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ANTIMICROBIAL PEPTIDES IN INNATE IMMUNITY: INTERACTIONS WITH ANTIBIOTICS AND EFFECTS OF POST-TRANSLATIONAL MODIFICATIONS

Salma Al Adwani

Karolinska Institutet

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Cover Image: Scanning electron microscopy image of extracellular morphological changes of *E. coli* after treatment with LL-37.

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Antimicrobial Peptides in Innate Immunity - Interactions with Antibiotics and Effects of Post-Translational Modifications

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By
Salma Al Adwani

Principal Supervisor:
Associate Professor Peter Bergman
Karolinska Institutet
Department of Laboratory Medicine
Division of Clinical Microbiology

Co-supervisor(s):
Professor Birgitta Agerberth
Karolinska Institutet
Department of Laboratory Medicine
Division of Clinical Microbiology

Professor Guðmundur H Guðmundsson
University of Iceland
Faculty of Life and Environmental Sciences

Professor Janne Johansson
Karolinska Institutet
Department of Biosciences and Nutrition

Opponent:
Associate Professor Joanna Koziel
Jagiellonian University, Krakow, Poland
Department of Microbiology

Examination Board:
Professor Ulrich Theopold
Stockholm University
Department of Molecular biosciences

Associate Professor Guro Gafvelin
Karolinska Institutet
Department of Clinical Neuroscience

Associate Professor Tulay Lindberg
Karolinska Institutet
Department of Dental Medicine

قال تعالى: «وَقُلْ رَبِّ زِدْنِي عِلْمًا»

سورة طه: الآية 114

“My Lord, increase me in knowledge.”

Taha (20:114), Quran

To my beautiful family

I love you always

ABSTRACT

Cationic antimicrobial peptides (AMPs) are important innate immunity factors contributing to the clearance of invading pathogens and immunomodulating immune responses. In this thesis, the focus is on AMPs of the cathelicidin family, i.e. the human LL-37 and the canine K9CATH. These peptides are positively charged at neutral pH and amphipathic in nature, making them prone to interact with negatively charged bacterial membranes. In this thesis, I have studied i) how the interaction between AMPs and bacteria can be modified by alterations of the LPS-structure of the bacteria and ii) how the host can regulate the function of AMPs, via a post-translational modification designated citrullination.

In part 1 of the thesis, I studied the potential cross-resistance between colistin and LL-37 in K. pneumoniae. Colistin is a cationic polypeptide antibiotic that shares a similar membrane-binding mechanism with LL-37; i.e. they both target the negatively charged components of the bacterial membrane. Resistance to colistin has appeared in bacteria due to modifications in the lipopolysaccharide (LPS) structure by reduction of the negative surface charge. The hypothesis of cross-resistance between colistin and LL-37 still remains debatable. In Paper I, we studied two clinical isolates of Klebsiella pneumoniae (Kpn), with similar genetic background but with different susceptibility to colistin. The colistin-resistant isolate (Col-R) had an insertion element in the mgrB gene, which caused the resistant phenotype. Interestingly, the Col-R isolate was more resistant to LL-37 in contrast to the colistin-susceptible isolate (Col-S) but only at concentrations above 50 μg/ml. However, there was no significant survival differences between Col-R and Col-S isolates in blood, serum nor in a zebrafish infection model. The findings of this study suggest that cross-resistance most likely plays a minor role during physiological conditions *in vivo*, where lower levels of LL-37 are present.

In part 2 of the thesis, I studied how the host, via citrullination, could affect the function of AMPs in various contexts. Peptidyl arginine deiminases (PADs) catalyze the conversion of the positively charged arginine residues into neutral citrulline residues in a process called citrullination or deimination. Notably, citrullination reduces the net charge of proteins and peptides and could thus, affect the biological functions of AMPs. Citrullinated LL-37 has not been detected in human samples and the knowledge on the functional and biophysical consequences of citrullination is limited. Paper II, describes a series of experiments characterizing the presence of citrullinated LL-37 in human bronchoalveolar lavage (BAL) fluid from the airways of healthy donors after exposure of LPS. We identified both native LL-37 and different variants of citrullinated LL-37 in the BAL samples. Citrullinated LL-37 had no antibacterial activity against Escherichia coli, interacted differently with LPS and had reduced affinity to anionic phospholipids. Finally, a net positive charge was shown to be essential for the antimicrobial activity of LL-37.

Finally, I expanded the studies on citrullination to another AMP, the K9CATH peptide, which is the only cathelicidin found in dogs. It has a broad antimicrobial activity against both gram-positive and gram-negative bacteria, it binds to LPS and has anti-inflammatory functions.

Similarly, to LL-37, K9CATH has a positive net charge at neutral pH due to lysine and arginine residues. PAD enzymes are found in all organisms studied, including dogs. However, it is unknown whether K9CATH is a substrate to PAD enzymes. In Paper III we report the that recombinant PAD2 and PAD4 citrullinated K9CATH at different degrees. Citrullination abrogated the antibacterial activity against gram negative bacteria and reduced the peptide's anti-inflammatory activity on LPS-induced macrophage stimulation.

In conclusion, my studies provide information on the interactions of cathelicidin peptides with bacterial membranes and the different host/microbe mechanisms, regulating the activity of the peptides. This information provides a platform for future research on the role of citrullination in health and disease.

LIST OF SCIENTIFIC PAPERS

I. **Effects of the antimicrobial peptide LL-37 and innate effector mechanisms in colistin-resistant *Klebsiella pneumoniae* with mgrB insertions.**
Hissa M. Al-Farsi, **Salma Al-Adwani**, Sultan Ahmed, Carmen Vogt, Anoop T. Ambikan, Anna Leber, Amina Al-Jardani, Saleh Al-Azri, Zakariya Al-Muharmi, Muhammet S. Toprak, Christian G. Giske and Peter Bergman
Frontiers in Microbiology (2019). DOI: 10.3389/fmicb.2019.02632

II. **Studies on citrullinated LL-37: detection in human airways, antibacterial effects and biophysical properties.**
Salma Al-Adwani, Cecilia Wallin, Melanie D. Balhuizen, Edwin J. A. Veldhuizen, Maarten Coorens, Michael Landreh, Ákos Végvári, Margaretha E. Smith, Ingemar Qvarfordt, Anders Lindén, Astrid Gräslund, Birgitta Agerberth & Peter Bergman
Scientific Reports (2020). DOI: 10.1038/s41598-020-59071-7

III. **Citrullination alters the antibacterial and anti-inflammatory functions of the antimicrobial peptide canine cathelicidin K9CATH *in vitro.***
Salma Al Adwani, Avinash Padhi, Harpa Karadottir, Cecilia Mörman, Astrid Gräslund, Ákos Végvári, Janne Johansson, Anna Rising, Birgitta Agerberth and Peter Bergman
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CONTENTS

LIST OF ABBREVIATIONS

AMP	Antimicrobial peptide
BAL	Broncheoaloveolar lavage
CAMP	Cathelicidin antimicrobial peptide
CD	Circular dichroism
CFU	Colony forming units
Col-R	Colistin-resistant
Col-S	Colistin-sensitive
COPD	Chronic obstructive pulmonary disease
CpG	Cytosine-phosphate-guanine oligodeoxynucleotides
DAMP	Damage-associated molecular patterns
DNA	Deoxyribose nucleic acids
IL	Inter-leukin
INF-γ	Interferon-gamma
Kpn	Klebsiella Pneumoniae
LC-MS/MS	Liquid Chromatography with tandem mass spectrometry
LPS	Lipopolysaccharide
LTA	Lipotechoic acid
LUVs	Large unilamellar vesicles
MAMP	Microbe-associated molecular pattern
MDR	Multidrug resistant
NET	Neutrophil extracellular traps
NLR	NOD-like Receptors
NO	Nitric oxide
NOD	Nucleotide oligomerization domain
PAD	Peptidylarginine deiminase
PRR	Pattern recognition receptors
PTM	Post-translational modifications
ROS	Reactive oxygen species
TFA	Trifluoroacetic acid
TLR	Toll like receptors
TNF-α	Tumor necrosis factor-alpha

1 INTRODUCTION

Mammals have evolved multiple defense mechanisms to survive in an environment filled of pathogens. Our immune system consists of a variety of cells and molecules that are divided into two systems, the innate and the adaptive immunity. Both systems are important in securing the integrity and defend the health of our bodies against infections and tissue injuries within the host. Innate immunity is found in every living organism, from prokaryotes to eukaryotes. It provides instant protection against intruders and requires less energy to spend. On the other hand, the adaptive immune system is important in vertebrates and most developed in mammals. It is unique since it has a memory that recognizes the already encountered pathogens and provide an enhanced protection against recurrent infections. Both systems are highly interconnected and interactions between the two systems are needed for a healthy and balanced immune response.

However, some pathogens have evolved ways of evading the mechanisms of our immune systems, resulting in diseases and thus the need of exogenous treatment approaches, such as antibiotics. In the past decades, the extensive use of antibiotics eventually led to emergence of resistance in some bacterial strains and this made it challenging to treat infections. Most importantly, public health concerns are increasing with the emergence of multidrug resistant (MDR) strains and their spread in both human and animal populations (1, 2). This has directed the scientific communities to search for safe alternatives to antimicrobials that are active against MDR strains, and not harmful to host cells (3). Endogenous antimicrobial peptides (AMPs) constitute a part of innate immunity and are known for their multiple functions against pathogens, such as direct killing or indirect killing by regulating the immune response to infections. Therefore, AMPs represent an attractive platform to develop novel therapies (3, 4). In addition, approaches of host-directed therapy by boosting the production of effective immune factors are gradually getting attention from the scientific community (5, 6).

The present thesis will discuss the innate immune system and AMPs. This thesis shed lights on innate immunity and AMPs and how AMPs interact with bacterial membranes. The first part covers the potential probability of cross-resistance between conventional antibiotics (with antimicrobial peptides' origin) with host antimicrobials. The second part describes how post-translational modifications (PTM), i.e. citrullination, affect the structure and functions of AMPs in the inflammatory micro-environment.

2 THE INNATE IMMUNE SYSTEM

The innate immune system is the first line of defense that can be rapidly activated by invading microorganisms. It consists of several barriers and effector cells empowering it with the capacity to defend, fight back and resolve infections successfully. Mechanical barriers, such as intact skin, the peristalsis movement of the gut and the nasal cilia are essential to hamper the ability of microbes to attach to the linings of the body. Chemical barriers, including sweat, lung surfactant, gastric acids and the mucus lining of the mucosal epithelia, are beneficial to inhibit colonization of unwanted microbes in the body. These linings are also populated with commensal microorganisms, which play a role in protecting the host from pathogenic microbes by competing for space and nutrition (7).

2.1 INNATE IMMUNE CELLS

The innate immune system is also armed of cellular and soluble components that are highly effective in eliminating pathogens or stopping them from spreading throughout the body. A majority of these cells are hematopoietic cells (except for epithelial cells) and they belong either to the myeloid lineage, such as granulocytes and macrophages, or to the lymphoid lineage, such as natural killer cells and innate lymphoid cells. They fight infections by direct killing, including phagocytosis and cytotoxicity or bacterial elimination via secreted effector components, such as AMPs and cytokines (8). Some immune cells have special functions, e.g. dendritic cells, which plays a central role in activating the adaptive immune system through presenting foreign antigens to B- and T-cells (9).

2.1.1 Epithelial cells

A part of the cellular compartment of innate immunity is composed of epithelial cells that are lining e.g. the gut and the respiratory system. Epithelial cells are connected to each other by tight junctions sealing the inner organs from invading microbes. In addition, they have receptors recognizing common conserved structures of pathogens, i.e. microbe-associated molecular patterns (MAMPs) (7, 10). These receptors are called pattern recognition receptors (PRRs) and include the toll like receptors (TLRs) as well as the intracellular receptors Nucleotide Oligomerization Domain (NOD)-like receptors. Activation of TLRs or NOD-like Receptors (NLRs) leads to release of cytokines, chemokines and AMPs, which collectively activate the adaptive immune-system, but also directly kill the invading bacteria by virtue of the direct bactericidal effects of AMPs (9, 11).

