antimicrobial resistance requires us to think differently about microbial infections. Antibiotic resistance is a growing global problem from both an economical and a societal point of view (Laxminarayan *et al.* 2013). Combating this problem is one of the major challenges for health care in the 21st century. A solution will likely require global approaches the problems surrounding antibiotic resistance, with both scientific and legislative contributions, and include efforts from agricultural and pharmaceutical industries, particularly in developing countries.

Additionally, new drugs are required to maintain a suitably large pool of treatment options against bacteria resistant against conventional drugs. In the drug discovery field, conventional target-based approaches to discover new antibiotics have failed to produce new classes of antibiotics so far (Payne *et al.* 2007). Future medicine will require intensive resistance monitoring (Ventola 2015) and inverse selection against resistance (Baym *et al.* 2016), while drug discovery will have to look for innovative methods to discover new classes of antimicrobials (Brown and Wright 2016).

Furthermore, alternative strategies other than looking for classical antibiotics should be considered. For example, promising results have been obtained by using directly lytic antibodies (LaRocca *et al.* 2009, Storek *et al.* 2018). These antibodies have low immunogenic properties and are extremely target specific (i.e. leaving no impact on the host microbiome), but their development has been challenging (Szijártó *et al.* 2018). Accruing evidence of immunological memory in the innate immune system could also lead to potential new therapies that boost the host's non-specific defence to kill pathogens rather than targeting the pathogen itself (Ifrim *et al.* 2014, Netea *et al.* 2016, Arts *et al.* 2018, Mitroulis *et al.* 2018).

Host defence peptides (HDPs) could be used as potential immunomodulatory molecules, as another way to treat microbial infections. The innate immune system of most animals expresses a wide variety of these peptides, which possess both antimicrobial and immunomodulatory properties (Hilchie *et al.* 2013, Mansour *et al.* 2014, Hancock *et al.* 2016). Their pleiotropic expression and targets, structural diversity, and their reactivity across species makes them interesting candidates for novel therapies or supplementation for existing treatments.

Host defence peptides have various genetic and structural classifications. The main classes are cathelicidins and defensins. Cathelicidins are characterized by a cathelin-like domain and defensins are defined by their intramolecular disulphide bonds (Yang *et al.* 2002, Ganz 2003). In addition to these, other smaller classes of HDPs can be distinguished [16, 17].

In this review, we will focus on cathelicidins, examine their various immunomodulatory functions, and their potential novel use for therapies.

Structural aspects

Cathelicidins have been characterized in many phyla of animals, including mammals, birds, reptiles, amphibians and some fish (Hemshekhar *et al.* 2016). The number of different functional genes encoding for cathelicidins differs greatly between animals, from only one gene in humans, mice, rats, dogs, and guinea pigs to several genes in pigs, cows, chickens, rabbits, horses, goats, and sheep, with pigs having 11 cathelicidin genes (Sang and Blecha 2009, Kościuczuk *et al.* 2012, Ageitos *et al.* 2017). It is not known whether there is a functional implication is for these species-specific cathelicidin repertoires.

Cathelicidins are encoded by genes consisting of 4 exons (Kościuczuk *et al.* 2012). Exon 1 encodes a 29 or 30 amino acid signal (AA) peptide. Exon 2 and 3 encode the conserved cathelin domain of 99 to 114 AA (Zanetti 2005). Exon 4 encodes the mature peptide of 12 to 100 AA, which possesses antimicrobial and immunomodulatory properties (Agier *et al.* 2015). Cathelicidins are produced aspro-peptides and stored inside granules. Upon activation of the cell, they are secreted and the N-terminal pro-domain which includes the cathelin domain is cleaved off to form the mature, biologically active peptide (Kościuczuk *et al.* 2012). Even though the cathelin domain is very conserved between species, the mature peptides are very diverse. Additionally, a single cathelicidin gene can also products of different lengths. The only human cathelicidin gene hCAP-18, for example, has been shown to yield at least 3 different mature peptides, of which LL-37 is the most commonly studied (Sørensen *et al.* 2003, Murakami *et al.* 2004) However, even without much homology at the sequence level, the mature cathelicidin peptides share certain physicochemical properties. Firstly, they are highly cationic with a charge that can vary

between +4 and +13 in physiological conditions (Coorens *et al.* 2017a). Secondly, many cathelicidins have an unstructured conformation in an aqueous environment but can adopt an α-helical structure in the presence of a membrane (Bommineni *et al.* 2007, van Dijk *et al.* 2009, Wieczorek *et al.* 2010). Other mature cathelicidin peptides may adopt β-sheet structures such as the cyclic protegrins with intrachain disulphide bonds. Some linear cathelicidin peptides are enriched in certain amino acids, e.g. tryptophan for bovine indolicidin, or proline and arginine for porcine PR39 (Zanetti 2005).

These commonalities are the main attributes resulting in their antimicrobial activity: the positive charge interacts with negatively charged bacterial membranes, while the hydrophobic residues can perturb the membrane and cause cell death. In contrast, the immunomodulatory nature of cathelicidins is usually more based on stereospecific interactions with a variety of receptors, depending on the cathelicidin (Sang and Blecha 2009), although in some cases the all-D enantiomer can still cause receptor activation, indicating a binding mechanism that is probably not due to interactions with a specific three-dimensional structure, as the naturally occurring peptide would be its mirror image (Tomasinsig *et al.* 2008).

Cathelicidins are constitutively expressed at low levels in epithelial cells, mucosal surfaces, and the skin, and are highly released in response to infections by particularly granulocytes and mononuclear phagocytes. For example, the average serum concentration of the human cathelicidin LL-37 is 1.18 µg/ml in serum of healthy individuals (Sørensen *et al.* 1997), but the local concentration upon neutrophil degranulation can be much larger (Bowdish *et al.* 2005a). For instance, LL-37 is present in broncho-alveolar lavage (BAL) at 5 µg/ml in healthy individuals but can be up-regulated to 30 µg/ml in BAL of cystic fibrosis patients (Sørensen *et al.* 1997, Schaller-Bals *et al.* 2002, Chen *et al.* 2004). The source of LL-37 in BAL is mostly neutrophil degranulation. This means that a gradient of cathelicidins will surround activated leukocytes at a site of infection.

Besides infection, there are other mechanisms regulating cathelicidin levels. For example, in *vitro* stimulation of epithelial cells and monocytes with the active form of vitamin D causes an increase in LL-37 levels (Liu *et al.* 2006, Yim *et al.* 2007), and exogenous vitamin D administration can lead to restoration of microbe-suppressed

levels of endogenous cathelicidins (Rode *et al.* 2017).

Based on their biochemical similarities, *de novo* design or adaptation of natural cathelicidins can be a source of novel drug candidates. Iteratively applied point substitutions, scrambling, and deletions of bovine cathelicidin bactenecin-2 led to an optimized immunomodulatory peptide IDR1018, which showed antimicrobial and anti-biofilm activity (Mansour *et al.* 2015), suppression of lipoteichoic acid (LPS)-induced TNFα production by human peripheral blood mononuclear cells (PBMCs) (Wieczorek *et al.* 2010), differentiation of M1/M2 intermediate macrophages (Pena *et al.* 2013), and protection from malaria in a preclinical mouse model (Achtman *et al.* 2012).

More recently, computational approaches combining characteristic features of length, charge and hydrophobicity has led to discovery of protein subdomains which could have the same functions as natural cationic HDPs. (Zanfardino *et al.* , Bosso *et al.* 2017, Pane *et al.* 2017) For example, a region in the poorly characterized human protein 11-beta-hydroxysteroid dehydrogenase type 3 (HSD 3) showed many HDP-like characteristics, including anti-biofilm and LPS neutralizing effects (Bosso *et al.* 2017).

Although *de novo* design of a new, functional cathelicidin is complicated, these results have shown that new HDP-like drug candidates can be synthesized; and that optimization of existing HDPs for specific purposes is possible.

Biological functions of cathelicidins

Cathelicidins are directly antimicrobial for many pathogens, including both Gram-positive and Gram-negative bacteria, fungi, parasites, and enveloped viruses *in vitro* (Figure 1) (Tossi *et al.* 1995, Xiao *et al.* 2006, Bommineni *et al.* 2007, Goitsuka *et al.* 2007, Wessely-Szponder *et al.* 2010, Veldhuizen *et al.* 2017). Cationic cathelicidins can bind and disrupt negatively charged membranes leading to cell death. These peptides can also cross membranes and target intracellular processes like RNA and DNA synthesis, impair functions of enzymes and chaperones, and can stimulate protein degradation (Kościuczuk *et al.* 2012, Mansour *et al.* 2014).

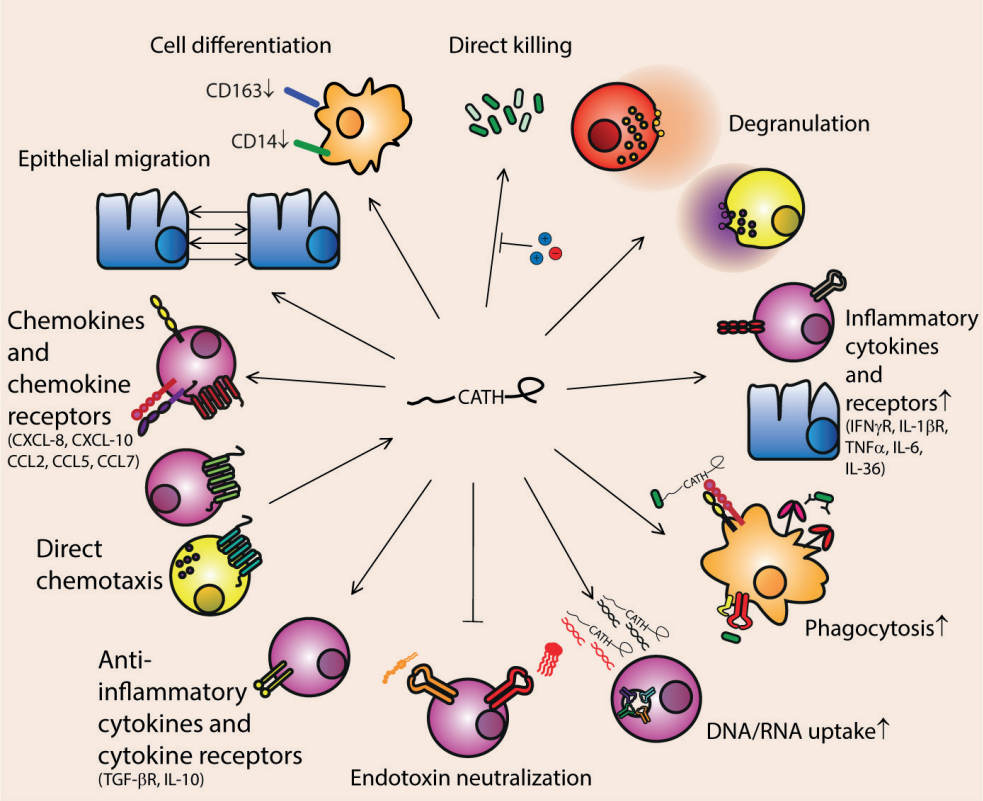

Figure 1: Summary of cathelicidin functions. Cathelicidins have direct killing activity against (among other pathogens) both Gram-positive and Gram-negative bacteria, but this activity is inhibited by the presence of salts. They can induce degranulation of neutrophils and mast cells, induce upregulation of inflammatory cytokines and cytokine receptors, enhance phagocytosis by opsonizing bacteria and upregulating bacterial recognition receptors, and enhance DNA/RNA uptake and thereby boost intracellular TLR signalling. Furthermore, they can inhibit endotoxin mediated activation of TLR 2 and 4 by binding to LTA and LPS, respectively, and directly induce upregulation of anti-inflammatory molecules IL-10 and TGF-βR. They are directly chemotactic for mast cells through the MrgX2 receptor, and for other cells through the FPLR1 receptor; but can also induce production of a variety of chemokines and chemokine receptors, including CCL2, CCL5, CCL7, CXCL8 (IL-8) and CXCL10. They can also induce migration of epithelial cells and thereby influence wound healing. Finally, cathelicidins can influence differentiation of cells, among others by polarizing macrophages to an inflammatory phenotype (M1).

However, the bactericidal effects of most cathelicidins are impaired under physiological circumstances by high salt concentrations, sugars, and other host or microbial factors. Salts, glycosaminoglycans and bacterial DNA present in the mucus of cystic fibrosis patients bind LL-37 and impair its bactericidal abilities, even at

high peptide concentrations (Scott *et al.* 2011). Restoring the abnormally high salt concentration in the BAL of CF patients to normal levels can also restore LL-37's killing capacity (Smith *et al.* 1996). Some cathelicidins, such as chicken chCATH-1, and chCATH-2, porcine PMAP-36, and PR-39, lose efficacy but retain some effectivity *in vitro* under physiological circumstances (Xiao *et al.* 2006, Coorens *et al.* 2017a, Veldhuizen *et al.* 2017).

Nevertheless, the antimicrobial effects of cathelicidins must not completely be disregarded. *In vivo* they are readily incorporated into neutrophil extracellular traps (NETs), where they stabilize the NET and perhaps contribute to the antimicrobial function (de Buhr *et al.* 2017, Hosoda *et al.* 2017). LL-37, along with other cationic peptides, has been shown to protect the NET from degradation by binding DNA in the NET and shielding it from bacterial nucleases (Neumann *et al.* 2014b). Additionally, coating indwelling medical devices such as catheters with immobilized or gel-trapped cathelicidins can prevent attachment of bacteria and formation of biofilms, which reduces the risk nosocomial of infections) (Onaizi and Leong 2011, Riool *et al.* 2017).

Considering the inhibitory effect of physiological conditions, most cathelicidins probably do not have direct bactericidal activities as their primary function *in vivo*. However, they still are important for prevention of microbial infections. Chicken chCATH-1, when administered intraperitoneally at concentrations of 10 mg/kg, protects mice from lethal methicillin resistant *Staphylococcus aureus* (MRSA) infection(Bommineni *et al.* 2014). mCRAMP deficient mice suffer from increased severity of streptococcal skin infections, although only to cathelicidin-susceptible streptococci. Virulent, resistant strains show no change in severity, indicating that direct killing could possibly be important here(Nizet *et al.* 2001, LaRock *et al.* 2015). Additive transgenic expression of porcine cathelicidin PR39 in normal mice ameliorates the infection's necrotic phenotype (Lee *et al.* 2005). *In ovo* treatment of 18 day old fertilized chicken eggs with D-chCATH-2 protects chicks from avian *E. coli* infections when they are challenged 7 days after hatching (Cuperus *et al.* 2016). The amount of peptide still present in the organs at that time is sufficiently low to conclude that the anti-infective properties of chCATH-2 are likely derived from immunomodulation.

These data together with the impaired bactericidal functions of cathelicidins under physiological circumstances suggest that cathelicidins rather act as immunomodulatory factors rather than as direct bactericidal agents.

Degranulation

Cathelicidins can stimulate degranulation of immune cells, which then release a myriad of pro-inflammatory and antimicrobial substances, including more cathelicidins (Figure 2). LL-37 stimulates mast cell degranulation via the MrgX2 receptor, which induces calcium mobilization and PI3K, AKT, ERK, and JNK activation (Gupta *et al.* 2016, Yu *et al.* 2017). This results in release of histamine and prostaglandin D2, which stimulate chemotaxis, diapedesis, and inflammation (Subramanian *et al.* 2011). Mast cell degranulation can be induced by 10µg/ml LL-37, which means that mast cell degranulation could be stimulated by LL-37 levels that can be present during infections (Schiemann *et al.* 2009, Yu *et al.* 2017). However, LL-37-induced degranulation of a human mast cell line can be inhibited by co-administration of Toll-like receptor (TLR-2) ligands (Zhang *et al.* 2016b). This raises the question of whether LL-37 also induces degranulation in the context of an infection.

LL-37, porcine PR-39 and PMAP-23, mouse mCRAMP, and chicken chCATH-1, chCATH-2, and chCATH-3 stimulate NO production in macrophages, but only in combination with unmethylated CpG-DNA (Baumann *et al.* 2014, Coorens *et al.* 2017a). LL-37 stimulates the production of reactive oxygen species (ROS) in neutrophils, most likely in a NADPH-dependent manner (Zheng *et al.* 2007). On the other hand, PR-39 inhibits NADPH oxidase activity, which impairs the oxidative bacterial killing of neutrophils (Shi *et al.* 1996). The PR-39 mediated NADPH inhibition could be a negative feedback loop to inhibit superoxide formation and prevent tissue damage.

Besides degranulation, 25µg/ml LL-37 induces neutrophil extracellular trap (NET) formation via the FPRL1 receptor(Neumann *et al.* 2014a). LL-37 and porcine PR39 can be interwoven in the NETs (Papayannopoulos and Zychlinsky 2009, de Buhr *et al.* 2017). Besides being possibly antimicrobial, these immobilized cathelicidins can prevent biofilm formation (Overhage *et al.* 2008).

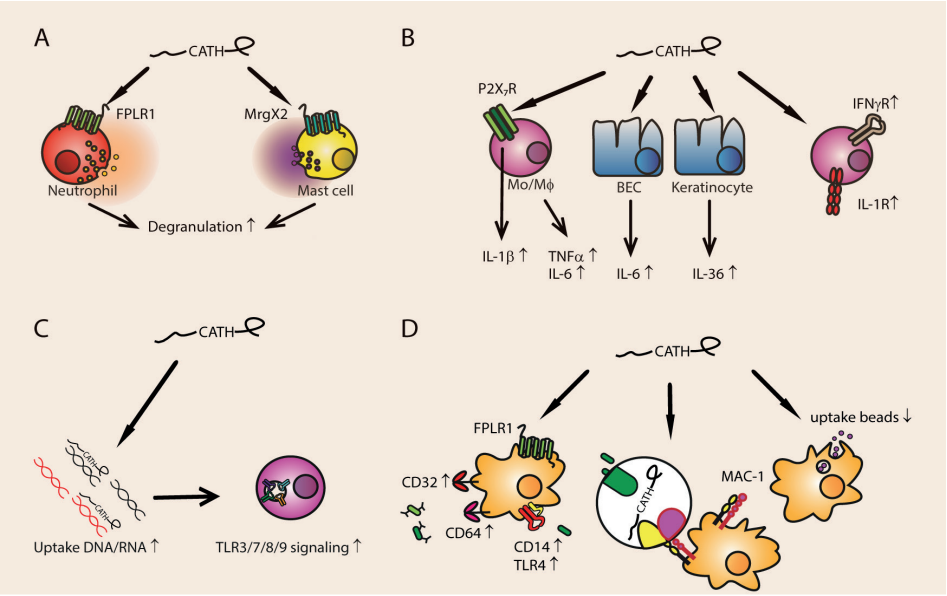

Figure 2: Pro-inflammatory functions of cathelicidins. **(A):** Neutrophil degranulation is stimulated through FPLR1, while mast cell degranulation is induced through MrgX2. **(B)** Cathelicidins induce production of IL-1β, TNFα and IL-6 by monocytes/macrophages through the $P2X_7$ receptor. Broncho-epithelial cells are induced to produce IL-6, and keratinocytes produce IL-36. Cathelicidins induce upregulation of IL-1R and IFN-γR. **(C)** Cathelicidins bind to extracellular DNA and RNA and increase extracellular nucleotide uptake. After the cathelicidins are degraded in the endosomal compartment, TLR3, 7, 8, and 9 signalling is increased. **(D)** Through FPLR1 signalling, FcγRs CD32 and CD64 on macrophages are increased, which enhances phagocytosis of opsonized bacteria. Additionally, TLR4 and CD14 are upregulated, which increases phagocytosis of Gram-negatives. Cathelicidins can also bind to pathogens and thereby opsonize directly through increased uptake by MAC-1 (CR3). In contrast, the uptake of latex beads is decreased by many cathelicidins.

Pro-inflammatory immune modulation

Cytokine and cytokine receptor expression

Cathelicidins regulate expression of pro-inflammatory cytokines and cytokine receptors (Figure 2). Human LL-37 (Coorens *et al.* 2015) and bovine bMAP28 (D'Este *et al.* 2012) upregulate TNFα production in murine macrophage cell line RAW264.7, and porcine PR-39 in porcine macrophage cell line 3D4/31 (Veldhuizen *et al.* 2014b). Chicken chCATH-2 induce IL-1β release from murine macrophages (Coorens *et al.* 2017b), and BMAP-28 stimulation results in activation of ERK1/2, P38 MAPK, and NF-κB, and subsequent IL-1β release, although the receptor is not known (D'Este

et al. 2012). IL-1β expression is increased by LL-37 through the $P2X_7$ receptor on monocytes (Elssner *et al.* 2004).

$P2X_7$ is a receptor for extracellular ATP which is released by damaged cells, and activation leads to inflammasome formation, processing of IL-1β and IL-18, and other downstream inflammatory processes (Tomasinsig *et al.* 2008, Wiley *et al.* 2011). LL-37 also induces the production of the inflammatory cytokine IL-36 (also of the IL-1-family) by human keratinocytes (Li *et al.* 2014). BMAP-28 and chCATH-2 increase IL-6 expression in RAW264.7 cells (Scott *et al.* 2002, D'Este *et al.* 2012, Coorens *et al.* 2017a) and bronchial epithelial cells (Pistolic *et al.* 2009). LL-37 upregulates the expression of cytokine receptors in murine macrophages, such as IL-1R and IFNγ-R (Scott *et al.* 2002).

It remains an open question whether induction of cytokine production happens *in vivo,* and if the increased levels are biologically relevant. For instance, LL-37 can increase TNFα expression in macrophages, but this effect is rather small and was only detectable at concentrations of 25 μg/ml LL-37 (Coorens *et al.* 2017a). Fifty μg/ml LL-37 was needed to induce upregulation of cytokine receptors on macrophages, which are concentrations that exceed the general LL-37 concentrations present in body fluids during inflammation, although these concentrations could possibly be reached locally (Scott *et al.* 2002). On the other hand, 10 to 20 μg/ml LL-37 stimulates the expression of IL-1β in monocytes, which lies within the LL-37 concentration range that could be established during infections (Elssner *et al.* 2004). Under non-inflamed physiological concentrations of 5 μg/ml, LL-37 is not able to increase expression of TNFα or IL-6 in human PBMCs (Medina Santos *et al.* 2016). Interestingly, LL-37 has different effects on various cell types, such as monocytes, dendritic cells (DCs), T cells, and B cells. Each of these cell types shows a different cytokine secretion pattern upon exposure to LL-37 at concentrations above 20μg/ml (Mookherjee *et al.* 2009).

So, the pro-inflammatory effects of cathelicidins *in vitro* depend on the cathelicidin used, the concentration of the peptide and the cell type studied. Furthermore, *in vivo* stimulation of inflammatory cytokine production by cathelicidins may not be significant, as normal concentrations of cathelicidins are low compared to *in vitro* studies. Although in inflammatory conditions the local concentration could

possibly be as high as *in vitro studies*, this would sooner be a result of inflammation rather than a driving factor. Therefore, the pro-inflammatory effects of cathelicidins should be seen as amplifying or modulating but not causing inflammation. Additionally, many cathelicidins are cytotoxic to mammalian cells at high concentrations (Johansson *et al.* 1998, Oren *et al.* 1999). Thus, any effects (particularly inflammatory) seen at very high concentrations could also be due to cathelicidin-related cellular damage.

Phagocytosis

Cathelicidins stimulate the uptake of pathogens via phagocytosis (Figure 2). LL-37 binds to bacteria and can simultaneously bind to complement receptor MAC-1 on monocytes and macrophages, thereby opsonizing the bacteria (Lishko *et al.* 2016, Zhang *et al.* 2016a). Furthermore, LL-37 and mCRAMP can indirectly enhance phagocytosis by human monocyte-derived macrophages through activating the FPRL1 receptor, which results in up-regulation of the Fcγ receptors CD32 and CD64, TLR4 and the TLR4 co-receptor CD14 (Wan *et al.* 2014). This way, LL-37 and mCRAMP stimulate phagocytosis of IgG-opsonized bacteria, and non-opsonized Gram-negative bacteria. Both blood monocyte-derived macrophages and cultured microglia of mCRAMP deficient mice indeed show reduced phagocytosis, and their bone-marrow derived macrophages have lower levels of CD14 and Fcγ-receptor expression (Wan *et al.* 2014, Kress *et al.* 2017).

On the other hand, a comparison of cathelicidins of various species showed that mCRAMP, K9CATH, chCATH-1, chCATH-2, and porcine PMAP-23 and PMAP-36 reduced uptake of latex beads by murine macrophages at concentrations ranging from 1.5 to 25 µg/ml, while equine eCATH-2 stimulated the uptake of latex beads (Coorens *et al.* 2017a). LL-37, eCATH-1, eCATH-3, chCATH-3, and PR-39 did not influence phagocytosis (Coorens *et al.* 2017a). This discrepancy between effects on phagocytosis by mCRAMP (among others) might be due to the difference between uptake of latex beads or phagocytosis of live bacteria. In contrast to beads, both the uptake and processing of live bacteria will induce immune signalling in the cell, which will cause activation of innate receptors and autocrine signalling through inflammatory cytokines (Underhill and Goodridge 2012). Additionally, the contrast between cathelicidin effects on phagocytosis shows that the unique features of one

cathelicidin cannot be generalized to others.

DNA- and RNA- mediated TLR activation

Cathelicidins can stimulate uptake of extracellular bacterial or self DNA and RNA (Figure 2). LL-37 and chCATH-2 bind to DNA released by lysed bacteria or damaged cells and increase its endosomal uptake by macrophages (Coorens *et al.* 2015). In the endosomal compartment the DNA-bound cathelicidin prevents TLR9 activation until it is degraded. The interaction between the DNA and the cathelicidins is probably electrostatic. However, mCRAMP, which has an identical charge as LL-37 (+6), does not induce enhanced DNA uptake and TLR9 activation in macrophages (Coorens *et al.* 2015). So, while binding can be electrostatic, other mechanisms are also responsible for the increased uptake of DNA.

LL-37 also stimulates CpG-DNA uptake and TLR9 activation in B cells and plasmacytoid DCs (pDCs), but not in T cells (Hurtado and Peh 2010). This effect was visible within 30 minutes using 6 µg/ml LL-37 in the presence of serum. This indicates that LL-37 could stimulate DNA uptake under physiological circumstances. This might actually be a driving factor in autoimmune disease like psoriasis, where LL-37 forms a complex e with self-DNA and activates pDCs (Lande *et al.* 2007).

DNA- and RNA-mediated TLR activation in pDCs and macrophages is a common feature of cathelicidins and has been demonstrated for a.o. eCATH-2, BMAP-27, BMAP-28, BMAP-34, Bac-1, PMAP-23, PMAP-36 and PR-39 (Baumann *et al.* 2014, Baumann *et al.* 2017, Coorens *et al.* 2017a). Porcine cathelicidins PMAP-23, PMAP-36 and protegrin 1 can potentiate the uptake of nucleotides in various forms, such as plasmid DNA, CpG DNA, genomic DNA, or RNA (Baumann *et al.* 2014). Additionally, both LL-37 and mCRAMP can enhance TLR3 signalling in a human lung cell line; but only LL-37 mediates this effect through FPLR1 (Singh *et al.* 2013).

The capacity of cathelicidins to stimulate TLR3 via dsRNA, TLR7 and TLR8 via ssRNA and TLR9 via dsDNA might be used in vaccine development against bacterial and viral diseases (Ganguly *et al.* 2009, Coorens *et al.* 2015). LL-37 does not stimulate TLR9 activation in B cells and pDCs when incubated with human DNA, which makes it suitable as a vaccine adjuvant (Hurtado and Peh 2010). However, it is important to investigate whether administration of exogenous cathelicidins does not elicit au-

to-immune reactions.

Anti-inflammatory immune modulation

Inhibition of endotoxin mediated TLR activation

Bacterial endotoxins, such as LPS, lipoteichoic acid (LTA), and flagellin are activators of TLR4, TLR2, and TLR5, respectively (Uematsu and Akira 2008). Systemic immune activation by endotoxins can be fatal, so inhibition of inflammatory reactions during sepsis is essential to protect the host from immune overactivation.

Cathelicidins can dampen endotoxin-mediated immune reactions by binding the endotoxin and preventing TLR signalling (Figure 3). Human LL-37, rabbit Cap-18, bovine Bac7, BMAP-28, ovine SMAP-29, and chicken chCATH-2 are able to bind directly to negatively charged LPS from Gram-negative bacteria (Hirata *et al.* 1994, Larrick *et al.* 1995, Ghiselli *et al.* 2003, Giacometti *et al.* 2004a, van Dijk *et al.* 2009, D'Este *et al.* 2012, Veldhuizen *et al.* 2014b, Veldhuizen *et al.* 2017). Binding to TLR4 ligands means that many cathelicidins, such as LL-37, mCRAMP, dog K9, PMAP-36, BMAP-27, BMAP-28, indolicidin, SMAP-29, and chCATH-1, chCATH-2, and chCATH-3 can inhibit LPS induced TNFα production in different leukocytes, such as macrophages, monocytes, and DCs (Giacometti *et al.* 2004a, Bowdish *et al.* 2005b, Mookherjee *et al.* 2006a, Nijnik *et al.* 2009, van Dijk *et al.* 2009, Coorens *et al.* 2017a).

Furthermore, LTA-induced TNFα production is inhibited by eCATH-2 and PMAP-23, but not K9CATH (Figure 3) (Scott *et al.* 2002, Coorens *et al.* 2017a, Veldhuizen *et al.* 2017). Interestingly, TLR2 activation by LTA induces LL-37 expression in human macrophages, which can in turn reduce the other LTA-induced inflammatory effects like TNFα and IL-6 production (Ruan *et al.* 2013).

Indolicidin and LL-37 inhibit LPS induced TNFα production at physiological concentrations of 1 to 5μg/ml (Bowdish *et al.* 2005b, Mookherjee *et al.* 2006a). Surprisingly, indolicidin, bMAP-27 and LL-37 inhibit LPS-induced TNFα production even when administered 1 hour after LPS exposure, but to a lower extent than co-administration (Scott *et al.* 2002, Bowdish *et al.* 2005b, Mookherjee *et al.* 2006b). Thus, inhibition of LPS-mediated TLR4 activation is not solely due to the binding of cathelicidins to LPS, but possibly also due to modification of cellular processes and/or epigenetics.

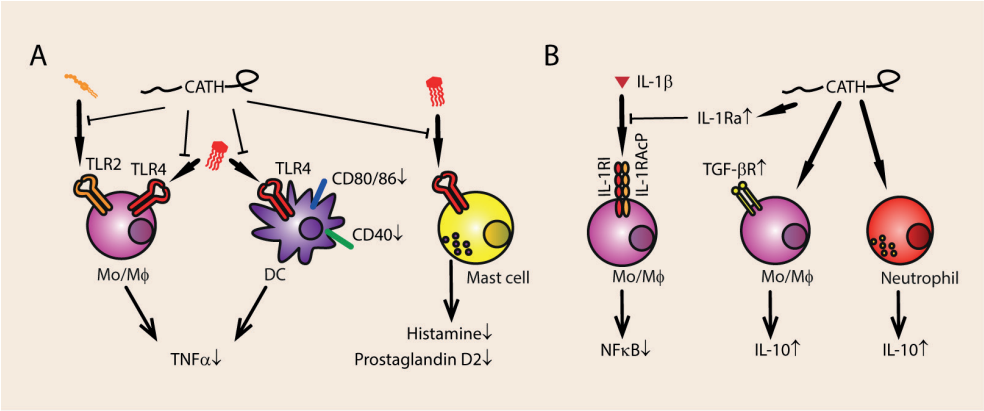

Figure 3: **Inhibition of endotoxin mediated TLR activation by cathelicidins**. **(A)** Cathelicidins can bind to LPS, preventing TLR4 activation, and thereby reducing TNFα production in monocytes, macrophages and DCs, and also reducing upregulation of CD80/86 and CD40 in DCs. This TLR4 blocking activity can also result in decreased release of histamine and prostaglandin D2 by mast cells. Additionally, cathelicidins can bind the TLR2 ligand LTA, and thereby preventing TNFα secretion by monocytes. **(B)** Cathelicidins can directly upregulate IL-10 production in monocytes and neutrophils, and induce upregulation of TGF-βR. Additionally, the upregulation of the IL1β antagonist IL-1Ra means that IL-1β induced NFκB activation can be reduced.

LL-37 and BMAP-27 can inhibit endotoxin-induced NFκB translocation to the nucleus in monocytes by increasing the amount of IκBα and by stimulating TNFα-induced protein 3 (TNFAIP-3), which has anti-inflammatory capacities (Mookherjee *et al.* 2006a, Mookherjee *et al.* 2006b). BMAP-28 inhibits not only TLR4 activation but also its internalization, which blocks its ability to stimulate TRAF, TRIF and IRF3 activation, resulting in less IFN-β expression (D'Este *et al.* 2012).

Furthermore, cathelicidins inhibit endotoxin mediated up-regulation of other pro-inflammatory cytokines, such as IL-12, IL-8, IL-6, IL-1β, and IFN-γ (Scott *et al.* 2002, Bommineni *et al.* 2007, Di Nardo *et al.* 2007, Nijnik *et al.* 2009, D'Este *et al.* 2012, van Dijk *et al.* 2016). mCRAMP inhibits LPS-induced up-regulation of co-stimulatory molecules, such as CD40, CD80, and CD86 on DCs (Di Nardo *et al.* 2007). LPS-induced macrophage activation and mast cell degranulation are also inhibited by LL-37 and rabbit CAP18 (Hirata *et al.* 1994, Ramos *et al.* 2011, Gupta *et al.* 2016). The presence of LPS inhibits the ability of LL-37 to induce Th1 responses in DCs (Kandler *et al.* 2006). Thus, LL-37 does not only block LPS-mediated pro-inflamma-

tory reactions, but LPS also blocks LL-37-mediated immune modulations. LL-37 and LPS can bind through electrostatic interactions and therefore possibly block each other's capabilities to stimulate cellular processes (Rosenfeld *et al.* 2006). The negatively charged, hydrophobic LPS molecules are a suitable binding partner for the cationic, amphipathic cathelicidins, although binding affinity does not necessarily indicate receptor inhibition (van Dijk *et al.* 2016).

In vivo, the biological function of cathelicidins released by neutrophils could be that a local high concentration of peptides directly kills bacteria, while simultaneously neutralizing endotoxins released from killed bacteria and thereby preventing excessive immune activation. Indeed, chCATH-2 and PMAP-36 can both kill *E. coli* and prevent immune activation under physiological conditions. LL-37, mCRAMP, chCATH1- and 3, K9CATH, eCATH-2 can only inhibit immune activation when the bacteria are non-viable (Coorens *et al.* 2017b). This points to a function for cathelicidins as immunomodulators rather than bactericidal effector molecules.

Secretion of anti-inflammatory cytokines

Besides inhibiting endotoxin-mediated pro-inflammatory cytokine production, cathelicidins stimulate the production of anti-inflammatory cytokines (Figure 3). chCATH-2 upregulates IL-10 in chicken PBMCs (Kraaij *et al.* 2017). LL-37 upregulates anti-inflammatory receptors such as TGF-βR, and stimulates the secretion of the anti-inflammatory cytokine IL-10 (Scott *et al.* 2002). LL-37 also simulates the release of IL-1Ra in neutrophils, an IL-1β antagonist (Zhang *et al.* 2008), and inhibits IL-1β and TNFα production by IL-32 induced inflammatory monocytes (Choi *et al.* 2014). At 5μg/ml, no IL-10 induction was mediated by LL-37 in isolated neutrophils or total PBMCs (Medina Santos *et al.* 2016). Therefore, it is unclear whether cathelicidins can increase expression of anti-inflammatory molecules sufficiently under physiological circumstances to elicit an effect *in vivo*. Rather, the neutralization of endotoxin seems to be the most relevant mode of anti-inflammatory action.

The systemic anti-inflammatory effects of cathelicidins are clearly demonstrated in *in vivo* infection models. Bovine BMAP-28 (2mg/kg) reduced sepsis mortality in mice when injected intravenously with *S. aureus*, to the same levels as the antibiotic imipenem (7mg/kg)(Giacometti *et al.* 2004a). Lethality was reduced from 100%

(control) to 30%. TNFα and IL-6 levels were also significantly reduced in the mice. Intraperitoneal administration of bovine Bac7 (1mg/kg), and ovine sMAP-29 also reduced septic mortality in rats, induced by intraperitoneal administration of live *E. coli*, to the same levels as the antibiotic polymyxin B (1mg/kg), reducing mortality from 100% to 20% for sMAP-29 and 27% for Bac7 (Ghiselli *et al.* 2003, Giacometti *et al.* 2004b). However, i.p. injection of both bacteria and peptide means the bacterial load could be lower due to direct bactericidal action of a locally high concentration of peptide. Endogenous mCRAMP production is induced by *Clostridium difficile*, but fails to protect the host from inflammatory damage, whereas adding exogenous LL-37 or mCRAMP reduces TNFα production induced by toxins (Hing *et al.* 2013). However, in a polymicrobial sepsis model, mCRAMP knockout mice had increased survival compared to the wild-type (Severino *et al.* 2017).

The anti-inflammatory effects of cathelicidins can also be seen in some immune diseases. Mice that lack mCRAMP showed more severe contact dermatitis responses than control mice (Di Nardo *et al.* 2007). Administration of intravenous mCRAMP (4 mg/kg) reduced the severity of the inflammation in the mCRAMP negative mice. mCRAMP negative mice also show more severe immune reactions during acute pancreatitis, having more TNFα production and tissue damage than wild type littermates (Deng *et al.* 2016).

These findings suggest that cathelicidin-derived compounds could provide a promising new therapy against severe infections, such as sepsis and immune diseases.

Chemotaxis

Cathelicidins can bind directly to chemoattractant receptors on immune cells and induce migration (Figure 4). Neutrophils are one of the cells responding to infection, and degranulation leads to a release of large amounts of cathelicidins. Therefore, a high local concentration of cathelicidins is found in inflammatory sites.

Human LL-37 has been shown to attract a variety of leukocytes, such as neutrophils, eosinophils (Tjabringa *et al.* 2006), monocytes and $CD4^+$ T cells via the FPRL1 receptor (Yang *et al.* 2000), and attracts mast cells via their MrgX2 receptors, resulting in ERK phosphorylation and Ca2+ mobilization (Niyonsaba *et al.* 2002, Subramanian *et al.* 2011). LL-37 does not attract monocyte-derived immature DCs, because they

downregulate FPRL1 during differentiation (Yang *et al.* 2001). The chemotactic ability of LL-37 is not hampered by serum, and LL-37 is able to attract monocytes (Yang *et al.* 2000), mast cells (Niyonsaba *et al.* 2002), and induces migration of epithelial cells (Shaykhiev 2005) at physiological concentrations of 5 µg/ml.

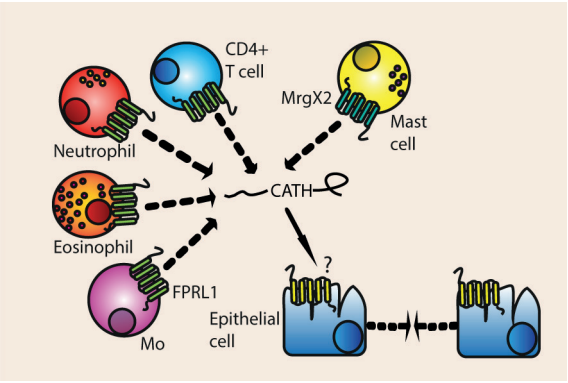

Figure 4: Direct induction of chemotaxis and cellular migration. Cathelicidins induce migration of T cells, neutrophils, eosinophils, and monocytes through the FPLR1 receptor. Mast cell chemotaxis is induced through the MrgX2 receptor. Additionally, epithelial cells are induced to migrate through unknown receptors.

It is remarkable that LL-37 can induce chemotaxis of different cell types via two distinct non-homologous receptors, MrgX2 and FPRL1. Therefore, one might wonder whether LL-37 or other cathelicidins are able to induce migration via other receptors that have not been investigated yet. Besides that, it is possible that some chemotactic effects of LL-37 have not been noticed yet due to use of cell lines, instead of blood isolated leukocytes. For instance, Bowdish et al. were not able to detect monocyte migration with the THP-1 cell line, whilst Yang et al. could observe LL-37 induced migration in blood derived monocytes (Yang *et al.* 2000, Bowdish *et al.* 2004).

Cathelicidins from other species can also attract different leukocytes even from different species, although their properties are often not as extensively studied as human LL-37. Mouse mCRAMP has been shown to induce chemotaxis of monocytes, macrophages, and neutrophils via the human FPRL1 and mouse FPR2 receptor. FPRL1 and FPR2 activation results in Ca2+ mobilisation and ERK phosphorylation in monocytes (Kurosaka *et al.* 2005). mCRAMP was also able to attract neutrophils

and monocytes in vivo at concentrations of 10µg/ml. Just like LL-37, mCRAMP is not able to attract monocyte-derived DCs.

Other cathelicidins that can mediate chemotaxis are bovine bMAP-28 (Kindrachuk *et al.* 2011) and chicken chCATH-1 (Bommineni *et al.* 2014) which attract neutrophils, bovine Bac2a that attracts monocytes and macrophages (Bowdish *et al.* 2005b), Bac7 in its pro-form can attract neutrophils (Verbanac *et al.* 1993) and rat rCRAMP which stimulates mast cells migration (Babolewska *et al.* 2014). Porcine PR-39 has been shown to stimulate neutrophil migration even at concentrations of 0.5 µg/ml (Huang *et al.* 1997). PR-39 and chCATH-1 were not able to induce migration of mononuclear leukocytes, such as monocytes, and alveolar macrophages (Huang *et al.* 1997, Bommineni *et al.* 2014).

The cathelicidins that are released at a site of infection (mainly by neutrophils) will attract other immune cells, which may then also secrete cathelicidins, resulting in a local high concentration of cathelicidins. This cumulative local concentration might even be high enough for the cathelicidins to be bactericidal, even in the presence of salt. Furthermore, the secreted cathelicidins will attract innate, but also adaptive immune cells and therefore help to induce proper immune responses against pathogens.

