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Stockholm 2009

Published by Karolinska Institutet.

ISBN 978-91-7409-681-1

Printed by
REPROPRINT AB
Stockholm 2009
www.reproprint.se
Gårdsvägen 4, 169 70 Solna

“New knowledge is the most valuable commodity on earth. The more truth we have to work with, the richer we become.”

Kurt Vonnegut

ABSTRACT

Antimicrobial peptides and proteins (AMPs) are effector molecules of innate immunity and are capable to kill a broad spectrum of microbes, i.e. bacteria, fungi and viruses. They are widespread in nature and have been found in almost all species of the animal kingdom, as well as in plants. The mammalian repertoire of antimicrobial peptides includes the defensins and the cathelicidins. Furthermore, several of the antimicrobial proteins are members of the S100 family. AMPs are predominantly expressed at exposed surfaces and in specific white blood cells. In the first part of this thesis, the presence of AMPs in material derived from the skin and gut of the fetus/neonate was investigated. Interestingly, the levels of AMPs of the gut were found to be induced after birth. Apart from being antimicrobial, AMPs are important in immunity by exhibiting additional functions, such as chemotactic, mitogenic and wound healing activities. Hence, in the second part of this thesis, novel immunological functions of several AMPs were discovered, further supporting their multifunctional role in host defense.

Vernix caseosa (vernix) is a lipid-rich material covering the skin of newborn babies and is suggested to serve as an anti-infective substance, both for the fetus during the last trimester of gestation, and for the neonate during the first days of life. In paper I, protein extracts of vernix were found to exhibit antimicrobial activity against three bacterial strains and one fungal strain. In a proteomic approach, we identified approximately 20 proteins possessing diverse effects implicated in innate immunity, such as opsonizing, anti-parasitic and anti-protease activities. In paper II, we reported that protein extracts of meconium and neonatal feces exhibit antimicrobial activity against gram-positive and gram-negative bacteria. We also identified several AMPs in both these extracts that most likely contribute to the observed antimicrobial activities. Interestingly, the levels of some AMPs were found at higher levels in feces than in meconium, suggesting an induction or release of AMPs after birth upon colonization and breast feeding. As demonstrated in paper III, physiological concentrations of hydrophilic components of breast milk induce the expression of the cathelicidin LL-37 in colonic epithelial cells after 48 h of stimulation. This novel role of breast milk may contribute to the protection against infections in the gut of breast-fed babies.

In paper IV of this thesis, LL-37 was found to inhibit biofilm formation of uropathogenic *Escherichia coli.* This inhibition is likely mediated by binding of LL-37 to the major curli subunit CsgA, preventing polymerization of CsgA to curli fibers. We demonstrated that biofilm of *E. coli* increased the resistance to cathelicidins, indicating that the inhibition of biofilm is a crucial anti-infective mechanism, making the bacteria more susceptible to killing. Furthermore, in paper V, we reported that neutrophil defensins 1-3 (HNP1-3) and heparin binding protein (HBP) enhanced macrophage phagocytosis of IgG-opsonized bacteria. The mechanism mediating this effect was found to be the release of IFNγ and TNFα, activating the macrophages in an autocrine manner, ultimately resulting in upregulation of Fc-receptors.

The anti-biofilm effect and the enhanced phagocytosis mediated by AMPs are novel effects, demonstrating that AMPs contribute to additional defense strategies that most likely are important in combating infections.

PUBLICATIONS

This thesis is based on the following articles, referred to in the text by their Roman numerals.

I. Tollin M., Bergsson G., **Kai-Larsen Y.**, Lengqvist J., Sjövall J., Griffiths W., Skuladottir GV., Haraldsson A., Jörnvall H., Gudmundsson G.H. and Agerberth B. Vernix caseosa as a multi-component defence system based on polypeptides, lipids and their interactions. *Cellular and Molecular Life Science*, 2005;62, 2389-2399

II. **Kai-Larsen Y**., Bergsson G., Gudmundsson G.H., Printz G., Jörnvall H., Marchini G. and Agerberth B. Antimicrobial components of the neonatal gut affected upon colonization. *Pediatric Research*, 2006;61, 530-536

III. **Kai-Larsen Y**., Cederlund A., Printz G., Yoshio H., Jörnvall H., Gudmundsson G.H. and Agerberth B. Enhanced expression of LL-37 by breast milk component(s) in human colonic epithelial cells. *Manuscript*

IV. **Kai-Larsen Y.**, Lüthje P., Chromek M., Wang X., Holm Å., Kádas L., Hedlund K-O., Johansson J., Chapman M.R., Jacobson S., Römling U., Agerberth B. and Brauner A. Uropathogenic *Escherichia coli* modulates immune responses and its curli fimbriae interact with the antimicrobial peptide LL-37. *Resubmitted to PLoS Pathogens*