2.1.2 Polymorphonuclear Granulocytes

The polymorphic nuclei and granules in the cytoplasm are the main characteristics of granulocytes. Neutrophils, eosinophils, basophils and mast cells are all considered granulocytes. Neutrophilic granulocytes or "neutrophils", are short-lived and they are the first cells recruited to the site of infection. They kill microbes intracellularly by engulfment and phagocytosis and they also release the content of the granules, such as AMPs and proteases, that help degrading the cell wall of microbes leading to their death. Neutrophils can also directly kill bacteria by generating reactive oxygen species (ROS) and nitric oxide (NO), which act as toxic substances on the bacterial membrane leading to its disruption (12, 13). Furthermore, during inflammatory conditions, neutrophils undergo a process of cell death leading to the formation of neutrophil extracellular traps (NETs). NETs are mainly composed of released DNA with a negative charge and decorated with positively charged proteins and peptides, including histones and AMPs, such as LL-37 (14).

2.1.3 Macrophages

The word "macrophage" means "big eater", i.e. they phagocytose invading microbes, apoptotic cells, cell debris and foreign entities. Macrophages are found circulating in the blood as monocytes but also reside in the parenchyma of tissues, where they recognize pathogens and maintain homeostasis in the tissues. Resident macrophages take various forms and names based on the tissue, where they reside, such as Kupffer cells in the liver and microglia in the brain and the spinal cord. Macrophages play an important role in the regulation of inflammation in order to avoid tissue damage and fibrosis due to persistent or excessive inflammation. Classically activated macrophages (M1-type) have the ability to increase inflammation, whereas alternatively activated macrophages (M2-type) are involved in anti-inflammatory and tissue repair processes (15). Macrophages have additional key functions in priming the adaptive immune-system through presenting antigens to lymphocytes and by secreting cytokines (16, 17).

3 INNATE IMMUNE RECOGNITION

The innate immune system can sense abnormal events, for example tissue damage or infections through PRRs (7), including TLRs and NLRs that are encoded in the germline of immune cells and epithelial cells. These receptors recognize conserved pathogen-associated molecular patterns (PAMPs) that are shared by large group of pathogens, such as microbial unmethylated DNA (CpG) or membrane components such as lipopolysaccharide (LPS) and subsequently,

trigger downstream effector mechanisms (7). Upon microbial exposure, these receptors initiate intracellular activation leading to secretion of pro-inflammatory cytokines and chemokines to attract immune cells to a site of infection. In addition, the innate immune system detects damage-associated molecular patterns (DAMPs) that are released after injury due to cell lysis and tissue damage that occur in the context of both infectious and sterile-tissue injury. The release of DAMPs activates PRRs and its downstream inflammatory pathway (18). The most important aspect of innate immune recognition is the ability to discriminate between self and non-self-antigens. Innate immune cells, such as NK cells can detect the host's own cells that are not healthy (infected with viruses or cancerous cells) and attack them. To avoid an immune response against host cells, this process is highly controlled through an inhibitory system using MHC class I molecules , which is upregulated on healthy cells and downregulated in infected cells (19).

4 ANTIMICROBIAL PEPTIDES (AMPS)

In mammals, AMPs play an important role in the defense mechanisms of the innate immune system against bacterial, fungal and viral infections. They are expressed by epithelial cells in the respiratory system, gastrointestinal tract and urogenital system. Furthermore, AMPs are expressed in immune cells such as neutrophils and macrophages (20). Most AMPs share some common characteristics such as i) the short length of 12 -50 amino acids residues, ii) a positive net charge and iii) an amphipathic character (one side is hydrophobic and the other side is hydrophilic or charged) (21, 22). Most AMPs provide immunity through direct killing of pathogens by disturbing the integrity of the microbial membrane. They also exhibit immunomodulatory activities, such as recruiting immune cells to the site of infection, neutralizing the inflammatory activity of LPS, stimulating wound healing and also stimulation of epithelial release of inflammatory factors (21). Defensins and cathelicidins are the two main families of AMPs in mammals (21). The structure of defensins is defined as anti-parallel β-sheets, which are stabilized with three intramolecular disulfide bonds (23). The defensins are subdivided into three groups; α-defensins are mainly expressed in neutrophils and Paneth cells of the small intestine, and β-defensins are found in epithelial cells. In addition, theta defensins are circular mini-defensins that are present in leukocytes of rhesus monkeys, but not expressed in humans due to a pseudo-gene (23, 24).

The other main AMP-family in mammals is the cathelicidin peptide family, which share a common N-terminal cathelin domain and an antimicrobial C-terminus of diverse sequence and length (25). Mature cationic peptides are generated by cleavage of the C-terminal domain and

they vary in their secondary structure; some adopt an α-helical structure, such as LL-37, whereas other peptides form β-sheet structures, such as the cyclic protegrin (26, 27). Cathelicidins have been described in many mammals, such as pigs, rabbits, mice and dogs. In humans, there is one cathelicidin gene (*CAMP*), which encodes hCAP-18 as a pro-protein, which is subsequently cleaved to release the mature peptide LL-37. This human peptide is found in granules of neutrophils and also expressed in epithelial cells (28).

In addition to defensins and cathelicidins, other small classes of peptides enriched for specific amino acids can be distinguished in mammals. It should also be mentioned that there are also antimicrobial proteins, such as lactoferrin, lysozyme and lipocalin (29, 30). However, in this review, the focus will be mainly on the human cathelicidin LL-37 and its orthologue in dog (K9CATH).

4.1 THE HUMAN CATHELICIDIN (LL-37)

The human cathelicidin LL-37 is expressed in both myeloid cells and epithelial cells and are encoded by the cathelicidin antimicrobial peptide (*CAMP)* gene. The gene is translated into the 18 kDa inactive proform; human cationic antimicrobial peptides (hCAP-18). Upon activation, hCAP-18 is cleaved extracellularly by proteinase-3 (31) leading to the generation of a mature peptide of 37 amino acid residues, with two leucine residues at the N-terminal end, hence the name LL-37 (32). LL-37 has a positive net charge of +6 at neutral pH due to the 11 basic residues of arginine and lysine. The secondary structure of LL-37 is random coil in pure water, but adopts an a-helical structure in the presence of phospholipid liposomes or lipid A (33) or in salt solution mimicking the body fluids, such as plasma and intracellular fluid (34). The antibacterial activity has been shown to correlate with the secondary structure of LL-37 (34). The distribution of the hydrophilic and hydrophobic residues gives the peptide its amphipathic character, which facilitates the interaction with target membranes.

4.1.1 Expression and regulation of LL-37

LL-37 is expressed in neutrophils and immune cells, such as monocytes, macrophages, NK cells (35), and mast cells. In addition, it is expressed in epithelial cells in direct contact with the environment, such as in the skin, gastrointestinal tract, lungs as well as in eccrine and salivary glands (16, 23). Notably, mature LL-37 peptide can be released from granulocytes and macrophages in response to infection. During inflammation LL-37 synthesis is upregulated in the skin, testis and in airway epithelia (33). The regulation of LL-37 expression is not only

controlled by inflammatory pathways, but also through vitamin D signaling and by endoplasmic reticulum (ER) stress (36, 37).

4.1.2 Biological activities of LL-37

LL-37 has a broad spectrum of antimicrobial activity, which has been demonstrated against gram negative bacteria, such as *S. typhimurium, E. coli*, and *P. aeruginosa*, as well as against gram positive bacteria including *L. monocytogenes, S. aureus* and *S. epidermidis* (38). Additionally, LL-37 shows antifungal activity against *C. albicans* and anti-viral properties against several viruses, including influenza virus and HIV-1 (39). Apart from its antimicrobial properties, LL-37 has additional functions, such as chemotactic activity on neutrophils, induction of cytokines and chemokines from epithelial cells, wound healing properties and angiogenesis (Fig.2.) (26).

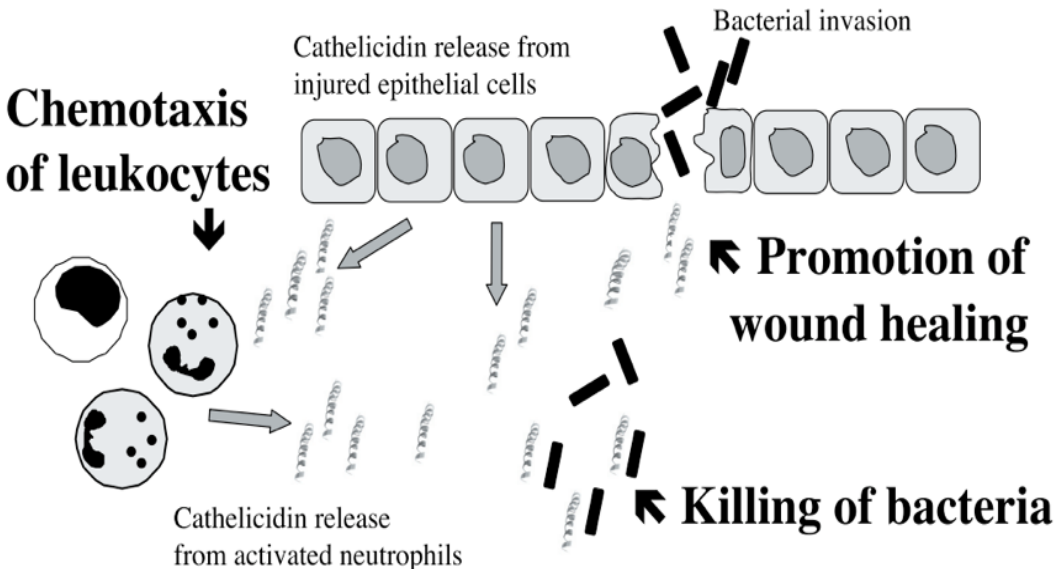

Fig. 2. Biological activities of the human cathelicidin LL-37 at epithelial surfaces. LL-37 participates in the recruitment of neutrophils and other circulating cells to the site of infections. LL-37 contributes to killing of invading pathogens and promotes repair of damaged tissue by inducing re-epithelialization and angiogenesis. Adapted from (26).

4.2 THE CANINE CATHELICIDIN (K9CATH)

AMPs play an important part of innate immunity in dogs. The two main families of peptides in dogs are composed of six defensins and one cathelicidin and they exhibit antibacterial activity

against bacteria and fungi. The mature peptide K9CATH is expressed in neutrophil granules and is made of 38 amino acid residues and shows 46% similarity to the human LL-37 (40) (Table 1). K9CATH show features of common cationic AMPs, such as positive charge (+6) at neutral pH and an a-helical structure in lipid-mimicking environments (40). It is highly effective against gram negative bacteria, such as *E. coli*, *P. aeruginosa*, as well as against gram positive bacteria, such as *S. aureus,* and methicillin-resistant *Staphylococcus. pseudintermedius* and to a lesser extent against methicillin-resistant *S. aureus*. Furthermore, it is active against yeast, such as *Malassezia pachydermatis* (41). In addition, K9CATH has been shown to inhibit LPS-induced macrophage activation (42). It also indirectly stimulates chemotaxis of immune cells, such as macrophages by upregulating expression of CXCL10 (42).

Bacterial pneumonia (bacterial infection of the lung) is a common clinical problem in dogs (43). Bacterial pneumonia in dogs are often caused by *E. coli*, *K. pneumoniae*, *Pasteurella* spp, *Mycoplasma* spp, and *S. pseudintermedius* (43). Treatment is based on the severity of the case, with antibiotics, such as amoxicillin, amoxicillin-clavulanic acid or doxycycline (43, 44). However, the frequent use of antibiotics may have contributed to the emergence of multi-drug resistant strains in dogs and humans worldwide. Bacterial resistance makes treatment more difficult and this urges the need to acquire alternative strategies to treat infections caused by multidrug-resistant bacteria (45). Both canine defensins and the single cathelicidin K9CATH are effective against methicillin-resistant strains (41). Thus, AMPs have been studied as alternative candidates for treatment, since the resistance against AMPs so far is low in these bacterial species (41).

Peptide	Sequence	Length	Charge
LL-37	LLGDFFRKSKEKIGKEFKRIVQRIKDFLRNLVPRTES	37	+6
K9CATH	RLKELITTGGQKIGEKIRRIGQRIKDFFKNLQPREEKS	38	+6

Table 1. Sequences of the human and canine cathelicidins.