Indirect chemotaxis

Besides their direct chemotactic functions, cathelicidins can indirectly stimulate chemotaxis by stimulating expression of chemokines and chemokine receptors (Figure 5). CCL2 is up-regulated in various leukocytes by human LL-37 (Scott *et al.* 2002, Bowdish *et al.* 2004), pMAP-36 (Coorens *et al.* 2017a), and chCATH-1, chCATH-2, and chCATH-3 (Kraaij *et al.* 2017). CCL2 especially attracts monocytes, T cells, and DCs towards the site of infection. CCL5, which stimulates chemotaxis of eosinophils, basophils, and T cells, is up-regulated by LL-37, canine K9CATH, and chCATH-2 (Coorens *et al.* 2017a). CXCL10 expression, which attracts macrophages, T cells, NK cells, and DCs, is induced by LL-37, mouse mCRAMP, canine K9CATH, and equine eCATH-3, (Coorens *et al.* 2017a) IL-8 production is increased by LL-37 (Scott *et al.* 2002, Bowdish *et al.* 2004, Tjabringa *et al.* 2006), porcine PMAP-23 (Veldhuizen *et al.* 2017) and PR-39 (Veldhuizen *et al.* 2014a), indolicidin (Bowdish *et al.* 2005b),

and chicken chCATH-2 (van Dijk *et al.* 2016). IL-8 stimulates neutrophil migration. CCL7 expression, also known as MCP-3, is upregulated by LL-37 and chCATH-2 (Scott *et al.* 2002, Bowdish *et al.* 2004, van Dijk *et al.* 2016).

Furthermore, 50 µg/mL LL-37 up-regulates the expression of chemokine receptors in mouse macrophages, such as IL-8 receptor, CXCR4, CCR2 and LFA-1 (Scott *et al.* 2002). LL-37 and chCATH-2 also upregulates mannose receptor MRC1 in chicken PBMCs, which is also important for chemotaxis (Kraaij *et al.* 2017).

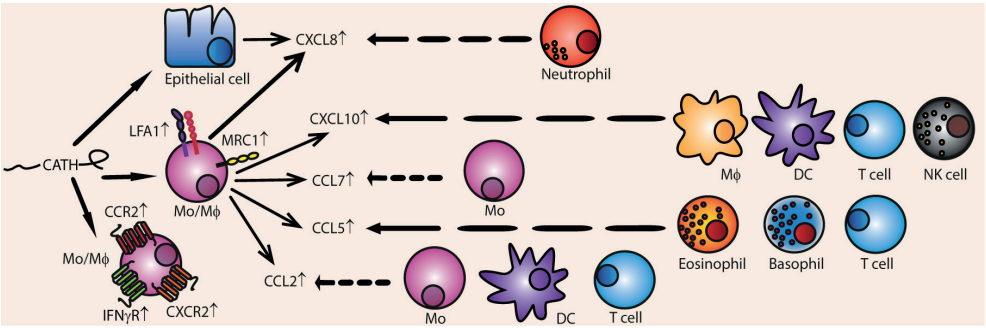

Figure 5: Cathelicidins indirectly induce chemotaxis by inducing the production of various chemokines and chemokines receptors. Cathelicidins can induce chemokine receptor production in monocytes of CCR2, CXCR2, IFNγ-R, MRC1, LFA1. Additionally, stimulated monocytes produce CCL2, CCL5, CCL7, CXCL10, and CXCL8 (IL-8). Epithelial cells can also be induced to produce CXCL8. The main cell types responding to the chemokines are depicted.

All in all, cathelicidins can indirectly induce influx of a great variety of innate and adaptive immune cells towards inflammatory sites by modulating chemokine and chemokine receptor expression. Up-regulation of the chemokine genes is induced via activation of EGFR, ERK and p38 MAPK pathways in *in vitro* models (Tjabringa *et al.* 2003, Bowdish *et al.* 2004). However, it is not clear whether cathelicidins greatly induce up-regulation of chemokines and chemokine receptors *in vivo*, since the concentrations cathelicidins that are needed to modify chemokine expression exceed the cathelicidin concentrations that are present in body fluids during infections. For instance, 50 to 100 µg/ml LL-37 was needed to up-regulate expression in IL-8, CCL2 (Scott *et al.* 2002) and CCL7 in monocytes (Bowdish *et al.* 2004) and IL-8 in epithelial cells (Tjabringa *et al.* 2003). On the other hand, 10µg/ml indolicidin or LL-37 can stimulate IL-8 expression in airway epithelial cells and mast cells, but the increase on a protein level is minimal (Bowdish *et al.* 2005b, Yu *et al.* 2017). Thus, it is controversial whether IL-8 is up-regulated by cathelicidins during infection, but

it is possible that local concentrations of cathelicidins during infections could reach high levels and could induce chemokine expression.

Thus, cathelicidins can act as direct chemoattractant for a variety of immune cells, and this effect is possibly enhanced by their indirect effect to induce up-regulation of chemokines and chemokine receptors on leukocytes, mediating an influx of immune cells to the site of infection.

Cell differentiation and proliferation

Cathelicidins can also modify immune responses on a broader level by influencing differentiation of immune cells and by enhancing expression of receptors in antigen presenting cells (APCs). 5 µg/mL of LL-37 is able to increase blood monocyte polarization of macrophages towards the pro-inflammatory M1 type (Figure 6) (van der Does *et al.* 2010). Administration of 10µg/ml LL-37 for 6 days to fully matured anti-inflammatory M2 macrophages even resulted in enhanced M1 cytokine secretion, such as IL-12p40, and diminishes secretion of M2 cytokines, such as IL-10 in the M2 macrophages (van der Does *et al.* 2010). LL-37 needs to be endocytosed before it can alter differentiation, but it is not known via which receptors it mediates its influence.

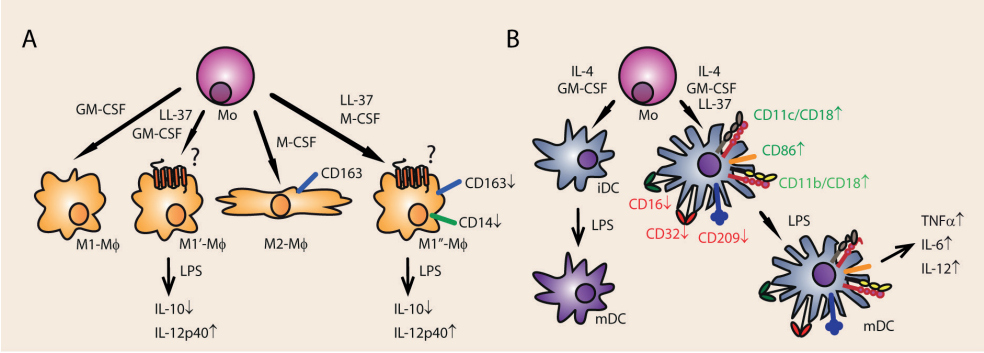

Figure 6: LL-37 effects on cell differentiation. (A) Exposure of M2 macrophages to LL-37 polarizes them into a more inflammatory (M1) phenotype, leading to a downregulation of CD163, CD14 and reduced secretion of IL-10. Additionally, already polarized M1 macrophages produce even more IL-12p40 when co-incubated with LL-37. **(B)** Human blood monocyte-derived DCs also become more inflammatory when exposed to LL-37, leading to a downregulation of CD16, CD32 and CD209, upregulation of CD11c/CD18, CD11b/CD18 (MAC-1), and co-stimulatory CD86. After maturation by LPS stimulation, the LL-37 exposed DCs could produce more TNFα, IL-6 and IL-12.

Besides that, cathelicidins influence the function and differentiation of DCs, and are therefore able to steer both the innate and adaptive immune responses. First of all, LL-37 stimulates the differentiation of monocytes towards immature DCs (iDCs) when administered for 24 hours at 50µg/ml (Davidson *et al.* 2004). FPR2 signalling (mCRAMP receptor) promotes DC maturation, while FPR2 knockout DCs have impaired maturation in response to LPS (Chen *et al.* 2014).

Furthermore, cathelicidins stimulate Th1 orientated reactions in DCs (Figure 6). Monocyte derived immature DCs express more HLA-DR and co-stimulatory CD86 when incubated with 30 µg/ml LL-37 for 12 hours (Bandholtz *et al.* 2006), which was also observed in chicken PBMCs stimulated with LL-37, chCATH-2 (Kraaij *et al.* 2017), and a truncated chCATH-1 analogue (Bommineni *et al.* 2014). Furthermore, LL-37 enhances the endocytic capacity of iDCs, and up-regulates phagocytic receptor expression, such as Fcy receptors CD16 and CD32, complement receptor CD11b/CD18 and CD11c/CD18 (Davidson *et al.* 2004). Besides that, LL-37 stimulates the production of co-stimulatory molecules and secretion of Th1 inducing cytokines in human monocyte derived DCs, such IL-12, IL-6, and TNFα (Davidson *et al.* 2004). It is not known how LL-37 induces these effects in DCs, but it appears that LL-37 is localized inside the nucleus after it has been endocytosed (Bandholtz *et al.* 2006). Since LL-37 can bind directly to RNA and DNA it is possible that LL-37 itself acts directly as a transcription factor or enhancer (Coorens *et al.* 2015).

Relating to this Th1 stimulating effect in DCs, there was an abundance of IgG1 antibody producing B cells and IL-4 producing $CD4^+$ T cells in mCRAMP KO mice, indicating a negative regulatory role for mCRAMP in type 2 immune response (Kin *et al.* 2011). Not much is known about Th17-related differentiation. LL-37 is found in elevated concentration in Th1/Th17 associated autoimmune skin diseases (Hwang *et al.* 2014, Thomi *et al.* 2018), and can be a T-cell auto-antigen in psoriasis (Lande *et al.* 2014).

Besides monocyte derived DCs, LL-37 influences follicular DCs (fDCs) (Figure 7). LL-37 activates the FPRL1 receptor on fDCs, which results in up-regulation of CXCL13, that attracts B cells via their CXCR5 receptor (Vissers *et al.* 2001, Kim *et al.* 2017). The production of B cell activating factor (BAFF) is also enhanced by LL-37. This leads to increased B cell proliferation and immunoglobulin secretion (Kim *et al.* 2017).

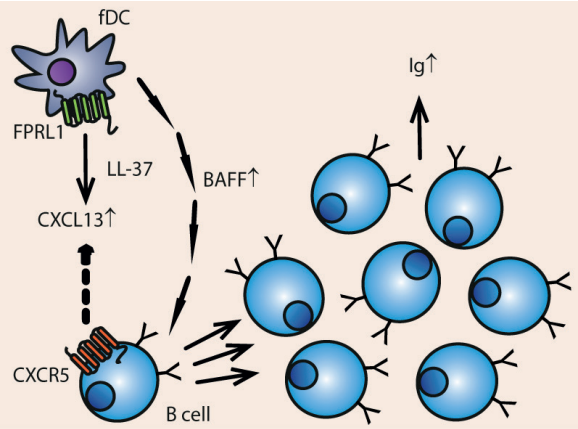

Figure 7: LL-37 induces B-cell attracting chemokines and enhances B-cell proliferation. fDCs produce CXCL13 through LL-37-induced FPLR1 signalling; this chemokine attracts B cells through the CXCR5 receptor. Additionally, the fDCs produce more B cell activating factor (BAFF), which enhances B cell proliferation and IgA production.

Most of the pro-Th1 induced functions of cathelicidins are hampered in the presence of LPS. LL-37 together with LPS does not elicit IL-6 and TNFα production by DCs and they do not up-regulate CCR7, CD86 or HLA-DR. Additionally, co-cultured T cells proliferate less and produce less IL-2 and IFN-γ (Kandler *et al.* 2006). Thus, LL-37 and LPS alone can both stimulate Th1 skewing responses in DCs, but when added together inhibit Th1 responses (Kandler *et al.* 2006). This is probably due to the endotoxin neutralizing properties of LL-37. The same interaction is seen between LL-37 in combination with LTA or flagellin (Kandler *et al.* 2006).

Besides that, administration of LPS together with LL-37 inhibits IgM and IgG2a production in B cells, B cell proliferation and class switching (Nijnik *et al.* 2009). LPS alone stimulates IgG2a antibody production in B cells. Again, the pro-Th1 effect of LL-37 and LPS alone seem to be neutralized when the two components are added together.

Since cathelicidins can stimulate Th1 reactions in the absence of LPS, they could be used as adjuvants in vaccines to boost adaptive immune responses. When mice were immunized with ovalbumin and chicken chCATH-1 and received a booster at day 14, the mice produced higher levels of IgG1 and IgG2a when challenged with ovalbumin on day 20 (Bommineni *et al.* 2014). Remarkably, when mouse mCRAMP

was administered to mice together with ovalbumin as antigen it induced up-regulation of IgG1, IgG2a, IgG2b, and IgG3, and stimulated production of IFNγ and IL-4 (Kurosaka *et al.* 2005). Thus, the immune reaction after mCRAMP stimulation did not show a specific polarization towards Th1, but rather consisted of both Th1 and Th2 responses.

Besides polarizing immune reactions, the DNA and RNA binding and subsequent TLR3, TLR7, TLR8 and TLR9 activating properties of cathelicidins can make cathelicidins suitable adjuvants (Coorens *et al.* 2017a). It is important to consider that presence of other TLR ligands, such as LPS or LTA in the vaccine can change the effects of cathelicidins on the induction of Th1 responses (Kandler *et al.* 2006). If this modulatory effect of cathelicidins can be harnessed to influence specific type immune response desired by the vaccine, cathelicidins can be very useful adjuvants.

Conclusions

Cathelicidins have a wide range of functions on many different cell types, but due to their pleiotropic effects therapeutic use also has its limitations. For instance, they have both pro-tumorigenic and anti-cancer effects, which depend on the tumor type (Piktel *et al.* 2016). Cathelicidin-derived compounds could be applicable in many kinds of therapies including pro-inflammatory in the case of vaccines or anti-inflammatory in the case of sepsis, but care must be taken to avoid tumour- or auto-immune inducing side effects.

From a therapeutic point of view, a marked difference exists between administering exogenous cathelicidins and inducing endogenous production. Stimulation of endogenous production will likely have fewer unforeseen side effects but is limited to the host's repertoire. For humans, who carry only one cathelicidin gene, this approach seems rather limited. Conversely, exogenous peptides can have any origin (including non-natural sources like *in silico* design), but will likely be more transient, and have less target-specific effects. Indeed, the specific effects of a cathelicidin are highly dependent on which cathelicidin is used in which concentration, the environmental parameters like salt concentration and inflammatory context, and which cell type is studied. Therefore, it is important to continue testing cathelicidins from diverse origins in different experimental setups.

Currently, the most efficient way of producing cathelicidins is to use traditional peptide synthesizers. This gives a lot of control over the sequence and allows use of unconventional amino acids. Given their antimicrobial potential, recombinant expression in bacterial species is a challenge. Some methods like bacterial production of fusion proteins or inteins do exist, for example in *E. coli* (Luan *et al.* 2014), *Bacillus subtilis* (He *et al.* 2015) or the yeast *Pichia pastoris* (Xing *et al.* 2016). The purified peptides often retain their antibiotic activity but are (in these examples) not tested for endotoxins or immunomodulatory activity. The LPS binding properties of cathelicidins makes purification of endotoxin-free solution especially challenging. Therefore, other recombinant expression systems might be more applicable, like plants or cell lines. Cathelicidins successfully expressed in plants include LL-37 and SMAP-29 (Morassutti *et al.* 2002, Jung 2013).

After exogenous *in vivo* administration, unmodified peptide is degraded readily by both host and microbial factors (Shinnar *et al.* 2003, Sieprawska-Lupa *et al.* 2004, Schiemann *et al.* 2009). If the therapeutic goal requires longer exposure time to the peptides, either multiple administrations are needed, or a slow-release system. Another possibility is to protect the mature peptide from degradation, for example by using unusual amino acids, D-enantiomers, or point mutations to limit the peptides' susceptibility to proteolytic degradation.

For therapeutic use as antimicrobials, immobilized cathelicidins are a good avenue to pursue. There are examples of immobilized peptides working effectively at preventing bacterial outgrowth (Costa *et al.* 2011, Riool *et al.* 2017). Immobilized peptides could lose some antibacterial activity depending on their length and orientation, but can also be more resistant to degradation (Costa *et al.* 2011). There are multiple chemical methods of coupling peptides using specific residues (deGruyter *et al.* 2017). In short, whereas direct (for example intravenous) application of cathelicidins as antimicrobials is probably not a very effective treatment, prevention of biofilms growing on medical devices can give major health benefits, since indwelling medical devices are a major source of nosocomial infections (Percival *et al.* 2015). Additionally, since cathelicidins stimulate wound healing (Yang *et al.* 2006, Carretero *et al.* 2008, Ramos *et al.* 2011) and angiogenesis (Li *et al.* 2000, Koczulla *et al.* 2003, Ramos *et al.* 2011), they could be used as treatment supplementation

for wounds at risk of infection like surgical wounds.

As immunomodulators, cathelicidins could also be used as vaccine adjuvants or additives. Their small size means they can easily be incorporated in already existing formulations. They attract immune cells to site of injection and polarize DCs and T-cells to specific types of immune reaction. Depending on the vaccine, this could be an answer for specific therapeutic questions. Cathelicidins could also be suitable as immunopotentiators on next generation nanoparticle vaccines (Zhao *et al.* 2014). However, not much is known on immunomodulation by immobilized cathelicidins.

In conclusion, cathelicidins are expressed in many different forms by most animals and have varied immunomodulatory functions. The specific effects are highly dependent on the specific cathelicidin, the concentration, presence of other factors and the inflammatory context. This diversity means they could potentially be applicable in a wide variety of immunomodulatory therapies.

Supplementary information

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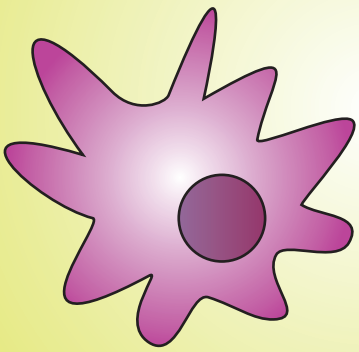

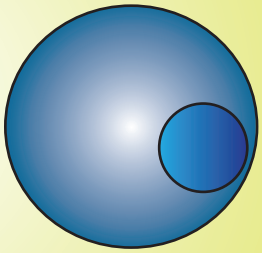

Chapter 3

The Cathelicidin CATH-2 efficiently neutralizes LPS- and *E. coli*-induced Activation of Porcine Bone Marrow Derived Macrophages

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Abstract

Infectious diseases in pigs cause monetary loss to farmers and pose a zoonotic risk. Therefore, it is important to obtain more porcine specific immunological knowledge as a measure to protect against infectious diseases, for example by exploring immunomodulators that are usable as vaccine adjuvants. Cathelicidins are a class of host defence peptides (HDPs) able to directly kill microbes as well as exert a diverse range of effects on the immune system. The peptides have shown promise as immunomodulatory peptides in many applications, including vaccines. However, it is currently unknown what the precise effect of these peptides is on porcine immune cells and whether peptides of other species might also have a strong immunomodulatory effect on porcine macrophages. Mononuclear bone marrow cells of pigs, aged 5-6 months, were cultured into M1 or M2 macrophages and stimulated with LPS or whole bacteria in the presence of host defence peptides (HDPs).

CATH-2 and LL-37 strongly inhibited LPS-induced activation of M1 macrophages, the inhibition of LPS-induced activation of M2 macrophages by HDPs was milder, showing that the peptides have selective effects on different cell types. Upon stimulation with whole bacteria, only CATH-2 could effectively inhibit macrophage activation, showing the potent anti-inflammatory potential of this peptide. These results show that porcine peptides are not necessarily the most active in a porcine system, and that CATH-2 is effective in a porcine system as an anti-inflammatory immune modulator, which can be used, for example, in inactivated pathogen vaccines.

Keywords: M1/M2 macrophages, cathelicidin, CATH-2, immunomodulation, porcine

Introduction

In 2019, around 850 million pigs were kept in farming worldwide (FAOstat 2019). Considering the threats to public health of both zoonotic disease and antibiotic resistance, there is a clear need for alternatives to antibiotics in livestock farming (Aarestrup 2012, Luiken *et al.* 2020, Duarte *et al.* 2021). Since to prevent is better than to cure, vaccination is the most effective method to reduce antibiotics use. However, not all porcine pathogens have broadly effective vaccines available. For instance, *Streptococcus suis*, while being one of the globally most damaging porcine pathogens, currently has no commonly available vaccine (Hernandez-Garcia *et al.* 2017, Segura *et al.* 2017, Segura *et al.* 2020).

While traditionally effective vaccines consist of live attenuated or inactivated pathogens, many modern experimental vaccines are based on subunit technology or DNA/RNA injection. Many of these have shown great efficacy in various animals and humans, for instance in the case of nucleotide injection for COVID-19 vaccines (Corbett *et al.* 2020, Polack *et al.* 2020), subunit vaccine for porcine circovirus 2 (PCV2) (Grau-Roma *et al.* 2011) or acellular vaccination for classical swine fever (Madera *et al.* 2016). However, many subunit vaccines lack the innate reactogenicity of whole cell or attenuated formulations. These, however, can in turn be too reactogenic and cause systemic inflammatory side effects (Coffman *et al.* 2010, Nanishi *et al.* 2020). For these reasons, vaccines can benefit greatly from a diverse selection of immune modulators to fine-tune the immune response. Cathelicidins are a promising option as immune modulator during vaccination.

Cathelicidins are host defence peptides (HDPs) from the vertebrate innate immune system, abundantly present in skin, respiratory and digestive tracts. These HDPs are constitutively expressed at low levels by epithelial cells and released in high concentrations by degranulation of immune cells in response to microbes or injury (Hancock *et al.* 2016, van Harten *et al.* 2018, Scheenstra *et al.* 2020). HDPs function as direct antimicrobials and can modulate the immune system, which includes up- or downregulating of immune related genes, acting as opsonizing or chemoattracting agents, and neutralizing inflammatory signals (Hancock *et al.* 2016, van Harten *et al.* 2018). HDPs have investigated successfully as vaccine components in the past (Kovacs-Nolan *et al.* 2009, Garlapati *et al.* 2011, Yu *et al.* 2016, Prysliak *et al.* 2017,

Schulze *et al.* 2017, Mookherjee *et al.* 2020), but not yet in pigs. Moreover, not much is known yet about HDP effects on porcine immune cells.

HDPs have been studied for immunomodulatory functions in multiple other species (Coorens *et al.* 2017a). The chicken cathelicidin CATH-2 has a strong affinity for LPS and inhibits Toll like receptor-4 (TLR-4) activation in mouse and human cells (van Dijk *et al.* 2009, van Dijk *et al.* 2016a, Scheenstra *et al.* 2019). Additionally, this peptide can enhance uptake of nucleic acids and thereby enhance TLR-9 signalling in mouse macrophages (Coorens *et al.* 2015) and porcine plasmacytoid dendritic cells (pDCs) (Baumann *et al.* 2014). When administered *in ovo*, the all-D-analogue of CATH-2 reduced mortality and infection morbidity in chickens from *E. coli* infection up to 7 days after hatch (Cuperus *et al.* 2016). This indicates an antimicrobial effect that persists beyond the longevity of the HDP itself and suggest that HDPs modulate the immune response. Although HDPs are natural effectors of the immune system, the immune modulatory functions of HDPs act cross-species (Pelillo *et al.* 2014, Schneider *et al.* 2016, Baumann *et al.* 2017, Coorens *et al.* 2017a, Scheenstra *et al.* 2019).

Eleven cathelicidins have been identified in pigs, in contrast to humans where only one cathelicidin, LL-37 has been identified. However, only very little is known about the effect of cathelicidins in pigs or on porcine cells. Porcine cathelicidins PMAP-23, PMAP-36 and PR-39 were shown to enhance nucleotide uptake and subsequent activation of porcine pDCs (Baumann *et al.* 2014). PR-39 induces IL-8 and to a lower extent TNFα expression by the porcine alveolar macrophage cell line 3D4/31 but does not inhibit LPS-induced activation like many other cathelicidins. In porcine 3D4/31 cells, PMAP-23 could only induce IL-8 production (Veldhuizen *et al.* 2014, Veldhuizen *et al.* 2017).

In this study, we aim to investigate the effects of HDPs on primary bone marrow derived porcine macrophages. Four HDPs were selected based on their described immunomodulatory effects: chicken CATH-2, human LL-37 and porcine PMAP-23 and PR-39, (Delfino *et al.* 2004, Kandler *et al.* 2006, Mookherjee *et al.* 2006a, Mookherjee *et al.* 2006b, Baumann *et al.* 2014, Veldhuizen *et al.* 2014, Cuperus *et al.* 2016, Schneider *et al.* 2016, Van Dijk *et al.* 2016b, Coorens *et al.* 2017a, Coorens *et al.* 2017b, Veldhuizen *et al.* 2017). The results indicate that especially CATH-2

shows strong anti-inflammatory capacities in porcine cells, both after LPS-induced activation as well as activation with live *E. coli*. Therefore, CATH-2 could potentially be used in bacterial vaccines where overstimulation of immune cells is problematic.

Materials and methods

Peptides

All peptides were synthesized by Fmoc chemistry on a chemical synthesizer (China peptides, Shanghai, China), and C-terminally amidated to improve stability (Table 1). Purity was >95% for each peptide. After lyophilization, the peptides were reconstituted in MilliQ water and further diluted in appropriate culture medium.

Table 1. Peptides' sequence and length/charge.

Peptide	Sequence	Length	Charge
CATH-2	RFGRFLRKIRRFRPKVTITIQGSARF-NH_2	26	+9
PMAP-23	RIIDLLWRVRRPQKPKFVTVWVR- NH_2	23	+6
LL-37	LLGDFFRKSKEKIGKEFKRIVQRIKDFLRNLVPRTES-NH_2	37	+6
PR-39	RRRPRPPYLPRPRPPPFFPPRLPPRIPPGFPPRFP-PRFP- NH_2	39	+10

Primary cell isolation, culture, and stimulation

Porcine bone marrow derived macrophages were grown as previously described (Gao *et al.* 2018) from bone marrow of 16 pigs in total, either Great Yorkshire, Large White or F1 of Large White and Nordic Landrace-, aged 5-6 months (70-80 kg) (van Beek SPF Varkens, Lelystad, The Netherlands). The animals are raised as SPF experimental animals, but not housed behind a barrier after arrival. The animals were allowed to acclimatize between 7 and 20 days, before being put under terminal anaesthesia. The animals were then used as educational models for complex surgical techniques for medical doctors and operating room staff. After training, bone

marrow was harvested by hip bone puncture by a trained veterinarian. All animals were used and kept under licence of the Central Laboratory Animal Committee of the Netherlands under the advice and guidelines of the animal ethical committee of Utrecht University. Mononuclear cells from bone marrow were isolated by Ficoll (GE Healthcare, Chicago, Illinois) density centrifugation. Mononuclear cells were seeded at a density of $5x10^4$ cells per well in a 96-wells plate, then cells from the same individual were cultured into M1- or M2-macrophages by exposure to 40 ng/mL recombinant porcine GM-CSF (BioTechne, Minneapolis, MN) or 30 ng/mL recombinant murine M-CSF (Peprotech, Rocky Hill, NJ), respectively in RPMI-1640 with 10% FCS and 100 U/mL penicillin/streptomycin (RPMI+/+) for 6 days. Then, the cells were stimulated with 100 ng/mL of LPS-EB from *E. coli* (Invivogen, San Diego, CA) for 24 h. When peptides were included, peptides were pre-mixed with LPS before addition to the cells. Supernatant was isolated and stored at -20°C for ELISA, and cells were detached with 0.5 mM EDTA in PBS, then analysed by flow cytometry.

Bacterial stimulation

E. coli ATCC 25922 was grown overnight in Mueller Hinton Broth (MHB) medium at 37°C, then inoculated in 10 mL fresh MHB and grown to mid-log phase for 2 h at 37°C on an orbital shaker. Bacterial density was measured at 620 nm and bacteria were diluted to 2.0 x 10^6 CFU/mL. Bacteria were killed by either 250 µM peptides, with 100 U/mL of penicillin/100 µg/mL streptomycin for 3 h at 37°C or by heating to 95°C for 1 h. Live bacteria used were kept on ice until stimulation. Efficiency of the different bacterial killing methods, as well as quantification of the viable bacteria used in the stimulation experiments were verified by plating out on TSA plates.

After killing, the bacterial solutions were pre-mixed with peptides with the exception of peptide-killed bacteria (as they already had peptides in solution), and added to macrophages in 1:100 dilution, leading to a final concentration of 2.5 µM peptides and/or an MOI of 2. After 2 h of stimulation, supernatant containing the bacteria was removed and the cells were washed with RPMI+/+ and incubated a further 22 h. Then, the cells and supernatant were harvested for analysis by flow cytometry and ELISA respectively.

Flow cytometry

Macrophages were detached by vigorous pipetting after a 5 min incubation with 0.5 mM EDTA in PBS at 37°C. After detachment, the cells were washed and kept in 0.5% w/v BSA in PBS on ice. After washing, the cells were stained for 20 min in the dark. The following antibodies were used: anti CD163-FITC mouse IgG2, anti-human CD14-PB (Bio-Rad, USA), aSWC3a-PE (Invitrogen, USA), recombinant CTLA-4-MuIgG-APC (Ancell, USA) which binds to the CD80/86 complex. Measurements were acquired with a FACS Canto II (BD Biosciences, Franklin Lakes, NJ). Analysis was performed with FlowJo v10 (BD Biosciences).

ELISAs

ELISAs were performed with porcine specific kits for TNFα, IL-10, IL-1β and IL-8 (R&D Systems, Minneapolis, MN) according to the manufacturer's recommendations.

Statistics

Statistical analyses were performed using Prism 8 (GraphPad, San Diego, CA), being paired T tests, mixed-model analysis with Tukey's correction for multiple comparisons or Friedman test with Dunn's correction for multiple comparisons. Results were considered significant if $p<0.05$ compared to the relevant control.

Results

Porcine bone marrow-derived M1 macrophages strongly increase their cytokine secretion upon LPS stimulation.

For this study, bone marrow cells were cultured into M1 macrophages as was previously set up and validated by our group (Gao *et al.* 2018). The cells were analysed using flow cytometry at day 7 of culture. Macrophages were first gated based on size (forward scatter (FSC)) and granularity (side scatter (SSC)) (Fig. 1A), followed by the expression of the porcine myeloid cell specific marker SWC3α (Fig. 1B). The macrophages were a homogeneous culture, expressing CD163 (Fig. 1C) and CD80/86 (Fig. 1E), and high levels of CD14 (Fig 1F). Most markers only slightly changed upon LPS stimulation (Fig. 1C-F). One individual showed a potent increase in CD80/86 expression, whereas the increase for the other individuals was less pronounced. LPS stimulation did significantly reduce CD14 expression in these cells (Fig 1F).

In contrast to the marker expression, cytokine expression was strongly induced upon LPS-stimulation. The M1 macrophages produced 10-1000-fold increased amounts of TNFα, IL-10, IL-1β and IL-8 compared to unstimulated controls (Fig 1G-J).

CATH-2, LL-37, and PMAP-23 inhibit LPS-induced stimulation of porcine M1 macrophages

The immunomodulatory properties of chicken CATH-2, porcine PMAP-23, human LL-37, and porcine PR-39 were tested. None of the peptides alone could induce significant changes on cytokine production or marker expression (Figure S1). Since pigs are genetically diverse animals, the interindividual variation in marker expression and cytokine production was substantial (Fig. 1 and S1). Therefore, the data showing immunomodulatory capacity by the peptides was normalized to the LPS-stimulated condition of each individual control.

With respect to the cell marker expression, stimulation of M1 macrophages with LPS in combination with LL-37 caused a dose-dependent decrease of CD163 expression compared to LPS alone (Fig. 2A), not seen for any of the other HDPs tested. LPS-stimulation together with PR-39 caused a significant increase of CD80/86 (Fig. 2B) and CD14 expression (Fig. 2C). PMAP-23 and CATH-2 showed a tendency to-

wards higher surface marker expression for CD163 and CD14, but this did not reach statistical significance.

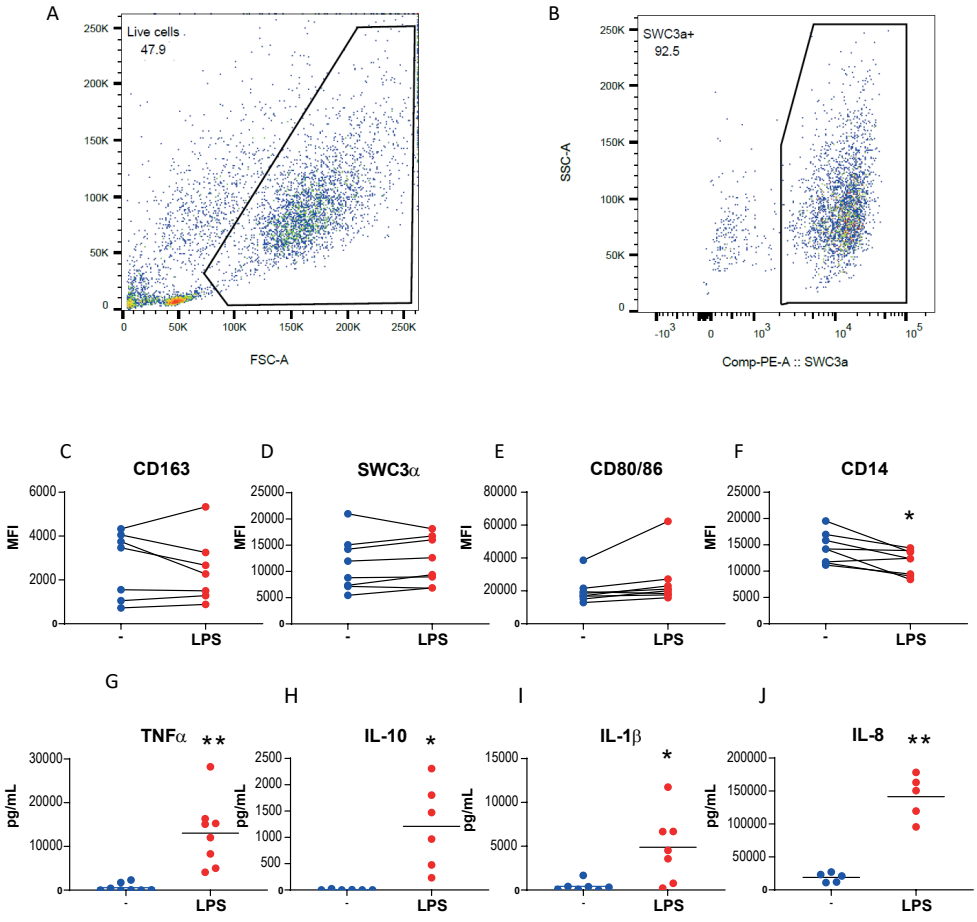

Figure 1: Porcine SWC3α+ M1 macrophages are CD163 and CD14 positive, and express large amounts of cytokines upon LPS stimulation. Porcine M1 macrophages are gated on FSC/SSC profile (A), then selected for the myeloid marker SWC3α (B). C-F The change in surface marker expression upon LPS stimulation was measured by the median fluorescence intensity (MFI) of cells within the SWC3α+ gate. G-J The levels of cytokines TNFα, IL-10, IL-1β and IL-8 was measured using ELISA. Individual pigs (N=3-6) are depicted in the graphs and paired T-test was used for statistical analysis (*=p<0.05, **= p<0.01, ***=p<0.001).

The effect of the peptides on LPS-induced cytokine expression was much stronger compared to the relative smaller differences in surface marker expression. LL-37, CATH-2, and, to a lesser extent PMAP-23, strongly inhibited the LPS-induced acti-

vation of M1 macrophages, demonstrated by a marked dose dependent decrease for all tested cytokines. For example, 2.5 µM CATH-2 reduced the LPS-induced TNFα production to 35%, while 2.5 µM LL-37 even reduced it to 15% of the original expression (Fig. 2D). PR39 strongly inhibited the expression of IL-10 (to 40%) and IL-8 (to 62%), but the expression of TNFα and IL-1β was unaffected by the peptide. This indicates a different mode of action of PR39 compared to CATH-2, LL-37, and PMAP-23 (Fig. 2A-G).

HDPs inhibit LPS induced activation in M2 macrophages

CD163 is reduced in both human (van der Does *et al.* 2010) and porcine M1 macrophages (Fig. 2A) after exposure to LL-37. Since anti-inflammatory M2 macrophages are characterized by a higher CD163 expression (Gao *et al.* 2018), the effect of the peptides was also tested on porcine M2 macrophages but, contrary to M1 macrophages, none of the peptides affected the CD163 expression (Fig. 3A). In contrast, LL-37 reduced CD80/86 expression by 25% at 2.5 µM (Fig. 3B). CD14 expression was also much higher in LPS stimulated M2 macrophages when co-stimulated with LL-37 and CATH-2, with the latter even doubling the average MFI (Fig. 3C).

Next, when observing cytokine expression in M2 macrophages, the LPS induced cytokine expression was reduced by the peptides. M2 macrophages are characterized by a high production of IL-10 (Gao *et al.* 2018)(Fig. S2). CATH-2 and LL-37 significantly inhibited LPS-induced TNFα production with 50%, although only at the highest HDP concentration tested (2.5 µM) (Fig. 3D). For PMAP-23 a small increase of the TNFα production was observed for some individuals, whereas in other individuals, expression was not changed. PR-39 had no effect on TNFα release. With respect to IL-10 expression only CATH-2 seemed to inhibit at 2.5 µM, but this failed to reach significance (Fig. 3E). Overall, no significant peptide effects were observed in IL-1β release in M2 macrophages (Fig. 3F). Finally, addition of 2.5 µM PMAP-23 or PR-39 significantly increased the LPS-induced IL-8 secretion by 132 % and 84 % on average, respectively (Fig. 3G). Taken together, the inhibitory effect of the peptides on M2 macrophages are present, but less pronounced than in M1 macrophages which often showed a larger relative reduction of cytokine production.

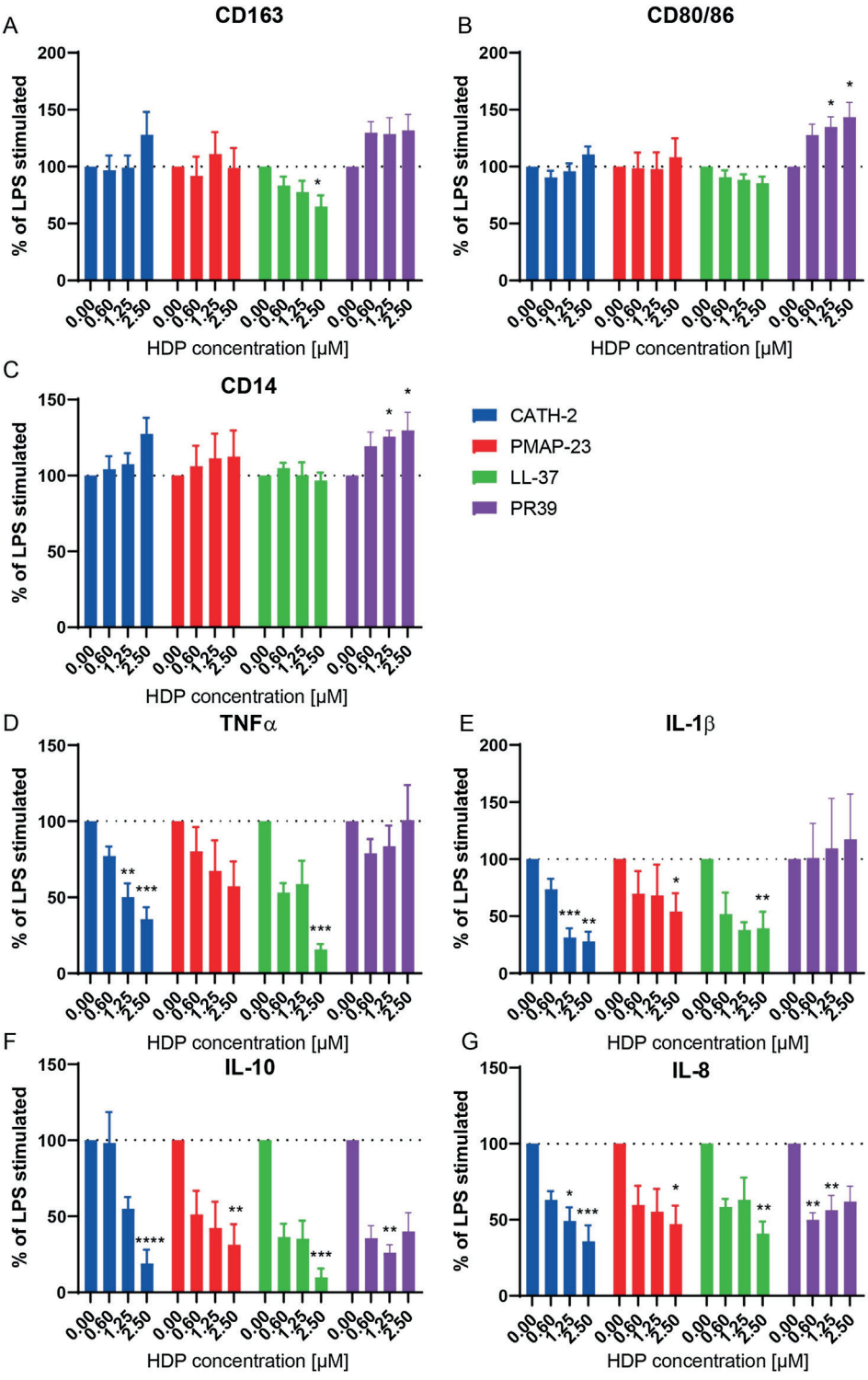
A
CD163
B
CD80/86
C
CD14
D
TNFα
E
IL-1β
F
IL-10
G
IL-8
% of LPS stimulated
HDP concentration [μM]
0.00
0.60
1.25
2.50
CATH-2
PMAP-23
LL-37
PR39

Figure 2: **CATH-2, LL-37, and PMAP-23 inhibit LPS-induced stimulation of porcine M1 macrophages**. M1 macrophages were stimulated with 100 ng/ml LPS in the presence and absence of HDPs. Changes in surface markers CD163, CD80/86 or CD14 (A-C) and cytokine expression of TNFα, IL-1β, IL-10 and IL-8 (D-G) were determined and normalized to the LPS-only control of that individual pig. Significant difference to the control was calculated by Friedman test with Dunn's correction for multiple comparison (*=p<0.05, **= p<0.01, ***=p<0.001, ****=p<0.0001). (N=5-7 individuals, results are shown as mean±SEM).