V. Soehnlein O., **Kai-Larsen Y**., Frithiof R., Sorensen O.E., Kenne E., Scharffetter-Kochanek K., Eriksson E., Herwald H., Agerberth B. and Lindbom L. Neutrophil primary granule proteins HBP and HNP1-3 boost bacterial phagocytosis by human and murine macrophages. *The Journal of Clinical Investigation*, 2008;118, 3491-3502

RELATED PUBLICATIONS NOT INCLUDED IN THE THESIS

1. **Kai-Larsen Y**. and Agerberth B**.** The role of the multifunctional peptide LL-37 in host defense. *Frontiers in Bioscience*, 2008; 13, 3760-3767

CONTENTS

LIST OF ABBREVIATIONS

AcN	Acetonitrile
AMP	Antimicrobial peptides and proteins
ATCC	American type culture collection
ASF	Airway surface fluid
B. megaterium	*Bacillus megaterium*
C. albicans	*Candida albicans*
CAMP	Cathelicidin antimicrobial peptide
CD	Circular dichroism
CF	Cystic fibrosis
CFTR	Cystic fibrosis transmembrane conductance regulator
CFU	Colony forming units
E. coli	*Escherichia coli*
ERK	Extracellular signal regulated protein kinase
EGFR	Epidermal growth factor receptor
FBS	Fetal bovine serum
FPRL-1	Formyl peptide receptor like-1
GAS	Group A streptococci
GBS	Group B streptococci
GM-CSF	Granulocyte-macrophage colony stimulating factor
HBD	Human β-defensin
HBS-EP	Hepes-buffered saline
HD	Human defensin
HBP	Heparin binding protein
HNP	Human neutrophil peptide
HPLC	High performance liquid chromatography
IC	Inhibitory concentration
IFNγ	Interferon γ
IRF-3	Interferon regulatory transcription factor-3
LB	Luria Bertani
LPS	Lipopolysaccharide
MALDI-MS	Matrix-assisted laser desorption/ionization-mass spectrometry
MAP	Mitogen-activated protein
mCRAMP	Mouse cathelicidin related antimicrobial peptide
MEK	Mitogen/extracellular signal protein kinase
MIC	Minimal inhibitory concentration
NEC	Necrotizing enterocolitis
NfκB	Nuclear factor κB
NK-cell	Natural killer-cell
NOD	Nucleotide-binding oligomerization domain
NLRs	NOD-like receptors
PAGE	Polyacrylamide gel electrophoresis
PAMP	Pattern associated molecular pattern
PRR	Pattern recognition receptor

P. aeruginosa	*Pseudomonas aeruginosa*
PMNs	Polymorphonuclear cells
PVDF	Polyvinylidene difluoride
S. aureus	*Staphylococcus aureus*
SDS	Sodium dodecyl sulfate
S. typhimurium	*Salmonella typhimurium*
TFA	Trifluoroacetic acid
ThT	Thioflavin T
TIR	Toll/interleukin-1 receptor
TLC	Thin-layer chromatography
TLR	Toll-like receptor
TNFα	Tumor necrosis factor α
UTRP-1	Uteroglobulin-related protein 1
VDR	Vitamin D receptor
Vernix	*Vernix caseosa*

1 BACKGROUND

1.1 THE MICROBE-HOST INTERPLAY

We live in a world rich in microbes. They are everywhere around us; on door nobs, on our clothes, on coins, in the food we eat and we carry them around. Humans and microbes are a good example of mutualism, meaning that we peacefully coexist in a symbiotic manner as a result of evolution on both sides. It is fascinating that the human body consists of 10 times more microbes (10^{14}) than our own cells (10^{13}) without being infected during normal circumstances. It is even more fascinating that we need all these organisms for our survival. In addition to providing us with vitamins, the normal flora offers protection against pathogens by occupying adherence sites and by producing substances that are antimicrobial against pathogenic strains [1]. However, this seemingly perfect balance of cooperation is sometimes disturbed, which may lead to disease because of changes in the microbial communities. We have evolutionarily developed a sophisticated immune system, which can be described as a synergistic interplay of several biological processes that protect us from, or eliminate, invading pathogens. Historically, the understanding of immunology started around two centuries ago when Edward Jenner 1796 discovered that individuals previously infected with vaccinia were protected against the human, often lethal, smallpox. This phenomenon was designated "vaccination", a term that is still utilized to describe immunization of individuals, providing protection against infectious agents. Later, at the end of the 19th century, Robert Kock isolated several microbial strains and demonstrated that they could cause disease. These discoveries led to further progress of vaccination for infectious diseases, such as rabies. During the same time (1890), it was found that serum from vaccinated individuals contained high levels of components that bind specifically to the corresponding inoculated microbe. These components were designated antibodies. With accumulating pieces of evidence, it became clear that the immune system is composed of an extremely complex mixture of millions components that cooperate and interact with each other.