5 ANTIMICROBIAL MECHANISMS OF ACTION

Similar to most cationic antimicrobial peptides, LL-37 kills bacteria by disrupting their membrane, resulting in lysis (28). Most of the antibacterial activity of LL-37 against bacteria

is attributed to its cationicity and hydrophobicity. The initial interaction with bacteria is through the electrostatic interaction of the positively charged peptide residues with the negatively charged components of the outer leaflet of the bacterial membrane, such as LPS or cardiolipin in gram negative bacteria or with lipotechoic acid (LTA) on gram positive bacteria. The hydrophobic side of LL-37 interacts with the hydrophobic headgroups of the lipids of the bacterial membrane, leading to a disturbed integrity of the membrane. The LL-37 peptide accumulates on the membrane according to the carpet-model (46), or form toroidal pores in the lipid bilayer (47). However, there is still a debate on the exact mechanistic model that the peptide utilizes to kill bacteria.

5.1 INTERACTIONS OF AMPS WITH LPS

The Gram-negative cell envelope is consisting of three layers, the inner membrane, the periplasmic membrane, and the outer membrane. The inner or the cytoplasmic membrane is usually composed of phospholipid bilayers. The periplasmic layer is the middle layer containing a thin matrix of peptidoglycan. The outer membrane is made of an inner leaflet composed of phospholipids and the outer leaflet is mainly composed of LPS (48). LPS, also known as endotoxin, is generally comprised of three parts, the inner hydrophobic part is lipid A, the middle part is the core oligosaccharide (core-OS), and the outer hydrophilic part is a chain of repeating units of O polysaccharide (O-antigen) (49). Most clinical strains of *Escherichia coli* (*E. coli*) have LPS, consisting of all three parts (smooth LPS), other strains, such as K-12, lack the O-antigen (rough LPS) and some strains lack both O-antigen and whole or part of the core-OS (deep rough LPS) (50). LPS functions as a protective layer that support viability of bacteria. LPS released from bacteria interacts with innate immune cells and can trigger an immune response. Furthermore, LPS could induce an excessive inflammatory response that might lead to the development of septic shock syndrome, which is a life-threating disease (51). LPS in general is an anionic molecule and so considered as an initial target for the activity of cationic AMPs due the electrostatic interaction. Apart from acting directly on the bacterial membrane leading to bacterial killing, it is widely known that AMPs neutralize the effects of released LPS by either a direct binding to the LPS molecule, preventing it from activating immune cells, such as macrophages (52). A recent study revealed that the LPS structure has a role in the sensitivity of bacteria, such as *E. coli,* to the activity of cationic AMPs. LPS mutant *E. coli* strains lacking sugar structures in the inner OS-core were more susceptible to a set of helical AMPs, including Cap18 a rabbit orthologue to human LL-37 peptide, compared to the wild-type strain, indicating the importance of LPS for the activity of AMPs (53).

6 IMMUNOMODULATORY ACTIVITIES OF CATHELICIDINS

AMPs including cathelicidins are additionally known as host defense peptides since they take part in the modulation of immune responses. Depending on the context, cell type and inflammatory stimuli, cathelicidins have either pro-inflammatory or anti-inflammatory properties (54). In addition, they chemoattract immune cells to the site of infections and they direct cell differentiation of immune cells. In this thesis we focus on the anti-inflammatory functions.

6.1 ANTI-INFLAMMATORY FUNCTIONS

Many bacterial membrane components, such as LPS of gram negative bacteria and lipoteichoic acid of gram positive bacteria, are ligands to TLR4 and TLR2, respectively (55). They activate TLR signaling, resulting in proinflammatory activation of leukocytes. Due to their overall negative charge LPS binds to cathelicidins such LL-37, K9CATH, mCRAMP and PMAP-36 and prevents production of pro-inflammatory cytokines such as LPS-mediated TNF-a production in leukocytes (56). LL-37 reduces LPS-induced nitric oxide (NO) released from macrophages and also protects mice injected with lethal concentrations of LPS (57). Furthermore, cathelicidins inhibit endotoxin-mediated up-regulation of pro-inflammatory cytokines, such as IL-6, IL-1b, and INFγ (56). Thus, interaction of cathelicidins and TLR ligands plays an important role in modulating pro-inflammatory responses via TLR activation.

7 COLISTIN

Colistin is a polypeptide antibiotic originally isolated from *Paenibacillus polymyxa* subsp. *colistinus* bacteria, which is found in soil (58). Colistin, also known as polymyxin E, is one of the primary classes of antibiotics known as polymyxins. Polymyxins consist of different compounds classified from A to E. It is effective against many Gram-negative bacteria by disturbing the integrity of the bacterial outer membrane. Colistin, which is positively charged, interact in an electrostatic mode with the negatively charged LPS and phospholipids of the bacterial membrane, where it displaces Mg^{2+} and Ca^{2+} from the cationic binding sites (59). This results in a detergent effect that disturbs the membrane integrity leading to leakage of cell content and eventually death of the bacteria (60). In addition, similar to AMPs, polymyxins bind and neutralized LPS molecules (61). In the clinic, colistin is considered to constitute the

last line resort in the treatment of infections caused by multidrug-resistant gram negative bacteria, such as *E. coli*, *K. pneumoniae and A. baumannii* (62).

7.1 CROSS-RESISTANCE BETWEEN LL-37 AND COLISTIN

Acquired resistance to colistin has been detected in many bacterial species. Resistance is primarily due to modifications of the LPS molecules, which lead to decreased overall negative charge and subsequently reduced binding affinity of colistin to the bacterial outer membrane (63). In recent years, an increasing number of colistin resistant pathogens has emerged in many parts of the world, with serious consequences for patients and health-care systems on a global scale. In addition, it has been speculated that widespread colistin therapy can induce cross-resistance towards AMPs. The consequences of expanded AMP-resistance among these bacteria could lead to even more severe infections, when the host's immune system becomes blunted. Indeed, a study on the human pathogen *A. baumannii* revealed a positive correlation between resistance to colistin and resistance to LL-37 (64). Colistin and LL-37 are both AMPs and share a similar binding mechanism with the bacterial cell wall. It should, however, be noted that the clinical implications of this potential cross-resistance are not clearly elucidated. Nevertheless, a detailed understanding of the molecular basis of the potential cross-resistance between AMPs and clinically used drugs, such as colistin, is important, as it can enhance the development of more efficient therapeutic AMPs.

8 CITRULLINATION

Citrullination or deimination of a peptide or protein is a post-translational modification (PTM) process, where the cationic arginine residues are converted into neutral citrullines in proteins (Fig. 3) (65). Citrulline is a non-coded amino acid and there is no corresponding tRNA. The enzymatic conversion of arginine to citrulline results in the loss of positive charge and an increase in mass of approximately 1 Da (66). This modification can influence the overall charge, the isoelectric point, the ionic and hydrogen bonds within a peptide or protein, as well as the interactions of peptides or proteins with other molecules (65).

8.1 PEPTIDYLARGININE DEIMINASE ENZYMES (PADS)

The citrullination process is catalyzed by the calcium-dependent peptidylarginine deiminase enzymes (PADs). In humans, there are five isotypes of PADs (PAD 1, 2, 3, 4, and 6) that are distributed throughout the body in a tissue-specific manner. The PAD-family is highly conserved and their encoding genes are clustered at chromosome 1p36.1, within a 300 kb region (67). These enzymes have different substrate specificity and tissue-specific expression.

PAD1 is mainly expressed in epidermis and uterus and one of its substrates is keratin K1. PAD1 is involved in the cornification of epidermal tissues. PAD2 is the most widely expressed member of the PAD family throughout the body. It is expressed in skeletal muscle, brain, spleen, secretory glands, neutrophils and macrophages (67, 68). The most common substrates for PAD2 are the myelin basic protein and vimentin in muscles and macrophages. PAD3 was detected in hair follicles and epidermis and it is involved in regulating epidermal functions through deiminiation of filaggrin and trichohyalin. PAD4 is found in immune cells, such as neutrophils, eosinophils and monocytes. It is localized mainly in the nucleus and citrullinates histones and other nuclear proteins, thus playing a role in gene regulation and NET-formation. PAD6 is expressed in ovaries, testis and eggs (65, 67). Overall, protein citrullination by PAD enzymes is involved in several physiological processes in gene regulation, innate immunity and reproduction systems, as well as in skin keratinization. Moreover, studies have shown the involvement of PADs in pathological conditions, such as tumorigenesis and autoimmune diseases, such as rheumatoid arthritis, Alzheimer disease and multiple sclerosis (69).

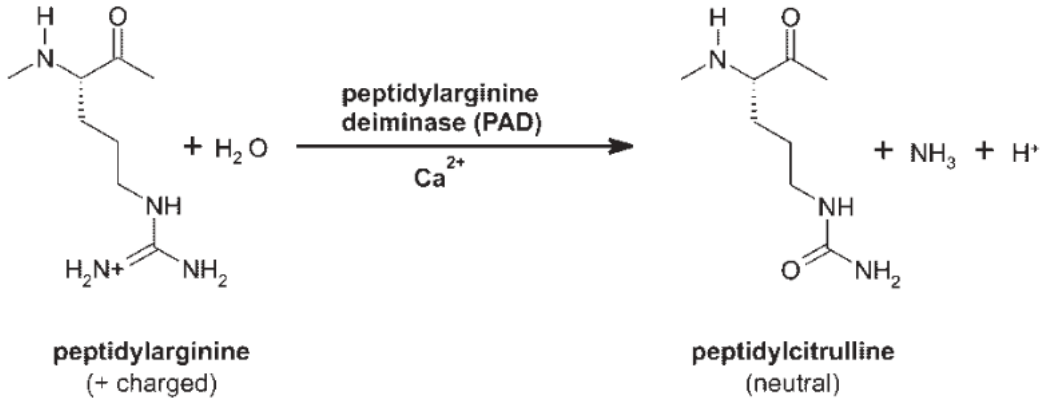

Fig.3. Citrullination of peptidylarginine by PAD. The arginine residue is converted to citrulline residue. Adapted from (67)

8.2 CITRULLINATION OF LL-37

Under inflammatory conditions, the human cathelicidin LL-37 is exposed to the post-translational activity of PADs. The possibility of citrullination of LL-37 by PAD-enzymes was first described *in vitro* by PAD2 and PA4 (70). LL-37 was citrullinated after incubation with recombinant PAD2 and PAD4. Both enzymes caused different degrees of citrullination on LL-37; PAD2 citrullinated all five arginine residues, whereas PAD4 only citrullinated three arginine residues at position 7, 29 and 34. Citrullination of LL-37 reduced the antibacterial

activity against bacteria associated with chronic obstructive pulmonary disease (COPD) including non-typable *H. influenzae*, *S. pneumoniae*, and *S. aureus.* Notably, citrullination did not affect the antimicrobial activity against *P. aeruginosa* to the same extent as the other bacteria. Citrullinated LL-37 caused leakage in anionic liposomes similar to the native peptide, suggesting that a reduction in charge was not fully detrimental for membrane disturbance by LL-37 (70). In addition, citrullination altered the immunomodulatory activities of LL-37 both *in vitro* and *in vivo*. A strong increase in chemotactic activity against macrophages was observed with citrullinated LL-37 compared to the native peptide, while an impaired capacity to block LPS-induced inflammatory cytokines in macrophages was detected (70, 71). Furthermore, citrullination of LL-37 abrogated its ability to prevent endotoxic shock in a mouse model of sepsis (71). The mechanism was shown to be caused by the inability of citrullinated LL-37 to inhibit the release of proinflammatory cytokines from macrophages stimulated by bacterial TLR ligands, such as LTA and poly(I:C) (71).

LL-37 has also been shown to facilitate delivery of free DNA from both pathogens and dying host cells to intracellular TLR-9 in human plasmacytoid dendritic cells (72, 73). In relation to this finding, a recent study reported that citrullinated LL-37 could not bind to the negatively charged DNA, which resulted in a reduced activation of dendritic cells and macrophages in response to bacterial DNA (74). This suggests that citrullination of LL-37 downregulates the immune response to cell-free DNA. Another recent study revealed the presence of auto-antibodies against LL-37 and its post-translationally modified forms, such as citrullinated and carbamylated LL-37 in sera and synovial fluid of psoriasis and psoriatic arthritis patients (75). Taken together, citrullination of LL-37 has direct effects on the regulation of innate immune responses. Therefore, it is important to further investigate the role of citrullination and its relation to diseases, such as rheumatoid arthritis, psoriasis, COPD and infections with pathogens.