CATH-2 inhibits M1 inflammation in whole bacterial cell stimulation as well as LPS stimulation

Since whole cell vaccines contain, besides LPS (for Gram-negative bacteria), a large mixture of other immune stimulatory components, we investigated the effect of *E. coli* stimulation of macrophages in the presence of peptides. Additionally, since HDPs can kill microbes directly, we assessed the effect of stimulation of macrophages with *E. coli* killed by HDPs, as well as by other methods such as heat treatment or incubation with antibiotics. Viable *E. coli* efficiently stimulated the M1 macrophages, as was shown by increased (15000 pg/mL) TNFα secretion compared to the unstimulated control (800 pg/mL; Fig. 4A). Using the same initial bacterial density, non-viable *E. coli* (both heat-killed, and penicillin/streptomycin killed) also significantly induced TNFα production compared to the non-stimulated control, although to a somewhat lower level than viable *E. coli.*

In contrast to heat and antibiotic treatment, addition of 2.5 μM CATH-2 together with live *E. coli*, also rendering the bacteria non-viable, reduced TNFα levels almost to non stimulated levels. PMAP-23 had no effect on bacterially induced TNFα production, while incubation of viable *E. coli* with LL-37 led to a slight induction in TNFα production (Fig. 4A).

Next, we wanted to know if peptides could similarly affect the activation of M1 macrophages by non-viable bacteria, since whole cell vaccines often contain inactivated microbes. Therefore, we incubated the macrophages with the heat and penicillin/streptomycin killed bacteria in the presence of CATH-2, PMAP-23, and LL-37. The already lower effect of heat-killed bacteria on TNFα levels could not be further reduced (Fig. 4B). Basically, when CATH-2 was present, TNFα levels were similar irrespective of the killing method of the bacteria. Contrary to the immunomodulatory effects observed for CATH-2, LL-37 was not effective in reducing TNFα induction by

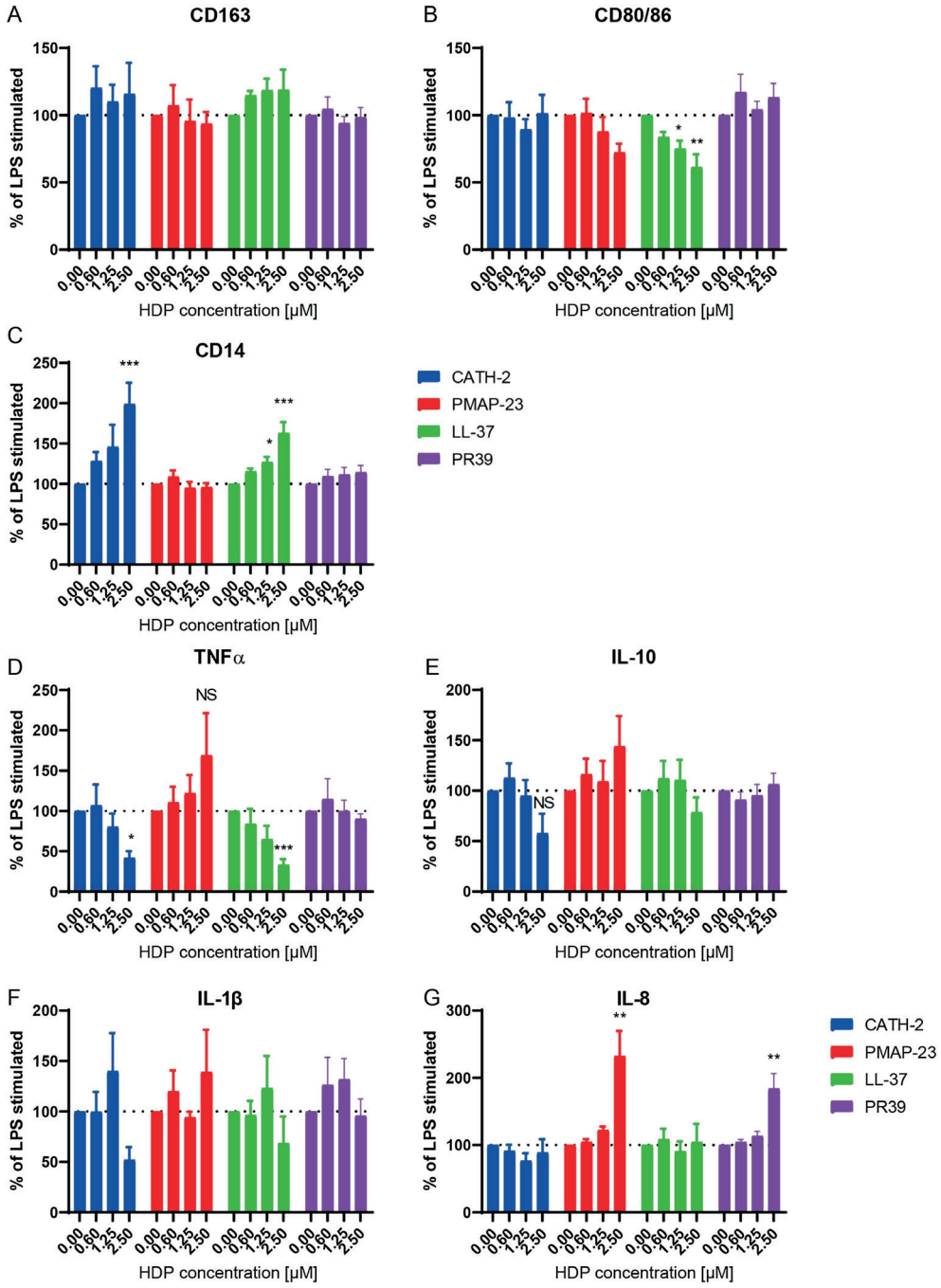

Figure 3: **HDPs show mild inhibition of LPS-induced stimulation of porcine M2 macrophages**. M2 macrophages were stimulated with 100 ng/ml LPS in the presence and absence of HDPs. Changes in surface markers CD163(A), CD80/86(B) or CD14(C) and cytokine

expression of TNFα(D), IL-1β(E), IL-10(F) and IL-8(G) were determined and normalized to the LPS-only control of that individual pig. Surface marker and cytokine expression levels are normalized to the LPS-only control of that individual pig. Significance was calculated by Friedman test of the raw data with Dunn's correction for multiple comparisons (*=p<0.05, **= p<0.01, ***=p<0.001, ****=p<0.0001). (N=5-7, results are shown as mean ± SEM)

any of the tested bacterial viability states and showed an increase to almost 2000 pg/ml, when LL-37 was added together with live bacteria (Fig. 4C). Additionally, no effect on TNFα production was observed when LL-37 was added to non-viable (heat or antibiotic treated) bacteria showing a similar TNFα levels as bacteria killed by antibiotics alone. Finally, PMAP-23 showed no inhibitory effects on bacterial stimulation of M1 macrophages irrespective of the viability of the bacteria or the method of killing of the non-viable E. *coli* (Fig. 4D).

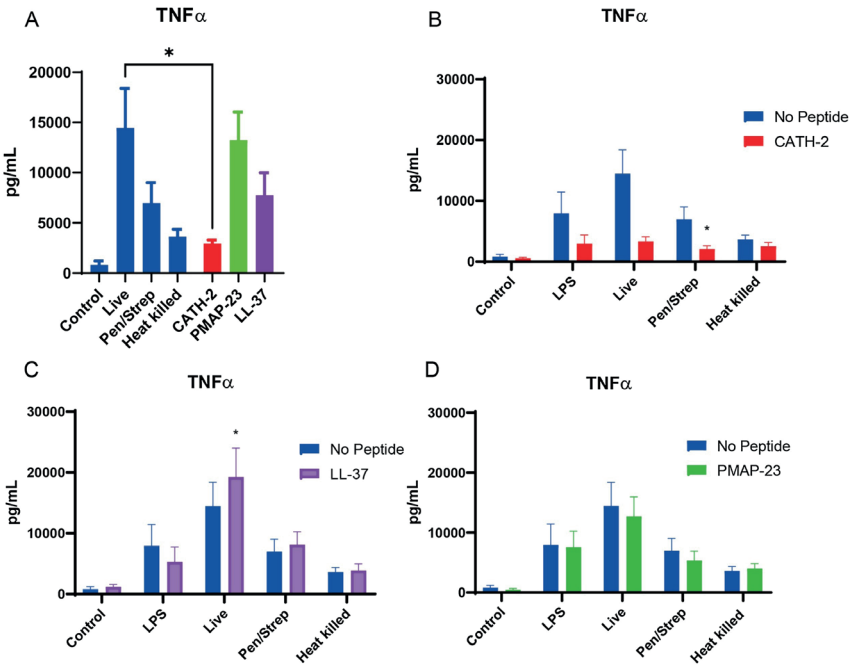

Figure 4: **CATH-2 can efficiently inhibit macrophage stimulation by viable and non-viable bacteria. A**. Porcine M1 macrophages were stimulated with LPS or bacteria, either live or killed by heat, antibiotics, or HDPs CATH-2, PMAP-23, and LL-37, then added to porcine M1 macrophages. **B-D** Bacteria or LPS were mixed with peptide or control in various viability states, then TNFα production of M1 macrophages was measured. Significance was calculated by Friedman test with Dunn's correction for multiple comparisons (*=p<0.05, **= p<0.01, ***=p<0.001, ****=p<0.0001). (N=4; results are shown as mean ±SEM)

Discussion

In the past, inactivated bacterial vaccines have led to good protection but they possess a risk of overactivation of the immune response as compared to subunit vaccines (Nanishi *et al.* 2020). On the other hand, subunit vaccines can result in possibly too little activation, and are prone to weaning protection, as has happened in the past with the vaccine for whooping cough (Mooi *et al.* 2001, Gilberg *et al.* 2002). In this case, overactivation caused by either whole cell vaccines or bacterial outer membrane vesicles could be tempered by formulation with HDPs (Balhuizen *et al.* 2021). Similarly, experimental vaccines for pigs could benefit from immunomodulatory peptides such as CATH-2.

Many porcine immune cell experiments are performed with alveolar macrophages, either directly isolated from pigs, or with the alveolar macrophage-derived cell line 3D4/31. However, alveolar macrophages do not produce NO in response to stimulation (Zelnickova *et al.* 2008), indicating that these cells might not be ideal for *in vitro* experiments. Therefore, a bone marrow-derived macrophage, culture system was recently set up by our group (Gao *et al.* 2018). These cells were responsive to stimulation by bacterial components, especially on the level of cytokine expression. The cells could be polarized into M1 and M2 macrophages, which responded comparable to human M1 and M2 macrophages (Fleetwood *et al.* 2007, van der Does *et al.* 2010, Gao *et al.* 2018). Additionally, bone marrow mononuclear cells can be easily cryopreserved, and large quantities can be stored from one individual. However, compared to cell lines, the variability in this system is higher. This is due to genetic variance in the outbred individuals, which makes the obtained results better translatable to the whole population, as genetic variability is present there, too.

Little is known about HDP activity in pigs. Previously obtained results focused particularly on porcine cathelicidins (Holani *et al.* 2016, Veldhuizen *et al.* 2017, Scheenstra *et al.* 2019) and porcine pDCs (Baumann *et al.* 2014). However, cathelicidins are known for broad activity across species barriers (Hancock *et al.* 2016, Coorens *et al.* 2017a, van Harten *et al.* 2018). It is therefore important to assess in depth the effect of HDPs without being limited to the target species' native HDPs. Here, we assessed CATH-2 and LL-37 alongside PMAP-23 and PR-39 in a porcine macrophage system. Surprisingly, the porcine peptides had the lowest immunomodulatory effect, de-

spite them being part of the natural innate immune system of pigs. It must be noted that not all pig HDPs were tested here, as pigs express 11 different cathelicidins in addition to a broad repertoire of defensins (Sang and Blecha 2009). In this work, CATH-2 actually functioned as the most potent anti-inflammatory HDP for pig cells, despite the fact that CATH-2 is a chicken cathelicidin.

LPS binding and subsequent steric hindrance of TLR-4 binding, and activation could be the main mechanism for peptide mediated neutralization. For example, CATH-2 is predicted to bind to LPS in a receptor-blocking manner. Truncations of CATH-2, that are too short to reach this part of LPS, are not able to inhibit LPS activation (van Dijk *et al.* 2016a). In this study, we found that CATH-2 was able to efficiently inhibit activation of macrophages by whole bacteria as well as LPS separately. Therefore, the modulation of effects that were observed for CATH-2 could be broader than only steric hindrance of TLR-4 activation.

Interestingly, in previous studies, LL-37 has been a better inhibitor of LPS activation of immune cells than CATH-2 (Coorens *et al.* 2017a, Scheenstra *et al.* 2019). This was shown by LL-37 having inhibitory effects at lower concentrations, while actually having lower affinity for LPS. However, in whole bacterial cell stimulation, LL-37 does not potently inhibit macrophage responses, especially in the presence of live bacteria. However, when macrophages were stimulated using dead bacteria, LL-37 is again a potent inhibitor. This indicates that although peptides can have similar inhibition profiles using LPS only, the viability state of bacteria is important to predict the immunomodulating effect of HDPs *in vivo*. In line with this: the immunomodulating effect of HDPs in vaccine preparations is likely very different depending on the type of vaccine (subunit, inactivated or attenuated) used, underlining the importance of studying immunomodulatory effects of HDPs in different conditions.

Surprisingly, the inhibitory effects of peptides did not always extend to all cytokines. For example, where CATH-2 had potent inhibitory effects on LPS-induced cytokine production in M1 cells, IL-8 production in M2 macrophages was not affected upon LPS stimulation. Likewise, where PR-39 inhibits IL-10 and IL-8 production in M1 macrophages, it has no effect on IL-1β release or TNFα production. Peptides evidently have differing ways of skewing inflammation, either by inhibiting specific cytokines or activation in general. It is therefore essential to thoroughly investigate

peptide effects in a particular system.

In conclusion, HDPs LL-37 and CATH-2 inhibit LPS-induced activation of porcine primary bone marrow derived macrophages. HDPs affect both M1 and M2 macrophages, although the inhibition is more effective for M1 macrophages. Only CATH-2 shows inhibition of live bacterial activation, which makes CATH-2 the most interesting candidate to add in the formulation of whole cell vaccines.

Competing interests

Declarations of interest: none.

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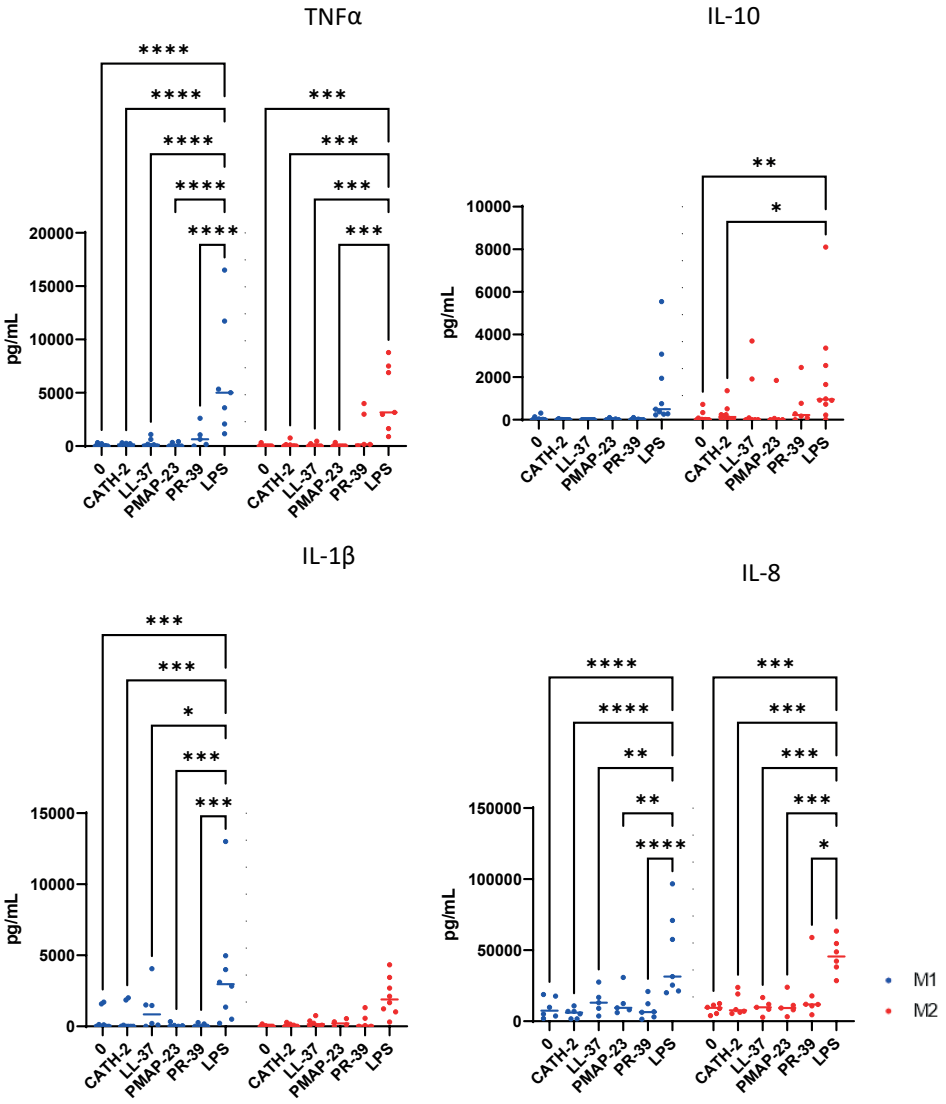
Abbreviations

BMDM - bone marrow derived macrophage
BSA - bovine serum albumin
ELISA - enzyme linked immunosorbent assay
FSC - forward scatter
MHB - Mueller Hinton broth
NFκB - nuclear factor κB
PCV2 - porcine circovirus 2
pDC - plasmacytoid dendritic cell
RPMI-1640 - Roswell Park Memorial Institute medium 1640
SSC - side scatter
TLR - Toll-like receptor
TSA - tryptic soy agar

Supplemental figures



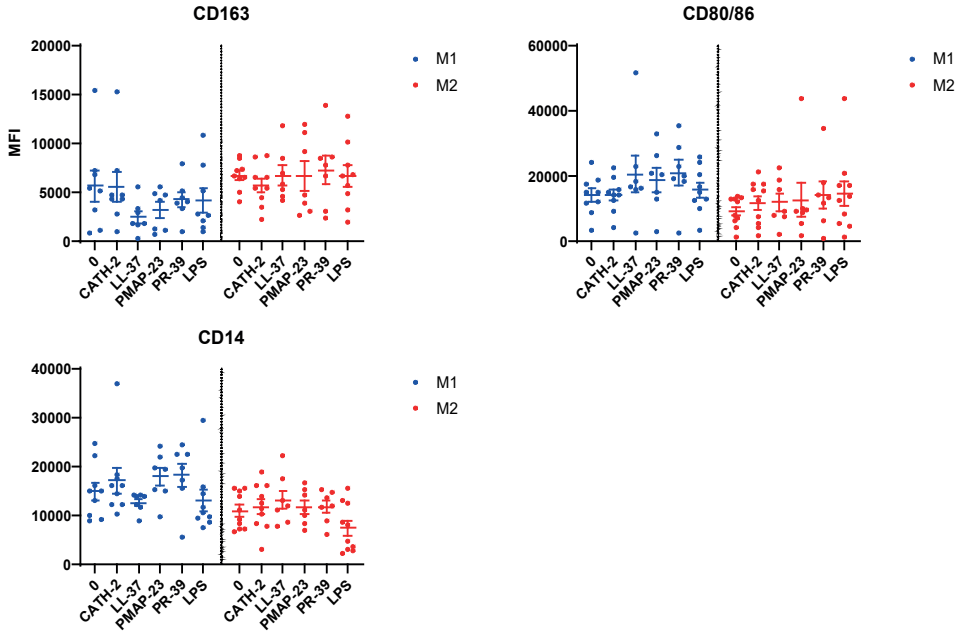


Figure S1: **Peptide alone does not change the macrophage marker expression or cytokine production.** Porcine M1 (blue) and M2(red) macrophages were incubated with either 2.5 µM peptide (CATH-2, LL-37, PMAP-23, or PR-39). Individual values are displayed with the mean ± SEM, N=6-10. Statistically significant differences compared to all other conditions with the group is calculated by a mixed-effects model with Tukey's correction for multiple comparisons.

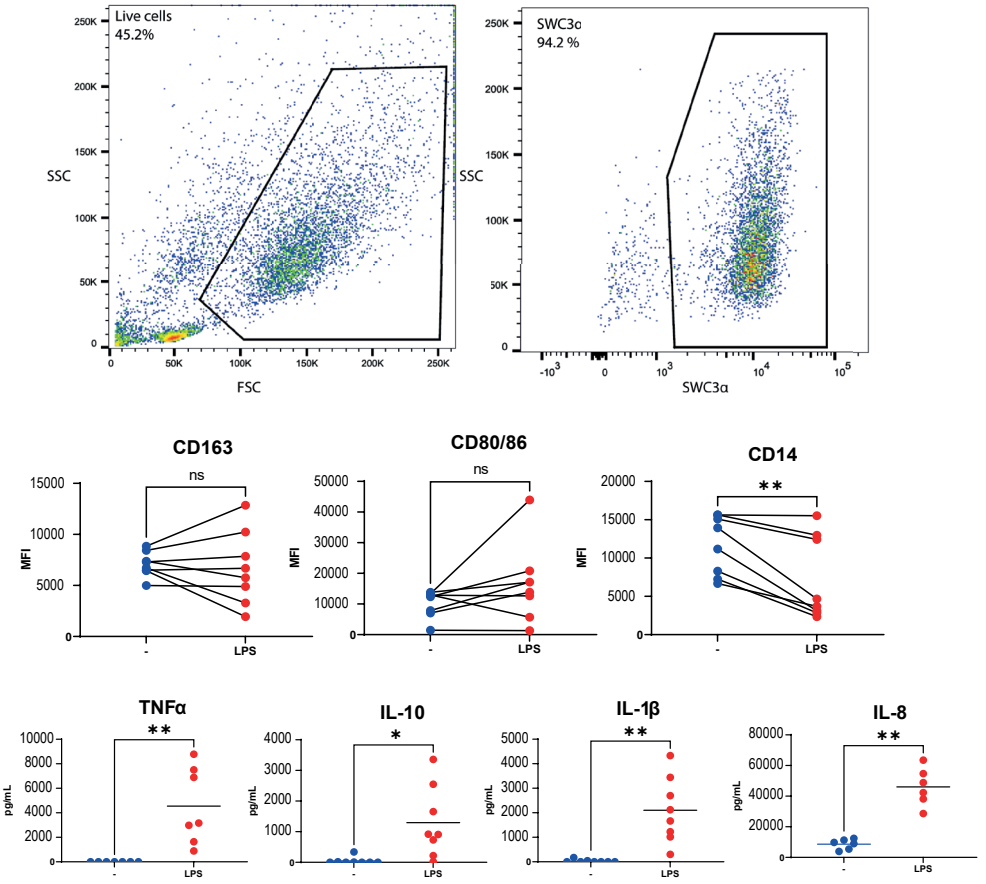


Figure S2: **Characterization of M2 macrophages shows production of cytokines and down-regulation of CD14 upon LPS stimulation.** M2 macrophages are typically gated on FSC/SSC profile then selected for SWC3α+. Median fluorescence intensity for CD163, CD80/86 and CD14 and Production of TNFα, IL-10, IL-1β and IL-8 is measured with or without LPS stimulation. ± SEM, N=6-10. Statistically significant differences compared to all other conditions with the group is calculated by a mixed-effects model with Tukey's correction for multiple comparisons.

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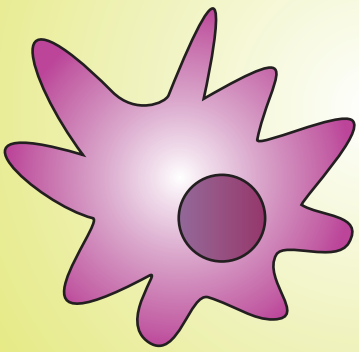

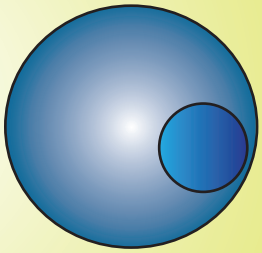

Chapter 4

Streptococcus suis evades HDP-mediated Killing

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Abstract

Streptococcus suis is a Gram-positive zoonotic pathogen that greatly affects pig health. Since antimicrobial resistance against multiple first-line antibiotics has been found in this bacterium, there is a clear need for novel antimicrobial compounds with anti-*S. suis* activity. Cathelicidins are a highly diverse class of host defence peptides from the vertebrate innate immune system with a broad antimicrobial activity and low rates of inducing resistance. We tested the antimicrobial effect of a variety of cathelicidins against *S. suis* and show that cathelicidins that originate from pigs have much lower activity against *S. suis.* Additionally, L-enantiomers of both CATH-2 and LL-37 are less active than D-enantiomers of the same peptide, with MBCs being twice as high or more for both L-peptides. This protective mechanism is not related to the bacterial capsule and is not affected by the addition of protease inhibitors. This protective effect can be transferred via *S. suis*-conditioned medium (Ss-CM) to *Staphylococcus aureus* which is normally sensitive to cathelicidin-induced killing. Finally, we show that the unknown protective mechanism remains over time, is thermally stable at 37°C and of a molecular mass of >3kD. These results show that *S. suis* can protect itself from various cathelicidins through an unknown mechanism and that cathelicidin D-enantiomers may be developed as effective antimicrobial therapeutics against *S. suis*.

Introduction

Streptococcus suis (S. suis) is a facultative anaerobic Gram-positive bacterium that causes porcine streptococcosis (Clifton-Hadley 1984, Staats *et al.* 1997, Dutkiewicz *et al.* 2017, Segura *et al.* 2020). Given the sporadic outbreaks and severe symptoms of *S. suis* infection, a public concern for this zoonotic pathogen has risen. The asymptomatic carriage rate of *S. suis* in pigs is very high ranging up to 80-100% (Staats *et al.* 1997, Zou *et al.* 2018). In pigs, most cases (67.1% in 2017) are reported in North America; where the annual costs to the pig industry reaches 300 million dollars (Dutkiewicz *et al.* 2017). Different biotic and abiotic factors increase the risk of *S. suis* infection. These include co-infections with viruses such as Porcine Reproductive and Respiratory Syndrome Virus (PRSSV), influenza virus, pseudorabies virus; and stress factors leading to immunosuppression such as poor housing, mixing and moving, and overcrowding (Goyette-Desjardins *et al.* 2014, Dutkiewicz *et al.* 2017). Through handling infected meat in slaughterhouses or through consumption of infected and undercooked pork products, humans can also get infected, leading to sporadic outbreaks (Gottschalk *et al.* 2010).

Curative treatment for *S. suis* infection is similar for both humans and pigs and consists of antibiotics administration (Yongkiettrakul *et al.* 2019), although pigs are often found dead before the infection is discovered. In a study of antimicrobial susceptibility of 262 *S. suis* strains, β-lactam antibiotic drugs such as cefotaxime and ceftiofur, vancomycin, chloramphenicol and florfenicol were the most effective therapeutic drugs (Yongkiettrakul *et al.* 2019). This bacterium was also found to be resistant to tetracyclines, azithromycin, clindamycin, and tiamulin. The most frequent genes shared among serotypes that contribute to *S. suis* resistance towards antibiotics involve tetracycline, macrolide, and aminoglycoside resistance (Gurung *et al.* 2015). These resistance genes were also present in strains which do not cause any symptoms in pigs, indicating that these strains can be reservoirs for the resistance genes (Seitz *et al.* 2016, Yongkiettrakul *et al.* 2019). Given the rise of antibiotic resistance, finding alternative treatment to antibiotics is necessary (Murray *et al.* 2022).

Host defence peptides (HDPs) are small protein components of the immune system that have antimicrobial properties towards bacteria, fungi, and viruses. HDPs are

generally rich in cationic and hydrophobic amino acids, which leads to an amphiphilic and positively charged peptide (van Harten *et al.* 2018, Lei *et al.* 2019). Two main classes of mammalian HDPs are defensins and cathelicidins. Cathelicidins are characterized by a precursor domain which resembles cathelin, while defensins are defined by their intramolecular disulphide bonds (Lei *et al.* 2019). The initial interaction of HDPs with bacterial membranes is electrostatic between the positively charged amino acids of the HDP and the negatively charged membrane components, such as lipopolysaccharide (LPS) for Gram-negative bacteria and lipoteichoic acid (LTA) for Gram-positive bacteria. Hydrophobic interactions will also contribute to the peptide membrane interaction and at a high enough concentration of HDP, permeabilization of the membrane occurs. Depending on the specific HDP, this permeabilization is achieved through either pore formation or a more general dissolving and thereby destabilization of the membrane. For a more detailed description of how HDPs permeabilize membranes the reader is referred to some excellent reviews on this subject (Shai 1999, Brogden 2005). Nonetheless, some HDPs have other (or additional) antimicrobial mechanisms in which they first cross the bacterial membrane and subsequently interact with intracellular targets (Beisswenger and Bals 2005). These peptides are often so-called proline-rich HDPs. These HDPs can kill Gram-negative bacteria by blocking enzyme activity like caspase-3 (Ramanathan *et al.* 2004), inhibiting protein and RNA synthesis, as has been shown for bovine HDPs Bac5 and Bac7 (Scocchi *et al.* 2009, Seefeldt *et al.* 2016, Mardirossian *et al.* 2018), and arrest of protein production by insect HDPs oncocin and apidaecin and porcine HDP PR-39 (Krizsan *et al.* 2015, Holani *et al.* 2016) . In addition to the antimicrobial properties, HDPs can also display strong immunomodulatory activities. These include regulation of chemotaxis, wound healing, phagocytosis, activation of immune cells, vascularization, and angiogenesis (van Harten *et al.* 2018, Mookherjee *et al.* 2020).

Since the antibacterial mechanism of action of most HDPs is membrane permeabilization, modifying the entirety of the bacterial membrane to adjust to HDPs would require time and a plethora of mutations. Indeed, many HDPs do not induce resistance over time (Veldhuizen *et al.* 2013, Magana *et al.* 2020, Van Eijk *et al.* 2020). However, activity of cathelicidins is often highly dependent on both the HDP and the specific species or strain of interest (Coorens *et al.* 2017). Although there is

some data available (Xie *et al.* 2019, Meurer *et al.* 2020), the specific activity of many HDPs against *S. suis* remain poorly understood. Here, we show that several porcine HDPs are relatively ineffective against *S. suis*, compared to their antibacterial activity towards other *Streptococci.* Additionally, the L-enantiomers of human LL-37 and chicken CATH-2 are less active than its corresponding D-enantiomer. This is not dependent on the presence of the bacterial capsule, cannot be improved by addition of protease inhibitors but can be transferred to other Gram-positive, sensitive strains through *S. suis*-conditioned media (CM). Finally, the peptides do not lose activity when incubated for 24 h in *S. suis*-CM.

Methods

Bacterial strains

S. suis strains P1/7, S10 and J28 are clinical isolates from human patients (Smith *et al.* 1999, de Greeff *et al.* 2011). *S. suis* strains S10 and J28 (S10 *cps*ΔEF) were provided by Dr. Jesus Arenas Busto. *Staphylococcus aureus* (*S. aureus*) ATCC29213 and *S. pyogenes* ATCC 19615 were obtained from the American Type Culture Collection (ATCC). *S. pneumoniae* 15F and 23F are clinical isolates from the Amsterdam Medical Centre and provided by Prof. Dr. Jos van Putten (van Selm *et al.* 2003). All *Streptococci* strains were grown on 5% sheep's blood tryptic soy agar (TSA) plates for 48 h at 37°C; or in Todd-Hewitt broth (THB)(Oxoid, United Kingdom) overnight in closed cap 15 mL culture tubes (Corning)(Gera and McIver 2013). *S. aureus* was grown on TSA plates overnight at 37°C or in Mueller Hinton broth (MHB) (Difco, United States). All strains were stored at -80°C in their liquid media containing 20% glycerol.

HDPs

All HDPs were synthesized by Fmoc chemistry followed by HPLC purification by Chinapeptides B.V. (Shanghai, China) (Table 1) with a purity of >95%.

Antimicrobial testing

Overnight cultures of all bacteria were diluted 1:100 in fresh medium and incubated at 37 °C for 3-4 h until mid-log phase was reached. Bacteria were spun down, resus-

Table 1. Characteristics of the HDPs used in this study

Peptide	Sequence	Length	Charge
L-CATH-2	RFGRFLRKIRRFRPKVTITIQGSARF-NH_2	26	+9
D-CATH-2	(all-D) RFGRFLRKIRRFRPKVTITIQGSARF-NH_2	26	+9
PMAP-23	RIIDLLWRVRRPQKPKFVTVWVR	23	+6
L-LL-37	LLGDFFRKSKEKIGKEFKRIVQRIKDFLRNLVPRTES	37	+6
D-LL-37	(all-D)LLGDFFRKSKEKIGKEFKRIVQRIKDFL-RNLVPRTES	37	+6
PR-39	RRRPRPPYLPRPRPPPFFPPRLPPRIPPGFPPRFP-PRFP	39	+10
PMAP-36	Ac-GRFRRLRKKTRKRLKKIGKVLKWIPPIVG-SIPLGCG	36	+13
SCR-CATH-2	CRIRFRTFVTKSGIRIPAGRRQRLFK-NH_2	26	+9

pended in fresh media and adjusted to 1 x 10^6 CFU/mL in appropriate media, then mixed 1:1 with a serial dilution of peptides in MilliQ water. After 3 h incubation at 37 °C, each individual bacterial suspension was serially diluted in PBS and 15 µL from each dilution was applied to Tryptic Soy agar plates (TSA) (Oxoid, UK) containing 5 % defibrinated sheep's blood (Oxoid, UK). After 48 h incubation at 37 °C (24 h for *S. aureus*), colonies were counted.

Where appropriate, 1 mM AEBSF (4-(2-aminoethyl) benzenesulfonyl fluoride hydrochloride) (Sigma-Aldrich, USA), 10 µM E64 (N-[N-(L-3-trans-carboxyoxiran-2-carbonyl)-L-leucyl]-agmatine) (Sigma-Aldrich, USA) and 10 µM Pepstatin A (Thermo-Fischer, USA) were added to the bacteria/HDP mixture directly prior to incubation.

S. suis conditioned media

Overnight cultures of *S. suis* or *S. aureus* were spun down, and the conditioned media (CM) or control medium was applied through a 0.2 µm Whatman filter (GE Life Science, US) to remove all bacteria. When indicated, the CM was filtered through a >3 kD Amicon Ultra spin filter column (Merck Millipore, Ireland) for 30 min at 4000 RPM (~2800 x *g*). The resulting 20x concentrated CM was subsequently used in antibacterial assays instead of fresh medium. Where appropriate, the CM or fresh

medium was heat-inactivated prior to peptide incubation by a 30 min incubation at 95 °C.

Time dependent incubation

HDPs were either 24 h or 3 h prior to the experiment or directly at the start of the antimicrobial assays as described above incubated in either *S. suis*-CM or fresh media at 37 °C. Prior to HDP addition, both fresh medium and *S. suis*-CM were stored at 4 °C.

Western blot

Bacterial cultures were incubated with CATH-2 for 3 h at 37 °C, then spun down and separated into pellet and supernatant fractions. Cells were lysed for 5 min at 37°C with cell lysis buffer (20 mM Tris-HCL (pH: 7.5), 150 mM NaCl, 1 mM Na_2EDTA, 1 mM EGTA, 1 % Triton, 2.5 mM sodium pyrophosphate, 1 mM β-glycerophosphate, 1mM N_3VO_4 and 1 µg/mL leupeptin). After addition of cell lysis buffer, sample buffer (30% glycerol, 10% β-mercaptoethanol, 1.2 % SDS, 0.04 % bromophenol blue, 0.15 M Tris-HCl pH 6.8) was added (1:1) and boiled for 5 min at 95 °C. Samples were loaded onto 18% polyacrylamide SDS-PAGE gels and run for 45 min at 28 mA with Tris-glycine running buffer (25 mM Tris, 0.2 M glycine, 0.1 % SDS).

After electrophoresis, the HDPs were transferred to a polyvinylidene difluoride (PVDF) membrane using Trans-Blot Turbo transfer system (Biorad). After 7 minutes of transfer, the PVDF membrane was washed with 20 mL 5 % blocking-buffer (5 % ELK in 5 mL of TBS-T (20 mM Tris, 150 mM NaCl, 0.1 % Tween-20)) and placed on a rolling incubator for 1 h at room temperature. After 1 h, the blocking buffer was washed away with 15 mL of TBS-T for 5 min on a gyratory rocker, repeated 3 times.

Next the PVDF membrane was put into a tube with 1 % ELK in 5 mL TBS-T and 1 µL polyclonal rabbit-anti-CATH-2 (van Dijk *et al.* 2009). The tube was incubated overnight at 4 °C on a rolling incubator.

The following day, the PVDF membrane was washed again 3 times for 5 minutes with 15 mL of TBS-T at RT. After 1 h incubation with horseradish peroxidase (HRP)-labelled goat anti-rabbit antibody (1:5000; Nordic, Tilburg, The Netherlands), membranes were washed twice in TBS-T, followed by a single 10 min wash step in TBS

buffer (20 mM Tris, 0.5 M NaCl, pH 7.5). Immunoreactive protein bands were detected using an Amersham ECL Western Blotting Detection Reagents kit (GE Healthcare Biosciences, Uppsala, Sweden).

Results

S. suis P1/7 is resilient to HDP-mediated killing

Since *S. suis* is primarily a porcine pathogen, several porcine HDPs were tested for their antimicrobial activity against *S. suis*. For this, *S. suis* P1/7 was exposed to HDPs PR-39, PMAP-23, and PMAP-36 for 3 h to determine the minimal bactericidal concentration (MBC). None of these HDPs showed complete killing of *S. suis*, with persisting bacteria remaining at concentrations as high as 40 µM for each of the peptides (Fig. 1A). PMAP-36 showed a complete lack of antibacterial activity as even the highest concentration did not result in partial reduction in viable bacteria. The porcine HDPs were slightly more effective in killing *S. pneumoniae* than *S. suis,* with each of the porcine peptides having an MBC of 20-40 µM (Fig. 1B). Both PMAP-36 and PR-39 were effective at killing *S. pyogenes* with MBCs at 1.25 µM, whereas PMAP-23 could not kill the different bacteria at the highest concentration tested (Fig. 1C). Similarly, PMAP-36 could effectively kill *S. aureus* at 10 µM, whereas PMAP-23 could not (Fig. 1D). Next, we were interested to see whether *S. suis* was also highly resistant to non-porcine peptides. Therefore, cathelicidins CATH-2 and LL-37 were tested in antimicrobial assays. LL-37 also did not kill *S. suis* at concentrations up to 40 µM (Fig. 1A), but CATH-2 showed a potent microbial inhibition at 10 µM and full killing at 40 µM (Fig. 1A). Additionally, it was tested whether the all-D amino acid enantiomers of these HDPs had similar antimicrobial activity against *S. suis*. Interestingly, D-CATH-2 showed much higher activity compared to its L-enantiomer with complete killing of *S. suis* at 5 µM. In contrast, both enantiomers were equally effective at killing *S. pneumoniae* (Fig. 1B) and *Streptococcus pyogenes* (Fig. 1C). The same increase in activity was observed for D-LL-37 compared to L-LL-37 with an MBC versus *S. suis* of 20 µM. A similar result was found for *S. pyogenes,* with 5 µM D-LL-37 killing all bacteria but 40 µM L-LL-37 failing to do so. Surprisingly for *S. pneumoniae,* the opposite order of activity was observed with a 4-fold higher MBC for the D-enantiomer of LL-37 (Fig. 1B). With this relatively small number of HDPs tested with such differing characteristics, activity of HDPs cannot be correlat

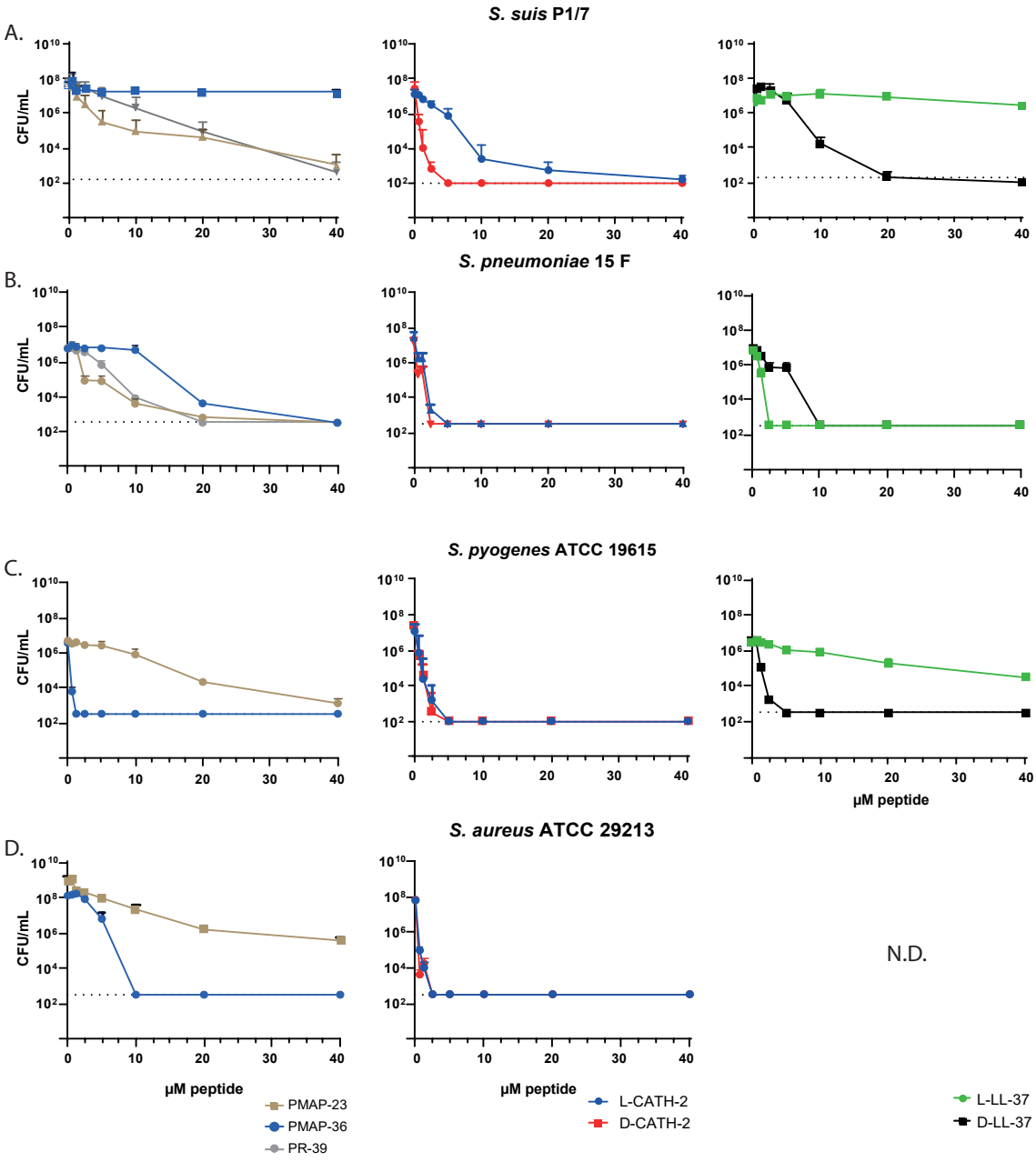

Figure 1: ***S. suis* P1/7 is resilient to cathelicidin-mediated killing.** Gram-positive bacteria *S. suis* P1/7 (A), *S. pneumoniae* 15F (B), *S. pyogenes* ATCC 19615 (C) and *S. aureus* ATCC 29213 (D) were incubated for 3 h with porcine cathelicidins PMAP-23, PMAP-36, or PR-39 (left); L-CATH-2 or D-CATH-2 (middle); or with L-LL-37 or D-LL-37 (right). Surviving bacteria after 3 h incubation is plotted as average CFU ±SEM (n=3).

ed to peptide sequence or chirality (D- vs L-peptides) but the effect is different for different bacterial species. Furthermore, results obtained for CATH-2, LL-37 and the porcine HDPs were consistent among representative strains of serotypes 1, 2, 7 and 9 (Fig. S1). Finally, a scrambled enantiomer of L-CATH-2 (SCR-CATH-2) which had the same amino acids but in a different sequence, so that it lacked amphipathic properties, was not effective against any strain of *S. suis* (Fig. S1A).

Deletion of capsule does not affect activity of D-CATH-2 or porcine HDPs but inhibits L-CATH-2 activity

S. suis seems to be more resistant to killing by LL-37, CATH-2, PMAP-36 and PR-39 compared to other Gram-positive bacteria. The capsule of *S. suis* is a protective barrier that shields the bacterium from various environmental factors including some HDPs (Cole *et al.* 2010, Tanabe *et al.* 2010) and therefore it was tested whether the presence of the capsule or capsule-bound proteins protects *S. suis* against HDP mediated killing. For this, *S. suis* J28, which has parts of the *cps*2E and cps2F genes replaced by an antibiotic resistance gene (Smith *et al.* 1999) and its isogenic control strain S10 with normal encapsulation were used. Despite some small differences (L-CATH-2 was even less effective against the capsule deficient mutant J28), the presence of the capsule of *S. suis* had no effect on the activity of HDPs. The porcine HDPs did not show a striking difference in activity against either strain (Fig 2A). Both L- and D-LL-37 showed a slightly better killing of unencapsulated *S. suis*, although this did not result in lower MBCs (Fig. 2B). The difference between the antimicrobial activity of L-CATH-2 and D-CATH-2 was retained, with D-CATH-2 having an MBC of 2.5 µM versus 20-40 µM for L-CATH-2 (Fig. 2C). Therefore, the observed high resistance of *S. suis* against HDP-killing cannot be explained by components of the bacterial capsule.

The difference in activity of L-peptides and D-peptides could possibly be explained by the presence of a protease secreted by *S. suis*. Therefore, *S. suis* was incubated with several inhibitors of different classes of proteases prior to antimicrobial assays. Neither pepstatin A, inhibiting aspartic proteases (Fig. 3A), nor E64, inhibiting cysteine proteases (Fig. 3B), could affect the antimicrobial activity of L-CATH-2. AEBSF, which inhibits serine proteases, slightly increased activity of L-CATH-2, however the activity of D-CATH-2 was also slightly increased (Fig. 3C). The same pattern was also

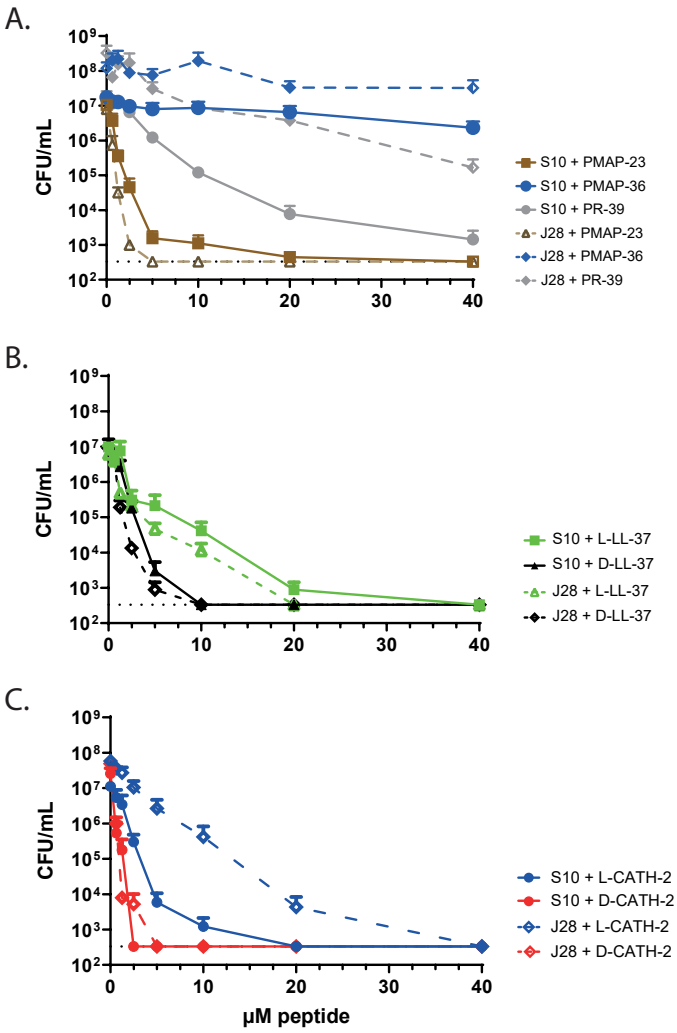

Figure 2: **Deletion of capsule does not affect activity of D-CATH-2 or porcine cathelicidins but inhibits L-CATH-2 activity.** *S. suis* S10 and its isogenic strain J28 (which lacks capsule) were incubated with porcine HDPs PMAP-23, PMAP-36 or PR-39 (A); L-LL-37 or D-LL-37 (B); or L-CATH-2 or D-CATH-2 (C). Surviving bacteria after 3 h incubation is plotted as average CFU +SEM (n=3).

observed for L-LL-37 and D-LL-37 with these protease inhibitors (Fig. 3D), indicating a possible side effect of AEBSF on *S. suis*. Furthermore, to detect potential proteolytic cleavage of peptide, L-CATH-2 was incubated with *S. suis* and subsequently detected on Western blot using specific anti CATH-2 antibodies. However, these experiments did not show a band shift nor obvious decrease of intensity of CATH-2

again indicating that it is unlikely that a protease inactivates CATH-2 (Fig. S2).

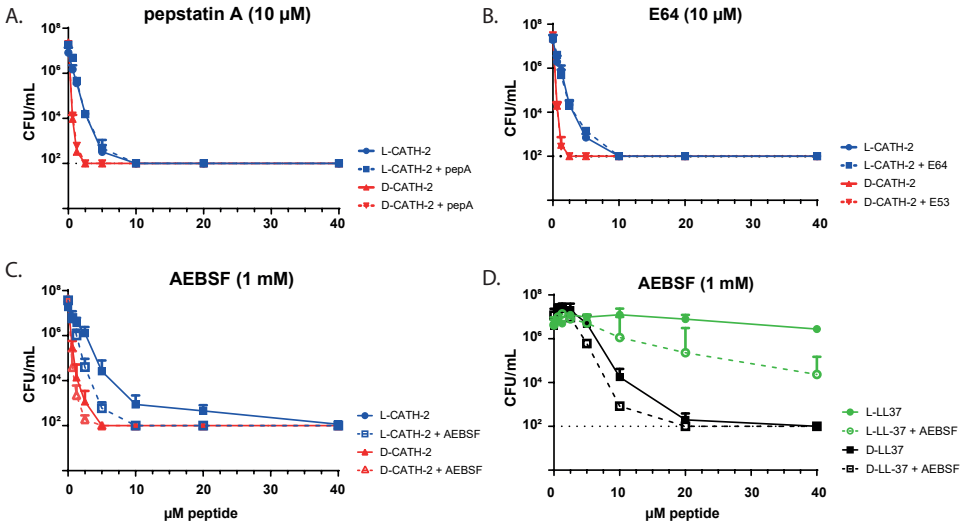

Figure 3: Protease inhibitor AEBSF slightly increases HDP activity, while Pepstatin A and E64 have no effect. *S. suis* P1/7 was incubated with L-CATH-2 or D-CATH-2 in the presence of 10 µM aspartyl protease inhibitor pepstatin A (A); or 10 µM cysteine protease inhibitor E64 (B); or *S. suis* was incubated with L-CATH-2 or D-CATH-2 (C) or L-LL-37 or D-LL-37 (D) with 1 mM serine protease inhibitor AEBSF. Surviving bacteria after 3 h incubation is plotted as average CFU ±SEM (n=3).

S. suis, *S. pyogenes* or *S. aureus*-CM reduces L-CATH-2 activity against *S. aureus* but not D-CATH-2 activity

To examine whether *S. suis* secretes other soluble compounds that inactivate HDPs, the peptides were incubated with an HDP-susceptible bacterium in the presence of various *Streptococcus*-conditioned medium (CM). *S. aureus* was determined to be a susceptible strain, with equal activities for L-CATH-2 and D-CATH-2 in fresh THB culture medium (Fig. 4A). *S. suis*-CM increased specifically the MBC of L-CATH-2, from 0.625 µM to 2.5 µM (Fig. 4B). Similarly but to a lesser extent, *S. pyogenes*-CM increased the MBC of L-CATH-2 but not for D-CATH-2. *S. pneumoniae*-CM led to complete killing of *S. aureus* in the absence of HDPs, likely due to production of bacteriocins (Dawid *et al.* 2007)(data not shown). However, also *S. aureus* CM, transferred to a fresh S. aureus suspension led to a similar shift in susceptibility towards L-CATH-2 (but not D-CATH-2) indicating that the cause of this shift in MBC is not a *Streptococcus* specific factor.

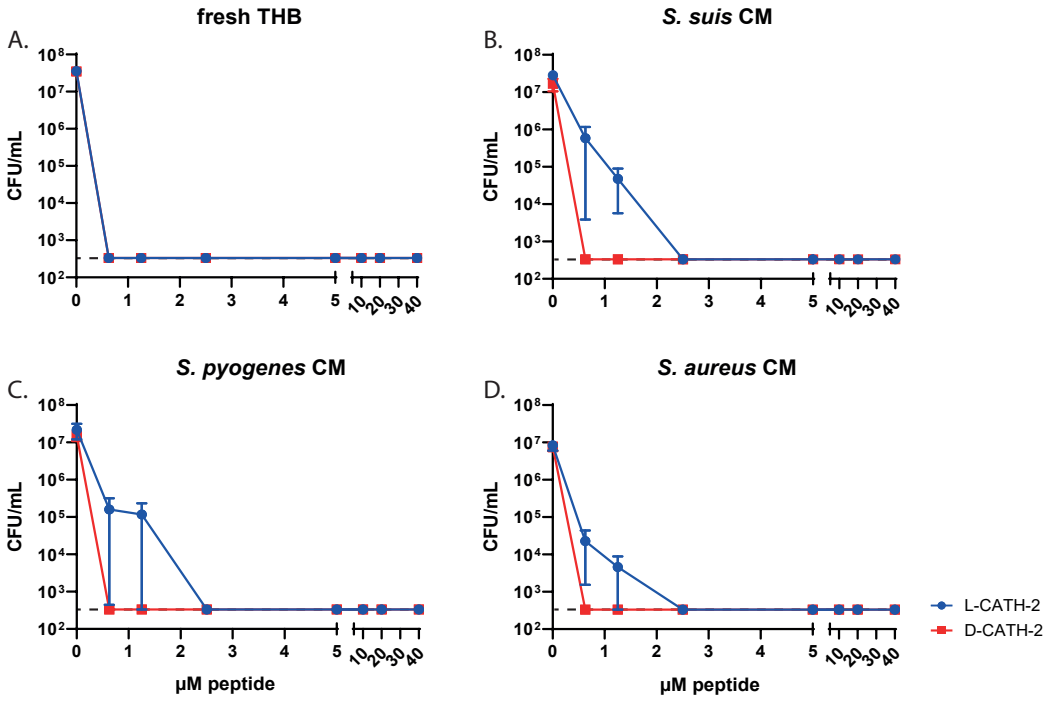

Figure 4: CM transfer of *S. suis, S. pyogenes* or *S. aureus* reduces L-CATH-2 activity towards S. aureus but not activity of D-CATH-2. *S. aureus* ATCC29213 was incubated with L-CATH-2 or D-CATH-2 in the presence of fresh THB (A); CM of *S. suis* P1/7 (B); CM of *S. pyogenes*(C); or CM of *S. aureus* (D); Surviving bacteria after 3 h incubation is plotted as average CFU +SEM (n=3).

S. suis secretes its protective compound into supernatant

If the HDPs are actively degraded by a *S. suis* secreted compound incubation in *S. suis*-CM would decrease the activity of peptides in a time-dependent manner. Therefore, both L- and D-CATH-2 were pre-incubated in *S. suis*-CM at 37 °C before addition to the *S. suis*. The activity of 2.5 µM L-CATH-2 decreased slightly in 24 h incubation in *S. suis* CM (Fig. 5A), and the activity of D-CATH-2 stayed the same. Neither peptide showed a difference in activity after 24 h incubation in THB (Fig. 5B). To test whether a different kind of protein could be present that inactivates or binds CATH-2, the CM was first concentrated on a 3 kD spin filter column, to increase the concentration of higher molecular weight targets. When the antimicrobial assay was performed in concentrated CM of *S. suis*, both L-CATH-2 and D-CATH-2 became less effective (Fig. S3). The MBC of L-CATH-2 shifted to 40 µM from 5 µM in

concentrated control medium (Fig. S3). Additionally, it was tested if the inhibiting component was heat labile. However, heat inactivation only changed the MBC of L-CATH-2 from 40 µM to 20 µM and did not change the MBC in heat-inactivated control media (Fig. S4).

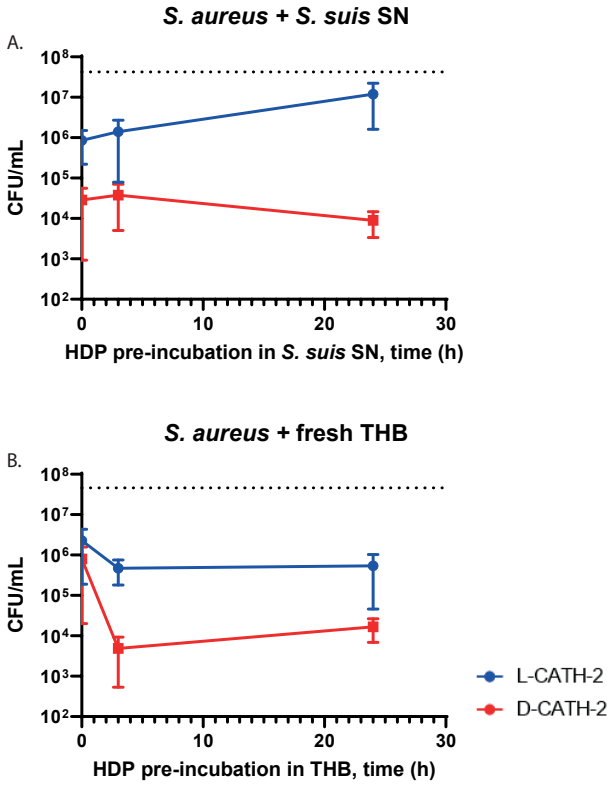

Figure 5: **Pre-incubation of L-CATH-2 in *S. suis* SN slightly decreased its activity against *S. aureus*.** L-CATH-2 and D-CATH-2 were incubated for 0, 3, or 24 h in CM of log-phase *S. suis* P1/7 (A) or THB only (B), before exposure to *S. aureus* for 3 h. Surviving bacteria after 3 h incubation is plotted as average CFU +SEM (n=3). The dotted line represents survival of *S. aureus* in the SN or THB in the absence of peptides.

Discussion

HDPs could be a promising treatment option for bacterial infections, as they possess both antimicrobial and immunomodulatory functions, and transferable resistance to killing by HDPs has been rare so far (Hancock *et al.* 2016, van Harten *et al.* 2018, Mookherjee *et al.* 2020). However, the genus *Streptococcus* possesses various innate mechanisms to protect itself from HDP mediated killing (for an excellent

review, see (LaRock and Nizet 2015)). Additionally, the killing capacity of HDPs has been shown to vary between peptides when directly compared to each other (Coorens *et al.* 2017). It is therefore essential to test how effective peptides are against a particular species and attempt to understand potential bacterial evasion mechanisms before HDPs can be considered as treatment.

No broad assessment has been made of the antimicrobial potential of HDPs against *S. suis.* Here, we chose a selection of porcine HDPs, as pigs are the bacterium's natural host (Clifton-Hadley 1984). None of the tested porcine HDPs were particularly effective against *S. suis*. This is an indication that the relatively low activity of these peptides could be a host adapted response. However, the HDPs were not very effective against *S. pneumoniae,* either. It could therefore be a streptococcal property, or a culture medium related property. The effect of the test medium on activity of HDPs is well known. For example, for PMAP-36 it was shown that the observed MBC against *S. aureus* changed from 0.3 µM in DMEM to 10 µM in MHB medium (Coorens *et al.* 2017). It should be noted that in the present study the activity of PMAP-36 against *S. suis* in DMEM was not tested. Furthermore, we wanted to know whether there was a general mechanism of the defence of S. *suis* against HDPs and whether this process would involve molecular recognition of HDPs. Both L-CATH-2 and L-LL-37 were less effective than its D-enantiomer against *S. suis* P1/7 (Fig. 1A). This is not the case for all species as both *S. pyogenes* and *S. aureus* are effectively killed to a similar extent by both L- and D-CATH-2. Therefore, it is likely that *S. suis* has a protective mechanism against the porcine HDPs and L-CATH-2 and L-LL-37 but not against D-CATH-2 and D-LL-37. It is at this point unclear whether the same mechanism underlies the evasion of killing by all HDPs, or whether *S. suis* has multiple contributing factors.

When assessing the influence of the capsule on cathelicidin activity, it was found that the differential effect of L-CATH-2 and D-CATH-2 against *S. suis* was retained in the capsule mutant (Fig. 2C). Surprisingly, L-CATH-2 was even less effective against the capsule mutant J28. It is possible that the negatively charged sialic acids of the capsular polysaccharide increase the initial interaction between bacterium and HDP and thereby the efficacy of the positively charged peptide (Van Calsteren *et al.* 2010). Moreover, the porcine HDPs and L-CATH-2 were slightly less effective against

the capsule mutant J28 (Fig. 2A). However, in these experiments, the starting concentration of capsule mutant was higher, which could have influenced the activity of the peptides, as there is less peptide available per individual bacterium. However, since the activity of the HDPs clearly did not improve by the absence of the capsule, it is unlikely that *S. suis* evades HDP-mediated killing through a capsule-related mechanism.

Since HDPs including L-CATH-2 are proteolytically vulnerable but D-CATH-2 is not (Molhoek *et al.* 2011), we suspected that *S. suis* can evade killing through production of a protease. For example, proteases ApdS and DPPIV from streptococci have been shown to degrade and inactivate LL-37 and PR-39, respectively (LeBel *et al.* 2018, Xie *et al.* 2019). However, we did not see a clear difference in activity when the bacteria were incubated in the presence of several protease inhibitors (Fig. 3). This assay does have an important disadvantage: a putative protease could be unaffected by the specific inhibitor. This cannot be excluded based on the data we collected. However, the fact that none of these inhibitors were effective is an indication that proteases are not involved in protection. There are further observations that make the presence of a protease as the cause of high resistance of S. suis towards HDPs unlikely. Detection of CATH-2 on a Western blot with a polyclonal antibody after incubation with *S. suis* did not lead to a band shift nor decrease of intensity, which could indicate that the peptide is still intact. The absence of a time dependent effect is not in line with the activity of a protease, although it should be noted that both Western blotting and time-dependency was only investigated for the CATH-2 peptides and not the porcine HDPs. Mass spectrometry could show definitively whether there is degradation of the peptides in the presence of *S. suis*.

Since proteases did not seem to be involved, it was investigated whether a secreted protein could be responsible for *S. suis* evasion. Therefore, CM from *S. suis* was concentrated to see whether this could transfer the protective capacity to a susceptible strain, in this case *S. aureus*. In this concentrated CM, L-CATH-2 was indeed less effective than D-CATH-2 (Fig. 4B). This difference was much larger than in the concentrated medium control (Fig. 4C) and additionally, heat inactivation of the conditioned medium could not restore the activity of the peptide in this environment (Fig. 5A). However, concentrated CM of *S. aureus* had the same effect.

This observation could have 2 different explanations: either concentration of CM has a profound effect on the activity of specifically L-CATH-2 (and not D-CATH-2) or both bacteria produce a secreted product that can protect against L-CATH-2 only. Since this effect is also observed after heat inactivation, it is unlikely that the factor is a *S. suis* protein, although heat-resistant proteins also exist. It is also unknown if both bacteria produce the same secreted product that inhibits L-CATH-2. This would require further experimentation. Considering the amphipathic properties of CATH-2, and its LPS binding property (Van Dijk *et al.* 2016, Scheenstra *et al.* 2019), the secreted factor could be a lipid or a lipidated protein. Treatment of the CM with various lipases could possibly give more insight on the mechanism of cathelicidin inactivation. However, it is unclear if this could explain the difference between L-CATH-2 and D-CATH-2 since in that case both peptides must have different binding properties to these lipids. Additionally, investigation of other components in these media such as nutrients, pH, cations, or DNA/RNA could also lead to better understanding of the observed results.

Previously, it has been shown that *S. pneumoniae* shed their capsule upon exposure to LL-37 through the protein LytA (Kietzman *et al.* 2016) in order to reduce the quantity of HDPs close to their surface (Beiter *et al.* 2008). For *S. pneumoniae,* D-LL-37 is less effective than L-LL-37 (Fig. 1B). It is possible that due to, for example, a higher proteolytic stability D-LL-37 is a better inducer of LytA. Furthermore, we see that for *S. suis*, L-LL-37 is not active whereas D-LL-37 shows moderate antimicrobial activity. Additionally, the activity of L-LL-37 can be improved by addition of AEBSF, a serine protease inhibitor. As *S. suis* is known to express a serine protease ApdS that degrades LL-37 (Xie *et al.* 2019), these results highlight that different species of *Streptococci* have different mechanisms to protect against antimicrobial peptides. The addition of AEBSF has a mild effect on the activity of D-LL-37 as well as both L-CATH-2 and D-CATH-2. At this time, we cannot exclude either a broadly active protease that has affinity for D-peptides, or the involvement of a protease that is required downstream of a protective protein. However, neither of these would explain the protective role the concentrated CM has.

In conclusion, *S. suis* evades killing of HDPs, including porcine HDPs and L-CATH-2 but not D-CATH-2. Particularly the inactivity of porcine HDPs could point towards

a host specific adaptation, as these would be the peptides the bacterium would encounter in its natural environment. Since these peptides display almost no sequence homology, a general mechanism would be expected, which could explain a partial inactivation of L-CATH-2. Although data suggests that the inhibiting compound could be a lipid or lipoprotein, it remains unclear at this time what the molecular basis of this mechanism is, but D-CATH-2 is not affected by this mechanism and retains a high antimicrobial activity in the presence of *S. suis* CM. Additionally, D-CATH-2 enhances the response of murine macrophages to *S. suis* (van Harten *et al.* 2021). However, it remains unknown whether D-CATH-2 is equally effective in treating *S. suis* infections of either humans or pigs. In pigs, treatment with HDPs as antimicrobials is unlikely, as the cost of peptide synthesis is high and curative treatment for infections in farm animals is rare. Nevertheless, D-CATH-2 could be a good therapeutic candidate for treatment of *S. suis* infections in humans, as it both kills the bacteria and improves the host's immune response, increasing the probability of successful treatment and reducing the probability of resistance development.

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Declarations of interest: none.

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Supplemental figures

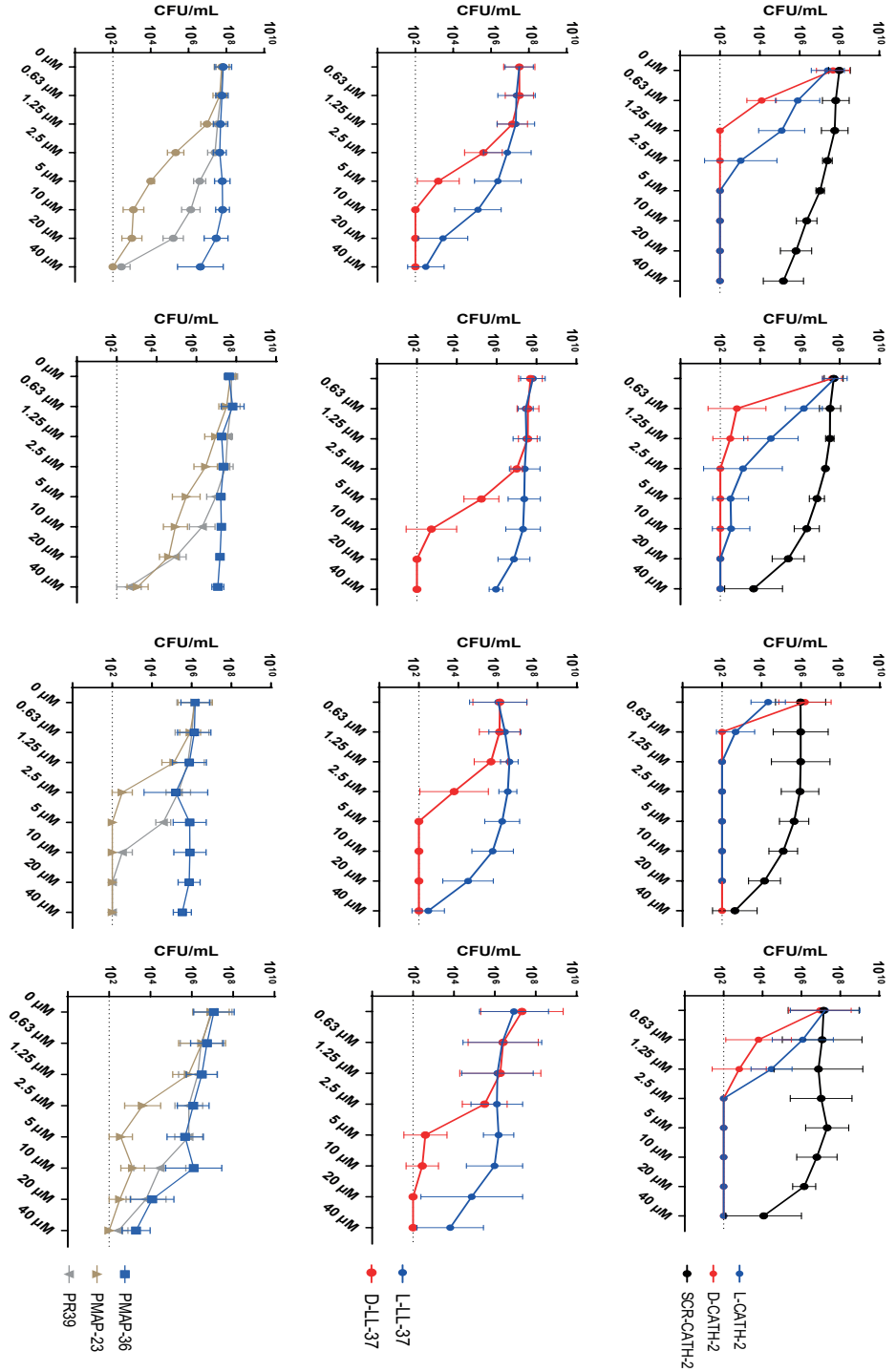

Figure S1: **Multiple serotypes of *S. suis* are resilient to cathelicidin mediated killing.** Reference strains of *S. suis* for serotype 1 (NCTC428), serotype 2 (P1/7), serotype 7 (15009) and serotype 9 (22083) were incubated for 3 h with L-CATH-2 or D-CATH-2 or a scrambled control of CATH-2 (top row); with L-LL-37 or D-LL-37 (middle row); or porcine cathelicidins PMAP-23, PMAP-36, or PR-39 (bottom row). Average CFU surviving after 3h of incubation are plotted as +SEM (n=3).

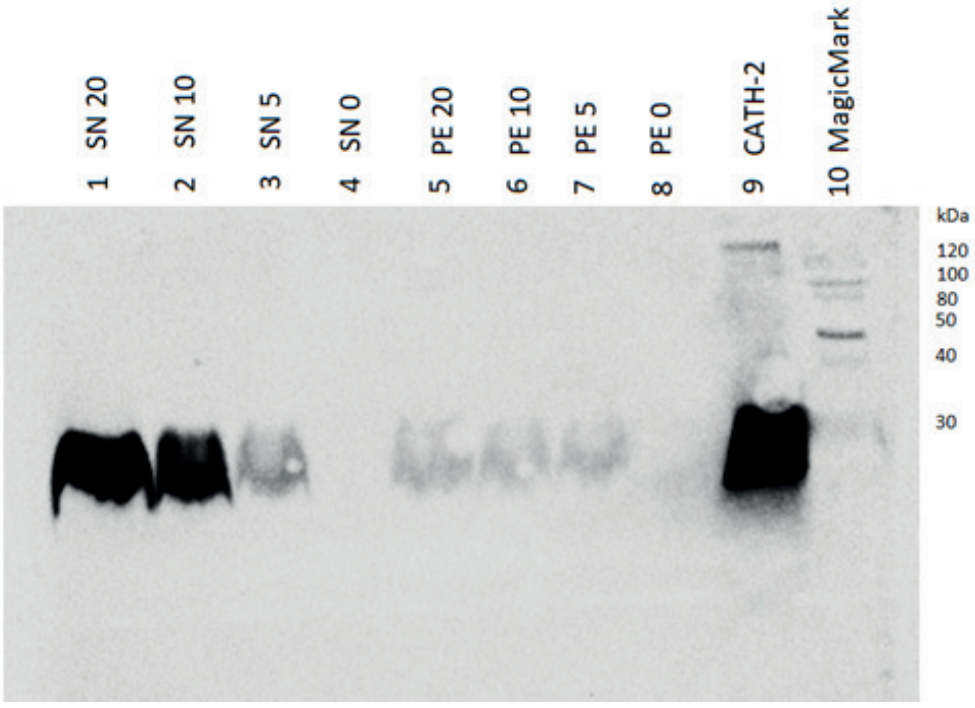

Figure S2: **Incubation of *S. suis* with increasing concentration of L-CATH-2 lead to saturation of the pellet but not to a shift in band width or intensity.** *S. suis* P1/7 was incubated for 3h in the presence of 0, 5, 10 or 20 µM CATH-2, then supernatant (SN) and pellet (PE) fractions were collected and processed for Western blot of CATH-2. 1,6 nmol CATH-2 in water is loaded as a control (9).

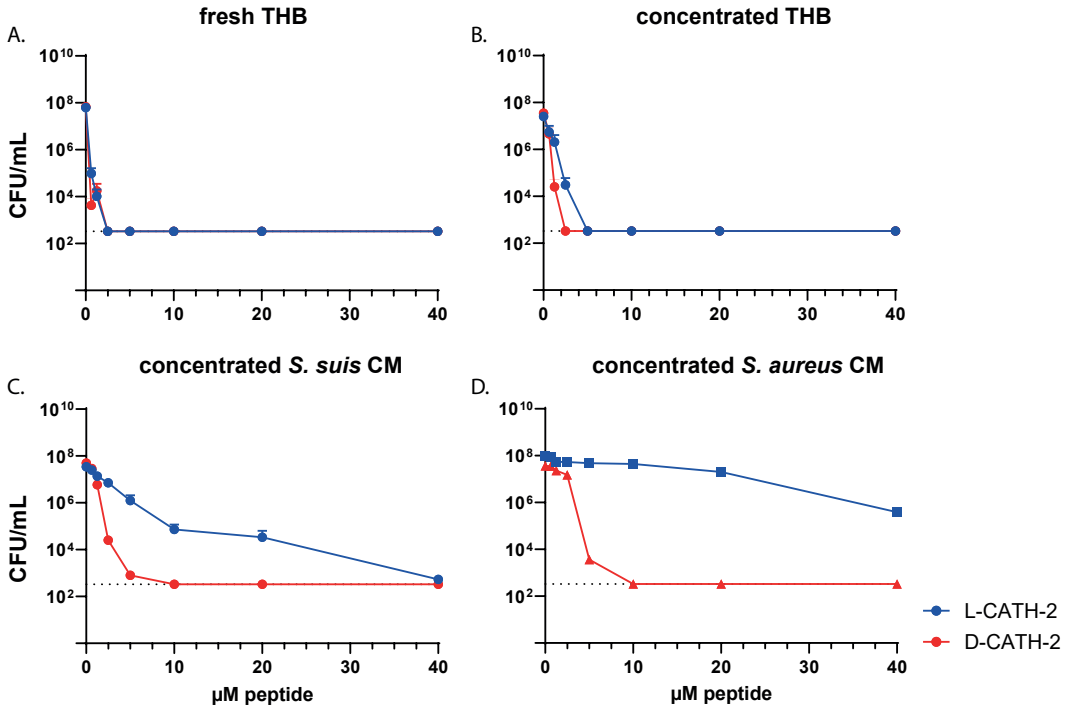

Figure S3**: Transfer of concentrated CM of *S. suis* as well as of *S. aureus* transfers protection against L-CATH-2 to *S. aureus*.** *S. aureus* ATCC29213 was incubated with L-CATH-2 or D-CATH-2 in the presence of fresh medium (A), 20x concentrated THB (B); CM of *S. suis* P1/7 (C); or CM of *S. aureus* ATCC29213 (D). Average CFU after 3 h incubation is plotted as ±SEM (n=3).

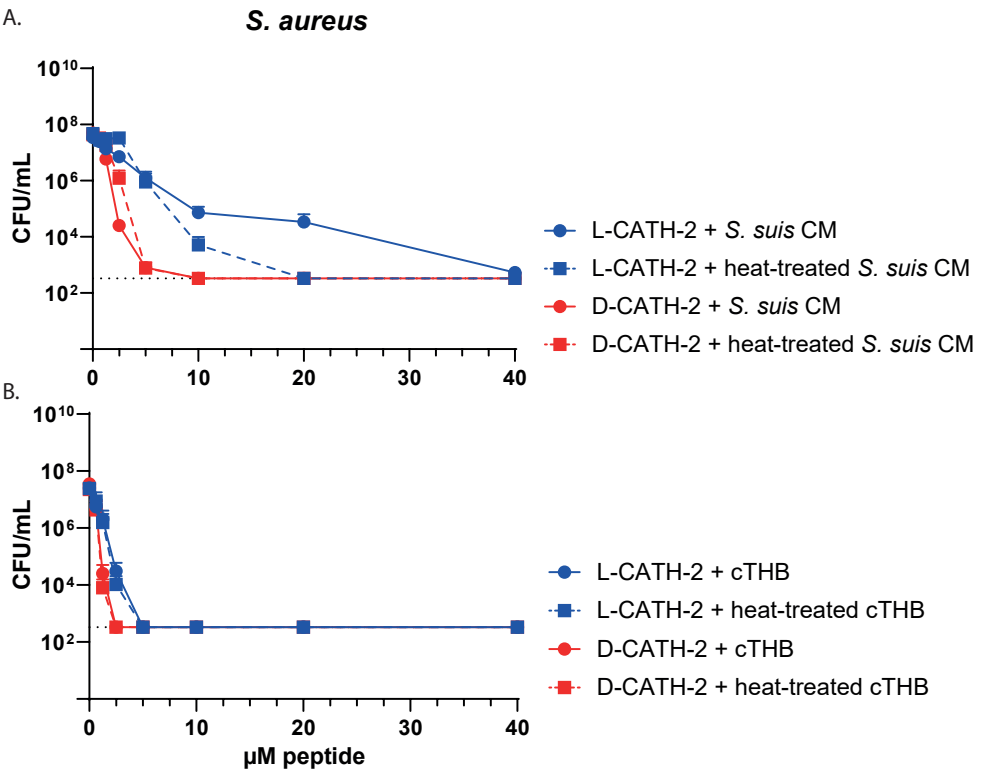

Figure S4: **Heat inactivation does not reduce protective capacity of concentrated CM *S. suis*.** *S. aureus* ATCC29213 was incubated with L-CATH-2 or D-CATH-2 in the presence of 20x concentrated CM of *S. suis* P1/7 grown overnight in THB with a heat-inactivated control (A); or 20x concentrated THB control with a heat-inactivated control (B). Average CFU after 3 h incubation is plotted as +SEM (n=3).

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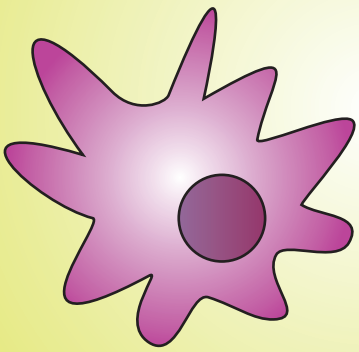

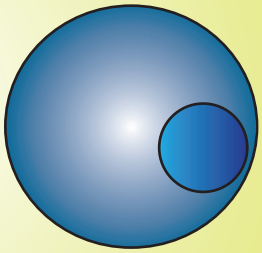

Chapter 5

Activation of Porcine Bone Marrow derived DCs by *E. coli* and *S. suis* is inhibited by Chicken Host Defence Peptide CATH-2

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Abstract

Streptococcus suis is an infectious Gram-positive bacterium that is a threat to both humans and pigs, exacerbated by antimicrobial resistance. Vaccinating pigs could be an effective solution but has thus far remained unsuccessful. Host defence peptides could play a part in modulating immune responses to vaccines, but not much is known about porcine dendritic cell responses to *S. suis.* To better characterize these, we cultured bone marrow derived cells from pigs into dendritic cells using GM-CSF and IL-4 and stimulated them with either LPS, *E. coli* or *S. suis* in the presence of peptides and measured the production of cytokines and the expression of cellular markers and quantified the uptake of GFP-labelled bacteria. These experiments showed that the cathelicidin CATH-2 is highly effective at neutralizing both LPS and bacterial activation of DCs. CATH-2 does not reduce uptake of *S. suis*. Conversely, the porcine cathelicidin PR-39 does not reduce activation of DCs but boosts uptake of unencapsulated *S. suis.* The results indicate that either peptide could potentially be used to modulate the DC response to a *S. suis* vaccine, to either reduce toxic overactivation or potentially increase antigen uptake and processing.

Introduction

Antibiotic resistance has become increasingly problematic in both veterinary and human practices. *Streptococcus suis* (*S. suis*), a Gram-positive bacterium with pigs as a natural host, can develop resistance against conventional antibiotics (Munita and Arias 2016, Seitz *et al.* 2016, Yongkiettrakul *et al.* 2019), providing a challenge to treat infected pigs. In adult pigs, carriage of *S. suis* is common and most adult pigs have formed natural immunity against environmental strains (Lowe *et al.* 2011). Clinical signs associated with infection consist of sepsis, arthritis, pneumonia, meningitis, and sudden death (Reams *et al.* 1994, Haas and Grenier 2018). The zoonotic potential of *S. suis* emphasizes the importance of finding effective treatments. An improvement of porcine health subsequently reduces the risk for human infection and prevents economic losses in pig industry (Haas and Grenier 2018). For pigs, an attractive alternative would be to develop a vaccine against *S. suis.* But despite many efforts and promising results in mouse models (Li *et al.* 2011, Zhou *et al.* 2015) candidate vaccines have not proven effective in pigs. Therefore, expanding the knowledge regarding the interaction of porcine immune cells with *S. suis* could help build a platform for further vaccine development.