8.3 CITRULLINATION IN DOGS (CANINES)

There are strong indications PAD enzymes exist in all mammals and they are involved in many physiological and pathological processes (67). A recent study showed that stimulation of canine neutrophils with LPS from *E. coli* resulted in the formation of NETs, generating hypercitrullination of histone H3 by PAD enzymes. This suggest that citrullination of histones by PADs is essential for cellular events during NETosis (76). In addition, PAD2 has been documented to be expressed in mammary tissues of female dogs. PAD2 citrullinated histones from mammary epithelial cells, suggesting a role in the regulation of lactation genes (77).

However, expression of PAD2 was reduced in mammary carcinoma from dogs (78). Further studies are needed to study effects of citrullination of K9CATH and its regulation during inflammation.

9 CONCLUSION

Bacterial resistance to conventional antibiotics is increasing worldwide in an alarming rate to both human and animal healthcare. AMPs may be studied in the context of multidrug resistant bacteria and cross-resistance may occur between LL-37 and colistin. However, the clinical implications need to be further studied. In addition, the role of PTM i.e. citrullination, has emerged as another and important regulatory circuit of AMP-function, which also deserved further studies.

10 AIM OF THE THESIS

The general aim of this thesis is to elucidate the role of host and pathogen factors, regulating the interactions of the cationic antimicrobial peptides with bacterial membranes. We therefore set out to study the following specific aims:

- To study the interactions of colistin-resistant *Klebsiella pneumonia* with the human antimicrobial peptide LL-37 and to shed light on the potential cross-resistance between LL-37 and the polypeptide antibiotic colistin. (**Paper I**)

- To detect citrullinated LL-37 in human biological samples and to study the effects of post-translational modification (citrullination) on the bio-physical properties of LL-37 and its interactions with bacterial membranes. (**Paper II**)

- To evaluate whether the effects of citrullination on LL-37 can be expanded to its canine orthologue K9CATH and study the effects of citrullination on the interactions between K9CATH, LPS and bacteria. (**Paper III**)

11 MATERIALS AND METHODS

This is a brief overview of the methods and materials used to obtain the results in **Papers I, II** and **III**, included in this thesis. For more detailed on the materials and methods, please refer to **Papers I, II** and **III** attached in this thesis.

11.1 MATERIALS

Bacterial strains: In **Paper I** we used clinical isolates of *K. pneumoniae* (Kpn) collected from Oman (n= 17). All of the Kpn isolates were resistant to carbapenems. Eight of the isolates were less susceptible to colistin due to *mgrB*-insertion and the other 9 isolates were more susceptible to colistin assessed by broth micro-dilution with colistin. In addition, two isolates from the above collection were selected and studied in depth at different conditions, these are colistin resistant OM124 (Col-R) and colistin sensitive OM322 (Col-S). They were selected based on their similar genetical background and difference in colistin sensitivity due to the presence of *mgrB*-insertion in Col-R isolate. Other strains were included in this study as controls, such as *K. pneumoniae* ATCC25955, *E. coli* D21 and *Proteus mirabilis* ATCC29245. In **Paper II** & **III**, we used *Pasturella multocida* (*P. multocida*) ATCC 6533 and a collection of *E. coli* strains with different LPS structures; wild type *E. coli* MG1655, the sequenced *E. coli* K-12 strain (DA5438) and its reconstructed LPS mutant strain (DA26796) with amino acid deletion at *rfaC* (del aa 195-199) (79), to study the antibacterial activities of LL-37 and K9CATH.

Peptides: Synthetic native LL-37 and K9CATH peptides and their citrullinated forms were purchased in a lyophilized form (Innovagen, Lund, Sweden). Prior to the experimental studies, peptides were dissolved in 0.1% TFA in water for bacterial work or in pure water for cell culture work and stored in aliquots at -20 °C until further use (**Paper I, II** & **III**).

Blood: Peripheral blood from healthy individuals was collected and used after dilution in RPMI-1640 medium for blood killing assays. In addition, serum was separated from blood and stored at -20 °C until further use for bacterial killing assay. Red blood cells from blood or buffy coat was also used to study hemolytic activities of AMPs (**Paper II** & **III**).

Zebrafish: Embryos of zebrafish were injected with bacteria and used as an *in vivo* infection model. They were used to study whether the resistance to colistin modified the virulence of Kpn and also to test their fitness *in vivo*.

Broncheoaloveolar lavage (BAL): In one of my studies, we had access to material from an experimental study in humans, where LPS was instilled into the lungs of healthy volunteers. The original data was published in (80, 81). Briefly, Bronchoscopy was used to deliver LPS (4

ng/kg) *Escherichia coli* (*E. coli*) 0113:H10 (Rockville, MD, USA) to lungs of healthy individuals. After 12 to 24 h, BAL fluid was collected and stored at -80 °C until further use.

Cell lines: Macrophage-like cell lines were used as in-vitro model to test the effects of peptides on LPS-mediated cell activation. Murine macrophages RAW264.7 cells (ATCC-TIB-71) are extensively used since they are easy to work with, give reproducible results and have good phagocytic capacity. We used them specifically to study the effects of K9CATH to block LPS-signaling and thus to provide a proof of concept for our hypothesis. In order to consider species-related factors, we used canine DH82 cells (ATCC CRL-10389)(82), which is derived from dog. Cells were seeded into 96-well cell culture plates in media supplemented with fetal bovine serum. After reaching confluency cells were washed once with PBS and then treated with peptides or LPS or a combination of treatments and supernatants were collected after 24 h and stored at -20 °C until further use. Supernatants were used to study levels of pro-inflammatory molecules, such as NO using the Griess assay or cytokines, as TNF-α using ELISA **(Paper III).**

11.2 METHODS

Bacterial killing assay: This method was used to study the effects of a certain drug or molecule on the growth or survival of bacteria. We used synthetic LL-37 peptides, K9CATH peptides, 40% diluted blood or serum in the studies included in this thesis. Bacteria were incubated with selected treatments, incubated at 37 °C in a shaker, serially diluted and then plated on blood agar plates overnight to measure colony forming units (CFU). Bacteria with media alone were used as negative controls in all experiments. (**Paper I, II & III**)

Zebrafish infection model: Zebrafish embryos develop innate immunity very early post fertilization but adaptive immunity takes longer time to develop (83). Thus, zebrafish embryos are good candidates to study interactions of colistin-resistant bacteria with innate immunity factors in an *in-vivo* model. In **Paper I**, we wanted to test whether the gain of an antibiotic resistance mechanism would cause a cost of fitness on Kpn strains *in vivo.* Therefore, we infected the embryos with 150-300 CFU of Col-R, Col-S or Kpn ATCC25955 as a control, using a microinjection procedure, where a volume of 1-2 nL was injected into the viable zebrafish embryo under a stereomicroscope. A group of embryos were injected with only E3 media as negative control. Infected embryos were either digested after injection to determine the number of bacteria in the embryos or left incubated at 30 °C for 72 h and any signs of disease or death were recorded (**Paper I**).

Peptide and protein extraction from BAL fluid samples: To isolate AMPs from BAL fluid, which is a complex matrix containing cells, salts, proteins and many other constituents, we first pooled BAL fluid from 10 healthy donors and centrifuged the mixture at 5000 rpm for 15 min. Next, supernatants were enriched for proteins and peptides using OASIS reversed-phase HLB columns that eliminated all salts. Proteins and peptides bound to the columns were then eluted with 80% acetonitrile in 0.1% TFA. Afterwards, the protein extracted samples were lyophilized to get rid of acetonitrile and resuspended in 0.1% TFA and stored at -20 °C until further use (**Paper II**).

Reversed-phase liquid chromatography: This method has been used in our group for many years. It has primarily been used to isolate AMPs from biological complex samples separated into fractions based on their hydrophobicity. Briefly, the BAL peptide/protein extract (0.5 mg) was separated on a Vydac C_8 (250 × 4.5 mm) reversed-phase column (HiChrom Ltd, UK) with a flowrate of 0.3 mL/min. Peptides trapped in the column were eluted with a gradient of acetonitrile in 0.1% TFA (0–20% for 16.6 min, 20–60% for 33.2 min and 60–80% for 66.2 min) and fractions were collected. The absorbance was monitored at 214 nm to detect peptide bonds and 280 nm to detect proteins. These fractions could later be used to detect specific AMPs with different methods, such as dot blot analysis, utilizing specific antibodies or further analysis with mass-spectrometry (LC-MS/MS) to detect the composition of the fractions. (**Paper II**)

***In vitro* PAD citrullination:** Recombinant PAD enzymes were used in this study to citrullinate the synthetic K9CATH peptides. PAD enzymes require high levels of calcium in order to function (84). The buffer used for this experiment contained (100 mM Tris-HCl, 5 mM $CaCl_2$, and 5 mM DTT, pH 7.6). We selected PAD2 and PAD4 in this study, since both enzymes are expressed by cells in the immune system and are expected to occur in close proximity to AMPs *in vivo* (85). Briefly, peptides (1 mg/ml) were incubated with the enzymes in a concentration of 50 units enzymes/mg of peptide, at different time points and the reaction was stopped by adding formic acid as described previously (74). Samples were stored at -20C until further use. Samples containing the mixture of peptides and enzymes were used along with LPS to study the effects of citrullination on the capacity to inhibit LPS-mediated macrophage activation. Peptide-enzyme mixtures were also tested for citrullination using mass-spectrometry analysis (**Paper III**).

Macrophage stimulation: Macrophage-like cell lines (murine RAW264.7 and canine DH82 cells) were used as *in-vitro* model to test the effects of peptides on LPS-mediated cell activation. Murine RAW 267.7 macrophage-like cell-line are extensively studied cell line and are excellent cells to us in proof of concept studies. In order to obtain a physiological system relevant for the K9CATH-peptide, which is the canine orthologue of LL37, we used the macrophage-like DH82 cell line. This is a cell line that originates from the neoplastic cells of canine malignant histiocytosis (82). Cells were seeded into 96-well cell culture plates in media supplemented with fetal bovine serum. After reaching confluency, cells were washed once with PBS and then treated with synthetic peptides (5 μM) or peptide (2.5 μM) /PAD enzyme mixture preincubated with LPS (100 ng/ml) and supernatants were collected after 24 h and stored at -20 °C until further use. To assess if synthetic peptides alone have any effects on LPS-mediated macrophage activation, cells were first stimulated with 5 μM peptides alone for 1 h, after which the peptides were washed away with PBS then stimulated with LPS as mentioned above. For all stimulation methods, supernatants were used to study pro-inflammatory molecules, such as NO using the Griess assay or cytokines as TNF-α using ELISA **(Paper III).**

11.3 ETHICAL CONSIDERATIONS

All of the studies in this thesis followed the ethical principles of the Declaration of Helsinki. Ethical approval was obtained from the Ministry of Health in Oman for the use of the bacterial isolates and the collection of (anonymized) patient data (MH/DG/R&S/32/2015) in **Paper I**. The procedure to draw blood from healthy volunteers was approved by the Regional Ethical Review Board in Stockholm (dnr 2019-02519) (**Paper I & II**). Patients and participants provided their written informed consent to participate in this study. The zebrafish study was approved by the Ethical Review Board, Stockholm Animal Research Committee (dnr 19204-2017) and by the Swedish Board of Agriculture (**Paper I**). The clinical study involving collection of BAL fluid from healthy individuals was performed in accordance with the Helsinki declaration after approval by the ethics committee, Regional Ethical Review Board in Gothenburg, Sweden (Dnr. 618-02, 065-04 and 683-07). Written informed consent was obtained prior to inclusion in the study (**Paper II**). The materials and methods in Paper III did not require any ethical permission since all experiments were *in vitro* only and only commercially available reagents were utilized.

11.4 STATISTICAL ANALYSES

All statistical analyses are described in the corresponding **Papers I-III**. GraphPad Prism software (GraphPad Software Inc., La Jolla, CA) were used for statistical analyses. In general, the distribution of data is determined and the number of groups different tests were used. T-test or Mann-Whitney U test was used to compare between two groups, whereas, ANOVA was used to compare between more than two groups. $P \leq 0.05$ was considered as statistically significant.