Dendritic cells are the central immune cells in the development of an effective immune response upon infection, but obviously also upon vaccination. *In vitro*, a pro-inflammatory response of multiple cytokines including TNFα, IL-12p70 was triggered by *S. suis* stimulation of murine bone marrow derived dendritic cells (BMDCs), through activation of Toll Like Receptor (TLR)2 (Lecours *et al.* 2012). TLR2 is known to recognize Gram positive-derived PAMPs, such as lipoteichoic acid (LTA), although a more recent study showed that *S. suis* LTA is not that good at activating TLR-2 in murine DCs (Gisch *et al.* 2018, Auger *et al.* 2019). A subsequent contributing factor that hampers an effective immune response is the capsular polysaccharide surrounding the cell wall of *S. suis* (Graveline *et al.* 2007, Meijerink *et al.* 2012). This capsule shields the bacterium from recognition and subsequent phagocytosis by innate immune cells. Therefore, dendritic cells exposed to encapsulated *S. suis* are inadequately activated, are unable to process antigen or fully upregu-

late their cell surface markers and have impaired cytokine production. All of these block establishment of protective immunity against *S. suis* (Smith *et al.* 1999, Segura *et al.* 2004).

Regarding vaccine development, proper adjuvants can help to boost the immunogenicity of an otherwise insufficiently active subunit vaccine (Coffman *et al.* 2010). For *S. suis*, using an adjuvant that can support dendritic cells in their recognition of *S. suis* could be key to promote vaccine efficaciousness. Promising adjuvants are the host defence peptides (HDPs) (Kindrachuk *et al.* 2009, Kovacs-Nolan *et al.* 2009a, Kovacs-Nolan *et al.* 2009b, Mackenzie-Dyck *et al.* 2014, van Harten *et al.* 2018, Scheenstra *et al.* 2020), which are part of the innate immune system of vertebrates. HDPs are small, amphipathic, and positively charged peptides (Zanetti 2005, van Harten *et al.* 2018). Two major families of HDPs are described: defensins, characterized by their three characteristic intramolecular disulphide linkages, and cathelicidins that are characterized by their precursor sequence that resembles cathelin. Both HDP families are antimicrobial against many multidrug resistant bacteria (Coorens *et al.* 2017b, Lei *et al.* 2019, Magana *et al.* 2020) but are increasingly appreciated for their immunomodulatory properties. For instance, the porcine cathelicidin PR-39 shows broad antimicrobial activity, induces *in vitro* cytokine production in macrophage cell lines, and exerts an anti-apoptotic effect in several cell types, including endothelial cells (Veldhuizen *et al.* 2014a, Holani *et al.* 2016). Despite its broad antimicrobial effect, PR-39 is not effective against *S. suis,* which produces a protease capable of degrading PR-39 (Jobin *et al.* 2005, LeBel *et al.* 2018).

Besides PR-39, there are 10 other porcine cathelicidins described: five protegrins (PG-1 to PG-5), 2 prophenins, and 3 porcine myeloid antimicrobial peptides (PMAP-23, PMAP-36, and PMAP-37) (Sang and Blecha, 2009; Wessely-Szponder *et al*., 2010). Many of these have proven antimicrobial and to some extent immunomodulatory activity (Scheenstra *et al.* 2019). Veldhuizen *et al*. (2017) showed that PMAP-23 can induce IL-8 production in a porcine macrophage cell line. PMAP-23 also lowered the uptake of latex beads by monocytes. PG-1, PMAP-23, PMAP-36 and PR-39 have been shown to

increase nucleic acid uptake in porcine plasmacytoid DCs and subsequent activation (Baumann *et al.* 2014).

For potential immunomodulation of porcine immune cells, the repertoire is not restricted to HDPs of the same species, since HDPs often show potent cross species reactivity (Coorens *et al.* 2017b). An interesting HDP for our studies is the chicken peptide CATH-2, which has multiple described immunomodulatory activities. For example, it is known to bind LPS, which prevents TLR-4 activation (van Dijk *et al.* 2009). The HDP also has direct effects on chicken immune cells without TLR ligands (Kraaij *et al.* 2017, Kraaij *et al.* 2020) and binds DNA, promoting uptake and TLR-9 signalling (Coorens *et al.* 2015). In addition, this HDP kills *S. suis* at relatively low concentrations (van Harten *et al.* 2021). The D-isomer form of CATH-2 (D-CATH-2), which cannot be proteolytically degraded, has shown to provide protective effects in chicken after *in ovo* injection against pathogenic *Escherichia coli* (*E. coli*) infection, even up to 7 days after hatching (Cuperus *et al*., 2016). When human macrophages were exposed to D-CATH-2, they gained a higher pro-inflammatory capacity through epigenetic reprogramming, which is a process called trained immunity (Netea *et al.* 2016, van Dijk *et al.* 2022)

For a large part, it is still unknown how porcine dendritic cells respond to most HDPs *in vitro,* especially in the context of the relevant pathogen *S. suis.* Here, we show that bone marrow derived DCs from pigs are efficiently inhibited by the HDPs in their response to LPS. Additionally, both L- and D-CATH-2 inhibit cytokine production of DCs in response to both *S. suis* S10 and its unencapsulated variant J28. However, of the peptides tested, only PR-39 could increase uptake of *S. suis*, showing its potential as vaccine adjuvant. Overall, these data provide more insight on the specific modulation by various HDPs in porcine DCs and could form the basis for more detailed applications of HDPs as a component of vaccines.

Methods

Peptides

All peptides were synthesized by Fmoc chemistry on a chemical synthesizer (China

peptides, Shanghai, China), and C-terminally amidated to improve stability (Table 1). Purity was >95 % for each peptide. After lyophilization, the peptides were reconstituted in MilliQ water and further diluted in appropriate culture medium.

Table 1. **Peptide sequence, length, and charge.**

Peptide	Sequence	Length	Charge
CATH-2 (L- or D-)	RFGRFLRKIRRFRPKVTITIQGSARF-NH_2	26	+9
PMAP-23	RIIDLLWRVRRPQKPKFVTVWVR	23	+6
LL-37 (L- or D-)	LLGDFFRKSKEKIGKEFKRIVQRIKDFL-RNLVPRTES	37	+6
PR-39	RRRPRPPYLPRPRPPPFFPPRLPPRIPPGFP-PRFPPRFP	39	+10

Cells

Porcine bone marrow derived cells were isolated as previously described (Gao et al., 2018) from bone marrow of 16 pigs in total, either Great Yorkshire, Large White or F1 of Large White and Nordic Landrace-pigs, aged 5-6 months (70-80 kg). (Van Beek SPF Varkens, Lelystad, The Netherlands). The animals were raised as SPF experimental animals, but not housed behind a barrier after arrival. The animals were allowed to acclimatize between 7 and 20 days, before being put under terminal anaesthesia. The animals were then used as educational models for complex surgical techniques for medical doctors and operating room staff. After training, bone marrow was harvested by hip bone puncture by a trained veterinarian. All animals were used and kept under licence of the Central Laboratory Animal Committee of the Netherlands under the advice and the approval and guidelines of the animal ethical committee of Utrecht University.

After isolation by hip bone puncture, the bone marrow was homogenized through a 70 µm cell strainer (Corning, Sigma-Aldrich). The cell suspension was pipetted onto a Ficoll density gradient of 1.077 g/ml (GE Healthcare Bio-Science AB, Sweden), followed by centrifugation at 900 x *g* for 20 min at room temperature with a slow start and brake.

The cells from the buffy coat were collected and washed with Dulbecco's phosphate buffered saline (dPBS) (GIBCO, Invitrogen). The washed cells were stored in foetal calf serum (FCS) (Bodinco B.V., Alkmaar, The Netherlands)/10% dimethyl sulfoxide (Sigma-Aldrich) at a concentration of 6 x 10^7 cells/ml in liquid nitrogen until further use.

Dendritic cell culture

Bone marrow cells from liquid nitrogen were slowly thawed and seeded into 96-well culture plates at a density of 0.5 x 10^6 cells per ml in Roswell Park Memorial Institute (RPMI) 1640 medium (GIBCO, Invitrogen), supplemented with 10% FCS and 1% 10 U/mL penicillin and 10 mg/mL streptomycin (Thermo Fisher Scientific), for 7 days at 37 °C, 5% CO_2. For bacterial assays, the antibiotics were left out of the cell culture medium.

Recombinant pig granulocyte-macrophage colony-stimulating factor (GM-CSF) (40 ng/ml) (Sanbio B.V., Uden, The Netherlands) and recombinant porcine IL-4 (40 ng/ml) (R&D systems, Minneapolis, MN, USA) were added to the wells to generate bone marrow-derived dendritic cells. Cells were fed on days 3 and 6 of culture with fresh medium containing GM-CSF and IL-4. After six days of culture, the supernatant was removed from the cells and replaced. Cells were stimulated with 100 ng/ml LPS from *Escherichia coli* 0111:B4 (Invivogen) as a positive control, or with bacteria (see below) in the presence of various concentrations of different cathelicidins.

The cells were stimulated at 37°C for either 24 h (LPS assays) or 2.5 h (bacteria). For the latter, the cell supernatant was collected and frozen at -20 °C for further analysis (ELISA), after 2.5 h. RPMI medium supplemented with FCS, GM-CSF, IL-4, and antibiotics (penicillin/streptomycin), was added to the cells for the remaining 21 h.

Bacterial assays

The *S. suis* isolate S10, its non-encapsulated mutant J28, and GFP-labelled *S. suis* were a gift from Dr. Jesús Arenas Busto, Utrecht University. 15 ml Todd Hewitt Broth (THB)(Oxoid Limited) medium was inoculated with *S. suis*-S10 or *S. suis*-J28 for 16 h at 37 °C in a small closed cap centrifuge tube (15 ml), to allow the bacteria grow in approximate anaerobic conditions (Gera and McIver 2013).

From this suspension, 300 µl was pipetted into 15 ml of fresh THB medium and the bacteria were grown to log-phase in 4 h (at 37 °C with shaking). After centrifugation (at 1200 x g for 10 min), the log-phase bacteria were resuspended in THB medium and the optical density (OD) was measured at 620 nm., The sample was diluted to obtain a solution of 5 x 10^6 bacteria per ml and washed twice with dPBS. For several assays, log-phase bacteria were heat-killed at 80 °C for 1 hour. The bacteria were resuspended in cell culture medium and added to the dendritic cells as described below.

As a control after each experiment, the number of viable bacterial was checked by serial dilution and subsequent spread plating onto tryptone soy agar (TSA)(Oxoid Limited) plates, supplemented with 5% sheep blood (Thermo Scientific, Oxoid Limited). The plates were incubated overnight at 37 °C and colonies were counted. T=0 plates were also counted to verify the MOI.

Flow cytometry

On day 7, after 24 h stimulation, the supernatant from the dendritic cell cultures was collected and frozen at -20 °C. Subsequently, the cells were detached from the culture plates using a brief incubation at 37°C with PBS supplemented with 0.5 mM EDTA. The cells were detached by vigorous pipetting, washed with FACS buffer (0.5 % BSA in PBS), and transferred to V-bottom plates, followed by centrifugation at 700 x g for 2 min. After washing, the cells were stained for 20 minutes (on ice) with labelled monoclonal antibodies (mAbs) for flow cytometry (FACSCantoII, BD Biosciences, San Jose, CA, USA).

The following mAbs were used: CD14-PB (clone TÜK4, AbD Serotech, dilution 1:100), SWC3a-PE (clone 74-22-15, Abcam, dilution 1:1000), Monocytes/Granulocytes mAb-PE (clone 74-22-15, Invitrogen, dilution 1:4000), SLA-II-FITC (clone 2E9/13, Thermo Scientific, dilution 1:1000), CD8α-AF647 (clone 76-2-11, BD Biosciences, dilution 1:500), CD4-PE-Cy7 (clone 74-12-4, BD Biosciences, dilution 1:1000), CD3ε-PerCP-Cy5.5 or CD3ε-AF647 (clone BB23-8E6-8C8, BD Biosciences, dilution 1:1000 or 1:250, respectively), and a soluble APC-labelled CTLA4-mouse immunoglobulin fusion protein for the detection of CD80/86 (Ancell, dilution 1:1000). The mAbs were diluted in FACS buffer. Control wells remained unstained to correct for

background fluorescence. After staining, cells were washed and resuspended in FACS buffer and kept on ice until FACS analysis.

Flow cytometry data was analysed using FlowJo analysis software V10 (FlowJo, Ashland, OR, USA). To compensate for spectral overlap, BDTM CompBead Anti-Mouse compensation particles (BD biosciences) were used, according to manufacturer's instructions.

ELISA analysis

Levels of IL-8 and TNFα in cell culture supernatant were measured by sandwich ELISA using ELISA Duoset kits (R&D systems, Minneapolis, MN, USA) according to the manufacturer's instructions. Absorbance was measured at 450 nm with a FLUOstar Omega microplate reader (BMG, Labtech GmbH) and corrected for absorbance at 540 nm.

Phagocytosis assays

GFP-labelled *S. suis*-S10 and GFP-labelled *S. suis* J28 were used for phagocytosis assays. The bacteria and dendritic cells were cultured and stimulated as described before and flow cytometry was used to visualize phagocytosed *S. suis*. Except for the SWC3α-PE mAb (clone 74-22-15, Abcam, dilution 1:1000) for gating purposes, no mAbs were added to the cells. Controls consisted of wells containing only dendritic cells, GFP-labelled S. suis-S10, or S. suis J28. To validate that the uptake of *S. suis* was caused by phagocytosis and not invasion, 1 µM Cytochalasin D (CytoD) (Sigma-Aldrich) was used 1 h before prior and during BMDC stimulation.

Statistical analysis

All data are expressed as mean ± SEM. The datasets were analysed for significance using the non-parametric Friedman rank sum test, due to skewed distributions of the data. A *p*-value < 0.05 was considered significant (*, $p<0.05$), with ** being $p < 0.01$ and *** being $p < 0.001$. Statistical analysis was performed using R studio software (RStudio Inc, Boston, USA). Experiments were repeated at least six times, unless indicated otherwise.

Results

After 6 days of culture, the bone marrow culture consisted routinely of 40-70% cells that had the expected FSC/SSC profile (Fig. 1A). From these, 95-100% expressed the myeloid marker SWC3a. Furthermore, the SWC3a+ cells were all positive for SLA-II, although not all cells expressed high levels of CD80/86. In routine analysis, all SWC3a+ cells were selected. LPS stimulation did not change the expression levels of either SLA-II or CD80/86 (Fig. 1B). However, stimulation with LPS led to a significant production of TNFα and IL-8 (Fig. 1C).

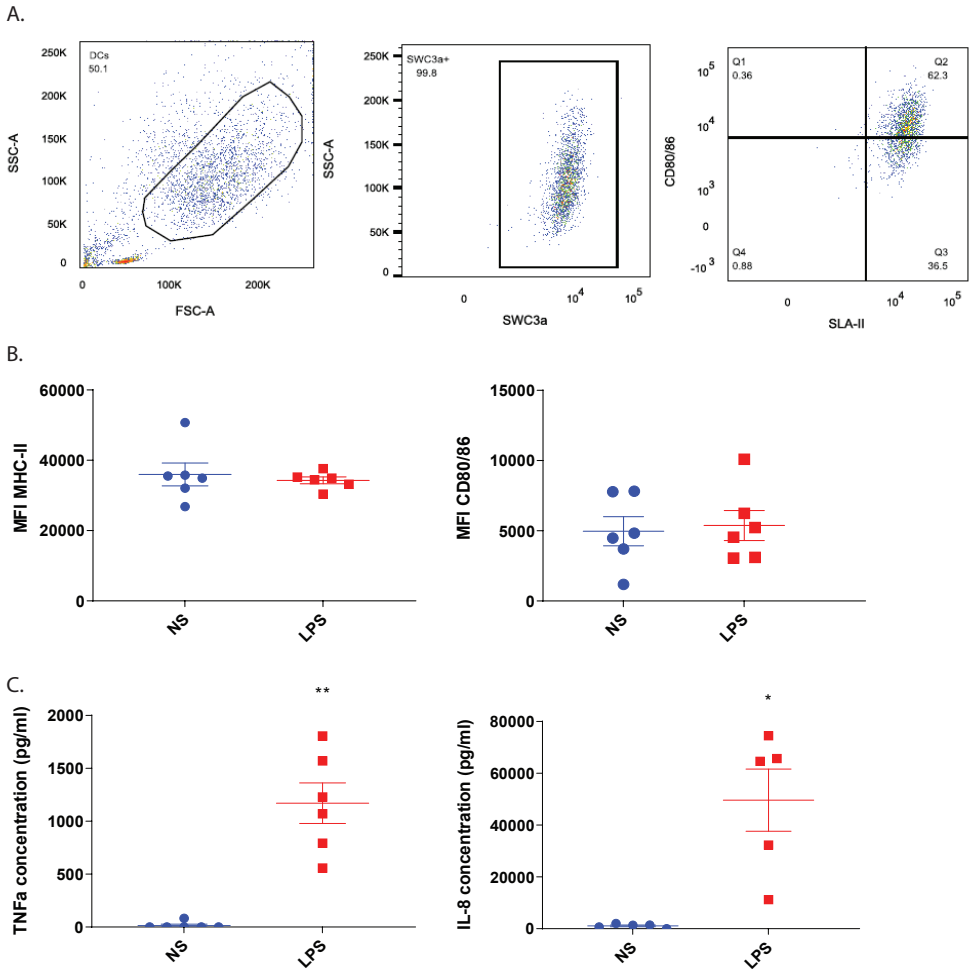

Figure 1: **Porcine bone marrow derived DCs are SLA-II+, CD80/86+ and express large amounts of TNFα and IL-8 upon activation with LPS.** (A) Standard FSC/SSC plot of DC culture after 7 days; the largest cell population is highly positive for the myeloid marker SWC3α. (B)

Cells do not significantly upregulate SLA-II and CD80/86 upon activation with LPS. (C) Cells produce large amounts of TNFα and IL-8 upon LPS stimulation. Data is shown as individual values (n=5-6), with bars denoting average ±SEM. Significance is calculated by paired T-test (P<0.05=*, P<0.01=**).

The effect of HDPs on porcine BMDCs has thus far been poorly characterized. HDPs can directly induce cytokine production in macrophages (Elssner *et al.* 2004, D'Este *et al.* 2012, Veldhuizen *et al.* 2014b, Coorens *et al.* 2015, Coorens *et al.* 2017c). Up to 2.5 µM of L-CATH-2 alone did not induce any cytokine production (Fig. S1). Furthermore, HDPs can inhibit LPS-induced activation of macrophages (Kandler *et al.* 2006, Veldhuizen *et al.* 2017, Scheenstra *et al.* 2019, van Harten *et al.* 2022). 2.5 µM L-CATH-2 significantly reduced LPS-induced production of TNFα and IL-8 by DCs in a dose dependent manner (Fig. 2A). Similarly, D-CATH-2 showed a strong inhibition of LPS-induced production of TNFα and IL-8 (Fig. 2B). PMAP-23 did not show an inhibition of cytokine production under these conditions (Fig. 2C) while PR-39 showed moderate inhibition of TNFα production of approximately 60% compared to the control, but LPS induced IL-8 production was not affected by PR-39 (Fig. 2D).

The HDPs were able to modulate the response to LPS, but bacteria contain a multitude of other stimuli for innate immune receptors besides LPS. As DCs need to take up bacteria for processing and subsequent presenting to adaptive immune cells, modulating the uptake could improve presentation efficiency and the subsequent adaptive immune response. Therefore, we investigated whether porcine DCs were able to take up GFP^+ *E. coli*, and whether this uptake could be modulated by the HDPs. In the absence of peptides, around 20% of DCs became GFP^+, (range from 10% up to 32%, Fig. 3A&B). At the highest concentration tested, both L-CATH-2 and D-CATH-2 could efficiently inhibit uptake of *E. coli* down to very low percentages (<5% on average)(Fig. 3A). Quantification of multiple individuals showed that this was repeatable despite variation in baseline uptake, and strongly dose dependent for L-CATH-2 (Fig. 3B). D-CATH-2 already showed maximum inhibition at concentrations as low as 0.625 µM. The same experiment using PMAP-23 led to a small increase in GFP^+ DCs at the highest concentration of HDP tested. PR-39 also seemed to increase the percentage of GFP^+ cells with a higher average than the control at all tested concentrations, although only statistically significant at 0.625 µM (Fig. 3B).

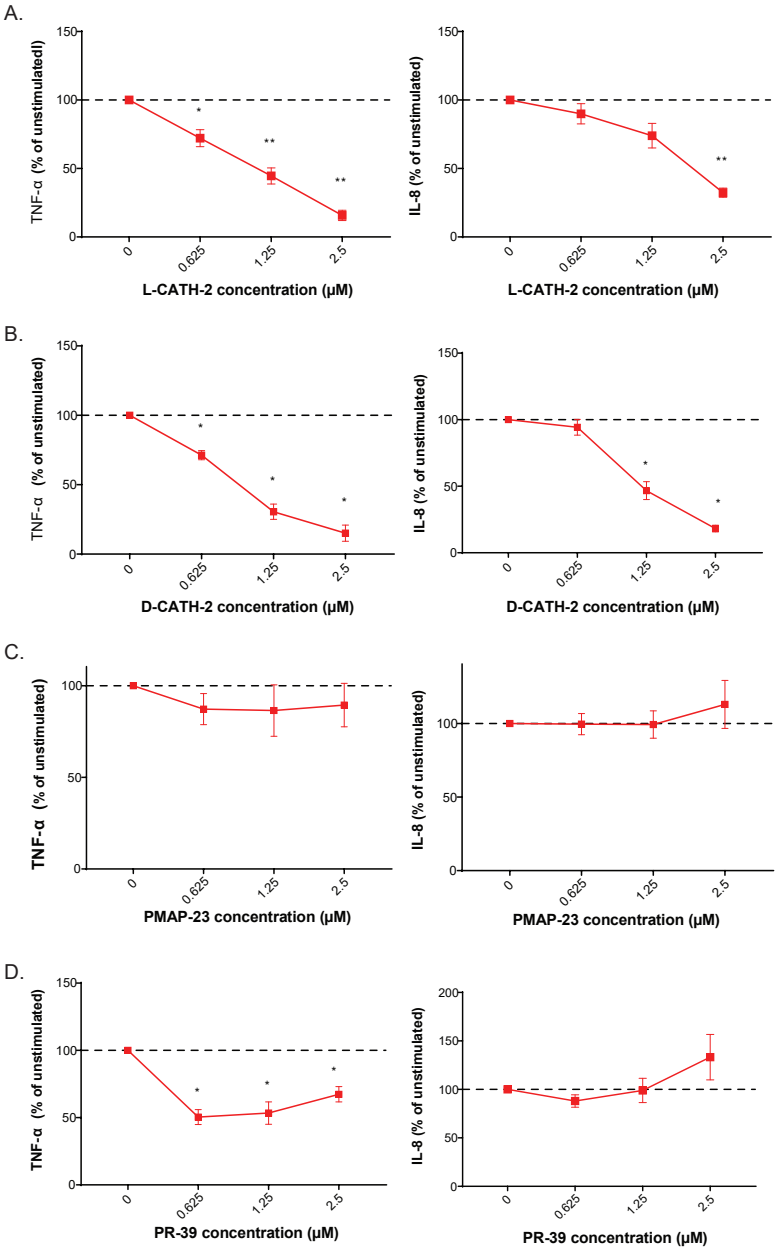

Figure 2: **Both L-CATH-2 and D-CATH-2 are effective at inhibiting response to both encapsulated and unencapsulated *S. suis*.** Cytokine production of TNFα and IL-8 by DCs is shown after stimulation with LPS. Peptides tested are L-CATH-2 (A), D-CATH-2 (B), PMAP-23 (C), and PR-39 (D). Data is shown as average ±SEM (n=5-7), with the dotted line showing control conditions. Significance is calculated by Friedman test with Dunnett's post-hoc test for multiple comparisons. Stars denote a significant difference with the 0 μM peptide/LPS control.

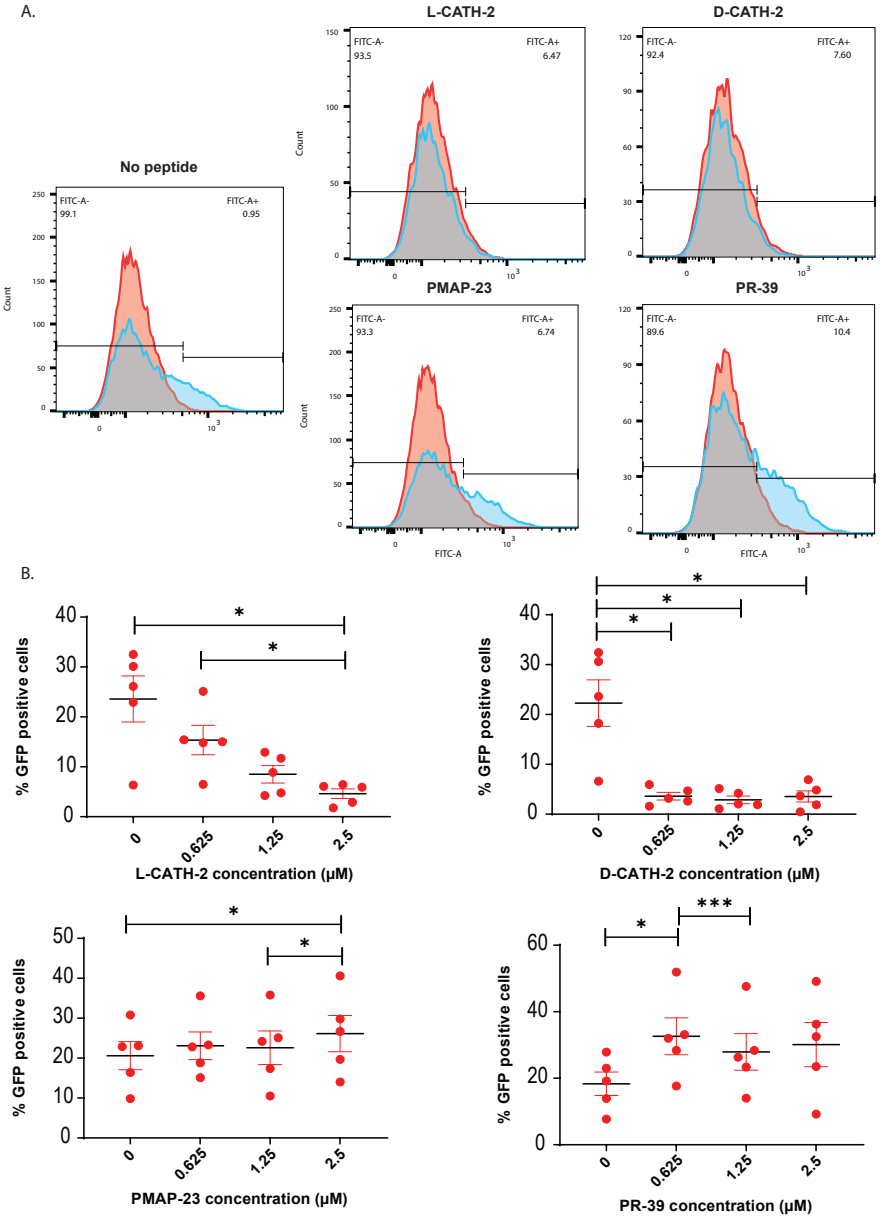

Figure 3: **CATH-2 leads to a potent inhibition of uptake of *E. coli.*** (A). Representative histogram overlays of DCs incubated with peptides and medium only (blue) and with fluorescent *E. coli* (red). (B)**.** The % of GFP⁺ DCs after incubation with fluorescent *E. coli* and concentrations of peptides are shown for L-CATH-2 (top left), D-CATH-2 (top right), PMAP-23 (bottom left) and PR-39 (bottom right). Individual values are shown (n=5) with the bars denoting average ± SEM. Significance is calculated by 1-way ANOVA with Tukey's post-hoc test for multiple comparisons. Unless otherwise indicated, stars indicate significant dif-

ferences with 0 µM peptide control. Absence of stars indicates no significant differences.

Since we were interested in the DC response to porcine pathogens in the context of a HDP containing vaccine against *S. suis*, we tested whether the DCs could take up GFP⁺ strains of S10 and J28. The S10 strain was not taken up, likely due to evasion of recognition by expression of the polysaccharide capsule (data not shown) (Smith *et al.* 1999). On the other hand, non-encapsulated J28 was taken up at a similar frequency as *E. coli,* with about 20-25% GFP⁺ cells on average (Fig. 4A&B). Contrary to the results with *E. coli,* L-CATH-2 and D-CATH-2 did not reduce the uptake or adhesion of the fluorescent *S. suis* J28 (Fig. 4A&B). PMAP-23 showed a tendency to increase the frequency of GFP⁺ DCs, but these results were not statistically significant (Fig. 4B). PR-39 did not increase the percentage of GFP⁺ cells.

The HDPs were able to modulate the percentage of DCs that take up *E. coli*, but for *S. suis* this process was less efficient. Therefore, we wondered whether the HDPs could modulate the cytokine production induced by *S. suis*. When DCs were stimulated with bacteria, only the unencapsulated strain of *S. suis* J28 could induce a significant change in CD80/86 (Fig. S2). *S. suis* J28 induced higher levels of TNFα than *S. suis* S10 at similar MOI (Fig. S3). L-CATH-2 was able to reduce both S10 and J28 induced production of TNFα and IL-8 to 50% of control (Fig. 5A). D-CATH-2 inhibited release of both cytokines much more substantially to <5% (Fig. 5B). PR-39 did not affect TNFα production but reduced particularly *S. suis* S10 induced IL-8 production (Fig. 5C).

5

Discussion

S. suis is a common Gram-positive commensal of pigs that can frequently cause outbreaks and disease among both pigs and humans(Dong *et al.* 2021, Maes *et al.* 2021). There is not yet a broadly effective vaccine available. Perhaps host defence peptides (HDPs) can increase the efficiency of vaccines, but there is not yet extensive knowledge of the effects of HDPs on porcine DCs, which are the first cells to act in generating a vaccine response. Therefore, in this study, porcine bone marrow derived DCs (BMDCs) were stimulated with *S. suis* and *E. coli*, and their response was characterized in the presence of various HDPs.

DCs *in vivo* are usually different from cultured DCs. In this study, BMDCs were cul

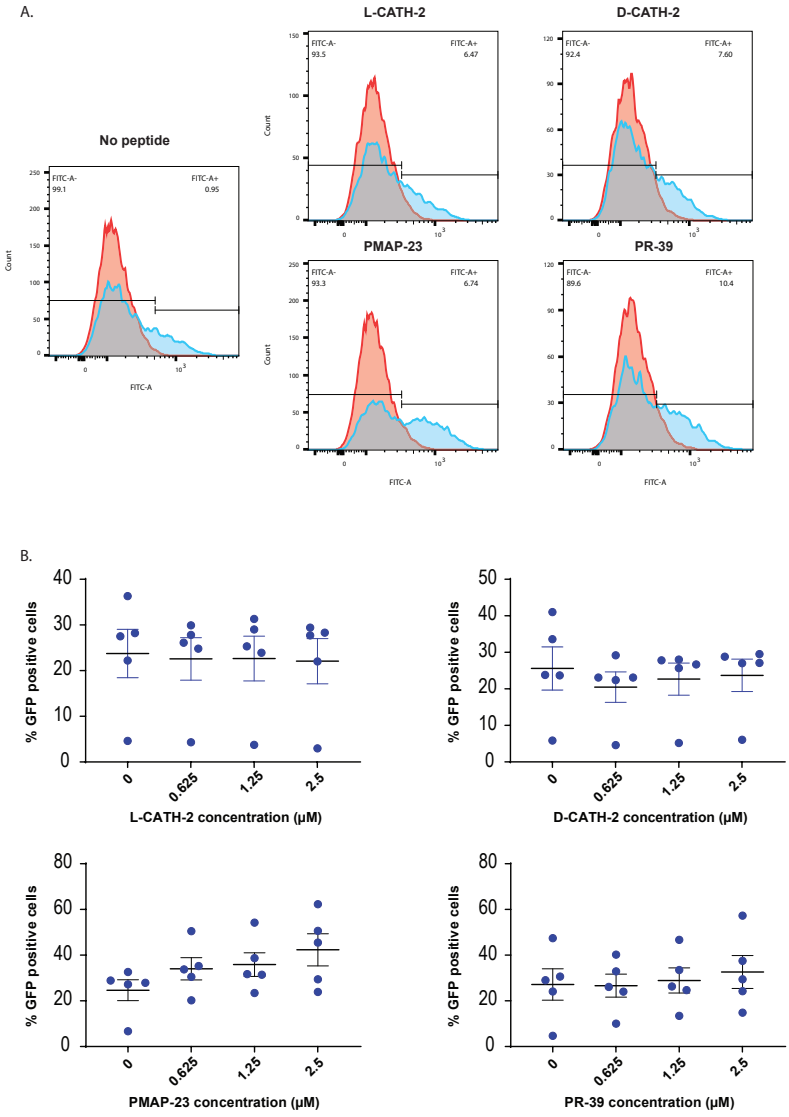

Figure 4: **PMAP-23 could lead to an increase of uptake of *S. suis,* although not statistically significant.** (A). Representative histogram overlays of DCs incubated with peptides and medium only (blue) and with fluorescent *E. coli* (red). (B)**.** The % of GFP⁺ DCs after incubation with fluorescent *E. coli* and concentrations of peptides are shown for L-CATH-2 (top left), D-CATH-2 (top right), PMAP-23 (bottom left) and PR-39 (bottom right). Individual values are shown (n=5) with the bars denoting average ± SEM. Significance is calculated by 1-way ANOVA with Tukey's post-hoc test for multiple comparisons.Stars indicate a significant difference with the no-peptide control (P<0.05=*, P<0.01=**). Absence of stars indicates no significant differences.

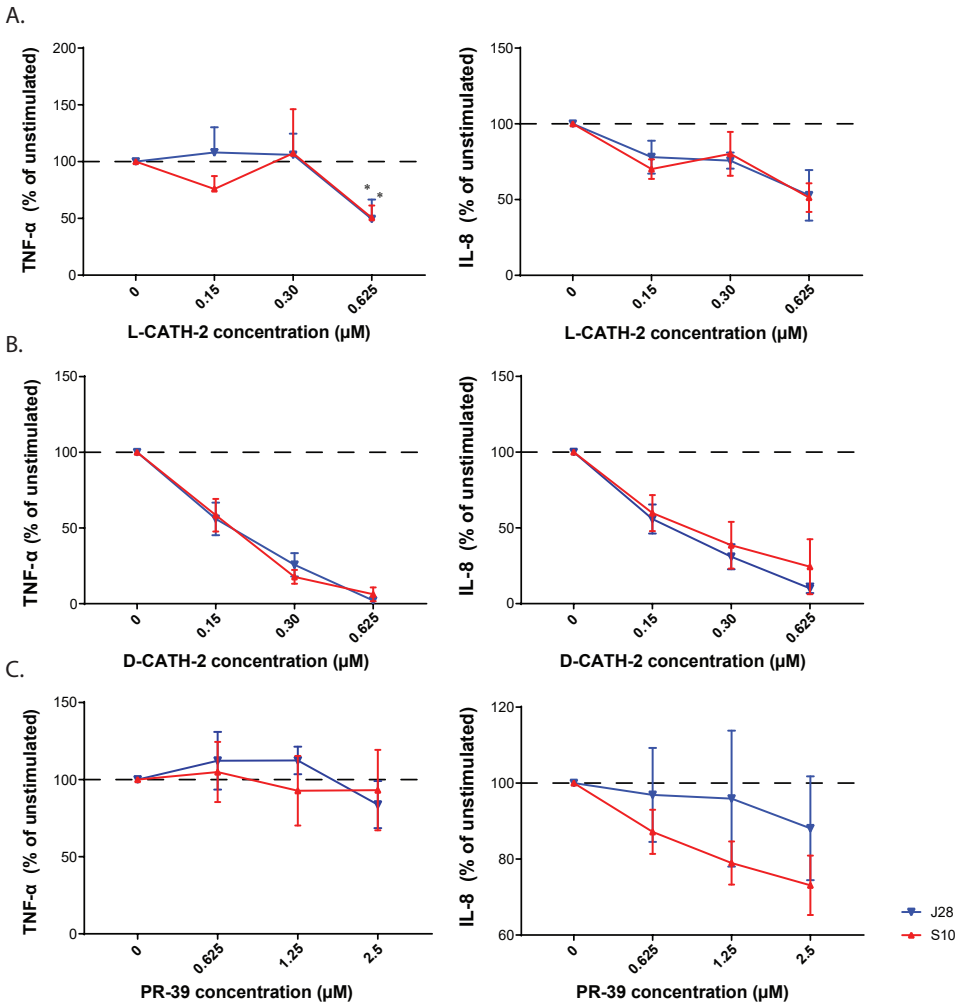

Figure 5: **Both L-CATH-2 and D-CATH-2 are effective at inhibiting the response to both encapsulated and unencapsulated *S. suis*.** Cytokine production by DCs is shown after stimulation with control, *S. suis* S10, J28 or LPS for different concentrations of peptides L-CATH-2 (A), D-CATH-2 (B), and PR-39 (C). Data is shown as individual values (n=5-7), with bars denoting average ±SEM. Significance is calculated by 2-way ANOVA with Dunnett's post-hoc test for multiple comparisons. Stars indicate a significant difference with the no-peptide control (P<0.05=*, P<0.01=**). Absence of stars indicates no significant differences.

tured in the presence of GM-CSF and IL-4. This is similar to protocols for porcine-monocyte derived DCs (Singleton *et al.* 2016) and human MoDCs, although for human DCs techniques have improved and specific subsets of interest can be directly isolated (Wimmers *et al.* 2014). There are other ways to grow porcine DCs, such as

from blood monocytes or from freshly isolated bone marrow, with GM-CSF alone or with IL-4, or in the presence of other compounds such as LPS or TNFα (Carrasco *et al.* 2001, Geervliet *et al.* 2020). In mouse, the classically known GM-CSF bone marrow derived DC culture contains up to 65% of immature macrophages, which addition of IL-4 could not improve (Helft *et al.* 2015). In pigs, the effect of IL-4 on porcine macrophages is to increase expression of CD203a (which hydrolyses extracellular ATP) and to suppress IL-12 (Sautter *et al.* 2018). It is unknown whether IL-4 has a similar effect on porcine BMDCs. There are also differences known when cells are cryopreserved or not, with fresh bone marrow cells being generally more responsive (Geervliet *et al.* 2020). In this study, the BMDCs do not really change their cell markers when stimulated with LPS. This is similar to other cryopreserved bone marrow DCs with the same LPS concentration (Mair *et al.* 2014, Summerfield *et al.* 2015, Geervliet *et al.* 2020). Considering markers, CD80/86 was observed to increase by *S. suis* J28 stimulation only. It is known that encapsulated *S. suis* are worse activators of DCs than non-encapsulated strains (Segura *et al.* 2004, Tanabe *et al.* 2010, Lecours *et al.* 2011). Therefore, it is not surprising that unencapsulated *S. suis* lead to higher CD80/86 expression. In addition, our BMDCs produced copious amounts of cytokines when stimulated with LPS and bacteria, which makes them suitable for *in vitro* testing. In short, the ideal culture method for porcine *in vitro* DCs is not yet definitively established, as each of these methods yields cells with DC-like properties.

When stimulated with LPS, the activation of DCs could be inhibited by both L-CATH-2 and D-CATH-2. These peptides are known to inhibit LPS responses (van Dijk *et al.* 2009, Van Dijk *et al.* 2016, Scheenstra *et al.* 2019). Interestingly, porcine cathelicidins have low modulating effects on LPS-stimulated DCs. This is similar to porcine macrophages co-stimulated with LPS and porcine cathelicidins (van Harten *et al.* 2022). This result indicates the *in vivo* mechanism of modulation of these peptides is probably not inhibition of LPS. Since L-CATH-2 and D-CATH-2 are good inhibitors of LPS stimulation, they might be a good component in a vaccine for pigs against Gram-negatives where overactivation is an issue.

Since particularly CATH-2 was effective at modulating LPS induced responses, we were interested to look at whole bacterial stimulation of DCs, as CATH-2 has

been previously demonstrated to modulate whole bacteria responses (Coorens *et al.* 2017d, van Harten *et al.* 2022). When DCs were stimulated with *E. coli*, both L-CATH-2 and D-CATH-2 showed a strong potential to inhibit *E. coli* uptake. For D-CATH-2, this already reached a maximum at 0.625 µM. At 0.625 µM, the *E. coli* are not killed by either form of CATH-2, but at 2.5 µM a 2 to 4 log reduction in bacterial viability is visible for L-CATH-2 and D-CATH-2, respectively (data not shown). This indicates that for D-CATH-2, maximum inhibition of uptake is reached when bacterial viability is unaffected. For L-CATH-2, inhibition of uptake is less binary than with D-CATH-2, and overlaps slightly with its antimicrobial activity. Previously, the immune activating potential of CATH-2-bacteria combinations was supposed to be inversely correlated with bacterial viability, also known as silent killing (Coorens *et al.* 2017a). In this case, it appears that the immune inhibition starts before the bacterial viability is affected. This means that, in supplement to the silent killing hypothesis, there could be a range of sublethal concentrations of HDPs, where the immune reaction is already dampened but bacteria are still viable. It is currently unknown whether this is true for more bacteria, more HDPs, and more cell types, which more extensive research could elucidate.

Furthermore, we saw similar neutralizing results for cytokine production induced by both *E. coli* and *S. suis*. This indicates that, if CATH-2 requires binding to a bacterial target before modulation, *S. suis* likely possesses such a target, although direct binding of *S. suis* by CATH-2 is not yet demonstrated. Additionally, although the baseline production of cytokines was higher with stimulation of unencapsulated bacteria, there were similar reductions in activations of DCs seen between encapsulated and unencapsulated bacteria. This indicates that the presence of the capsule cannot protect the bacterium from CATH-2. Conversely, it also means that CATH-2's target cannot be the capsule or a capsule bound antigen, but could be a membrane bound, excreted or intracellular target. It is, however, still possible that modulation occurs on a signalling level downstream of initial activation of the DCs, for instance if CATH-2 inhibits the MyD88-induced signalling pathway (Weighardt and Holzmann 2008). We have seen before that CATH-2 can bind LTA of *S. aureus* and can reduce LTA-induced activation (Coorens *et al.* 2017b, van Harten *et al.* 2021); however, LTA from *S. suis* is likely not the main contributing immune activating component, but rather various lipoproteins (Gisch *et al.* 2018). The exact molecular mechanism of

CATH-2 modulation of *S. suis*-induced activation is therefore still unknown.

Antigen uptake by DCs is essential for processing and subsequent establishment of adaptive immunity. The capsule of *S. suis* impairs recognition by both human DCs (Meijerink *et al.* 2012) and porcine DCs (Lecours *et al.* 2011). When looking at uptake of fluorescent *S. suis*, we see that the J28 strain is taken up better than the encapsulated strain. This is again in agreement with the lower activation and recognition of *S. suis* S10. CATH-2 does not increase or reduce uptake of GFP in DCs (Fig. 4A&B), despite its potent modulatory capacity in these assays. However, the GFP signal does not differentiate between adhesion, invasion or uptake of bacteria. Selectively quenching the fluorescent signal of attached (but not intracellular) bacteria with trypan blue did not work to selectively turn off attached bacteria (data not shown). Therefore, it is still possible that CATH-2 can change the proportion of bound bacteria versus internalized. PMAP-23 showed a tendency towards higher uptake (or adhesion) of unencapsulated *S. suis,* although this did not reach statistical significance. This could lead to a more efficient processing of *S. suis* antigen. However, vaccination studies with unencapsulated *S. suis* only did not lead to a good protective response (Wisselink *et al.* 2002). It is possible that PMAP-23 as an adjuvant may induce the immune response to a higher level, but this remains to be investigated. PMAP-23 modulation of cytokine response was not studied and should be performed in further research. Nevertheless, PMAP-23 could potentially be useful as an adjuvant-like vaccine additive to increase bacterial delivery to DCs.

In conclusion, CATH-2 displays a broad inhibition of both LPS and *S. suis* induced activation of porcine DCs. This might prove essential in attuning a vaccine response, when overactivation and side effects are an issue. This must not be detrimental to its immune stimulatory capacity, however, which must be thoroughly investigated. This includes the effect of CATH-2 on porcine T cells in general, and the DC-T cell interaction in particular. Furthermore, we saw a potential increase in uptake of *S. suis* J28 induced by PMAP-23. This could mean PMAP-23 might be useful as a tool to enhance delivery of antigen or other compounds to porcine DC, although more research into this HDP is required. It is currently unknown how cell-specific this uptake is. PMAP-23 could be a more general stimulant of uptake or work specifically for *S. suis*. Nevertheless, both CATH-2 and PMAP-23 display promising properties as

vaccine components and could lead to new strategies in inducing broad protective immunity against *S. suis* in pigs.

Acknowledgements

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Supplemental figures

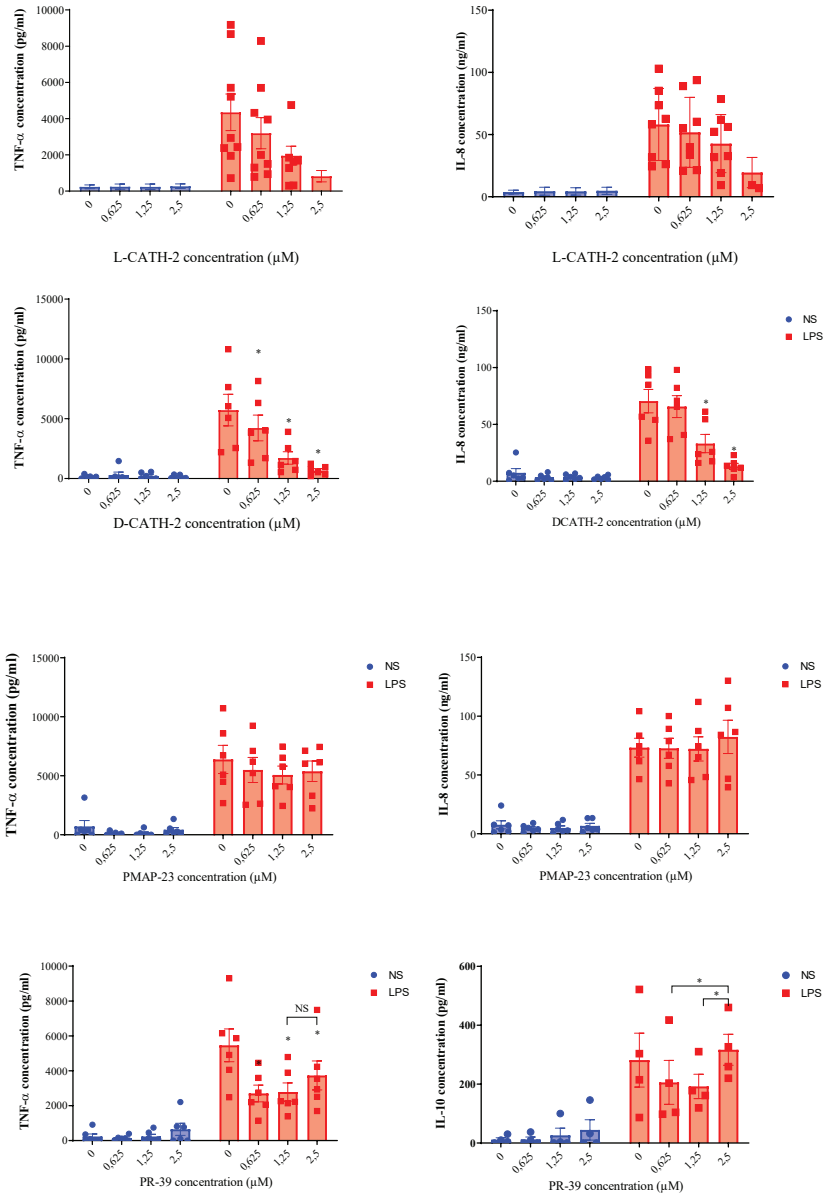

Figure S1: **Both L-CATH-2 and D-CATH-2 are effective at inhibiting the response to both encapsulated and unencapsulated *S. suis*.** Cytokine production of TNFα and IL-8 by DCs is shown after no stimulation (NS) or stimulation with LPS. Peptides tested here are L-CATH-2, D-CATH-2, PMAP-23, and PR-39. Data is shown as individual values (n=5-7), with bars denoting average ±SEM. Significance is calculated by 2-way ANOVA with Dunnett's post-hoc test

for multiple comparisons. Stars indicate a significant difference with the no-peptide control (P<0.05=*, P<0.01=**). Absence of stars indicates no significant differences.

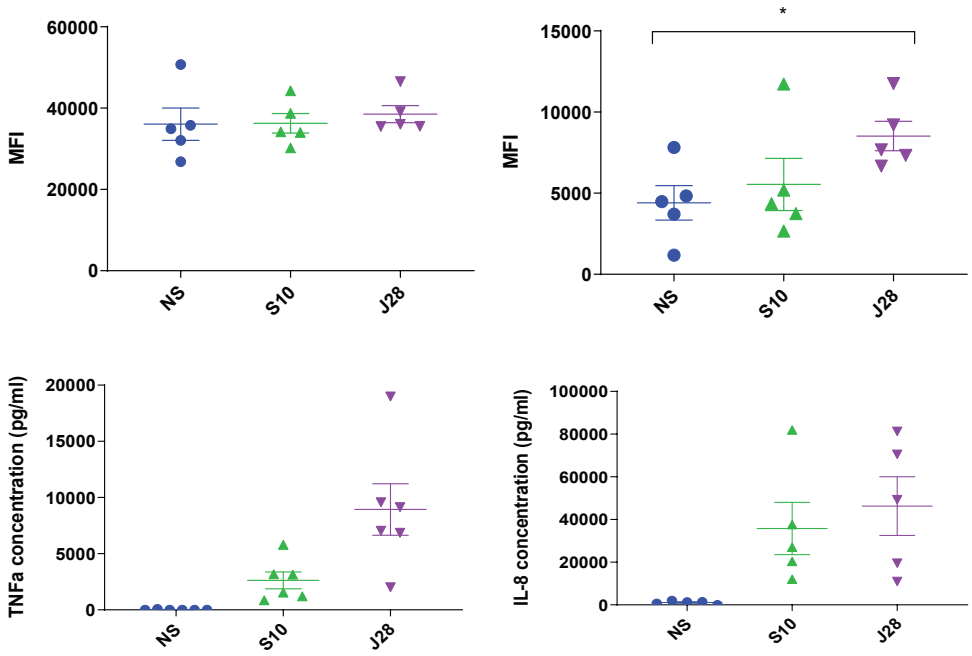

Figure S2: **BMDCs produce TNFα and IL-8 upon stimulation with S10 and J28.** Cells do not significantly upregulate SLA-II, but CD80/86 is upregulated upon activation with J28. Cells produce large amounts of TNFα and IL-8. Data is shown as individual values (n=5-6), with bars denoting average ±SEM. Significance is calculated by RM one-way ANOVA with Tukey's post host test for multiple comparisons. Unless otherwise indicated, Stars indicate a significant difference with the no-peptide control (P<0.05=*, P<0.01=**). Absence of stars indicates no significant differences.

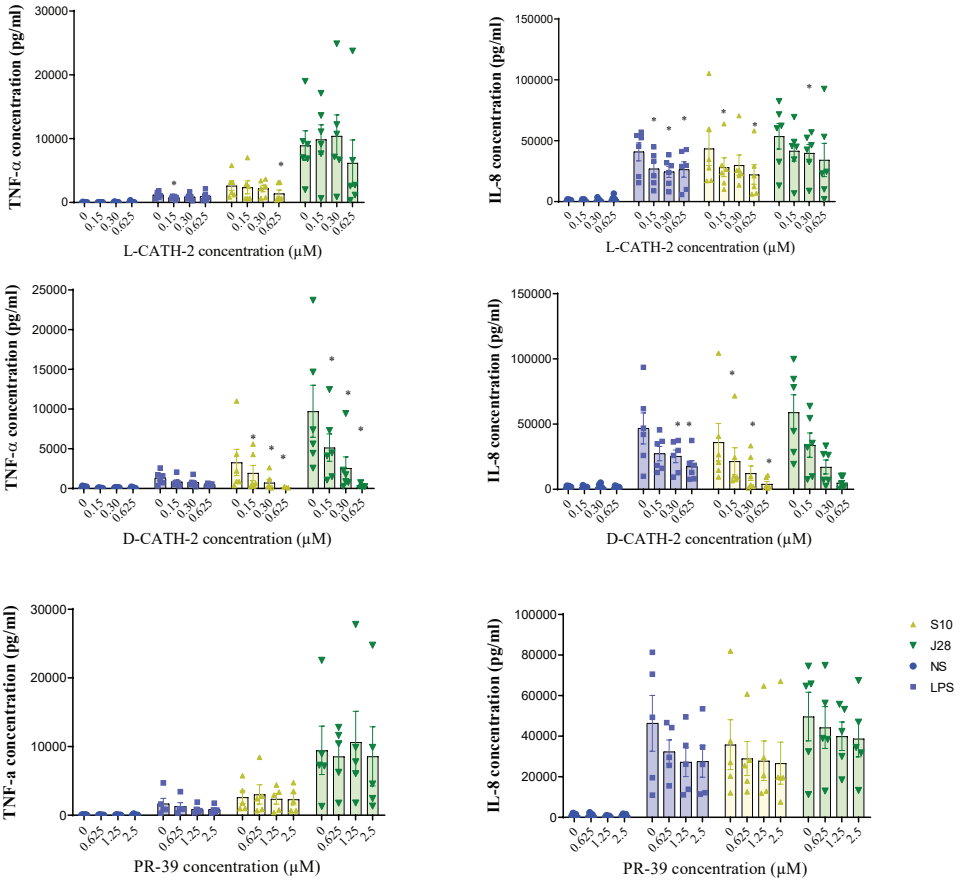

Figure S3: **Both L-CATH-2 and D-CATH-2 are effective at inhibiting response to both encapsulated and unencapsulated *S. suis*.** Cytokine production by DCs is shown after stimulation with control, *S. suis* S10, J28 or LPS for different concentrations of peptides L-CATH-2, D-CATH-2, and PR-39. Data is shown as individual values (n=5-7), with bars denoting average ±SEM. Significance is calculated by 2-way ANOVA with Dunnett's post-hoc test for multiple comparisons.

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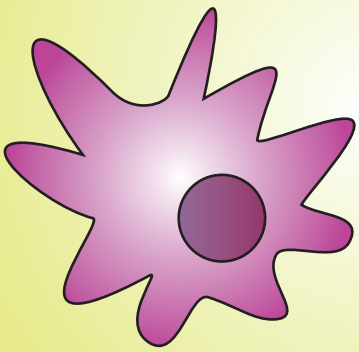

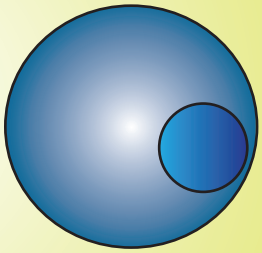

Chapter 6

D-enantiomers of CATH-2 enhance the response of macrophages against *Streptococcus suis* serotype 2

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Abstract

Introduction: Due to the increase of antibiotic resistant bacterial strains, there is an urgent need for development of alternatives to antibiotics. Cathelicidins can be such an alternative to antibiotics having both a direct antimicrobial capacity as well as an immunomodulatory function. Previously, the full D-enantiomer of chicken cathelicidin-2 (D-CATH-2) has shown to prophylactically protect chickens against infection 7 days post hatch when administered *in ovo* three days before hatch.

Objectives: Further evaluate D-CATH-2 in mammals as a candidate for an alternative to antibiotics. In this study, the prophylactic capacity of D-CATH-2 and two truncated derivatives, D-C(1-21) and D-C(4-21), was determined in mammalian cells.

Methods: Antibacterial assays; immune cell differentiation and modulation; cytotoxicity, isothermal titration calorimetry; *in vivo* prophylactic capacity of peptides in an *S. suis* infection model.

Results: D-CATH-2 and its derivatives were shown to have a strong direct antibacterial capacity against four different *S. suis* serotype 2 strains (P1/7, S735, D282, and OV625) in bacterial medium and even stronger in cell culture medium. In addition, D-CATH-2 and its derivatives ameliorated the efficiency of mouse bone marrow-derived macrophages (BMDM) and skewed mouse bone marrow-derived dendritic cells (BMDC) towards cells with a more macrophage-like phenotype. The peptides directly bind lipoteichoic acid (LTA) and inhibit LTA-induced activation of macrophages. In addition, *S. suis* killed by the peptide was unable to further activate mouse macrophages, which indicates that *S. suis* was eliminated by the previously reported silent killing mechanism. Administration of D-C(1-21) at 24h or 7 days before infection resulted in a small prophylactic protection with reduced disease severity and reduced mortality of the treated mice.

Conclusion: D-enantiomers of CATH-2 show promise as anti-infectives against pathogenic *S. suis* for application in mammals.

Introduction

Since the discovery of penicillin in 1928, antibiotics have saved billions of lives around the world (Fraenkel 1998). However, due to emergence of antibiotic resistance, the development of novel antibiotics is urgently needed (Tacconelli *et al.* 2018). A promising alternative to antibiotics are host defence peptides (HDPs). Cathelicidins are an important family of HDPs, which play an important role in the innate immune response to infections (Zasloff 2002, Mookherjee *et al.* 2020, Scheenstra *et al.* 2020). Cathelicidins are characterized by their highly conserved precursor cathelin-domain, but the active, mature peptides are highly variable in sequence and structure (Vandamme *et al.* 2012, Scheenstra *et al.* 2020). They are strongly upregulated during infection (Horibe *et al.* 2013, Ruan *et al.* 2013) and despite their variable sequence, almost all cathelicidins show strong antimicrobial activity against many different bacteria (Coorens *et al.* 2017b), viruses (Peng *et al.* 2020, Xing *et al.* 2020), fungi (Oshiro *et al.* 2019, van Eijk *et al.* 2020), and parasites (Rico-Mata *et al.* 2013, Mahdavi Abhari *et al.* 2019). This antimicrobial activity is based on electrostatic membrane interactions, which makes resistance development less likely, as lipid targets can not easily mutate. In addition, many peptides have intracellular targets as well (Bechinger and Gorr 2017). Besides their direct multivalent antimicrobial activities, the immunomodulatory functions of these peptides make them of special interest for potential clinical applications (Mookherjee *et al.* 2020).

One of the best studied immunomodulatory functions of cathelicidins is neutralization of the capsular structures lipopolysaccharides (LPS) or lipoteichoic acid (LTA). These capsular structures decorate the outer membrane of Gram-negatives or the peptidoglycan layer of Gram-positives, respectively, and induce potent immune responses through TLR-4 or TLR-2, which leads to strong overactivation in sepsis patients (Weighardt and Holzmann 2008). Cathelicidins can prevent this overstimulation of the immune system (Rosenfeld and Shai 2006, Coorens *et al.* 2017b). Besides this inhibition of LPS- and LTA-induced cell activation, cathelicidins can also enhance the uptake of DNA and thereby increase TLR-9 activation (Coorens *et al.* 2015, Schmidt *et al.* 2015, Lee *et al.* 2019), induce chemokine and cytokine release, and are involved in phagocytosis (Marin *et al.* 2019). In addition, cathelicidins can

skew macrophage differentiation towards a more pro-inflammatory phenotype (van der Does *et al.* 2010). Taken together, the indirect immune modulatory effects might contribute better protection against infections than the direct antimicrobial activity. Injection of cathelicidins in chicken eggs three days before hatch, reduced the morbidity and bacterial load when the chickens were infected seven days post hatch. The low concentration of peptide and the long period of time between administration and infection suggests that the immunomodulatory activity of CATH-2 exerted the protective effect (Cuperus *et al.* 2016).

Streptococcus suis (*S. suis*) is a Gram-positive facultative anaerobe bacterium that is found in almost all pigs as a commensal of the respiratory microbiota. It can also cause invasive infections in young piglets, such as sepsis, meningitis, endocarditis, and may cause sudden death (Votsch *et al.* 2018). In addition, *S. suis* is a zoonotic agent that can cause sepsis and meningitis in humans and is the prevalent cause of meningitis in several Southeast Asian countries (Kerdsin *et al.* 2016). The bacterium is encapsulated with a large polysaccharide capsule to prevent phagocytosis-dependent clearing (Segura *et al.* 2004). Up to 35 various capsular serotypes of *S. suis* have been identified so far, of which serotype 2 is found most often in diseased pigs worldwide, followed by serotype 9 and 3, although regional differences in prevalence do occur. In human cases serotype 2 is the most prevalent (Goyette-Desjardins *et al.* 2014). Many *S. suis* strains carry resistance genes, most likely introduced due to prophylactic use of antibiotics in livestock industry (Goyette-Desjardins *et al.* 2014, Hernandez-Garcia *et al.* 2017).

To determine putative application of cathelicidins in prevention and treatment of *S. suis* infections in mammals, the direct antibacterial capacity of the full D-enantiomer of chicken cathelicidin-2 (D-CATH-2) and two derivatives were determined. In addition, the capacity of D-CATH-2 and its derivatives to skew mouse bone marrow-derived dendritic cells towards a more macrophage-like phenotype was assessed. The peptides were tested whether they could bind LTA and thereby inhibit LTA-induced activation. In addition, D-CATH-2 and its derivatives were examined for *S. suis* killing without inducing an excessive immune reaction upon infection. Finally, mice were injected with the D-CATH-2 truncated derivative D-C(1-21) 24h before *S. suis* infection, to observe effects on the immune response and disease severity

caused by S. *suis.*

Material and Methods

Peptides, bacterial strains, and experimental animals

The 26 amino acid full D-enantiomer of chicken CATH-2 (RFGRFLRKIRRFRPKVTITIQGSARF-NH_2) (D-CATH-2) with a net charge of 9+ and two truncated derivatives (D-C(1-21), 8+ and D-C(4-21), 7+) were used in this study. The peptides were synthesized by Fmoc-chemistry at China Peptides (CPC scientific, Sunnyvale, CA, USA) and purified by reverse phase high-performance liquid chromatography to a purity of > 95%. Lyophilized peptides were dissolved in endotoxin free water.

S. suis serotype 2 strains P1/7, D282, S735, and OV625 were used in this study. All strains have been previously characterized.(de Greeff *et al.* 2011) Bacterial strains were grown overnight at 37°C from glycerol stocks in Todd-Hewitt broth (THB, Oxoid Ltd., London, UK) before use.

Seven- to ten-week-old Crl:CD-1 mice (both male and female) were purchased from Charles River (Germany). All mice were kept under specific pathogen-free conditions with free access to food and water under the guidelines for animal experimentation as approved by the Dutch central authority for scientific procedures on animals (CCD, License number: AVD108002015175).

Antibacterial activity

S. suis serotype 2 strains P1/7, D282, S735, and OV625 were grown into mid-logarithmic phase for 3-4 hours at 37 °C in THB, after which bacteria were centrifuged at 1200 x *g* for 10 minutes at 4 °C and resuspended in fresh THB. Bacterial concentration was determined by measuring optical density at 620 nm with an OD of 1.0 being equivalent to $1x10^8$ colony forming units (CFU)/mL. $1x10^6$ CFU/mL *S. suis* was mixed with different concentrations of D-CATH and derivatives (0.63 – 40 µM) and incubated for 3 h at 37 °C. Ten-fold dilutions were prepared and spread in Tryptic Soy agar (TSA) plates containing 5% (v/v) defibrinated sheep blood (Oxoid) and colonies were allowed to grow for 48 h. Minimal Bactericidal Concentration (MBC) was defined as ≤ 100 CFU/mL (2 log CFU/mL), the detection limit of the assay.

Cell culture and flow cytometry

Murine bone marrow cells, isolated from the femur and tibia of both hindlegs, were stored in foetal calf serum (FCS) (Corning, NY, USA) containing 10% DMSO (Sigma-Aldrich, MO, USA) in liquid nitrogen. Cells were grown at a concentration of $5x10^5$ cells/mL in RPMI-1640 without phenol red (Thermo Fisher Scientific, MA, USA) supplemented with 10% FCS and 1% penicillin/streptomycin (Thermo Fisher Scientific). Bone marrow-derived macrophages (BMDM) and bone marrow-derived dendritic cells (BMDC) were cultured by adding 20 ng/mL murine recombinant M-CSF or GM-CSF (Peprotech, NJ, USA) respectively. Where indicated, cells were supplemented with 1.25 µM peptide at day 1, which was replaced by fresh medium at day 2. The medium of all cells was replaced by fresh medium without antibiotics at day 3. At day 6 cells were stimulated with 1 µg/mL LTA from *S. aureus* (LTA-SA) (Invivogen, CA, USA) or with the different *S. suis* strains at a multiplicity of infection (MOI) of 0.2. Medium containing *S. suis* was removed after 2 h and replaced by medium containing 200 µg/mL gentamycin (Sigma) and left for an additional 22 h. After 24 h, medium was collected and stored at -20 °C for cytokine measurements. Cells were incubated for 5 min with 0.5 mM EDTA in PBS after which they were resuspended by vigorous pipetting and used for flow cytometry. Cells were resuspended in flow cytometry buffer (PBS/0.5% BSA, Sigma) and kept on ice during the whole procedure. Cells were stained with antibodies (**Table 1**) for 20 minutes, washed and measured using the BD FACSCantoII (BD Biosciences, NJ, USA) and analysed with FlowJo software (Ashland, OR, USA).

Splenocyte activation

Mice were killed using CO_2 suffocation after which the spleens were harvested. Spleens were digested with digestion buffer (1.5 WU/mL liberase TL grade, 100 Units/mL recombinant DNase I, both Roche, Basel, Switzerland) for 30 minutes at 37 °C and meshed through a 40 µm filter (BD Biosciences) to prepare a single cell solution using PBS/0.5 mM EDTA wash buffer. The red blood cells were lysed using an isotonic ammonium chloride buffer (155 mM NH_4Cl, 10 mM $KHCO_3$, 0.1 mM EDTA) for 5-10 minutes on ice, washed 1x with PBS, after which the cells were counted and resuspended in high glucose DMEM

Table 1. Antibodies used for flow cytometry. All antibodies were diluted 1000x in flow cytometry buffer prior to use (CD19 and CD335 were used in a 500x dilution).

Antigen	Clone	Label	Manufacturer
MHC-II	M5/114.15.2	FITC	eBioscience
CD11c	HL3	PE	BD Biosciences
Sirp-α	P84	PerCP-eFluor710	eBioscience
CD19	1D3	PE-Cy7	BD Biosciences
CD8α	53-6.7	APC	BD Biosciences
CD11b	M1/70	APC-Cy7	BD Biosciences
CD24	M1/69	eFluor450	eBioscience
CD86	GL-1	PerCP	BioLegend
F4/80	BM8	APC	eBioscience
Ly6C	HK1.4	eFluor450	eBioscience
CD4	RM4-5	AF488	BD Pharmingen
CD62L	MEL-14	PE	eBioscience
CD335	29A1.4	PerCP-Cy5.5	BD Biosciences
CD44	IM7	PE-Cy7	BD Biosciences
CD3e	145-2C11	APC-Cy7	BD Pharmingen
CD25	eBio3C7	eFluor450	eBioscience

(Thermo Fisher scientific) supplemented with 10% FCS. $5x10^5$ splenocytes were added per well in a U-bottom 96-wells plate (Corning). Total splenocytes were stimulated with 1 µg LTA-SA or the different *S. suis* strains at an MOI of 0.2. After 2 h, the supernatant was collected (by centrifugation at 700x *g* for 2 minutes) and the cells were resuspended in 100 µl fresh medium supplemented with 200 µg/mL gentamycin and left for an additional 22 h. After 24 h, medium was collected and stored at -20 °C for cytokine measurements.

Cell viability and activity

WST-1 reagent (Roche) was used for determination of the metabolic activity and

thereby cell viability of BMDCs and BMDMs as well as for cell activity of activated splenocytes. In both cases, 100 µL fresh medium containing 10% WST-1 was added to the cells and incubated at 37 °C. After 30-60 minutes, colorimetric changes were measured at 450 nm using a FLUOstar Omega microplate reader (BMG Labtech GmbH, Ortenberg, Germany). The metabolic activity is depicted as a percentage with the untreated BMDCs/BMDMs or unstimulated splenocytes set to 100%.

ELISA

TNFα, IFN-γ, IL-1β, and IL-6 were measured in supernatant (diluted in PBS/1% BSA if needed) using a Duoset ELISA kit (R&D systems, MN, USA). ELISAs were performed according to the manufacturer's instructions. Colorimetric changes were measured at 450 nm using a FLUOstar Omega microplate reader (BMG) with a correction for background signal at 570 nm.

NO production

NO production in the supernatant was measured using the Griess assay. 50 µL undiluted supernatant or standard was mixed with 50 µL 5% phosphoric acid (Sigma Aldrich) and 1% sulphanilamide (Merck, NJ, USA) and incubated for 5 min in the dark. Subsequently, 50 µL 0.1% N-(1-Naphthyl)ethylenediamine (NED (MERCK)) was added and left for an additional 5 min in the dark. Colorimetric changes were measured at 550 nm using a FLUOstar Omega microplate reader (BMG) and plotted to the standard curve in a 4-parameter fit.

Isothermal calorimetry (ITC)

The interaction between the D-CATH-2 peptides and LTA-SA was tested using isothermal titration calorimetry (ITC). All ITC experiments were performed on a Low Volume NanoITC (TA instruments - Waters LLC, New Castle, USA). Peptide solution or 37.2 µM LTA-SA was prepared in MilliQ: dPBS (Gibco) in a 3:1 ratio. The chamber was filled with 164 µl LTA-SA and the peptide was loaded in the 50 µL syringe. Every 300 seconds, 1.99 µL peptide was titrated into the chamber at 37°C. Data was analysed using the Nano Analyze software (TA instruments-Waters LLC). The data of three experiments was averaged and an independent model was used to determine

the peptide-LTA interaction.

In vivo infection experiment

Upon arrival, mice were allowed to acclimatize for at least 7 days before the start of the experiment. The experiment was performed as depicted in **Figure 5A.** The experiment was repeated twice to obtain in total 4 mice in the control groups and 12 mice in the infection groups. At day 1, mice were subcutaneously injected in the neck region with 1 mg/kg D-C(1-21) in PBS/cholesterol or with PBS/cholesterol alone. Cholesterol was added 5% v/v, 2 mg/ml in ethanol. The peptide and control groups were blinded to avoid any influence by the researchers. After 24 h (group 1) or after 7 days (group 2), mice were intraperitoneally infected with 10^7 CFU *S. suis* P1/7 in THB or with THB alone as control. 24h after infection, a few drops of blood were collected via cheek puncture for bacterial count. During the infection phase of the experiment, mice were checked every 12h in the acute phase of disease (first 48h) and thereafter daily until the end of the study. A cumulative clinical score was given to the mice as measure of disease using several parameters as depicted in **Table 4**, according to Seitz *et al (Seitz et al. 2012).* When a mouse obtained a clinical score of 2 for a minimum of 3 out of 8 scoring points two days in a row, or in case of severe weight loss (> 20%), the mouse was euthanized for animal welfare reasons (humane end point (HEP)) and the organs were collected for bacterial counts as described hereafter. Seven days post infection all mice were sacrificed for further analysis. Mice were anesthetized using isoflurane and 1 mL blood was drawn via heart puncture, followed by cervical dislocation. The peritoneum was flushed with 5 mL PBS/0.5 mM EDTA and diluted in 10 mL ice cold PBS/0.5% FCS. Spleen, lungs, liver, lymph nodes (axillary, inguinal, and mesenteric), brain, kidney and bone marrow were collected and stored in ice cold PBS. All organs, except the bone marrow and lymph nodes were weighed using a Sartorius microbalance. The peritoneal lavage (PTL) samples were counted using the Countess II Automated Cell Counter (Invitrogen, CA, USA). The lungs, liver, brain, and kidney were meshed through a 40 µm filter (BD Biosciences) with 5 mL PBS to obtain single cell suspensions. Spleen and lymph nodes were digested with digestion buffer (1.5 WU/mL liberase TL grade, 100 Units/ml recombinant DNase I, both Roche) for 30 minutes at 37°C and meshed through a 40 µm filter using 5 mL PBS/0.5 mM EDTA. The red blood cells of the

blood and spleen were lysed using an isotonic ammonium chloride buffer (155 mM NH_4Cl, 10 mM $KHCO_3$, 0.1 mM EDTA) for 5-10 min on ice, washed 1x with PBS and were resuspended in FACS buffer (PBS/0.5% BSA). Bone marrow samples were flushed out the femur and tibia of both legs with 5 mL PBS and filtered through a 40 µm filter. A sample of the blood, bone marrow, spleen, peritoneal lavage, and lymph nodes was taken and stained for 30 min with different antibody panels (**Table 1**) and measured using the BD FACS Canto-II and analysed with FlowJo V8. Of the lungs, liver, brain, kidney, spleen, and peritoneal lavage samples, a 10-fold serial dilution was prepared, and the samples were plated on TSA plates containing 5% (v/v) defibrinated sheep blood. The colonies were allowed to grow for 48 h at 37°C. The number of colonies were counted, with ≤ 100 CFU/mL (2 log CFU/mL) as detection limit of the assay and calculated as CFU/mg organ.

Ethics statement

All mice were kept under specific pathogen-free conditions with free access to food and water under the guidelines for animal experimentation as approved by the Dutch central authority for scientific procedures on animals (CCD, License number: AVD108002015175).

Statistics

Samples were compared to no-peptide-controls using two-way ANOVA with the Dunnett post-hoc test. Samples were paired for cell culture samples. *=p≤0.05; **=p≤0.01; ***=p≤0.001; ****=p≤0.0001.

Results

D-CATH-2 and its derivatives efficiently kill several S. suis type 2 strains in both THB and RPMI+FCS

Antimicrobial activity of D-CATH-2 and its derived peptides was assessed against 4 different *S. suis* serotype 2 strains. The MBC of the three peptides for the *S. suis* strains was 2.5-5 µM in THB medium (**Figure 1A** and **Table 2**). However, most of the subsequent assays were performed in cell culture medium RPMI+10% FCS, which contains serum proteins and cationic ions that can influence the activity of cathelicidins.(Veldhuizen *et al.* 2017, Scheenstra *et al.* 2019) Therefore, the MBC of

D-CATH-2 and its derived peptides was also tested in RPMI+10%FCS medium. The activity of D-CATH-2 and D-C(1-21) slightly increased to 0.6-2.5 µM, whereas the MBC of the shortest peptide, D-C(4-21), remained stable at 2.5 µM (**Figure 1B** and **Table 2**).

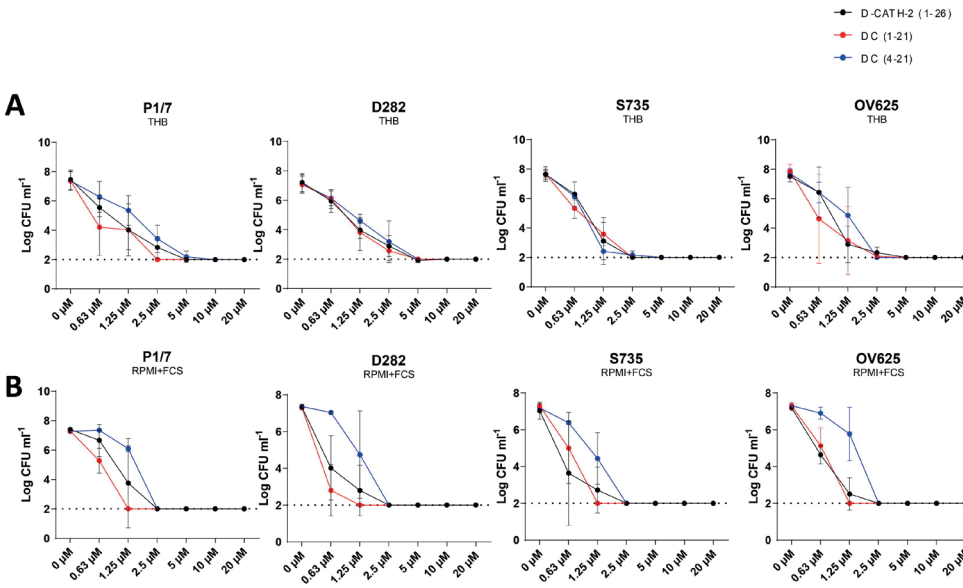


Figure 1. D-CATH-2 and its derivatives efficiently kill several *S. suis* type 2 strains in both THB and RPMI+FCS. Antibacterial activity of D-CATH-2 and its derivatives against 10^6 CFU/mL *S. suis* type 2 strains (P1/7, D282, S735, and OV625) was tested using a colony count assay in both THB medium **(A)** and RPMI+FCS **(B)**. 2 log CFU/mL was set as detection limit for the experiment. Data is plotted as average +/- SEM (N=3-4).

Table 2. MBC values of D-CATH-2 killing *S. suis* strains MBC values for the different peptides depending on the bacterial strain and the medium.

	THB			RPMI + 10% FCS		
	D-CATH2	DC(1-21)	DC(4-21)	D-CATH2	DC(1-21)	DC(4-21)
P1/7	2.5-5	2.5	5-10	1.25-2.5	1.25	2.5
S735	1.25-2.5	2.5	2.5	0.6-2.5	1.25	2.5
D282	2.5-5	2.5-5	2.5-5	0.6-2.5	0.6-1.25	1.25-2.5
OV625	1.25-5	0.6-5	1.25-2.5	1.25-2.5	1.25	2.5

D-CATH-2 and its derivatives inhibit LTA-SA- or S. suis-induced immune cell activation by binding to LTA

Natural (L-)CATH-2 is known to inhibit LPS and LTA activation of a murine macrophage cell-line.(Coorens *et al.* 2017b) However, it is unclear whether the all D-enantiomer of CATH-2 is also capable of inhibiting LTA-induced activation of primary cultured murine BMDMs and BMDCs. In addition, cathelicidins can in some instances be cytotoxic to mammalian cells at higher concentrations.(Scheenstra *et al.* 2019) Therefore, murine BMDMs and BMDCs were exposed to D-CATH-2, D-C(1-21) and D-C(4-21) added at either day 1 or day 6 of culture, to observe effects of the peptides on both cell viability and differentiation.

BMDMs were relatively sensitive to addition of D-CATH-2 and its derivates, especially to D-C(1-21) (**Figure S1A**), showing a marked reduction in metabolic activity at 5 µM. BMDCs had some reduced viability, starting from 2.5 µM peptide, with no difference between the three peptides (**Figure S1B**). Both BMDMs and BMDCs were less sensitive if the peptides were added at day 1 of the culture, with only a small reduction in viability at 5 µM **(Fig S1C and D**), although a similar slight reduction in viability was seen for D-C(1-21). Flow cytometry analysis also showed a decrease in BMDM purity, with a lower percentage of cells expressing the macrophage marker F4/80. Those BMDMs that did survive had an increased F4/80 expression and reduced MHC-II (**Figure S1E**). A minor reduction in live BMDCs is only visible at 5 µM, without affecting the other cell markers (**Figure S1F**).

To analyse the effect of peptides on bacterial stimulation of macrophages, four different strains of *S. suis* serotype 2 were mixed with 1.25 µM peptide and added to BMDMs at day 6 of culture. Stimulation of BMDMs with peptide did not influence the percentage of live macrophages in culture, as shown by flow cytometry. Bacterial stimulation of BMDMs showed a typical upregulation of activation markers, like MHC-II, CD86 and CD38. However, this upregulation was strongly inhibited by all three peptides for all four bacterial strains (**Figure 2A**). Similar results were found for BMDCs (**Figure S2A**). In addition, the secretion of TNFα and IL-6 seemed to be lowered in the presence of the peptides, although the results were not significant (**Figure 2B**). To study the influence of the peptides on *S. suis*-induced activation in a more complex system, total splenocytes from mice were activated *ex vivo*. Besides

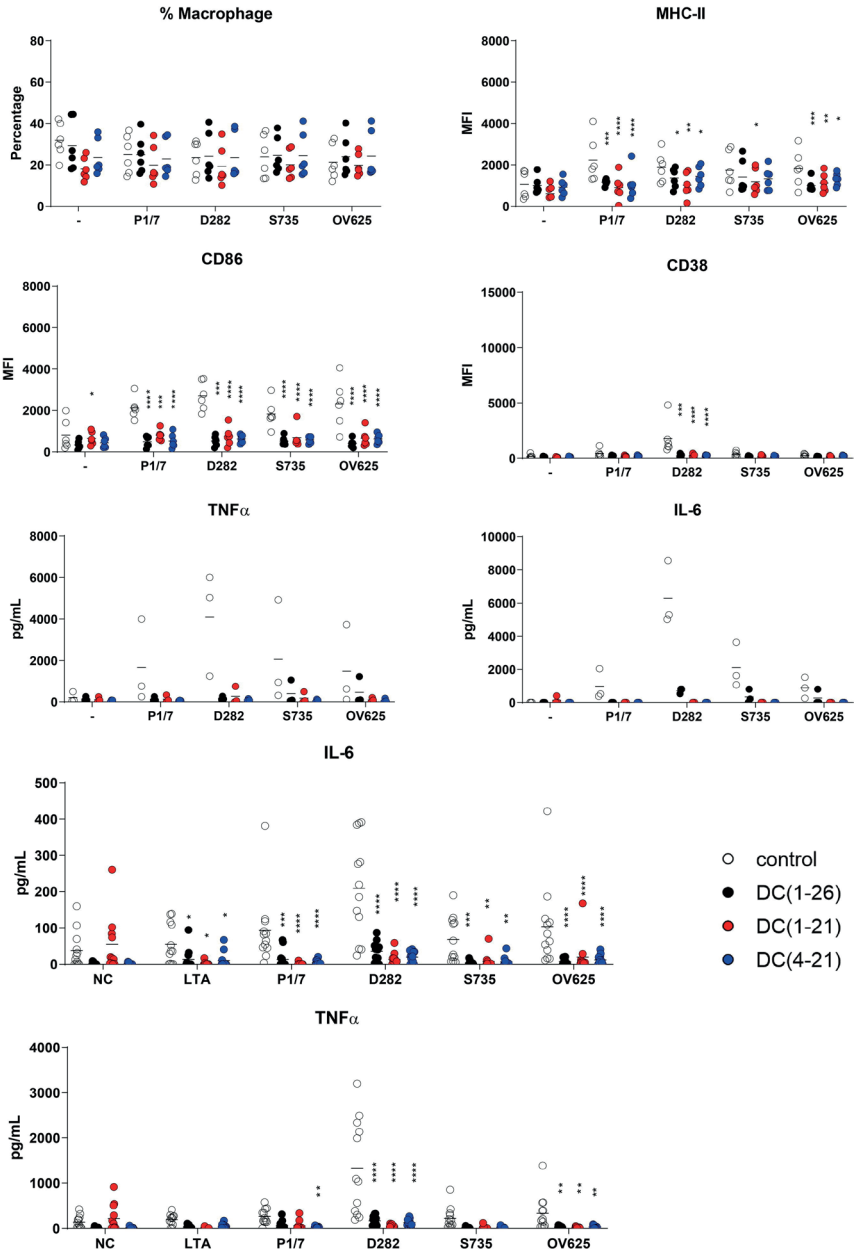


Figure 2. D-CATH-2 and its derivatives inhibit LTA-SA- or *S. suis*-induced activation.