12 RESULTS AND DISCUSSION

For more details on the results and discussion, please refer to **Papers I, II** and **III.**

Paper I: ***Effects of the Antimicrobial Peptide LL-37 and Innate Effector Mechanisms on Colistin-Resistant Klebsiella pneumoniae with mgrB Insertions***

Colistin, also known as polymyxin E, is a polypeptide antibiotic that is cationic in charge and attacks the negatively charged bacterial membrane (86). Due to extensive and unregulated use of colistin in clinical and animal farming settings many bacteria developed resistance to this drug, including *Klebsiella pneumoniae* (Kpn) (87). Colistin is the last resort antibiotic that is used in the clinic to treat multi-drug resistant bacteria and therefore emergence of colistin resistant bacterial strains is of most concern and a huge threat to humans worldwide. Colistin and LL-37 are both cationic in charge and they initially target the negatively charged parts of the bacterial membrane (60). Therefore, there is a potential risk of cross-resistance between colistin and LL-37, but previous data on this topic is not conclusive.

In this study we compared two clinical Kpn carbapenem-resistant isolates that were genetically similar but differed in their colistin sensitivity, one isolate OM124 (Col-R) was colistin resistant (MIC 16 μg/ml) and the other isolate OM322, was colistin sensitive (Col-S) (MIC less than 1 μg/ml). Based on our gene sequencing data it was revealed that OM124 isolate had an inactivation with an insertion element in the *mgrB* gene as a likely mechanism of acquired colistin-resistance. The alterations in the *mgrB* gene, which encodes a negative feedback regulator on the PhoPQ system, is associated with LPS modifications leading to a reduction of the negative charge of LPS and thus, cause a phenotypic colistin resistance (88).

To test the hypothesis that there is cross-resistance between colistin and LL-37, we performed a colony count assay. Notably, at 100 μg/ml of the LL-37 peptide, the Col-R isolate was significantly less susceptible to LL-37 when compared to Col-S (p = 0.0098). This was followed by comparing the effect of 100 μg/ml LL-37 against additional Col-R isolates (n = 8) with *mgrB* gene insertion elements and against Col-S isolates (n = 9) lacking this insertion. Indeed, these results indicate that there is cross-resistance between colistin and LL-37 in Col-R strains, but only at high concentrations (above 50 μg/ml).

Mutations in genes that cause antibiotic resistance usually target important biological and physiological properties in the bacteria, which might have negative side effects on the growth and survival in normal environments lacking antibiotics (89). Therefore, we investigated if the gain of colistin resistance caused a loss of fitness in relation to factors in the innate immune

system. We used whole blood, which provides a physiological condition, since it contains a complex mixture of immune cells and factors, such as white blood cells, platelets and proteins of the complement system. We found that colistin-resistance in Col-R strain OM124 did not exhibit any cost of fitness compared to Col-S OM322 strain. They were both similarly sensitive to be killed by whole blood and the complement system (human serum). In order to translate these results to an *in vivo* system, we tested the Col-R and Col-S strains in an infection model of zebrafish embryos. We selected this model since it is an established model of the innate immune system. Interestingly, in the zebrafish the adaptive immune cells do not mature until 4 to 6 weeks after hatching, whereas the innate immune cells are available as soon as one day post-fertilization (90). Based on our experiments, we found no differences in survival nor in pathogenicity between the Col-R and Col-S strains. These results suggest that mutations in the *mgrB* gene due to insertion elements had no effects on bacterial fitness in relation to the innate immune system. Based on these results we concluded that the observed cross-resistance at high LL-37 concentrations most likely play a limited role during physiological conditions *in vivo.*

Paper II: *Studies on citrullinated LL-37: detection in human airways, antibacterial effects and biophysical properties*

During inflammation the level of activated PAD enzymes increase, which result in citrullination of various arginine-rich proteins and peptides, including the positively charged histones. In the same inflammatory milieu, plenty of AMPs are released from neutrophils and epithelia. Due to the close proximity of LL-37 to PAD enzymes and the presence of arginine residues in LL-37, it is highly likely that LL-37 will become citrullinated. However, the impact of citrullination on the biophysical and biological properties of LL-37 is not fully explored. Therefore, in this study we aimed to detect citrullinated forms of the LL-37 peptide in human BAL fluid obtained from healthy volunteers after intra-bronchial exposure to *E. coli* LPS as a proof-of-concept on the occurrence of citrullination on LL-37 *in vivo*. First, a peptide-protein extract was made from pooled BAL-fluid collected from the healthy volunteers. Next, the extract was separated using reversed phase HPLC and fractions were collected and further analyzed by dot-blot analysis with a monoclonal antibody against native LL-37 and a polyclonal antibody against LL-$37_{Cit5.}$ Notably, we were able to detect both native LL-37 as well as LL-37_{Cit5} in the fractions. To rule out unspecific immunoreactivity, we employed the sensitive analysis of targeted LC-MS/MS of selected BAL fractions. Indeed, we could confirm that both native LL-37 and citrullinated LL-37 were present in BAL fluid samples. Previous studies suggested the presence of the citrullinated form of LL-37 in biological samples, but the results were not fully confirmed with LC-MS/MS and thus, not fully conclusive. Therefore,

our study is the first to actually unequivocally demonstrate the presence of citrullinated LL-37 in a biological sample from humans. Nevertheless, the methods used (dot blot analysis and LC-MS/MS) did not give any quantitative measurements of LL-37 and its citrullinated form(s). However, these results represent a valuable proof on the existence of LL-37 citrullination in human airways.

The interactions of LL-37 to membranes is highly dependent on both electrostatic and hydrophobic interactions (46). Any disturbance in these features might have an effect on the biological functions of the peptide. Citrullination leads to reduction in the overall positive charge of the peptide, which potentially could reduce its interactions with bacterial membranes. Thus, we tested the antibacterial activity of multiple synthetic variants of citrullinated LL-37 against *E. coli*. The native peptide exhibited potent antibacterial activity against *E. coli* in a dose-dependent manner. However, the fully citrullinated peptide had no antibacterial activity, even at concentrations as high as 80 μM. Citrullinating one or more arginine residues affected the antibacterial activity of LL-37 dramatically. Via electron microscopy studies we showed that, in contrast to native LL-37, fully citrullinated LL-37 had no effects on the intracellular structure of *E. coli*. Next, we tested whether the peptides could bind to the surface of bacteria using fluorescently labelled peptides and confocal microscope. Notably, LL-37_{Cit5} failed to bind to the surface of *E. coli*, whereas the native peptide exhibited a clear binding to the bacterial surface. We concluded from these results that the positive charge of LL-37 is a key factor for its binding to bacterial membranes and consequently for its antibacterial activity.

In addition, we studied the interaction of native and fully citrullinated LL-37 with different components of the bacterial membrane. Using the isothermal titration calorimetry analysis, we determined a more enthalpy-driven interaction between native LPS molecules compared to fully citrullinated LL-37 peptides. Mass spectrometry analysis showed that the binding affinity of LL-37_{Cit5} to the anionic phospholipid micelles containing POPG was reduced compared to native LL-37. Furthermore, we studied the secondary structure of peptides upon interaction with negatively charged large unilamellar vesicles (LUVs). Based on the CD spectra, peptides mixed with LUVs exhibited an increased helical content of both native and citrullinated LL-37 with an overall higher helical content of citrullinated LL-37. In addition, the helical content of both peptides slightly increased upon interaction with an increasing concentration of LPS molecules. Taken together, citrullination impaired the antibacterial activity of LL-37. However, our data indicated that the interaction of LL-37 with LPS and phospholipids were not affected by citrullination. One way to explain this unexpected finding is that the interaction between LL-37 and LPS/phospholipids could be through hydrophobic interactions but this

interaction is insufficient for the disturbance of the bacterial membrane. In fact, citrullinated LL-37 is slightly more hydrophobic than native LL-37 and could thus, interact more strongly with LPS. However, this interaction does not seem to be involved in bacterial killing, since $LL37_{Cit5}$ was inactive against gram-negative bacteria.

Paper III: *Citrullination alters the antibacterial and anti-inflammatory functions of the antimicrobial peptide canine cathelicidin K9CATH in vitro*

K9CATH is a cationic AMP found in dogs (canine=K9) sharing similarities with human LL-37 by virtue of its positive charge at neutral pH, its ability to adopt a helical secondary structure as well as its antibacterial and anti-inflammatory functions (40, 42). K9CATH contains five arginine residues, similarly to LL-37 and could thus be subjected to citrullination *in vivo.* PAD enzymes are found in many animal species such as bony fish, frogs, and chickens, suggesting that citrullination has an important mechanism in nature (67). Given the need for additional models to study cathelicidin biology and citrullination, we designed a study to test the impact of citrullination on the dog cathelicidin K9CATH.

In this study, to test whether K9CATH is prone to be citrullinated, we exposed the synthetic peptide K9CATH to human recombinant PAD2 and PAD4 enzymes at different time-points (30 – 240 min). Subsequently, using a LC-MS/MS analysis we first used synthetic peptides of K9CATH and $K9CATH_{Cit5}$ to study their ionization and fragmentation characteristics as a reference and then we continued with the *in-vitro* citrullinated K9CATH. We found that both PADs catalyzed citrullination of K9CATH starting from 30 min after incubation and the level of citrullinated variants increased with time. Interestingly, we found that PAD2 citrullinated 4 arginine residues starting from 60 min, whereas PAD4 citrullinated only 3 arginine residues after 120 min. These results indicated that the PAD2 enzyme was more efficient in citrullinating K9CATH than the PAD4 enzyme. Next, we set out to test the effects of citrullination on the anti-inflammatory activity of K9CATH peptides exposed to PAD2 or PAD4. Peptides were incubated with LPS (100 ng/ml) before their addition to macrophages (RAW264.7 cells). After 24 h incubation, cell supernatants were used to study the anti-inflammatory activities of the peptides. In contrast to native K9CATH, exposing K9CATH to the activity of PAD2 reduced the capacity to neutralize LPS-mediated NO production. However, PAD2 had no effects on TNF-α and IL-6 release by macrophages. We observed that exposing the peptide to PAD4 had no effects on its ability to neutralize LPS-mediated pro-inflammatory effects on macrophages.

Next, we studied the effects of citrullination on the antibacterial activity of K9CATH. Based on the colony count assay against gram negative *E. coli* and *P. multocida* we found that citrullination profoundly affected K9CATH ability to inhibit bacterial growth even at the high concentration of 80 μM. Full citrullination affected the ability of the K9CATH peptide to cause leakage of the bacterial membrane in both *E. coli* and *P. multocida.* In addition, we hypothesized that citrullinated K9CATH potentially could cause killing of *E. coli* with different LPS-structures. Thus, we obtained a collection of *E. coli* with different LPS components: *E. coli* 25922 had a complete LPS-structure, *E coli* MG1655 had an LPS-structure with no O-antigens, and *E. coli* DA26796 lacked the outer core-OS and O-antigen of LPS. Interestingly, $K9CATH_{Cit5}$ was not able to kill any of these strains, despite their different LPS-structures. These results suggest that the electrostatic interaction between the peptide and bacteria is a key factor for the antibacterial activities of K9CATH. This finding validates the previous observations of citrullinated LL-37 in **Paper II**.

Next, we studied whether the degree of citrullination had any impact on the anti-inflammatory activity exerted by synthetic K9CATH peptides. To that end, we stimulated murine and canine macrophage cell lines with LPS (100 ng/ml) pre-incubated with different peptides for 24 hours, including native and citrullinated K9CATH and LL-37 peptides (5 μM). We found that the ability of citrullinated peptides to neutralize LPS-mediated proinflammatory activity was reduced in comparison to native K9CATH or native LL-37. The level of reduction in this activity was influenced by the number of citrullinated arginine residues in each peptide.

Finally, the hemolytic activity of the fully citrullinated peptide was tested against human red blood cells. We found that in contrast to the high hemolysis rate observed for the native peptide, the citrullinated peptide did not cause hemolysis to cells even at high concentrations (80 μM).