Mouse BMDM cells were cultured for 6 days before they were activated with different *S. suis* serotype 2 strains at an MOI of 0.2. Bacteria were mixed for 5 minutes with 1.25 µM D-CATH-2 or its derivatives before stimulation. After 24 h of stimulation, cells were analysed

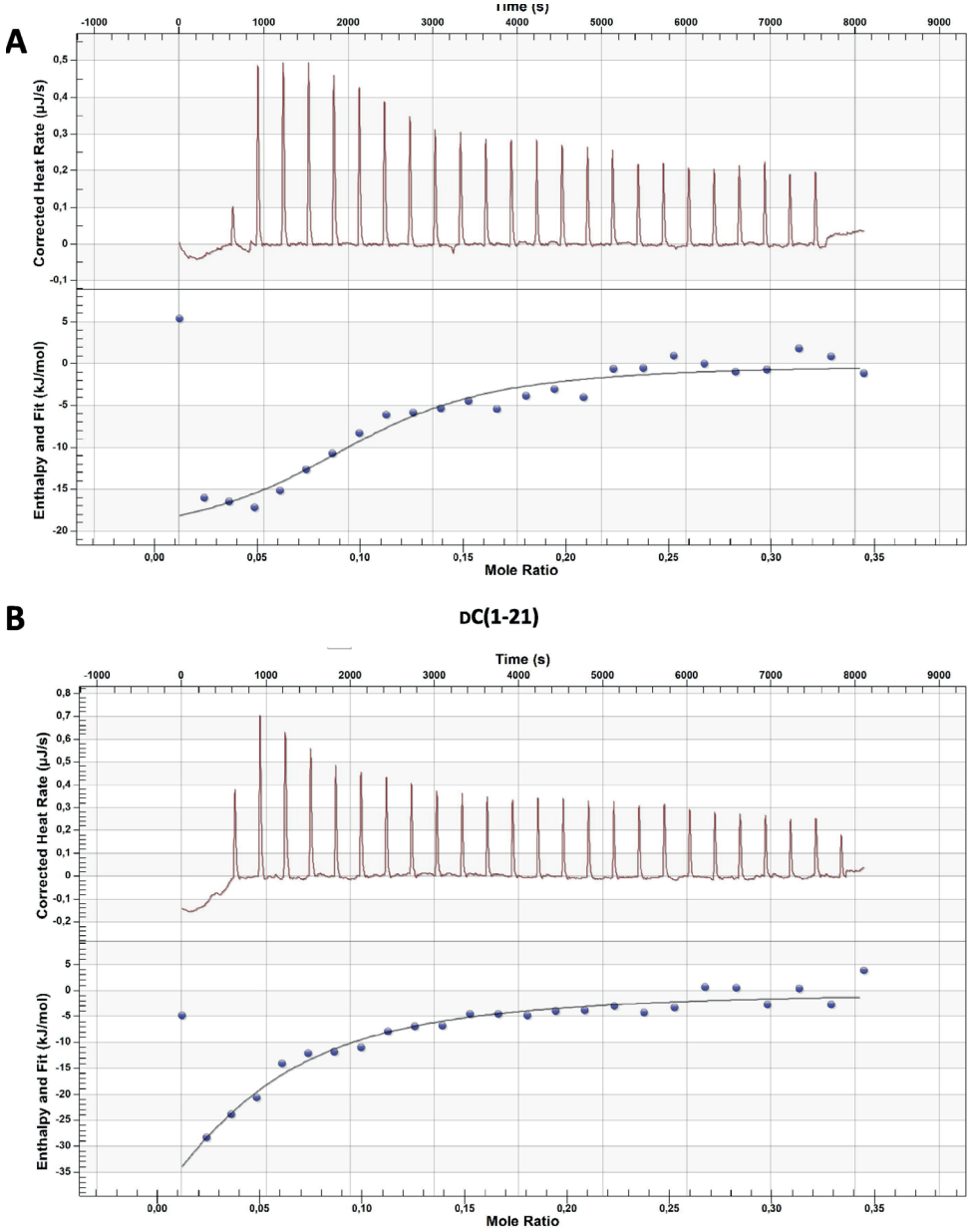
by flowcytometry plotting the median fluorescence index (MFI) **(A)** and cytokine expression was measured **(B)**. 5*10^5 splenocytes, freshly isolated from WT mice using a digestion buffer followed by filtering through a 40 µM cell filter, were activated with different *S. suis* type 2 strains premixed with 5 µM D-CATH-2 or its derivatives at an MOI of 0.2. After 24 hours of stimulation, secreted cytokines were measured using ELISA **(C)**. Data is plotted as average +/- SEM (N=3-6). Samples were compared to no-peptide-controls using two-way ANOVA with the Dunnett post-hoc test. Samples were paired for cell culture samples. *=p≤0.05; **=p≤0.01; ***=p≤0.001; ****=p≤0.0001.

live *S. suis* bacteria, purified LTA was used for activation. In the presence of peptides, neither LTA nor whole *S. suis* bacteria were able to activate the splenocytes, shown by the inhibition of TNFα and IL-6 secretion (**Figure 2C**).

To study whether the inhibitory effect on activation by the peptides was due to a direct interaction of the peptides with LTA, the LTA binding capacity of peptides was tested using isothermal titration calorimetry (ITC). All three peptides inhibiting LTA-induced activation showed direct binding to LTA with a dissociation coefficient K_d ranging from 2-10 µM. Interestingly, D-C(1-21) showed weaker binding compared to the other two peptides, with a higher K_d and less peptide binding to one LTA molecule (**Figure 3 and Table 3**), although the three peptides are equally efficient in inhibiting LTA- and *S. suis*-induced activation.

Table 3. Overview of ITC results of the binding capacity of 200 µM d-CATH-2, dC(1-21) or d-C(4-21) to 37.2 µM LTA-SA. K_d – dissociation coefficient (µM); n – number of peptide molecules binding to one LPS molecule; ΔH – enthalpy changes; -ΔS – entropy changes.

	D-CATH2	DC(1-21)	DC(4-21)
K_d (µM)	3.039	10.22	2.12
n	0.543	0.207	1.209
ΔH (kJ/mol)	-21.16	-85.64	-16.73
ΔS (J/mol·K)	37.39	-180.6	54.68


A
Time (s)
Corrected Heat Rate (µJ/s)
Enthalpy and Fit (kJ/mol)
Mole Ratio
B
DC(1-21)
Time (s)
Corrected Heat Rate (µJ/s)
Enthalpy and Fit (kJ/mol)
Mole Ratio

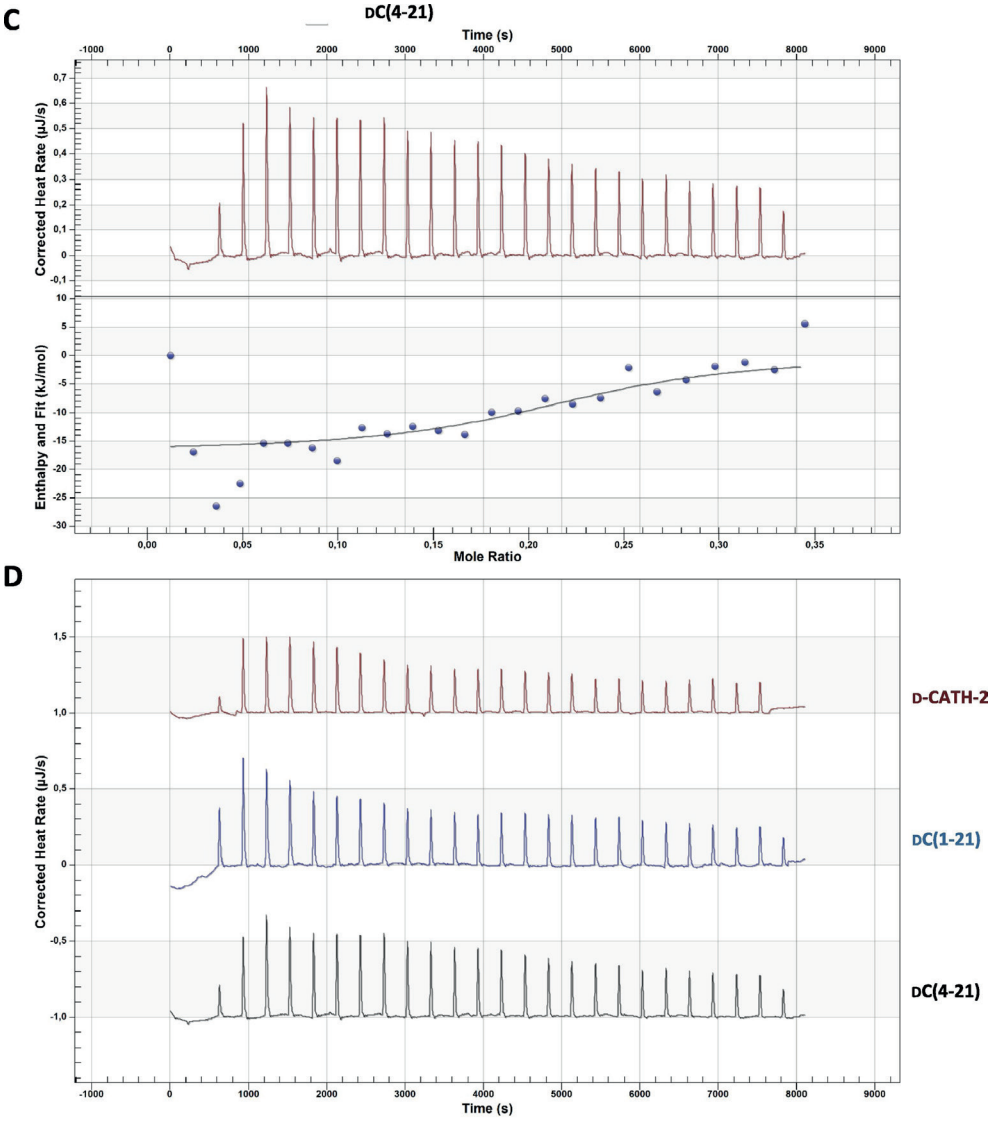


Figure 3. D-CATH-2 and its derivatives bind to LTA.

Thermodynamic binding capacity of 200 µM D-CATH-2 **(A)**, D-C(1-21) **(B)**, and D-C(4-21) **(C)** to 37.2 µM LTA-SA was measured using isothermal titration calorimetry (ITC). Every 300 seconds, 1.99 ml peptide solution was titrated into 164 ml LTA solution. The corrected heat rate (µJ/sec) is plotted (top panel) and normalized integrated heat was plotted against the molar ratio between LTA and the peptide (lower panel). Experiments (N=3) were averaged before plotting and fitting an independent model. The corrected heat rate of D-CATH-2, D-C(1-21), and D-C(4-21) is depicted for comparison **(D)**.

D-CATH-2 and its derivatives increase BMDM culture efficiency

To further study the effect of D-CATH-2 and its derivatives on macrophages, cells were exposed to the peptides at day 1 of culture for 24 h. The differentiation efficiency of the BMDMs was enhanced by the early exposure of the peptides, shown by a higher percentage of cells expressing F4/80 at day 6, which was most pronounced for D-C(1-21) (**Figure 4A**). However, the activity of the cells was not changed based on levels of the activation markers MHC-II, CD86 and CD38 (**Figure 4A**), nor was there any difference in cytokine expression by the peptide treated cells compared to non-treated cells (**Figure 4B**).

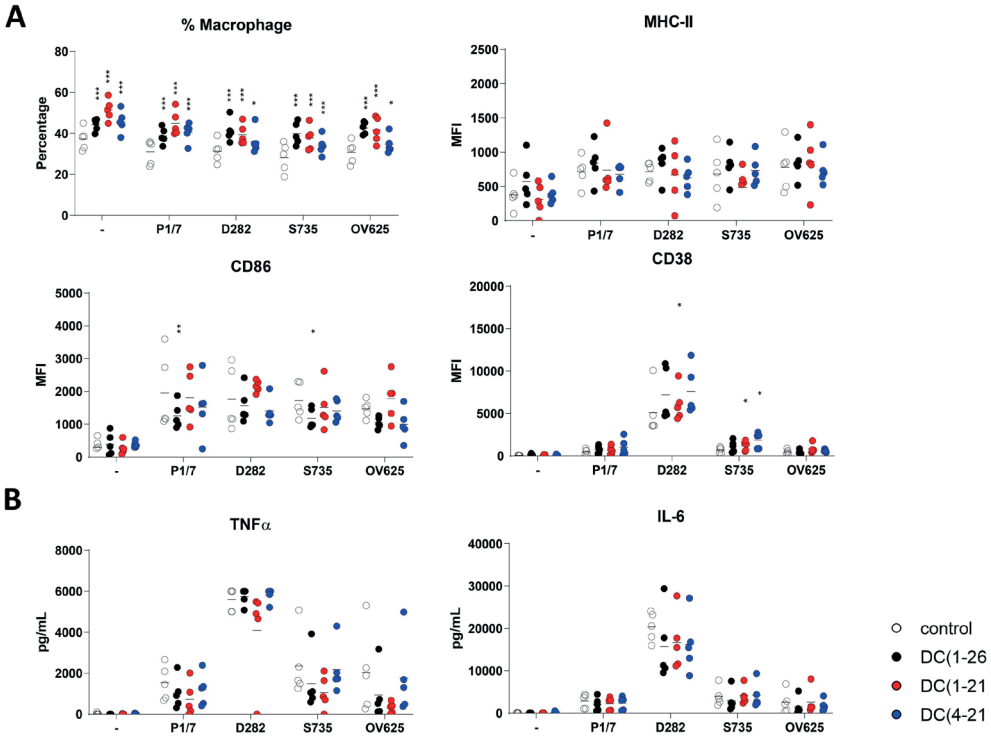


Figure 4. D-CATH-2 and its derivatives increase BMDM efficiency and slightly enhance the activation by *S. suis* serotype 2 strains.

Mouse BMDM cells were cultured for 6 days. At day 1, 1.25 µM D-CATH-2 or its derivatives were added for 24 hours. At day 6 the cells were activated with different *S. suis* type 2 strains at an MOI of 0.2. Bacteria were mixed for 5 minutes with 1.25 µM D-CATH-2 or its derivatives before stimulation. After 24 hours of stimulation, cells were analysed by flowcytometry showing median fluorescence index (MFI) **(A)** and cytokine expression was measured **(B)**. Data is plotted as average +/- SEM (N=3-6). Samples were compared to no-peptide-controls

using two-way ANOVA with the Dunnett post-hoc test. Samples were paired for cell culture samples. *=p≤0.05; **=p≤0.01; ***=p≤0.001; ****=p≤0.0001.

Similar results were found in the BMDC culture when exposing the cells to the peptides on day 1 of the culture for 24h. Although the percentage of BMDCs at day 7 did not change, there was also no difference in the expression of the activation markers. However, the macrophage marker F4/80 was increased, indicating a slight skewing towards macrophage like cells (**Figure S2B**).

D-C(1-21) reduces the clinical symptoms of S. suis P1/7 in mice

Previously, our group has shown that *in ovo* injection of D-CATH-2 three days before hatch protects chickens for seven days post hatch against infection.(Cuperus *et al.* 2016) Since addition of D-CATH-2 both enhanced the efficiency of the murine BMDM culture and balanced the inflammatory response, we questioned whether injection of D-C(1-21) could boost the immune response in mice as well. Therefore, mice were injected subcutaneously with 1 mg/kg D-C(1-21) at day 1 and were subsequently infected with 10^7 CFU/mL *S. suis* P1/7 intraperitoneally either 24h or 7 days post peptide injection. Mice were weighed twice a day during the acute phase of infection, and then daily until 7 days post infection (**Figure 5A**). Both peptide-treated mice and control mice lost approximately 8% bodyweight up to 48 hours post infection, then started to gain weight again, in both the 24 h (**Figure 5B**) or 7 d post peptide injection groups (**Figure 5E**). In addition, a cumulative clinical score was given twice a day during the acute phase of infection and daily during the chronic phase to the mice using a scoring table (**Table 4**). A small reduction of cumulative clinical score for mice was shown in the late stage of disease, if mice were infected 24 hours post peptide injection (**Figure 5C**). Similarly, if infected 7 days post peptide injection a reduction in cumulative clinical score was visible at the acute phase of disease (**Figure 5F**). In addition, treated mice had a higher chance of survival when infected either 24 hours (**Figure 5D**) or 7 days post peptide injection (**Figure 5G**).

Bacterial counts in the different organs were determined as well. Twenty-four hours post infection, all mice, treated or not, had *S. suis* bacteria in the bloodstream at a level of 10^5-10^6 CFU/mL (**Figure S3A**). After 7 days, most mice were able to deplete all bacteria from the blood, which was more efficient in 24h D-C(1-21) treated mice

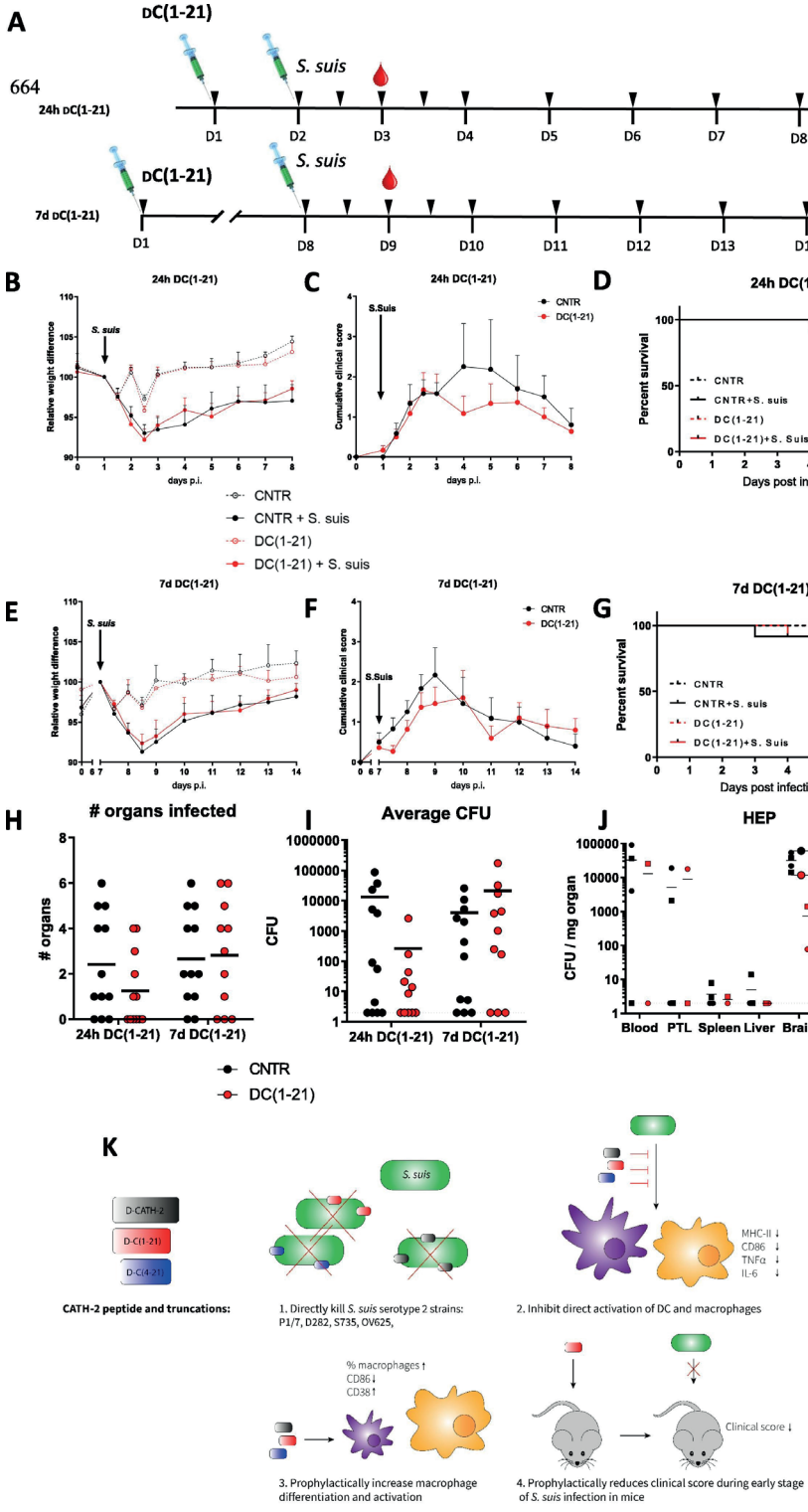

A
664
24h DC(1-21)
S. suis
D1
D2
D3
D4
D5
D6
D7
D8
D9
7d DC(1-21)
D1
D8
D9
D10
D11
D12
D13
D14
D15
B
24h DC(1-21)
Relative weight difference
days p.i.
C
24h DC(1-21)
Cumulative clinical score
S.Suis
CNTR
DC(1-21)
D
24h DC(1-21)
Percent survival
Days post infection
CNTR
CNTR+S. suis
DC(1-21)
DC(1-21)+S. Suis
CNTR
CNTR + S. suis
DC(1-21)
DC(1-21) + S. suis
E
7d DC(1-21)
F
7d DC(1-21)
G
7d DC(1-21)
H
organs infected
organs
24h DC(1-21)
7d DC(1-21)
I
Average CFU
CFU
J
HEP
CFU / mg organ
Blood
PTL
Spleen
Liver
Brain
Kidney
Lung
K
D-CATH-2
D-C(1-21)
D-C(4-21)
CATH-2 peptide and truncations:
S. suis
1. Directly kill S. suis serotype 2 strains: P1/7, D282, S735, OV625,
MHC-II ↓
CD86 ↓
TNFα ↓
IL-6 ↓
2. Inhibit direct activation of DC and macrophages
% macrophages ↑
CD86 ↓
CD38 ↑
3. Prophylactically increase macrophage differentiation and activation
Clinical score ↓
4. Prophylactically reduces clinical score during early stage of S. suis infection in mice

Figure 5. Prophylactic D-C(1-21) s.c. injection reduces the clinical symptoms of *S. suis* P1/7 in mice.

Shown is a schematic overview of the *in vivo* experimental set up. At day 1, all mice were subcutaneously injected with D-C(1-21) or a control in the neck region. Either after 24 hours (24 h D-C(1-21)) or 7 days (7 d D-C(1-21)) the mice were intraperitoneally injected with 10^7 CFU *S. suis* P1/7 or only THB. Twenty-four hours after infection, a few drops of blood were collected and at 7 days post infection the mice were sacrificed for analysis. The black arrows indicate the moment of animal welfare evaluation by weighing and score for clinical symptoms **(A)**. The relative weight difference is depicted for 24 h D-C(1-21) **(B)** and 7 d D-C(1-21) **(E)**. The cumulative clinical score of 8 different parameters is shown for 24 h D-C(1-21) **(C)** and 7 d D-C(1-21) **(F).** Survival curves are shown and bacterial counts in different organs of mice reaching HEP are depicted for 24 h D-C(1-21) **(D)** and 7 d D-C(1-21) **(G)**. The number of organs per mouse in which *S. suis* bacteria were found **(H)** and the average CFU per organ per mouse **(I)** is shown for both groups. The bacterial burden in organs of mice which reached the HEP before the end of the study, with circles depicting mice infected 24 hours post peptide injection and squares depicting mice infected 7 days post peptide injection **(J)**. Results are depicted as mean +/- S.E.M. (CNTR n=4, CNTR+*S. suis* n=12, D-C(1-21) n=4, and D-C(1-21)+*S. suis* n=12). (**K**) Results of the paper are graphically summarized.

compared to untreated mice, where no mice had bacteria left in the bloodstream (**Figure S3B**). More cells were present in the peritoneal lavage after infection, but not different for treated or untreated mice (**Figure S3C**), nor were differences in specific cellular subsets found with flow cytometry (data not shown). Moreover, most mice were able to clear the bacteria in the peritoneum at similar rates in the treated and untreated groups (**Figure S3E**). The spleens of *S. suis* infected mice were enlarged, showing that the infection model was efficient at inducing a systemic immune response, however, no differences in spleen size were found between peptide treated and untreated mice (**Figure S3D**). Only minor differences were found in the number of *S. suis* present in different organs (**Figure S3E**). However, counting the number of organs in which bacteria could be found, showed that 24 h D-C(1-21) treated mice had generally less *S. suis* positive organs (**Figure 5H**) and lower total CFU counts (**Figure 5I**) compared to the untreated mice. Although these results were not statistically significant, the differences are striking. Moreover, the lower bacterial counts were not found for 7-day D-C(1-21) treated mice. In addition, D-C(1-21) treated mice reaching the HEP before the end of the study showed less bacterial counts, especially in the brain, pointing towards a less severe course of disease (**Figure 5J**). The immune cells were analysed for the different organs; however, no differences were found between treated and untreated mice (**Figure S4A-C**).

Table 4. Clinical scoring parameters for cumulative scoring of *S. suis*-infected mice. The cumulative clinical score was defined as the sum of the clinical scoring for eight parameters. Mice were euthanized for animal welfare reasons (humanized end point (HEP)) when they endured severe clinical signs (defined as: 2 days in a row a score of 2 on 3 of the 8 points) or in case of severe weight loss (>20%).

	Score		
	0	1	2
Body weight	Constant or gain	>5% weight loss	> 20% weight loss
Coat	Flat and glossy	Rougher	Bloated
Breathing	Rhythmic	Rapid	Rapid and abdominal
Dehydration	Normal skin elasticity	Reduced skin elasticity	Persisting skin fold
Bearing	Normal	Curved back	Huddled
Eyes	Normal	Moderately squeezed	Squeezed and swollen
activity	Normal	Reduced activity	Apathy
locomotion	Normal	Reduced coordination	Unsteady, apraxia

Discussion

Cathelicidins have both an antimicrobial as well as an immunomodulatory function. Due to this dual function combined with the aspecific and non-proteinaceous molecular target of the peptides, bacteria are less likely to develop resistance and therefore cathelicidins are an interesting candidate as alternative to antibiotics. Chicken CATH-2 has been previously shown to have a good antibacterial activity against a wide variety of bacterial strains (Molhoek *et al.* 2010, van Dijk *et al.* 2012, Banaschewski *et al.* 2017, Scheenstra *et al.* 2019). However, the activity against the Gram-positive encapsulated *S. suis* was, up to now, unknown. *S. suis* is a porcine commensal bacterium which can also cause invasive infections in pigs (Votsch *et al.* 2018) and sepsis and meningitis in humans (Kerdsin *et al.* 2016). Of the 35 known different serotypes, serotype 2 is found to be the most common cause of *S.* suis in-

fection in pigs and humans (Goyette-Desjardins *et al.* 2014). Therefore, in this study, we investigated the possibility to use chicken CATH-2 as an alternative to antibiotics for *S. suis* infection. To enhance the stability of cathelicidins *in vivo*, the L-amino acids were substituted by D-amino acids to prevent proteolytic cleavage (Molhoek *et al.* 2011). In addition, two shorter D-CATH-2 variants were designed.

The full D-enantiomer D-CATH-2 and both its shorter derivatives showed an equally strong killing capacity of four clinically relevant strains of *S. suis* serotype 2 in bacterial medium. However, the salt concentration of the assay medium can strongly influence the activity of cathelicidins. Most cathelicidins lose antibacterial activity at increasing cation concentrations, which is especially important *in vivo* (Lee *et al.* 1997, Travis *et al.* 2000, Cox *et al.* 2003). Increasing salt concentrations can influence the secondary structure of α-helical cathelicidins, which may lead to loss of activity (Durr *et al.* 2006). In addition, other factors such as the presence of serum can reduce their activity, possibly to protect mammalian cells from collateral damage during infection (Johansson *et al.* 1998). However, increased activity was also found for many peptides in the presence of serum containing culture medium depending on the bacterial strain (Coorens *et al.* 2017b). Therefore, the MBC of D-CATH-2 and both its shorter derivatives was also tested in culture medium containing 10% FCS. The antibacterial activity against the four tested *S. suis* strains of D-CATH-2 and D-C(1-21) was slightly enhanced in culture medium, whereas D-C(4-21) appears to be more sensitive to increased salt or serum concentrations, which makes the shortest peptide slightly less favourable as direct antimicrobial drug candidate. However, as this study focuses only on serotype 2 strains, the peptide might still be suitable as antimicrobial for other serotypes (Schultsz *et al.* 2012, Goyette-Desjardins *et al.* 2014). In the immunomodulation experiments there was little to no difference between any of the tested enantiomers. Compared to full length, D-C(1-21) reduced the MHC-II expression of DCs when stimulated with several serotypes, and slightly reduced CD38 expression. Conversely, the full-length peptide reduced CD86 expression when administered during culture, whereas the truncations had no effect. The differences observed between the various enantiomers were overall mild. Therefore, we selected D-C(1-21) as the most promising peptide, as it has the most potent antimicrobial activity, equivalent immunomodulatory activity compared to the other peptides, and is slightly shorter than the full-length peptide.

Inhibition of LPS-induced, and to a lesser extent LTA-induced activation, is a widely studied immunomodulatory aspect of cathelicidins. In this study, LTA-induced activation was strongly inhibited by all three peptides, and this can be partially explained by direct binding to LTA. The dissociation coefficient to LTA is with 2-10 µM much higher than previously shown for L-CATH-2 to LPS with a dissociation coefficient of 0.08 µM (Scheenstra *et al.* 2019), indicating a stronger binding to LPS than to LTA. However, since D-CATH-2 was highly efficient at inhibiting LTA-induced activation, it might also be that the inhibition of LPS- and LTA-induced activation of macrophages occur with different mechanisms. In addition, in this study LTA from *S. aureus* was used to induce TLR-2 activation, while *S. suis* LTA by itself does not potently induce TLR-2 dependent activation in DCs (Gisch *et al.* 2018). It is therefore likely that the inhibition of *S. suis* induced activation observed is through another mechanism, potentially by binding to other lipoproteins.

In addition to inhibition of LTA-induced activation, D-CATH-2 and its derivatives strongly inhibit *S. suis*-induced activation when bacteria are killed by peptides prior to activation of the cells. This inhibition is as efficient for macrophages as for dendritic cells, without affecting cell markers and relative cell numbers. This silent killing was also found for *E. coli* (Coorens *et al.* 2017c), *P. aeruginosa* (Banaschewski *et al.* 2017, Coorens *et al.* 2017a), and *S. aureus* (Banaschewski *et al.* 2017, Schneider *et al.* 2017), when bacteria were killed by L-CATH-2, but not by exposure to heat or antibiotics. Inhibition was also not observed if the peptide concentration remains below bactericidal concentrations. This viability-dependent regulation of immune activation by cathelicidins balances the strength of the immune response, prevents overactivation by killed bacteria and thereby reduces the collateral damage of an unrequired immune response.

Since D-CATH-2 and its derivatives strongly inhibited LTA-induced activation, improved the efficiency of macrophages, and efficiently and silently killed the four *S. suis* strains, the next step was to study the possible protective effect of the peptides *in vivo*. Previously, it was shown that *in ovo* administration of D-CATH-2 protects chickens up to 7 days post hatch from *E. coli* infection (Cuperus *et al.* 2016), suggesting immunomodulation by the peptide as the main mechanism of protective action. In this study injection of D-C(1-21) reduced disease severity and increased

the survival rate, although the differences are relatively small compared to the *in ovo* experiments mentioned above. It is unclear if this difference related to species difference where these peptides may have less activity in mammalian models. However, the human cathelicidin LL-37 has been proven to be beneficial in infection studies in mice before. Intravenous administration of LL-37 in septic mice improved the survival and reduced the bacterial load in the blood and peritoneum (Hu *et al.* 2016, Hosoda *et al.* 2017). However, LL-37 was administered either just before (Hosoda *et al.* 2017) or immediately after (Hu *et al.* 2016) induction of sepsis. Still, these models most likely show not only a direct effect of LL-37 on the infection, but also some immunomodulation, since 2 µg LL-37 per mouse is too low for solely direct antibacterial killing. In addition, mice are not the natural host for *S. suis*, which results in a relatively high bacterial burden needed to establish infection. The preventive effects in pigs could be much higher and should still be investigated. Moreover, in such a model, more detailed parameters such as colonization efficiency in the intestine and tonsils could be measured, which provides a much more complete overview of effects that are important *in vivo.*

Another improvement in activity of D-CATH(1-21) in our model might be achieved by the timing of peptide administration. Administration during embryonic phase would resemble the *in ovo* administration better, but embryonic peptide administration will be difficult and unethical in mammalian farm animals. A possible solution might be to administer a hormonally active form of vitamin D (1,25(OH)2D) to the mother. This has been shown to increase the placental cathelicidin expression (Akoh *et al.* 2017), which might reduce the risk of infection (Akoh *et al.* 2018). Lastly, the bacterial species might also explain the difference in protection. It was demonstrated in a wax moth model that a sea snake cathelicidin (HcCATH) protects better against *P. aeruginosa* infection than against *S. aureus* infection.(Carlile *et al.* 2019) However, more research should be performed to improve the understanding about the immunomodulatory functions of cathelicidins.

Conclusion

Taken together, this study showed a direct antibacterial effect of chicken catheli-

cidin-2 (D-CATH-2) and its shorter derivatives on different *S. suis* strains as well as an immunomodulatory effect *in vitro* by skewing bone marrow-derived dendritic cells towards a more macrophage-like phenotype. In addition, the peptides are able to reduce the inflammatory response *in vitro* for LTA and live *S. suis* bacteria. The immunomodulatory effect *in vivo* is present but should be further optimized to improve D-CATH-2 for potential clinical use.

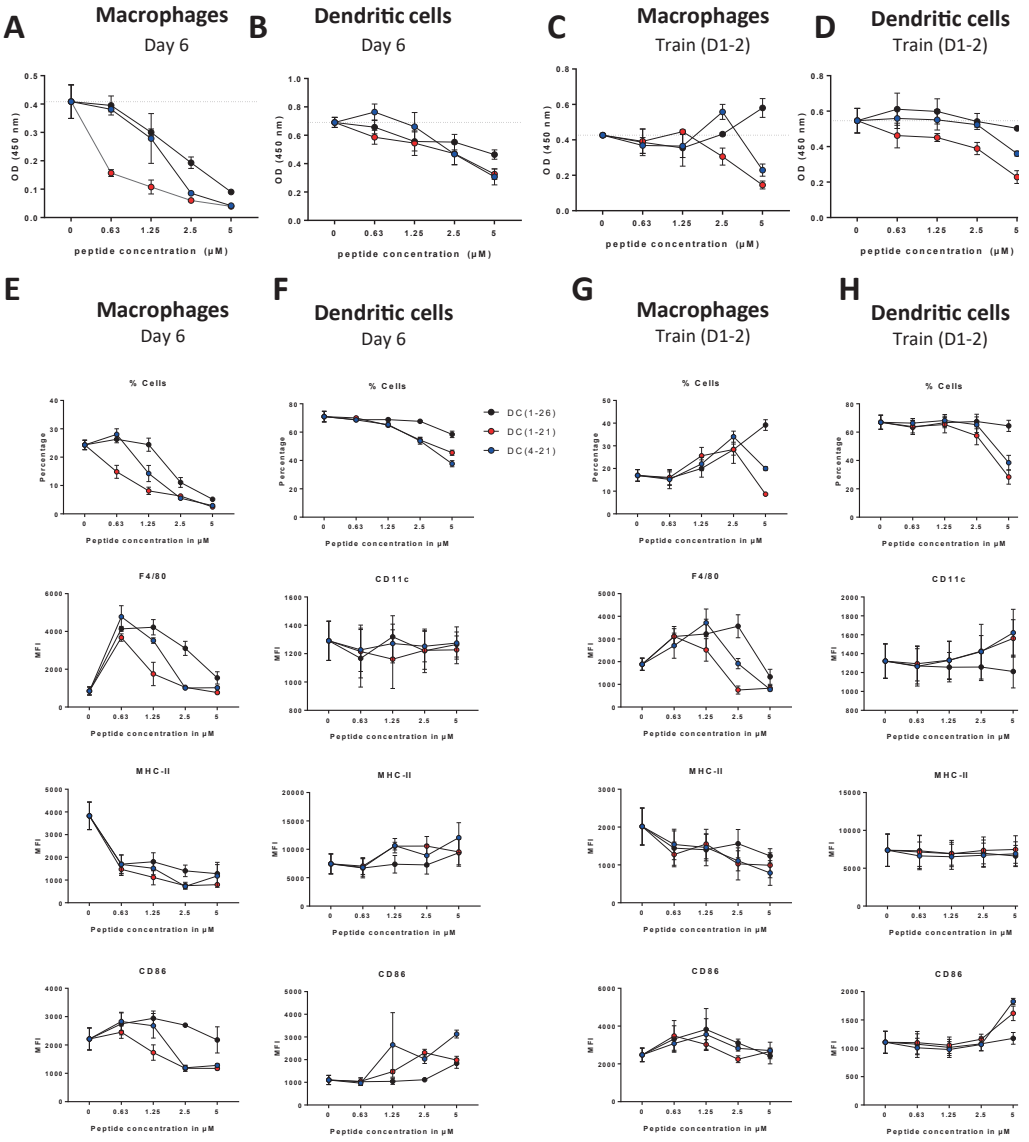
Conflict of interest

The authors report no conflict of interest

Abbreviations

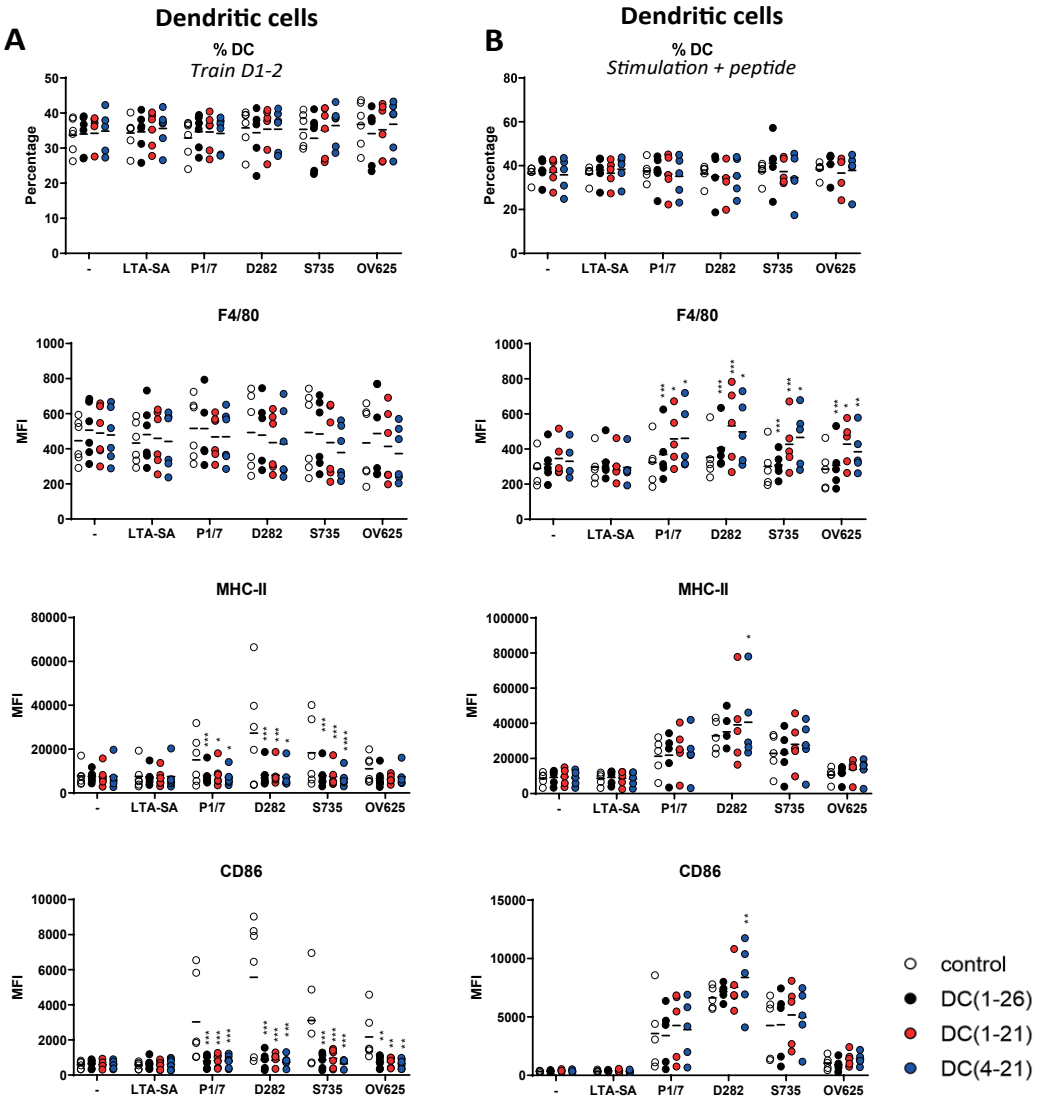
BMDC - bone marrow dendritic cells
BMDM - bone marrow derived macrophages
BSA - bovine serum albumin
DNA - deoxyribose nucleic acid
EDTA - Ethylenediaminetetraacetic acid
ELISA - enzyme-linked immunosorbent assay
FCS - foetal calf serum
HDP - host defence peptide
IFN-γ – interferon γ
IL – interleukin
ITC - isothermic calorimetry
LPS - lipopolysaccharide
LTA - lipoteichoic acid
MBC - minimal bactericidal concentration
PBS - phosphate buffered saline
RPMI - Roswell Park Memorial Institute-1640
THB - Todd-Hewitt broth
TLR - Toll-like receptor
TNFα – Tumour necrosis factor
WST-1 - water soluble tetrazolium salt

Supplemental figures



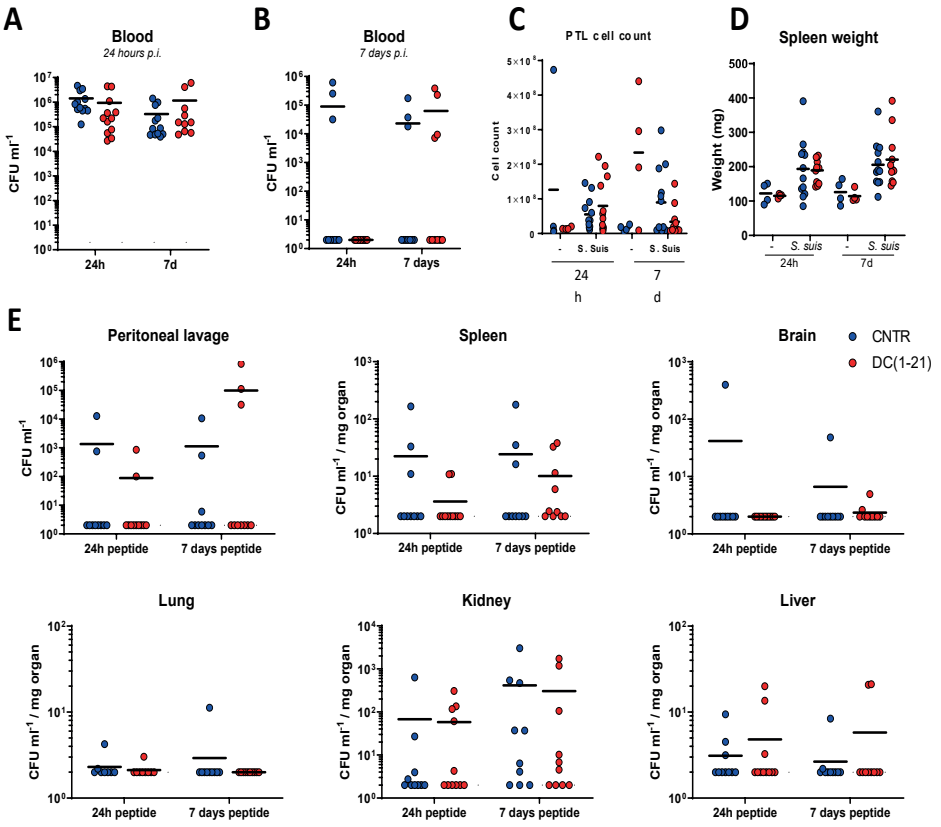
Supplementary figure 1. Peptide titration on BMDM and BMDCs for cytotoxicity.