Based on our results, we concluded that citrullination of K9CATH profoundly affected the biological activities of the peptide due to loss of the overall positive charge. However, further studies in animal models are required to better understand the role of citrullination in physiological and pathological conditions. Furthermore, the field of PAD regulation using PAD-inhibitors to treat autoimmune diseases and other inflammatory disorders is gaining much interest in the recent years. Consequently, additional molecular and mechanistic studies on PAD regulation are warranted.

Overall, **Paper I-III** describe which factors that may define the interactions between antimicrobial peptides and bacterial membranes. In **Paper I**, we reported the bacterial side of this interaction, where potential cross-resistance between colistin and LL-37 was studied. The

mgrB-mutation in certain bacteria cause colistin-resistance by reducing the negative charge of the LPS-structure, which subsequently impair the interaction between colistin and the bacterial surface and cause phenotypic resistance to this drug. The same mechanism appears to be involved in how LL-37 interact with bacterial membranes. In **Papers II** and **III, I** shifted focus to the host and studied how endogenous enzymes (PADs) could reduce the net charge of the peptide, via citrullination of arginine-residues. Notably, the loss of the net charge in both LL-37 and the dog orthologue K9CATH, severely impaired the antibacterial effect of these peptides. Thus, citrullination is a powerful regulator of AMP-function. Most of the experiments in paper II and III were performed *in vitro*. However, it should be noted that we could identify citrullinated LL-37 in human BAL-fluid, which strongly indicates that citrullination of AMPs plays a role also *in vivo*.

13 CONCLUSIONS

The main findings of the presented work can be summarized as following:

1. There is cross-resistance between colistin and LL-37 at concentrations above 50 µg/ml in Kpn clinical isolates with colistin-resistance due to *mgrB*-insertion. No differences in the survival rate between the Col-R and Col-S isolates in blood, serum or in the zebrafish infection model were observed. (**Paper I**)

2. Citrullinated variants of LL-37 are present in human airways. Citrullination abrogates the antibacterial activity of LL-37. The positive charge of LL-37 is essential for bacterial killing. (**Paper II**)

3. The K9CATH peptide is prone to be citrullinated by PAD enzymes *in vitro*. In addition, citrullination impaired the antibacterial and LPS-neutralization activities of K9CATH. (**Paper III**)

14 FUTURE PERSPECTIVES

Increasing emergence of bacterial resistance to conventional antibiotics is a global concern in the human and animal healthcare, leading to an increase in the need for novel antimicrobial drugs (22). Research on the use of AMPs as novel antibiotics is gaining much attention in the scientific field. These peptides are of great interest by virtue of their broad-spectrum antibacterial activities against several pathogens. In addition, AMPs with different mechanism of action on pathogens make the development of resistance difficult. Cationic AMPs mainly target the negatively charged membranes of bacteria but in addition they target anionic intracellular components, such as ribosomes and DNA, thereby inhibiting the growth of target pathogens (91, 92). The application of AMPs as therapeutics are mainly by direct administration of the AMP to the patient, either systematic or topically. For example, Polymyxin B (topical treatment) and colistin (systemic or inhaled) are examples of peptide antibiotics that are used in the clinic. The other proposed way to exploit AMPs in the fight against infections, involves boosting of the innate immune system to induce AMP-expression using nutrients, such as Vitamin D_3 and phenylbutyrate (93). However, there is a rising argument in the scientific community on the risk of emergence of bacterial resistance to specific AMPs due to continuous exposure to bacteria and selection of resistant mutants. There is a risk that resistance against one AMP may lead to cross-resistance to other innate immune AMPs or even to conventional antibiotics, such as colistin (94). Therefore, there is a need to consider this risk when designing novel therapies involving AMPs. Our findings on cross-resistance between colistin and LL-37 at high concentrations in clinical colistin-resistant Kpn-isolates may have several interesting implications. First, increased levels of LL-37 during pathological conditions, such as infections might lead to selection pressure for colistin-resistant strains. Therefore, it would be interesting to study the virulence of colistin-resistant strains in infections models of healthy and inflamed animal models. It would also be interesting to study the survival of those strains in blood and plasma isolated from patients with high circulating levels of LL-37. On such disease is psoriasis, where the level of LL-37 is dramatically elevated in serum and may reach as high as 1366 µg/ml (95). If the cross-resistance has clinical importance, our results may lead to special considerations when colistin is administered to patients with elevated levels of LL-37, especially in intensive care units where colistin-resistant Kpn are thriving. This risk of pre-existing colistin-resistance could be high in these patients, and other treatment options should be chosen.

On the host side, AMPs undergo several PTMs via different pathways. In recent years, a great interest has been focused on citrullination of proteins and peptides in association with

autoimmune and chronic inflammatory diseases, such as rheumatoid arthritis and also associated with some types of cancer (96–98). In our studies on citrullinated LL-37 and K9CATH, we found that citrullination affect their biological functions, such as direct antibacterial activity. The results presented here may serve as a starting point for future studies on the role of AMP citrullination in health and disease in humans and animals. In fact, there are ongoing efforts to use PAD inhibitors as a way to regulate excessive activation of these enzymes in chronic diseases (99–101). However, the physiological functions of PAD enzymes in innate immunity, particularly on AMPs, are not fully understood. Thus, it is of great interest to study the effects of citrullination, not only on the antibacterial and immunomodulatory functions of AMPs, but also on additional biological functions of AMPs, such as angiogenesis, wound healing and anticancer properties. This would give us a better picture to evaluate the effects of PAD enzymes and to learn how to use PAD inhibitors in therapy. Further investigations using *in vivo* systems of PAD-deficient animal models or patients with altered expression of PADs (induced or down-regulated) could facilitate such studies. Since PAD enzymes are found in many animal species (67), it would be great to expand the studies of citrullination to other living organisms, such as zebrafish, birds and reptiles. These organisms have relatively less complex immune systems compared to mammals and they are easy to manipulate genetically. In addition, PAD deficient cell-lines or PAD-labelled cells could be obtained from these organisms for better visualization and localization. The half-life of these organisms is shorter than mammals and also cheaper to keep and maintain these model organisms in a laboratory set up. A recent study on halibut fish proved the presence of PAD enzymes and that innate immune factors, such as complement proteins C3 and C4 can be citrullinated (102). It would be interesting to detect citrullinated AMPs in the zebrafish, for example the defensin peptides. This would enable studies on the effects of citrullination *in vivo* and *in vitro*. Since cathelicidins have not yet been detected in zebrafish, there is also a great opportunity to dig deeper into the zebrafish genome and search for this gene or close orthologues. Although the fish-model is much different from humans, there are many conserved genes and shared mechanisms between the two species. Therefore, it is important to perform basic research in simple model organisms and learn about the fundamental mechanisms behind the function of PADs and the details of innate immunity. This will open doors to new research areas and provide a better and deeper understanding of human and animal biology.

14.1 FUTURE PERSPECTIVES – A PERSONAL NOTE

My background is in animal and veterinary sciences and I am planning to continue along this path both in research and education. I have thought of several aspects that I have learned from my PhD work and would like to implement in my future career. In Oman, camels are highly precious animals since they represent the Omani and the Arabic heritage. They are used mainly for racing but also for their meat and milk. These animals are unique and strong, they tolerate the harsh environment in the desert but they also endure many infectious diseases. In addition, their immune system is quite unique compared to other domestic animals. However, it is not fully explored, in particular there is a lack of knowledge about the innate immune system in camels. My plan is to start to search for cathelicidins and other AMPs in camels. I will use the fractionation of biological samples using the reversed phase-HPLC method and then test each fraction for their antibacterial activity using a simple inhibition zone assay. Depending on the results we could send the positive fractions for protein and peptide detection via electro spray mass-spectrometry (LC-MS/MS). In addition, I would like to study PAD enzymes and their contribution to the innate immunity of camels. More specifically I would like to study the role of PADs in the process of NETosis. Another aspect that I would like to explore is to study the usage of antibiotics as food additives in animal farms and relate it to the emergence of antibiotic resistant bacteria in animals and humans.

Finally, I am also interested in wild animals and the migratory birds in Oman. Due to the unique geographical features and location of Oman, millions of migrating birds transit in Oman every year. It would be interesting to study the presence of antibiotic resistant strains in these birds and to relate it to the microbiota in resident birds and animals. This will be important to know from an environmental point of view.

In the end, this PhD-period has been quite a journey and I have learned lots of laboratory skills and research methods that will be very useful in my future career.

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16 REFERENCES

1. Laxminarayan, R., Duse, A., Wattal, C., M Zaidi, A. K., L Wertheim, H. F., Sumpradit, N., Vlieghe, E., Levy Hara, G., Gould, I. M., Goossens, H., Greko, C., So, A. D., Bigdeli, M., Tomson, G., Woodhouse, W., Ombaka, E., Quizhpe Peralta, A., Naz Qamar, F., Mir, F., Kariuki, S., qar Bhutta, Z. A., Coates, A., Bergstrom, R., Wright, G. D., Brown, E. D., and Cars, O. (2013) Antibiotic resistance-the need for global solutions. *Lancet Infect. Dis.* **13**, 1057–1098

2. Moodley, A., Damborg, P., and Nielsen, S. (2014) Antimicrobial resistance in methicillin susceptible and methicillin resistant Staphylococcus pseudintermedius of canine origin: Literature review from 1980 to 2013. 10.1016/j.vetmic.2014.02.008

3. Mansour, S. C., Pena, O. M., and Hancock, R. E. W. (2014) Host defense peptides: front-line immunomodulators. *Trends Immunol.* **35**, 443–450

4. Hilchie, A. L., Wuerth, K., and Hancock, R. E. W. W. (2013) Immune modulation by multifaceted cationic host defense (antimicrobial) peptides. *Nat. Chem. Biol.* **9**, 761–768

5. Kulkarni, N. N., Yi, Z., Huehnken, C., Agerberth, B., and Gudmundsson, G. H. (2015) Phenylbutyrate induces cathelicidin expression via the vitamin D receptor: Linkage to inflammatory and growth factor cytokines pathways. *Mol. Immunol.* **63**, 530–539

6. Ottosson, H., Nylén, F., Sarker, P., Miraglia, E., Bergman, P., Gudmundsson, G. H., Raqib, R., Agerberth, B., Strömberg, R., Nylen, F., Sarker, P., Miraglia, E., Bergman, P., Gudmundsson, G. H., Raqib, R., Agerberth, B., and Stromberg, R. (2016) Potent Inducers of Endogenous Antimicrobial Peptides for Host Directed Therapy of Infections. *Sci Rep*. **6**, 36692

7. Medzhitov, R., and Janeway, C. A. (1997) Innate immunity: impact on the adaptive immune response. *Curr. Opin. Immunol.* **9**, 4–9

8. Galli, S. J., Borregaard, N., and Wynn, T. A. (2011) Phenotypic and functional plasticity of cells of innate immunity: Macrophages, mast cells and neutrophils. *Nat. Immunol.* 10.1038/ni.2109

9. Janeway, C. A., and Medzhitov, R. (2002) INNATE IMMUNE RECOGNITION. *Annu. Rev. Immunol*. **20**, 197–216

10. Janeway, C. A. (1989) Approaching the asymptote? Evolution and revolution in immunology. in *Cold Spring Harbor Symposia on Quantitative Biology*, 10.1101/sqb.1989.054.01.003

11. Medzhitov, R. (2007) Recognition of microorganisms and activation of the immune response. *Nature*. **449**, 819–826

12. Babior, B. M., Kipnes, R. S., and Curnutte, J. T. (1973) Biological defense mechanisms. The production by leukocytes of superoxide, a potential bactericidal agent. *J. Clin. Invest.* 10.1172/JCI107236

13. Yang, Y., Bazhin, A. V., Werner, J., and Karakhanova, S. (2013) Reactive oxygen species in the immune system. *Int. Rev. Immunol.* 10.3109/08830185.2012.755176

14. Brinkmann, V., Reichard, U., Goosmann, C., Fauler, B., Uhlemann, Y., Weiss, D. S., Weinrauch, Y., and Zychlinsky, A. (2004) Neutrophil extracellular traps kill bacteria. *Science*. **303**, 1532–5

15. Mills, C. D., Kincaid, K., Alt, J. M., Heilman, M. J., and Hill, A. M. (2000) M-1/M-2 Macrophages and the Th1/Th2 Paradigm. *J. Immunol.* 10.4049/jimmunol.164.12.6166