Mouse BMDM **(A, C, E, G)** and BMDCs **(B, D, F, H)** were cultured for 6 days with GM-CSF and M-CSF respectively. Different concentrations were added at day 6 **(A, B, E, F)** or the cells were primed with different concentrations peptides at day 1-2 **(C, D, G, H)**. At day 7, cell viability was tested using WST-1 reagent, with no-peptide-control set to 100% viability (shown as a dashed line) **(A-D)** and cells were analyzed by flow cytometry for cell marker expression **(E-H)**. Results are depicted as mean +/- S.E.M. (n=3).



Supplementary figure 2. Dendritic cell primed by peptides have increased macrophage markers.

Mouse BMDCs were cultured for 6 days with M-CSF and either primed with 1.25 µM peptides at day 1-2 **(A)** or 1.25 µM peptide was added at day 6 during stimulation **(B)**. Cells were analyzed by flowcytometry for cell marker expression **(C, D)**. Results are depicted as mean +/- S.E.M. (n=6).

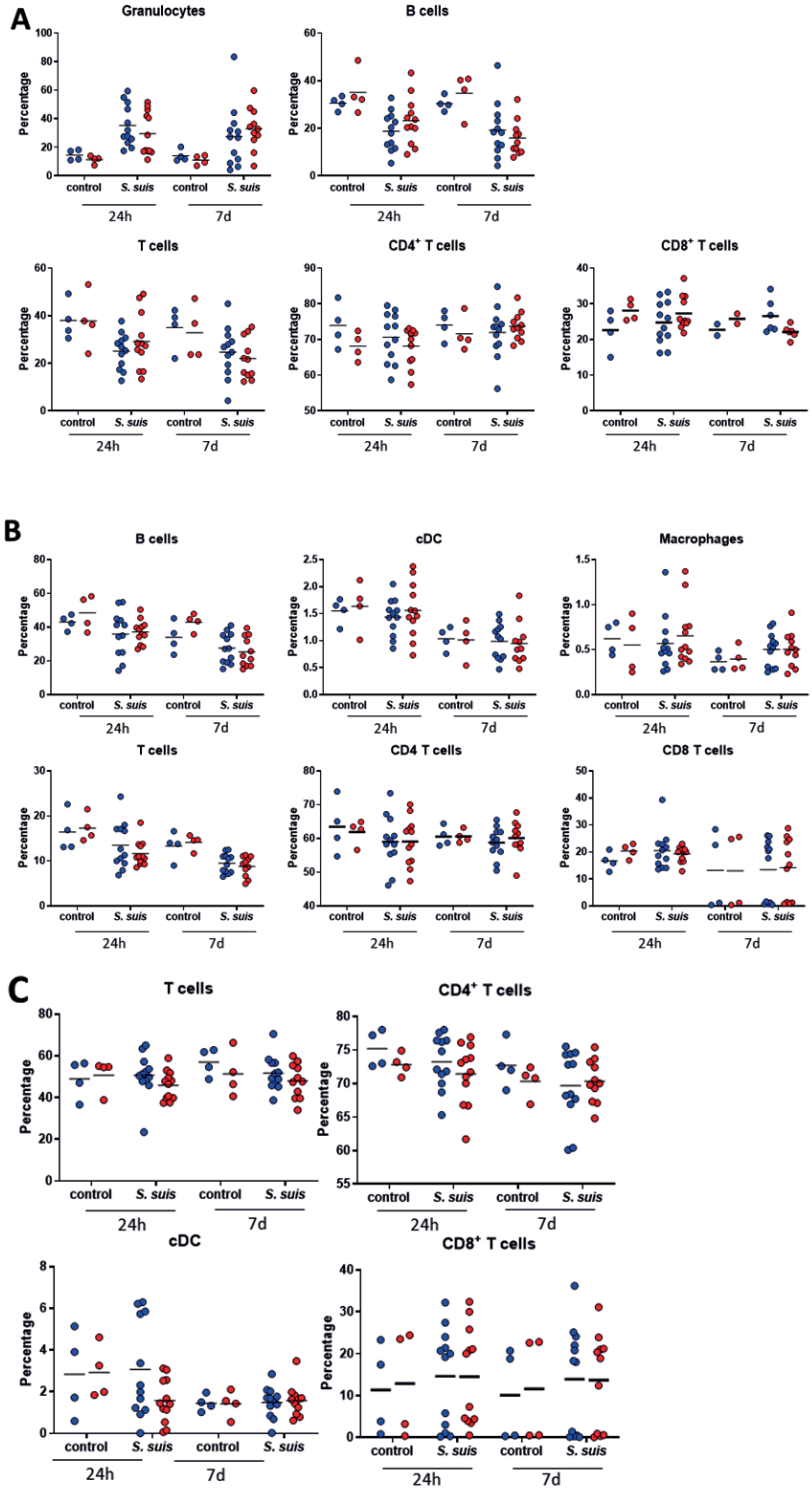


Supplementary figure 3. Bacterial counts in the blood and different organs of *S. suis* infected mice.

Blood was drawn via cheek puncture 24h after infection **(A)** and via heart puncture after 7 days **(B)** and plated on TSA/5% sheep blood plates for bacterial counts **(B)**. The peritoneum was flushed at day 7 and cells present in the peritoneal lavage (PTL) were counted **(C)**. The spleens were weighed **(C)**. The single cell suspension of the different organs was plated on TSA/5% sheep blood plates for bacterial counts. CFU per mg organs was calculated **(E).**

Supplementary figure 4. Flow cytometry analysis of *S. suis* infected mice.

Mice were sacrificed 7 days post infection and fully analyzed. Single cell suspensions of the organs were prepared and analyzed using flow cytometry. A selection of the results of the blood **(A)**, spleen **(B)**, and the axillary lymph nodes **(C)** are depicted.


A
Granulocytes
B cells
T cells
CD4+ T cells
CD8+ T cells
B
B cells
cDC
Macrophages
T cells
CD4 T cells
CD8 T cells
C
T cells
CD4+ T cells
cDC
CD8+ T cells
Percentage
control
S. suis
24h
7d

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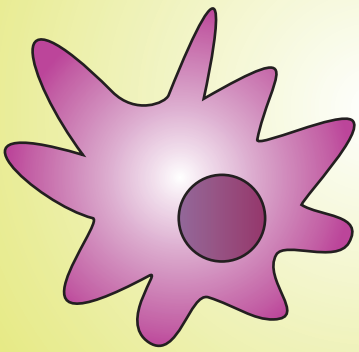

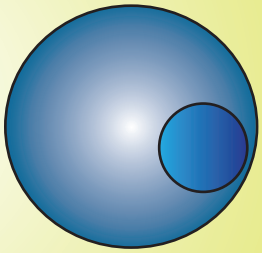

Chapter 7

General Discussion

Pigs are abundant, intensively farmed animals that suffer from numerous bacterial diseases. One such bacterial pathogen is *Streptococcus suis* (*S. suis*), which can also cause harmful infection in humans (Haas and Grenier 2018, Votsch *et al.* 2018) and has shown resistance to multiple therapeutic antibiotics (Yongkiettrakul *et al.* 2019). A potential solution to treat *S. suis* infections could be the application of host defence peptides (HDPs). These HDPs are interesting as potential antibacterial therapy, since they are able to both directly kill bacteria, and modulate immune cells, which could improve host response to infection or vaccination. In addition, bacteria are less able to develop genetically transferable resistance to HDPs (Bechinger and Gorr 2017). HDPs are widely studied in macrophages and DCs (Wan *et al.* 2014, Hu *et al.* 2016, Coorens *et al.* 2017a, Coorens *et al.* 2017c, van Dijk *et al.* 2022), but for pigs, the innate immunity toolbox is not very big. The application of HDPs in the context of *S. suis* infection, specifically in pigs and in the context of the interaction with cells of the immune system, is not broadly explored.

The scope of this thesis is to better characterize the effect of HDPs on LPS- and bacteria-induced activation of porcine immune cells, as well as on bacterial survival, with a special emphasis on *S. suis*. Furthermore, the potential application of HDPs as vaccine components is explored. **Chapter 2** provides a clear overview of the immunomodulatory capacity of HDPs in general. In **chapter 3,** it is shown that the chicken cathelicidin CATH-2 inhibits the inflammatory reaction of porcine macrophages in the presence of LPS or *E. coli,* while several porcine cathelicidins were less capable of inhibition. In **chapter 4**, it is demonstrated that *S. suis* can protect itself from antimicrobial effects of porcine peptides and to some extent from L-CATH-2, but not from D-CATH-2. In **chapter 5**, it is shown that CATH-2 similarly inhibits both LPS and bacteria-induced inflammation of porcine bone marrow derived DCs (BMDCs). Again, on DCs, the porcine peptides were ineffective at modulating both LPS as well as bacteria-induced stimulation, although stimulation of BMDCs with the porcine peptide PR-39 leads to an increased uptake of unencapsulated *S. suis*. Finally, D-CATH-2 had a small prophylactic effect in a mouse model of *S. suis* infection (**chapter 6**). In this chapter obtained results will be discussed in the light of the aims stated, as well as the limitations and opportunities offered.

BMDMs

In chapter 3, the effects of various peptides on the immune activation of porcine bone marrow derived macrophages were assessed. This particular model is based on the M1/M2 dichotomy by culturing macrophages from bone marrow mononuclear cells in the presence of GM-CSF or M-CSF, respectively (Gao *et al.* 2018). However, macrophages *in vivo* are a very diverse type of cell, with a variety of functions depending on their surrounding tissues, and *in vitro* macrophages reflect that diversity (Geissmann *et al.* 2010). For our BMDMs, the method we used is not the only way to culture macrophages or macrophage-like cells. Other groups have developed culture methods for porcine macrophages with similar characteristics. When porcine myeloid cells were isolated based on SWC3α expression from PBMCs, incubation with GM-CSF also led to a M1-like phenotype (Sautter *et al.* 2018). Sautter *et al.* described an upregulation of MHC-II, CD40 and CD11a and downregulation of CD1 and CD203a was observed. In the paper that established our method, upregulation of MHC-II was also found, but no change in CD203a expression was measured, while the other markers were not determined (Gao *et al.* 2018). These differences could be explained by the different origin of the SWC3α expressing cells, as we used bone marrow mononuclear cells and not myeloid blood cells, and as such might contain a subpopulation of SWC3α+ that is not present in blood, like common myeloid progenitor cells. Additionally, our cells were stored in liquid nitrogen, which reduces cell viability, but might hit certain populations harder than others. Considering other methods of M2 macrophage polarization, Kapetanovic *et al.* examined M-CSF derived bone marrow derived macrophages, which resulted in SWC3α^{+}, CD14^{+}16^{+} cells that were CD163$_{lo}$ or CD163^{-} (Kapetanovic *et al.* 2012), while ours are explicitly high in CD163, which is a hallmark of M2 macrophages (Gao *et al.* 2018, van Harten *et al.* 2022). This could potentially be explained by their use of human-derived M-CSF, whereas our studies used mouse-derived M-CSF. In swine blood, macrophages are generally defined as CD172a^{+} CD1$^{+/-}$ CD4^{-} CD11R1^{-} CD14$^{+/-}$ CD16^{+} MHC-II$_{lo}$ CD80/86$_{low}$ (Summerfield and McCullough 2009, Fairbairn *et al.* 2011), with CD163 expressed on both macrophages and monocytes (Fairbairn *et al.* 2013). In contrast, the BMDMs we used were CD172α^{+}CD80/86$_{hi}$ and variable in CD163 (Gao *et al.* 2018). In each of these studies differences can be pointed out, but all cells can likely be considered macrophages with a slightly different phenotype. Indeed, when comparing *in vitro*

cultured macrophages to macrophages isolated from liver, blood, brain and other tissues, M1 and M2 cultured macrophages do not necessarily express groups of genes that determine whether a macrophage is M1 or M2, and the genes that do get activated are not stably expressed over time (Hume 2008, Geissmann *et al.* 2010, Yona *et al.* 2013, Hume and Freeman 2014, Xue *et al.* 2014). This means that the terms M1 and M2 are likely more indicative of the macrophage culture conditions, rather than that it reflects a type of cell that can be found in the body. So essentially, the BMDMs seem to match the macrophages that are described in the literature, but it seems challenging to directly translate the functioning of these macrophages to macrophages that are present in a live pig. Rather, these results should be interpreted as a model for innate immune cells of the pig. Additionally, a good *in vitro* model improves screening capacity for immune regulatory compounds for an animal, and ultimately reduces the number of experimental animals needed, which is highly beneficial especially in large animals.

BMDCs

Similar to macrophages, dendritic cells are also diverse both *in vivo* and *in vitro*. In pigs, *in vivo* as Flt3+ cells, with SWC3α present on all DCs except cDC2 (which are Flt3+ SWC3α-). However, monocyte derived DCs lack Flt3 expression (Summerfield *et al.* 2015)monocytes,macrophages,bovine,ovine,porcine,canine,feline,equine</keyword></keywords><dates><year>2015</year></dates><accession-num>25387110</accession-num><urls><related-urls><url>https://www.annualreviews.org/doi/abs/10.1146/annurev-animal-022114-111009</url></related-urls></urls><electronic-resource-num>10.1146/annurev-animal-022114-111009</electronic-resource-num></record></Cite></EndNote>. Monocytes differ from these MoDCs by expressing CD11b (Moreno *et al.* 2010). Although their heterogeneity is not as pronounced as that of macrophages, the phenotype of *in vitro* cultured BMDCs and their marker expression depend highly on the animal used and the protocol applied. Chapter 5 describes the effects of HDPs on BMDCs that were grown in the presence of GM-CSF and IL-4, which uses similar cytokines as a protocol for human monocyte-derived DCs (moDCs) (Banchereau *et al.* 2000). Interestingly, porcine cells grown from bone marrow in the presence of GM-CSF alone are also often called DCs, which could also be called M1 macrophages

(Carrasco *et al.* 2001, Lecours *et al.* 2011, Martelet *et al.* 2017, Geervliet *et al.* 2020). Functionally, these cells express high levels of MHC-II and co-stimulatory markers, which indicates DC-like properties. However, these cells are also highly phagocytic, can kill invading microbes and express large quantities of inflammatory cytokines, which indicates macrophage-like properties (Pérez-Ortega *et al.* 2021, van Harten *et al.* 2022). In mouse bone marrow DC cultures, it was shown that these bone marrow derived DC cultures grown with GM-CSF alone contain a subpopulation of immature macrophages (up to 65%) (Helft *et al.* 2015). Further addition of IL-4 did not significantly change the purity of the culture (Helft *et al.* 2015). This is an indication that *in vitro* cultures may not be as homogeneous as many assume, although extensive gating experiments have not been performed for porcine bone marrow cultures. After the initial culture, many protocols for porcine DCs include an activation or priming step prior to the stimulation that is the objective of the experiment. These protocols include TNFα (Carrasco *et al.* 2001, Chamorro *et al.* 2004), or LPS (Chang *et al.* 2008). It is unclear whether these cultures contain more DCs or better-quality cells. Therefore, porcine DC research would benefit from a more standardized protocol for DC culture. Until that time, papers discussing primary DCs from pigs should include an experimental validation step. In the case of chapter 5, the DCs were almost uniformly SWC3α^{+}, highly positive for MHC-II and CD80/86, and expressed both cytokines and chemokines after LPS stimulation, which makes them suitable for both our experiments and comparison to existing literature.

When it comes to *in vitro* cultured cells, many culture methods yield a heterogenous population of cells of which it is often unclear whether they should be defined as macrophages, DCs, both or neither. In my opinion, functional characterization of *in vitro* cultured cells is the relevant discourse about these cells. DCs and macrophages are defined by the expression of markers or transcription factors, but ultimately do share several functions; DCs are primarily APCs but are capable of phagocytosis (Meijerink *et al.* 2012). Likewise, macrophages are extremely specialized phagocytes and experts at tissue remodelling, but they are also able to present antigens and activate adaptive immune cells (Barker *et al.* 2002, Muntjewerff *et al.* 2020). Especially when these cultures contain multiple different populations, it is not vital to define *in vitro* cultured cells as macrophages or DCs but characterize them based on the assay that is used. Other scientists have also proposed that classifying cells from the mono-

nuclear phagocytic system based on ontology is more relevant *in vivo* (Guilliams *et al.* 2014).

Effects of HDPs on porcine immune cells

HDPs have been shown to have various immunomodulatory effects *in vitro* on immune cells, including inhibiting LPS induced activation, promoting nucleic acid uptake, inducing chemotaxis and regulating expression of cytokines and cytokine receptors (van Harten *et al.* 2018). In this work, the tested HDPs that originate from pigs were remarkably ineffective at inhibiting activation of porcine cells, both BMDMs and BMDCs, as described in chapters 3 and 5. Porcine derived HDPs PMAP-23 and PR-39 neither reduced LPS activation of porcine macrophages nor did they inhibit LPS activation of porcine DCs. These results are surprising, as these HDPs are a native part of the pig immune system and would therefore be expected to have a modulating function, based on the extensive number of immunomodulatory functions of similar HDPs in other species. Under homeostatic conditions, HDPs in human tissues range from 0.2 to 6 µM depending on the tissue, while under extreme conditions such as psoriasis up to 300 µM HDP can be detected (Ong *et al.* 2002, Scheenstra *et al.* 2020). It is possible that the HDPs tested in chapters 3 and 5 have more immunomodulating properties at higher concentrations, although *in vivo* concentrations are not well established for porcine HDPs. Additionally, we tested only PMAP-23, PMAP-36 and PR39, while pigs express at least 11 cathelicidins as well as multiple defensins. Some of these other HDPs could be more active in the immunomodulation assays that we used. Furthermore, we assessed mostly the LPS neutralization and bacterial uptake as modulatory function of these peptides. It is possible that these tested peptides have other functions *in vivo* such as increasing nucleotide uptake or modulating other innate stimuli, directly upregulating cytokines or receptors in other cell types, and acting as chemokines, all of which require different assays than those used in this thesis. For example, porcine cathelicidins PMAP-23, PMAP-36 and PR-39 can form a complex with nucleic acids like DNA, and thereby enhance uptake and subsequent TLR-9 signalling in porcine pDCs, and induce a strong IFN-α response (Baumann *et al.* 2014). PMAP-23 and PR-39 induce IL-8 expression in both porcine IPEC-J2 and 3D4/1 cells, while PMAP-23 reduces the uptake of latex beads by freshly isolated porcine monocytes (Veldhuizen *et al.* 2017). These result contrast with the

poor activity of the HDPs in our assays and show that the choice of cell and assay have a strong effect on the outcome. It is therefore vital that assumptions about the activity of HDPs are not extrapolated from individual experiments, but rigorously tested in a target species, cell type and activity assay.

In contrast to the porcine peptides, chicken CATH-2 was remarkably effective at neutralizing the LPS-induced immune activation as well as bacteria-induced immune activation of both porcine BMDMs and BMDCs. Although CATH-2 is a chicken cathelicidin, it has previously been shown to bind directly to LPS and thereby prevent TLR-4 stimulation on mouse RAW264.7 and human derived macrophage-like THP-1 cells (van Dijk *et al.* 2009, Van Dijk *et al.* 2016, Scheenstra *et al.* 2019). Considering that CATH-2 binding activity is necessary for its LPS neutralization, it is possible that CATH-2 sterically interferes with the binding of porcine TLR-4 to LPS. On the other hand, a peptide's affinity for LPS does not always correlate to modulation efficiency (Scheenstra *et al.* 2019). In fact, sometimes lower affinity leads to better modulation. More research is needed to determine whether HDPs affect other layers of modulation beyond steric hinderance of TLR-4, particularly in downstream signalling pathways.

S. suis and HDPs

Besides being poor immunomodulators as shown in chapters 3 and 5 and above, the porcine HDPs were not very effective antimicrobials against *S. suis* (chapter 4). *S. suis* typically colonizes the upper respiratory tract but can cross this epithelium and enter the bloodstream from where it can cross the blood brain barrier and colonize the cerebrospinal fluid (CSF). In mammalian respiratory epithelia (which includes humans and pigs), HDPs are usually constitutively expressed at low levels (Nizet *et al.* 2001, Schauber *et al.* 2003, Tjabringa *et al.* 2003, Shaykhiev 2005). These epithelial conditions are what is mimicked by the conditions set in our assay, although the HDP concentration could increase in infectious conditions. During infection, invading streptococci encounter the epithelia and interact with locally produced HDPs *in vivo*. As an example of such an interaction, the human pathogen *S. pneumoniae* recognizes human LL-37, which is constitutively expressed by lung epithelial cells, to detect proximity to the epithelial surface. After coming in contact with the human respiratory epithelia, the bacterium can shed its capsule, which then both acts as a decoy for LL-37 and increases adhesion and invasion of the epithelia by the bacteria

(Kietzman *et al.* 2016). However, there is no evidence for capsule shedding for *S. suis* so far, and in addition, the bacterium lacks the gene LytA that *S. pneumoniae* uses for shedding (Kietzman *et al.* 2016, Gaiser *et al.* 2019). We have shown that the lack of the capsule in *S. suis* J28 does not change the effect of porcine peptides, so therefore the lack of antimicrobial activity is most likely not related to the presence of a capsule. Therefore, the way that *S. suis* protects itself from the constitutive expression at epithelial surfaces is not yet known.

The chicken HDP CATH-2 performed remarkably well in killing *S. suis* (chapter 4). In other assays, CATH-2 has consistently proven to be a good antimicrobial (Schneider *et al.* 2016a, Coorens *et al.* 2017b, Scheenstra *et al.* 2019). Although CATH-2 was consistently less active against *S. suis* than its D-analogue, the antimicrobial activity of CATH-2 should not be dismissed. It is possible that whatever mechanism protects *S. suis* against porcine HDPs, works to a lesser extent against CATH-2 and not against D-CATH-2. Combined with its potent anti-inflammatory effect (chapter 3 and 5), CATH-2 could be a useful dual antimicrobial and antisepsis drug against bacterial infections. However, as curative treatment for these bacterial infections is likely only interesting for humans and potentially companion animals, more research would have to be performed focussing on CATH-2 effects on cells of those species.

In the immune system of vertebrates, naturally occurring host defence peptides are exclusively composed of L-amino acids. Bioavailability of peptides might be reduced due to proteolytic breakdown, as many peptides are susceptible to proteolysis *in vitro*, but data *in vivo* proteolysis is scarce, although some tracking experiments have been performed. When experimental peptide SET-M33L was injected intravenously in mice, various tissues including lung, spleen, liver and kidney showed uptake within 30 min, where it persists for up to 24 h, whereas plasma clearance was almost complete by 60 min (Brunetti *et al.* 2016). When synthetic peptide IDR-1018 was intraperitoneally administered to mice, peak blood concentration was reached after 60 minutes, and the labelled peptide could also be found in brain tissue up to 4 h after delivery (Bolouri *et al.* 2014). To protect peptides from proteolysis, D-amino acids can be used to synthesize peptides, as these are often not recognized by the L-amino acid specific proteases present in blood and other tissues (Hong *et al.* 1999). However, these D-peptides are mirror images of the normal peptides and can therefore lose

potential receptor mediated functions. In our experiments, D-peptides repeatedly outperformed their L-analogues in immunomodulation of porcine cells and killing of streptococci. This could be due to better proteolytic stability, although breakdown of L-CATH-2 could not be shown. It would be interesting to see whether D-analogues of the porcine peptides have similar poor antimicrobial function. Furthermore, a more complete picture of possible proteolytic breakdown of L-peptides could show whether proteases play a key role in escaping HDP-mediated killing. Finally, it remains unclear whether proteolysis is responsible for L-peptides being less effective, or whether there is another process involved.

HDPs potential as therapeutics for pigs

The data shown in chapter 4 expand our knowledge of *S. suis* killing by cathelicidins. It was found that particularly D-CATH-2 is effective at killing and seems to evade the protective mechanism seen for the porcine peptides and to a lesser extent L-CATH-2. However, this study was done with mice, which are not the real target of *S. suis* infections. Additionally, *S. suis* infections do not naturally occur through intraperitoneal invasion, but rather through invasion of respiratory epithelia. A better model with pigs and a, for instance, nasal route of infection would be preferred as a disease model. However, despite administration of peptide at a different site and time than the *S. suis,* a reduction of disease severity could still be observed. Combined with the anti-inflammatory properties of CATH-2 described in Chapter 3 and 4, we could speculate that D-CATH-2 may be a good HDP to protect pigs against *S. suis*.

An important downside of HDPs as drug candidates for farm animals is the current cost of synthesis. For humans, currently approved clinical application of HDPs is limited to a handful, which are nisin A, gramicidin, polymyxins, daptomycin and melittin, and are mostly in use as alternatives to antibiotics (Dijksteel *et al.* 2021). The use of peptides made entirely from D-amino acid for treatment, especially for pigs, is currently not feasible. However, mammals do make their own HDPs, and can therefore be induced by other, cheaper compounds, for example through feed additives. For example, LL-37 production in human cells can be induced through butyrate (Schauber *et al.* 2003) or vitamin D_3 (Mallbris *et al.* 2005). This approach is highly dependent on application, as the induction of HDPs would need to occur at sufficient concentrations at the site of infection. Furthermore, for many therapeutics, combination

therapies of multiple classes of antibiotics are now increasingly common (Sullivan *et al.* 2020). In this thesis, only individual peptides were tested. It is possible that combinations with multiple HDPs or with other therapeutic drugs like antibiotics lead to synergistic effects. For some HDPs, synergy with existing drugs has already been shown, for instance as vaccination with HDPs, CpG oligonucleotides and polyphosphazene as adjuvants lead to higher rates of antibody production against a model antigen in cattle compared to traditional carriers (Kovacs-Nolan *et al.* 2009).

The question posed in the introduction of this thesis was whether peptides could be used for any kind of treatment of *S. suis* in pigs, either as antimicrobials or vaccines-additive. Vaccines are usually less effective at inducing protection than natural infections. This is among other things because in natural infections all necessary innate activation is present (Coffman *et al.* 2010, Nanishi *et al.* 2020). The issue with waning immunity after vaccination can be clearly seen in the case of the *Bordetella pertussis* vaccine (Wendelboe *et al.* 2005). In first instance, an inactivated whole cell vaccine was used which induced protective immunity, although it came with side effect such as fever. Therefore, an acellular vaccine was introduced, which eliminated these side effects and seemed to induce similar protection. However, it was shown later that the acellular vaccine not only led to waning immunity over the course of 4 to 12 years, but also induced a Th2 response while Th1 or Th1/Th17 are preferred, and fails to induce tissue resident T memory cells (Wendelboe *et al.* 2005, Wilk *et al.* 2019). This shows that a third generation of bacterial vaccines must be developed to prevent or halt waning immunity and to prevent unwanted side effects at the same time. To achieve this, the use of immunomodulators, which could be added to vaccines to either boost or dampen the immune response, can be envisioned.

In the studies performed in this thesis, CATH-2 generally served as an inhibitor of LPS-induced activation. If CATH-2 would be added to a vaccine containing LPS (like whole cell vaccines), in theory it would inhibit inflammation which is required for establishing an immune response. However, in the broadest sense, this could be useful for applications that have unwanted levels of inflammation causing side effects, such as inactivated whole cell vaccines have. Furthermore, other cell wall mimicking vaccines like outer membrane vesicles could be investigated in combination with these peptides (Balhuizen *et al.* 2021). It remains rather speculative how HDPs could

influence vaccine efficacy. The effect of HDPs on a wide variety of processes remains poorly characterized. For some HDPs, an increase in phagocytosis was observed (chapter 4)(Wan *et al.* 2014, Zhang *et al.* 2016), which could lead to more efficient antigen presentation in DCs, provided the DCs are efficiently targeted by the HDPs *in vivo*. Other pathways, such as antigen processing, cross presentation, induction of B cell response, and the polarization of T helper populations remains even poorer understood. Additionally, for vaccines it is important that there is no immune response generated against the peptides, as that would undercut the main goal of vaccination.

The question posed in the introduction of this thesis was whether HDPs could be used for any kind of treatment of *S. suis* in pigs, either as antimicrobials or vaccines-additive. The best-known immune memory is adaptive immunity. However, the innate immune system can also develop memory-like properties (Ifrim *et al.* 2014, Netea *et al.* 2016). For example, BCG vaccination of humans leads to protection against unrelated virus infections (Arts *et al.* 2018). Likely, this process occurs due to epigenetic changes in metabolic pathways in innate immune cells that make subsequent activation easier (Domínguez-Andrés *et al.* 2019). Since peptides are part of the innate immune system, it is possible that they too can induce innate memory. As HDPs like D-CATH-2 can increase immune responses in chickens (Cuperus *et al.* 2016) and zebrafish (Schneider *et al.* 2016b) beyond the life time of the HDPs, it is speculated that HDPs could work through a similar mechanism. Recently, some evidence has been provided for innate immune training by HDPs, specifically of human macrophages by D-CATH-2 analogues (van Dijk *et al.* 2022), although for pigs there is no data. D-CATH-2 analogues worked as prophylaxis in a mouse model of *S. suis* infection, although they could not fully protect the mice. Currently, prophylactic treatment is much more applicable for pigs than curative treatment. Two major questions remain, however. Firstly, how does D-CATH-2 or analogues function as vaccine additives? In other words, what effects do these peptides have (if any) on antigen processing and presentation, and on the skewing of the Th response? And secondly, what is the effect on local versus systemic immunity, and on the generation of protective immunity and memory T cells? These questions depend highly on the vaccine, on the antigen, on the eventual formulation and on the pathogen, and the answers can only

be found experimentally by testing a vaccine containing D-CATH-2.

A highly explored function of HDPs is modulation of extracellular TLRs such as TLR-4 and TLR-2. However, HDPs have been demonstrated to have another potent layer of TLR modulation. HDPs can complex with nucleic acids and improve uptake and subsequent signalling of intracellular TLRs such as TLR-7, TLR-8, and TLR-9 (Lande *et al.* 2007, Ganguly *et al.* 2009, Coorens *et al.* 2015, Baumann *et al.* 2017, Takahashi *et al.* 2018). By complexing, the HDPs can structure the nucleic acids to provide optimal spacing for TLR activation, as these HDP-nucleic acid complexes fit very well in the TLR groove (Lee *et al.* 2019, Lee *et al.* 2020). Although for pig TLRs this mechanism has not yet been demonstrated, the increased uptake of nucleic acids and subsequent TLR-9 signalling has been observed in porcine pDCs (Baumann *et al.* 2014). Although we did not investigate TLR-9 signalling in our macrophages nor DCs, this TLR-9 route could be another avenue to explore HDP incorporation in vaccines. Since nucleic acid vaccines have been successfully applied for the first time recently with COVID-19 vaccines (Corbett *et al.* 2020, Polack *et al.* 2020), this avenue could prove a promising new strategy, although nucleic acid vaccines for pigs are not widely available.

As antimicrobials, the HDPs tested in this thesis are probably not very suited in pigs. There are already other antimicrobials against bacteria like *S. suis*, but CATH-2 works fairly well, and the chance of protective resistance developing is low. However, curative antimicrobials for pigs are not commonly used for financial reasons. A different strategy that can be employed for these peptides is induction of native HDP production, although these would likely only work for infections of the intestinal tract. Using some other (cheap) compound, inducing HDP production could lead to a better protected animal. Considering that the porcine peptides were not very useful against, *S suis*, this strategy probably has limited chance of success. HDPs as antimicrobials could have some important advantages, though. Firstly, the mechanism of killing through membrane interaction rather than protein targets makes it more unlikely that resistance development occurs. Secondly, the often-dual function of antimicrobial and immunomodulatory makes it so that over inflammation maybe does not occur so soon. These properties make HDPs still a potential good antimicrobial for human application, where still more curative drugs are needed, and they are allowed

to be expensive. For HDPs in pigs, it is more likely that either incorporation in vaccines or application as boosters of innate immunity are the way forward. Hopefully, the results obtained here help achieve the goal of better health for pigs.

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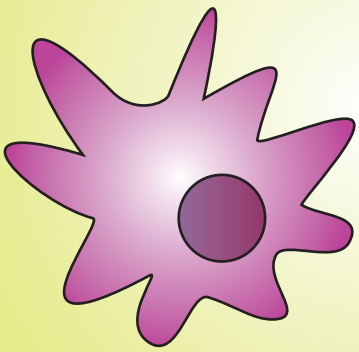

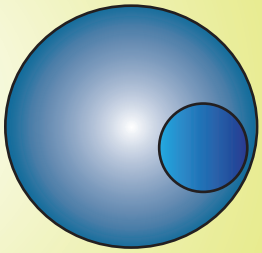

Appendices

Nederlandse samenvatting

Wereldwijd leven er bijna 1 miljard varkens, die door allerlei ziekmakende bacteriën en virussen worden bedreigd. Eén van deze varken-specifieke pathogenen is de bacterie *Streptococcus suis*. Tegen deze bacterie is geen effectief vaccin beschikbaar, waardoor jaarlijks vele varkens ziek worden, wat ook weer risico voor mensen met zich meebrengt. Behandeling met antibiotica is mogelijk, maar bij varkens moet infectie dan tijdig worden gediagnosticeerd en deze behandeling brengt risico op antimicrobiële resistentieontwikkeling met zich mee. Een mogelijke oplossing voor *S. suis* infecties zouden de 'host defence peptides' (HDPs) kunnen zijn. HDPs zijn bewezen antimicrobieel zonder overdraagbare resistentie te induceren, en hebben een variëteit aan immuunmodulatoire functies die ze wellicht als vaccincomponent geschikt maken. Er is echter een tekort aan specifieke kennis over HDP-activiteit in celsystemen van het varken, en al helemaal in de context van een *S. suis* infectie. In deze thesis wordt getracht dit tekort aan te vullen.

In hoofdstuk 1 wordt de achtergrond van de thesis geïllustreerd. De immunologie van het varken, de problematiek van *S. suis* in de varkenshouderij en het risico op antimicrobiële resistentie worden verder toegelicht. Ook is er aandacht voor het immuunsysteem in het algemeen: de ontwikkeling ervan, de verschillende cellulaire en moleculaire componenten en de specifieke eigenaardigheden die varkens hebben waardoor hun immuunsysteem anders is dan van andere veel bestudeerde zoogdieren.

In hoofdstuk 2 worden de eerdergenoemde eigenschappen van HDPs verder uitgelegd aan de hand van een uitgebreid literatuuronderzoek. De moleculaire structuur van HDPs wordt toegelicht, en er wordt stilgestaan bij alle verschillende biologische functies die HDPs kunnen hebben. Aan het eind van dit hoofdstuk worden enkele aanbevelingen gedaan voor de therapeutische toepassingen van HDPs.

In hoofdstuk 3 wordt getoond hoe HDPs, en dan met name het kippen-HDP CATH-2, effectief is in het remmen van de immuunrespons van varkensmacrofagen. Een aantal HDPs waaronder CATH-2, maar ook LL-37 en PMAP-23, werden gebruikt om de immuunrespons van varkensmacrofagen die uit beenmerg zijn gekweekt te moduleren. Elk van deze peptiden is in staat om LPS-gemedieerde activatie van de macro-

fagen te remmen maar alleen CATH-2 is in staat om *E. coli* geactiveerde macrofagen te moduleren. Deze ontdekking wijst erop dat CATH-2 als activatie-remmende HDP geschikt zou kunnen zijn voor varkens, mits de therapie dus om verlaging van de immuunrespons vraagt. Dit zou van belang kunnen zijn voor bijvoorbeeld vaccins die effectief zijn maar veel bijwerkingen opleveren.

In hoofdstuk 4 wordt beschreven hoe *S. suis* een opmerkelijke weerstand heeft tegen HDPs uit het varken, maar veel minder tegen het eerdergenoemde CATH-2. Er is een uitgebreide screening uitgevoerd die de activiteit van verschillende HDPs onderling vergelijkt in antimicrobiële activiteit tegen *S. suis* maar ook tegen verwante Gram-positieve bacteriën. *S. suis* wordt relatief slecht gedood door de varkens-HDPs, en is daarnaast beter bestand tegen de L-enantiomeren van CATH-2 en LL-37 dan tegen D-enantiomeren van dezelfde peptiden. Er wordt aangetoond dat deze weerstand niet is gerelateerd aan de bacteriële capsule, maar kan wel worden overgedragen aan een vatbare bacterie door middel van geconditioneerde groeimedia. De moleculen die verantwoordelijk zijn voor de weerstand van *S. suis* zijn waarschijnlijk groter dan 3 kilodalton en zijn niet hittegevoelig. Deze laatste bevinding maakt het niet erg waarschijnlijk dat één of meerdere eiwitten verantwoordelijk zijn voor de bescherming van *S. suis*. Om het precieze werkingsmechanisme van de weerstand tegen HDPs op te helderen is meer onderzoek nodig.

In hoofdstuk 5 wordt getoond dat ditzelfde CATH-2 effectief is in het remmen van de activering van dendritische cellen van het varken in respons op zowel *E. coli* als *S. suis*. Het panel aan HDPs dat eerder gebruikt is werd onderzocht op immuunmodulerende effecten op varkens DCs. Het HDP PR-39 verlaagt de LPS geïnduceerde immuunreactie licht. HDPs van het varken lijken de opname van *E. coli* licht te verhogen, maar hebben geen effect op *S. suis.* Tot slot wordt aangetoond dat CATH-2 de DC-activiteit effectief verlaagd zonder effect te hebben op de efficiëntie van de opname van de bacteriën. Dit betekent dat CATH-2 in een vaccin tegen *S. suis* mogelijk bijwerkingen zou kunnen verlagen zonder de antigeenverwerking aan te tasten, alhoewel dat nog uitgebreid onderzocht moet worden.

In hoofdstuk 6 wordt beschreven hoe CATH-2 en enkele afgeleide peptiden muizen kunnen beschermen tegen *S. suis* infectie. Na profylactische toediening van een kort D-CATH-2 peptide in het nekvel 1 dag voor infectie blijkt dat de muizen op de

piek van de belasting een lagere klinische score en dus mildere symptomen hebben, maar niet geheel kunnen worden beschermd tegen ziekte. Met name het feit dat de timing en locatie van infectie anders is dan van de toediening van HDPs betekent dat verkorte vormen van D-CATH-2 wellicht als profylaxis tegen *S. suis* of andere bacteriën kunnen worden gebruikt. Het is wel belangrijk dat dit nog uitgebreid in varkens wordt getest.

In hoofdstuk 7 worden de eerdere hoofdstukken besproken in de context van de doelstellingen die in hoofdstuk 1 zijn geformuleerd. Alles bijeen wijst het in het proefschrift beschreven onderzoek erop dat CATH-2 goed werkt als anti-inflammatoir en antimicrobieel middel bij varkens, en dat het dus wellicht geschikt zou zijn als alternatief antibioticum of als vaccincomponent bij een vaccin dat te veel immuunreactie induceert. Voor behandeling van *S. suis* zouden HDPs profylactisch kunnen werken, met de kanttekening dat dit nog in varkens onderzocht moet worden.

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CV

Roeland M. van Harten was born on September 9th 1991 in Leiden, The Nether-

lands. He finished high school at the Stedelijk Gymnasium in Leiden in 2009, after which he went on a gap year to South Africa as a research volunteer at the Centre for Dolphin Studies and the Knysna Elephant Park. He then completed his Bachelor's in Biology in Utrecht in 2015 and continued with the Master's in Molecular and Cellular Life Sciences. He did internships at Utrecht University in the Medicinal Chemistry group of Dr. Nathaniel Martin (now Professor at Leiden University) and in the group of Peter Leadley, Herschel Smith Professor of Biochemistry at the University of Cambridge. He wrote his Master's thesis titled "Multidrug-Resistant Enterococcal Infections: New Compounds, Novel Antimicrobial Therapies?" under supervision of Dr. Anthoni Hendrickx, member of the Medical Microbiology group at the Utrecht University Medical Centre. The thesis work was carried out at the Molecular Host Defence research group at the Faculty of Veterinary Medicine of Utrecht University under supervision of Professor Henk Haagsman. The PhD project was part of Bac-Vactory, a public-private partnership funded by NWO-TTW. Several chapters have already been published in scientific journals.

List of publications

van Harten, R. M., E. J. A. Veldhuizen, H. P. Haagsman and M. R. Scheenstra (2022). "The cathelicidin CATH-2 efficiently neutralizes LPS- and E. coli-induced activation of porcine bone marrow derived macrophages." Veterinary Immunology and Immunopathology 244: 110369.

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