16. Gasteiger, G., D'Osualdo, A., Schubert, D. A., Weber, A., Bruscia, E. M., and Hartl, D. (2017) Cellular Innate Immunity: An Old Game with New Players. *J. Innate Immun.* **9**, 111–125

17. Beutler, B. (2004) Innate immunity: an overview. *Mol. Immunol.* **40**, 845–859

18. Mogensen, T. H. (2009) Pathogen recognition and inflammatory signaling in innate immune defenses. *Clin. Microbiol. Rev.* 10.1128/CMR.00046-08

19. Hato, T., and Dagher, P. C. (2015) How the Innate Immune System Senses Trouble and Causes Trouble. *Clin. J. Am. Soc. Nephrol.* **10**, 1459–1469

20. Ganz, T. (2003) The Role of Antimicrobial Peptides in Innate Immunity. *Integr. Comp. Biol.* **43**, 300–304

21. Zasloff, M. (2002) Antimicrobial peptides of multicellular organisms. *Nature*. **415**, 389–395

22. Mookherjee, N., Anderson, M. A., Haagsman, H. P., and Davidson, D. J. (2020) Antimicrobial host defence peptides: functions and clinical potential. *Nat. Rev. Drug*

Discov. **19**, 311–332

23. Lehrer, R. I., and Ganz, T. (2002) Defensins of vertebrate animals. *Curr. Opin. Immunol.* **14**, 96–102

24. Lichtenstein, A. (1991) Mechanism of mammalian cell lysis mediated by peptide defensins: Evidence for an initial alteration of the plasma membrane. *J. Clin. Invest.* 10.1172/JCI115310

25. Zanetti, M., Gennaro, R., and Romeo, D. Cathelicidins: a novel protein family with a common proregion and a variable C-terminal antimicrobial domain. *FEBS Lett.* **374**, 1–5

26. Zanetti, M. (2005) The role of cathelicidins in the innate host defenses of mammals. *Curr Issues Mol Biol*. **7**, 179–196

27. Zanetti, M., Gennaro, R., Scocchi, M., and Skerlavaj, B. (2000) Structure and biology of cathelicidins. in *Advances in Experimental Medicine and Biology*, 10.1007/0-306-46831-x_17

28. Kai-Larsen, Y., and Agerberth, B. (2008) The role of the multifunctional peptide LL-37 in host defense. *Front Biosci*. **13**, 3760–3767

29. Bugla-Płoskońska, G., Kiersnowski, A., Futoma-Kołoch, B., and Doroszkiewicz, W. (2009) Killing of gram-negative bacteria with normal human serum and normal bovine serum: Use Of lysozyme and complement proteins in the death Of Salmonella strains O48. *Microb. Ecol.* 10.1007/s00248-009-9503-2

30. Kjeldsen, L., Cowland, J. B., and Borregaard, N. (2000) Human neutrophil gelatinase-associated lipocalin and homologous proteins in rat and mouse. *Biochim. Biophys. Acta - Protein Struct. Mol. Enzymol.* 10.1016/S0167-4838(00)00152-7

31. Sørensen, O. E., Follin, P., Johnsen, A. H., Calafat, J., Tjabringa, G. S., Hiemstra, P. S., and Borregaard, N. (2001) Human cathelicidin, hCAP-18, is processed to the antimicrobial peptide LL-37 by extracellular cleavage with proteinase 3. *Blood*. **97**, 3951–9

32. Gudmundsson, G. H., Agerberth, B., Odeberg, J., Bergman, T., Olsson, B., and Salcedo, R. (1996) The human gene FALL39 and processing of the cathelin precursor to the antibacterial peptide LL-37 in granulocytes. *Eur J Biochem*. **238**, 325–332

33. Gennaro, R., and Zanetti, M. (2000) Structural features and biological activities of the cathelicidin-derived antimicrobial peptides. *Biopolymers*. **55**, 31–49

34. Johansson, J., Gudmundsson, G. H., Rottenberg, M. E., Berndt, K. D., and Agerberth, B. (1998) Conformation-dependent antibacterial activity of the naturally occurring human peptide LL-37. *J Biol Chem*. **273**, 3718–3724

35. Agerberth, B., Charo, J., Werr, J., Olsson, B., Idali, F., Lindbom, L., Kiessling, R., Jö, H., Wigzell, H., and Gudmundsson, G. H. (2000) *The human antimicrobial and chemotactic peptides LL-37 and-defensins are expressed by specific lymphocyte and monocyte populations*, [online] www.bloodjournal.org (Accessed February 4, 2019)

36. Park, K., Elias, P. M., Oda, Y., Mackenzie, D., Mauro, T., Holleran, W. M., and Uchida, Y. (2011) Regulation of cathelicidin antimicrobial peptide expression by an endoplasmic reticulum (ER) stress signaling, vitamin D receptor-independent pathway. *J. Biol. Chem.* **286**, 34121–30

37. Modlin Philip Liu, L. T., Stenger, S., and Tang, D. H. (2007) the Induction of Cathelicidin Is Dependent on Mycobacterium tuberculosis Antimicrobial Activity against Cutting Edge: Vitamin D-Mediated Human. *J Immunol Ref.* **179**, 2060–2063

38. Turner, J., Cho, Y., Dinh, N. N., Waring, A. J., and Lehrer, R. I. (1998) Activities of LL-37, a cathelin-associated antimicrobial peptide of human neutrophils. *Antimicrob Agents Chemother*. **42**, 2206–2214

39. Vandamme, D., Landuyt, B., Luyten, W., and Schoofs, L. (2012) A comprehensive summary of LL-37, the factotum human cathelicidin peptide. *Cell Immunol*. **280**, 22–35

40. Sang, Y., Teresa Ortega, M., Rune, K., Xiau, W., Zhang, G., Soulages, J. L., Lushington, G. H., Fang, J., Williams, T. D., and Blecha, F. (2007) Canine cathelicidin (K9CATH): Gene cloning, expression, and biochemical activity of a novel pro-myeloid antimicrobial peptide. *Dev. Comp. Immunol.* **31**, 1278–1296

41. Santoro, D., and Maddox, C. W. (2014) Canine antimicrobial peptides are effective against resistant bacteria and yeasts. *Vet. Dermatol.* **25**, 35-e12

42. Coorens, M., Scheenstra, M. R., Veldhuizen, E. J., and Haagsman, H. P. (2017) Interspecies cathelicidin comparison reveals divergence in antimicrobial activity, TLR modulation, chemokine induction and regulation of phagocytosis. *Sci Rep*. **7**, 40874

43. Dear, J. D. (2014) Bacterial pneumonia in dogs and cats. *Vet. Clin. North Am. Small Anim. Pract.* **44**, 143–159

44. Lappin, M. R., Blondeau, J., Boothe, D., Breitschwerdt, E. B., Guardabassi, L., Lloyd, D. H., Papich, M. G., Rankin, S. C., Sykes, J. E., Turnidge, J., and Weese, J. S. (2017) Antimicrobial use Guidelines for Treatment of Respiratory Tract Disease in Dogs and Cats: Antimicrobial Guidelines Working Group of the International Society for Companion Animal Infectious Diseases. *J. Vet. Intern. Med.* **31**, 279–294

45. van Duijkeren, E., Catry, B., Greko, C., Moreno, M. A., Pomba, M. C., Pyorala, S., Ruzauskas, M., Sanders, P., Threlfall, E. J., Torren-Edo, J., and Torneke, K. (2011) Review on methicillin-resistant Staphylococcus pseudintermedius. *J. Antimicrob. Chemother.* **66**, 2705–2714

46. Oren, Z., Lerman, J. C., Gudmundsson, G. H., Agerberth, B., and Shai, Y. (1999) Structure and organization of the human antimicrobial peptide LL-37 in phospholipid membranes: relevance to the molecular basis for its non-cell-selective activity. *Biochem J.* **341 (Pt 3**, 501–513

47. Henzler Wildman, K. A., Lee, D. K., and Ramamoorthy, A. (2003) Mechanism of lipid bilayer disruption by the human antimicrobial peptide, LL-37. *Biochemistry*. **42**, 6545–6558

48. Silhavy, T. J., Kahne, D., and Walker, S. (2010) The bacterial cell envelope. *Cold Spring Harb Perspect Biol*. **2**, a000414

49. Whitfield, C., and Trent, M. S. (2014) Biosynthesis and Export of Bacterial Lipopolysaccharides. *Annu. Rev. Biochem.* **83**, 99–128

50. and, D. S. S., and McIntosh*, T. J. (2000) The Lipopolysaccharide Barrier: Correlation of Antibiotic Susceptibility with Antibiotic Permeability and Fluorescent Probe Binding Kinetics†. 10.1021/BI000810N

51. Beutler, B., and Rietschel, E. T. (2003) Innate immune sensing and its roots: The story of endotoxin. *Nat. Rev. Immunol.* **3**, 169–176

52. Rosenfeld, Y., Papo, N., and Shai, Y. (2006) Endotoxin (lipopolysaccharide) neutralization by innate immunity host-defense peptides: Peptide properties and plausible modes of action. *J. Biol. Chem.* **281**, 1636–1643

53. Ebbensgaard, A., Mordhorst, H., Aarestrup, F. M., and Hansen, E. B. (2018) The role of outer membrane proteins and lipopolysaccharides for the sensitivity of escherichia coli to antimicrobial peptides. *Front. Microbiol.* 10.3389/fmicb.2018.02153

54. Hancock, R. E. W., Haney, E. F., and Gill, E. E. (2016) The immunology of host defence peptides: Beyond antimicrobial activity. *Nat. Rev. Immunol.* **16**, 321–334

55. Kawai, T., and Akira, S. (2010) The role of pattern-recognition receptors in innate immunity: Update on toll-like receptors. *Nat. Immunol.* **11**, 373–384

56. Van Harten, R. M., Van Woudenbergh, E., Van Dijk, A., Haagsman, H. P., Van Harten, R. M., Van Woudenbergh, E., Van Dijk, A., and Haagsman, H. P. (2018) Cathelicidins: Immunomodulatory Antimicrobials. *Vaccines*. **6**, 63

57. Larrick, J. W., Hirata, M., Balint, R. F., Lee, J., Zhong, J., and Wright, S. C. (1995) Human CAP18: a novel antimicrobial lipopolysaccharide-binding protein. *Infect Immun*. **63**, 1291–1297

58. BENEDICT, R. G., and LANGLYKKE, A. F. (1947) Antibiotic activity of Bacillus polymyxa. *J. Bacteriol.*

59. Dixon, R. A., and Chopra, I. (1986) Leakage of periplasmic proteins from Escherichia coli mediated by polymyxin B nonapeptide. *Antimicrob. Agents Chemother.* 10.1128/AAC.29.5.781

60. Zahedi, A., Hossein, B. &, Kafil, S., Bialvaei, A. Z., and Kafil, H. S. (2015) Colistin, mechanisms and prevalence of resistance Review Colistin, mechanisms and prevalence of resistance. *Curr. Med. Res. Opin.* 10.1185/03007995.2015.1018989

61. Warren, H. S., Kania, S. A., and Siber, G. R. (1985) Binding and neutralization of bacterial lipopolysaccharide by colistin nonapeptide. *Antimicrob. Agents Chemother.* 10.1128/AAC.28.1.107

62. Landman, D., Georgescu, C., Martin, D. A., and Quale, J. (2008) Polymyxins revisited. *Clin Microbiol Rev*. **21**, 449–465

63. Olaitan, A. O., Morand, S., and Rolain, J. M. (2014) Mechanisms of polymyxin resistance: Acquired and intrinsic resistance in bacteria. *Front. Microbiol.* 10.3389/fmicb.2014.00643

64. Napier, B. A., Burd, E. M., Satola, S. W., Cagle, S. M., Ray, S. M., McGann, P., Pohl,

J., Lesho, E. P., and Weiss, D. S. (2013) Clinical use of colistin induces cross-resistance to host antimicrobials in Acinetobacter baumannii. *MBio*. **4**, e00021-13

65. György, B., Tóth, E., Tarcsa, E., Falus, A., and Buzás, E. I. (2006) Citrullination: A posttranslational modification in health and disease. *Int. J. Biochem. Cell Biol.* **38**, 1662–1677

66. Rogers, G. E., Harding, H. W. J., and Llewellyn-Smith, I. J. (1977) The origin of citrulline-containing proteins in the hair follicle and the chemical nature of trichohyalin, an intracellular precursor. *BBA - Protein Struct.* 10.1016/0005-2795(77)90250-1

67. Vossenaar, E. R., Zendman, A. J. W., van Venrooij, W. J., and Pruijn, G. J. M. (2003) PAD, a growing family of citrullinating enzymes: genes, features and involvement in disease. *BioEssays*. **25**, 1106–1118

68. Zhou, Y., Chen, B., Mittereder, N., Chaerkady, R., Strain, M., An, L. L., Rahman, S., Ma, W., Low, C. P., Chan, D., Neal, F., Bingham 3rd, C. O., Sampson, K., Darrah, E., Siegel, R. M., Hasni, S., Andrade, F., Vousden, K. A., Mustelin, T., and Sims, G. P. (2017) Spontaneous Secretion of the Citrullination Enzyme PAD2 and Cell Surface Exposure of PAD4 by Neutrophils. *Front Immunol*. **8**, 1200

69. Wang, S., and Wang, Y. (2013) Peptidylarginine deiminases in citrullination, gene regulation, health and pathogenesis. *Biochim. Biophys. Acta - Gene Regul. Mech.* **1829**, 1126–1135

70. Kilsgard, O., Andersson, P., Malmsten, M., Nordin, S. L., Linge, H. M., Eliasson, M., Sorenson, E., Erjefalt, J. S., Bylund, J., Olin, A. I., Sorensen, O. E., and Egesten, A. (2012) Peptidylarginine deiminases present in the airways during tobacco smoking and inflammation can citrullinate the host defense peptide LL-37, resulting in altered activities. *Am J Respir Cell Mol Biol*. **46**, 240–248

71. Koziel, J., Bryzek, D., Sroka, A., Maresz, K., Glowczyk, I., Bielecka, E., Kantyka, T., Pyr, K., Svoboda, P., Pohl, J., and Potempa, J. (2014) Citrullination Alters Immunomodulatory Function of LL-37 Essential for Prevention of Endotoxin-Induced Sepsis. *J. Immunol.* **192**, 5363–5372

72. Zhang, X., Oglecka, K., Sandgren, S., Belting, M., Esbjorner, E. K., Norden, B., and Graslund, A. (2010) Dual functions of the human antimicrobial peptide LL-37-target

membrane perturbation and host cell cargo delivery. *Biochim Biophys Acta*. **1798**, 2201–2208

73. Lande, R., Gregorio, J., Facchinetti, V., Chatterjee, B., Wang, Y.-H. Y. H. Y. H. Y.-H., Homey, B., Cao, W., Wang, Y.-H. Y. H. Y. H. Y.-H., Su, B., Nestle, F. O., Zal, T., Mellman, I., Schröder, J.-M. M., Liu, Y.-J. J., and Gilliet, M. (2007) Plasmacytoid dendritic cells sense self-DNA coupled with antimicrobial peptide. *Nature*. **449**, 564–569

74. Wong, A., Bryzek, D., Dobosz, E., Scavenius, C., Svoboda, P., Rapala-Kozik, M., Lesner, A., Frydrych, I., Enghild, J., Mydel, P., Pohl, J., Thompson, P. R., Potempa, J., and Koziel, J. (2018) A Novel Biological Role for Peptidyl-Arginine Deiminases: Citrullination of Cathelicidin LL-37 Controls the Immunostimulatory Potential of Cell-Free DNA. *J Immunol*. **200**, 2327–2340

75. Frasca, L., Palazzo, R., Chimenti, M. S., Alivernini, S., Tolusso, B., Bui, L., Botti, E., Giunta, A., Bianchi, L., Petricca, L., Auteri, S. E., Spadaro, F., Fonti, G. L., Falchi, M., Evangelista, A., Marinari, B., Pietraforte, I., Spinelli, F. R., Colasanti, T., Alessandri, C., Conti, F., Gremese, E., Costanzo, A., Valesini, G., Perricone, R., and Lande, R. (2018) Anti-LL37 antibodies are present in psoriatic arthritis (PsA) patients: New biomarkers in PsA. *Front. Immunol.* **9**, 1–16

76. Li, R. H. L. H., Ng, G., and Tablin, F. (2017) Lipopolysaccharide-induced neutrophil extracellular trap formation in canine neutrophils is dependent on histone H3 citrullination by peptidylarginine deiminase. *Vet. Immunol. Immunopathol.* **193**–**194**, 29–37

77. Cherrington, B. D., Morency, E., Struble, A. M., Coonrod, S. A., and Wakshlag, J. J. (2010) Potential Role for Peptidylarginine Deiminase 2 (PAD2) in Citrullination of Canine Mammary Epithelial Cell Histones. *PLoS One*. **5**, e11768

78. Cherrington, B. D. D., Mohanan, S., Diep, A. N. N., Fleiss, R., Sudilovsky, D., Anguish, L. J. J., Coonrod, S. A. A., and Wakshlag, J. J. J. (2012) Comparative Analysis of Peptidylarginine Deiminase-2 Expression in Canine, Feline and Human Mammary Tumours. *J. Comp. Pathol.* **147**, 139–146

79. Linkevicius, M., Sandegren, L., and Andersson, D. I. (2013) Mechanisms and fitness costs of tigecycline resistance in Escherichia coli. *J. Antimicrob. Chemother.* **68**, 2809–2819

80. Smith, M. E., Stockfelt, M., Tengvall, S., Bergman, P., Lindén, A., and Qvarfordt, I. (2017) Endotoxin Exposure Increases LL-37 - but Not Calprotectin - in Healthy Human Airways. *J. Innate Immun.* **9**, 475–482

81. Glader, P., Smith, M. E., Malmhäll, C., Balder, B., Sjöstrand, M., Qvarfordt, I., and Lindén, A. (2010) Interleukin-17-producing T-helper cells and related cytokines in human airways exposed to endotoxin. *Eur. Respir. J.* **36**, 1155–1164

82. Wellman, M. L., Krakowka, S., Jacobs, R. M., and Kociba, G. J. (1988) A macrophage-monocyte cell line from a dog with malignant histiocytosis. *Vitr. Cell. Dev. Biol.* **24**, 223–229

83. Davidson, A. J., and Zon, L. I. (2004) The "definitive" (and 'primitive') guide to zebrafish hematopoiesis. *Oncogene*. **23**, 7233–7246

84. Mondal, S., and Thompson, P. R. (2019) Protein Arginine Deiminases (PADs): Biochemistry and Chemical Biology of Protein Citrullination. *Acc. Chem. Res.* **52**, 818–832

85. Vossenaar, E. R., Radstake, T. R., van der Heijden, A., van Mansum, M. A., Dieteren, C., de Rooij, D. J., Barrera, P., Zendman, A. J., and van Venrooij, W. J. (2004) Expression and activity of citrullinating peptidylarginine deiminase enzymes in monocytes and macrophages. *Ann Rheum Dis*. **63**, 373–381

86. Poirel, L., Jayol, A., and Nordmanna, P. (2017) Polymyxins: Antibacterial activity, susceptibility testing, and resistance mechanisms encoded by plasmids or chromosomes. *Clin. Microbiol. Rev.* **30**, 557–596

87. Liu, Y.-Y., Wang, Y., Walsh, T. R., Yi, L.-X., Zhang, R., Spencer, J., Doi, Y., Tian, G., Dong, B., Huang, X., Yu, L.-F., Gu, D., Ren, H., Chen, X., Lv, L., He, D., Zhou, H., Liang, Z., Liu, J.-H., and Shen, J. (2016) Articles Emergence of plasmid-mediated colistin resistance mechanism MCR-1 in animals and human beings in China: a microbiological and molecular biological study. 10.1016/S1473-3099(15)00424-7

88. Cannatelli, A., Giani, T., D'Andrea, M. M., Pilato, V. Di, Arena, F., Conte, V., Tryfinopoulou, K., Vatopoulos, A., and Rossolini, G. M. (2014) MgrB inactivation is a common mechanism of colistin resistance in KPC-producing klebsiella pneumoniae of clinical origin. *Antimicrob. Agents Chemother.* **58**, 5696–5703

89. Andersson, D. I., and Levin, B. R. (1999) The biological cost of antibiotic resistance.

Curr. Opin. Microbiol. **2**, 489–493

90. Lam, S. H., Chua, H. L., Gong, Z., Lam, T. J., and Sin, Y. M. Development and maturation of the immune system in zebrafish, Danio rerio: a gene expression profiling, in situ hybridization and immunological study. 10.1016/S0145-305X(03)00103-4

91. Le, C.-F., Fang, C.-M., and Sekaran, S. D. (2017) Intracellular Targeting Mechanisms by Antimicrobial Peptides. *Antimicrob. Agents Chemother.* **61**, e02340-16

92. Sonawane, A., Santos, J. C., Mishra, B. B., Jena, P., Progida, C., Sorensen, O. E., Gallo, R., Appelberg, R., and Griffiths, G. (2011) Cathelicidin is involved in the intracellular killing of mycobacteria in macrophages. *Cell. Microbiol.* **13**, 1601–1617

93. Mily, A., Rekha, R. S., Kamal, S. M., Arifuzzaman, A. S., Rahim, Z., Khan, L., Haq, M. A., Zaman, K., Bergman, P., Brighenti, S., Gudmundsson, G. H., Agerberth, B., and Raqib, R. (2015) Significant Effects of Oral Phenylbutyrate and Vitamin D3 Adjunctive Therapy in Pulmonary Tuberculosis: A Randomized Controlled Trial. *PLoS One*. **10**, e0138340

94. Dobias, J., Poirel, L., and Nordmann, P. (2017) Cross-resistance to human cationic antimicrobial peptides and to polymyxins mediated by the plasmid-encoded MCR-1? *Clin. Microbiol. Infect.* **23**, 676.e1-676.e5

95. Ong, P. Y., Ohtake, T., Brandt, C., Strickland, I., Boguniewicz, M., Ganz, T., Gallo, R. L., and Leung, D. Y. M. (2002) Endogenous Antimicrobial Peptides and Skin Infections in Atopic Dermatitis. *N. Engl. J. Med.* **347**, 1151–1160

96. Darrah, E., and Andrade, F. (2018) Rheumatoid arthritis and citrullination. *Curr. Opin. Rheumatol.* **30**, 72–78

97. Vossenaar, E. R., Nijenhuis, S., Helsen, M. M., van der Heijden, A., Senshu, T., van den Berg, W. B., van Venrooij, W. J., and Joosten, L. A. (2003) Citrullination of synovial proteins in murine models of rheumatoid arthritis. *Arthritis Rheum*. **48**, 2489–2500

98. Brentville, V. A., Vankemmelbeke, M., Metheringham, R. L., and Durrant, L. G. (2020) Post-translational modifications such as citrullination are excellent targets for cancer therapy. *Semin. Immunol.* **47**, 101393

99. Ledet, M. M., Anderson, R., Harman, R., Muth, A., Thompson, P. R., Coonrod, S. A., and Van de Walle, G. R. (2018) BB-Cl-Amidine as a novel therapeutic for canine and feline mammary cancer via activation of the endoplasmic reticulum stress pathway. *BMC Cancer*. **18**, 412

100. Witalison, E. E., Thompson, P. R., and Hofseth, L. J. (2015) Protein Arginine Deiminases and Associated Citrullination: Physiological Functions and Diseases Associated with Dysregulation. *Curr Drug Targets*. **16**, 700–710

101. Mohanan, S., Cherrington, B. D., Horibata, S., McElwee, J. L., Thompson, P. R., and Coonrod, S. A. (2012) Potential role of peptidylarginine deiminase enzymes and protein citrullination in cancer pathogenesis. *Biochem. Res. Int.* **2012**, 895343

102. Magnadóttir, B., Bragason, B. T., Bricknell, I. R., Bowden, T., Nicholas, A. P., Hristova, M., Guðmundsdóttir, S., Dodds, A. W., and Lange, S. (2019) Peptidylarginine deiminase and deiminated proteins are detected throughout early halibut ontogeny - Complement components C3 and C4 are post-translationally deiminated in halibut (Hippoglossus hippoglossus L.). *Dev. Comp. Immunol.* **92**, 1–19