The immune system consists of two branches, the innate and adaptive immunity, which differ in respect to antigen recognition, mechanism of elimination, and cell types. The innate (also called the natural immune system) is the first line of defense, is constantly present and is non-specific. In contrast to the innate, the adaptive immune

system is specific towards the invading pathogen and takes several days to become fully activated. The adaptive immunity includes activation of lymphocytes, resulting in the generation of antibodies that are directed to microbial antigens. In general, the innate immunity helps the organism to shield the body from microbial invasion, whereas the adaptive immune system eliminates the microbes after invasion and prevents reinfection with the same pathogen. These two systems are tightly linked and regulated to eachother with the ultimate goal of eliminating the invading pathogen. Still the picture of the immune system is far from complete. However, every piece of the puzzle increase our possibility to be a step ahead the pathogens and hence avoid disease [2].

1.1.1 Innate immunity

Although humans are persistently exposed to an enormous amount of microbes, infections are fortunately rare, partly due to effective innate immune responses with crucial functions in the immediate defense against pathogenic microbes. Innate immunity is present in most multicellular organisms, even in the most primitive types, such as plants, and is evolutionarily the most ancient form of immune defense [2]. Without a functioning innate immunity, we are extremely susceptible to life-threatening infections [3].

1.1.1.1 Components of innate immunity

Innate immunity is a structural and immunological barrier, which serves as an efficient protection against microbial invasion. The structural barrier consists of epithelial cells joined with tight junctions that together form rigid walls. These can be found in the skin and in tubular structures, such as the gut, respiratory and uroepithelial tracts, where they sparate the internal environment from the non-sterile, external milieu. In addition to serving as a physical barrier, epithelial linings also produce molecules that can kill microbes or at least inhibit their growth. Among these are antimicrobial peptides and proteins (AMPs) and digestive enzymes that degrade microbial proteins. Normally, microbes cannot cross the epithelial layers. However, wounds or disruptions can allow microorganisms to reach underlying tissues, where they can replicate. In deeper tissues, the complement system and residing innate effector cells, i.e. macrophages, neutrophils, also called polymorphonuclear cells (PMNs), and dendritic cells are present to fight the invading pathogens. In acute inflammation, PMNs are the first

immunological cells to appear at the site of infections [4]. Thereafter, monocytes are recruited from the blood stream to the affected tissue, where they are differentiated to macrophages. During migration from the blood to specific tissues, PMNs release their granule products, such as AMPs [5] that are able to modulate the function of neighbouring immunological and endothelial cells [6, 7]. The innate leukocytes have receptors recognizing structures that are shared by invading pathogens and are critical for their survival but distinguishable from human components. The host receptors are referred to as pattern recognition receptors (PRRs) and the ligands for these receptors are pathogen-associated molecular patterns (PAMPs) [8].

1.1.1.2 PRRs and PAMPs

There are several different PAMPs that can be recognized by PRRs. Examples of PAMPs include the conserved components bacterial flagellin, lipopolysaccharides (LPS), peptidoglycan and lipoteichoic acids, mannose, viral RNA and glucans from fungal cell walls [9]. PRRs can be membrane-bound or soluble in the cytoplasm of various cells. The membrane bound PRRs are the mannose receptor [10] and the toll like receptors (TLRs), of which 10 human members (TLR1-10) have been characterized [11]. TLRs are most abundant on macrophages, PMNs and dendritic cells with specificity to different PAMPs. Microbial crosslinking facilitates dimerization of these receptors, activating the cytoplasmic toll/interleukin-1 receptor (TIR) domain. This leads to activation of the MAP kinase pathway with subsequent NFκB and IRF-3 signaling [11]. Ultimately, this can result in the release of pro-inflammatory cytokines [11] and AMPs [12] that can kill noxious agents and elicit a strong immune response by recruitment/activation of additional immunological cells. It has been demonstrated that IL-1α and flagellin induce NFκB activation, resulting in the production of the antimicrobial peptide HBD-2 [13, 14]. In contrast to TLRs and the mannose receptor present in membranes, the NOD-like receptors (NLRs), i.e. NOD1 and NOD2, are abundant in the cytosol, where they can sense microbial components [15]. Recently it was shown that a point mutation in the gene encoding NOD2 is associated with Crohn's disease [16].

1.1.1.3 Phagocytosis

An efficient manner for the host to eliminate pathogens is to engulf the invader, a process called phagocytosis (means "to eat" in greek). Phagocytosis is a complex process starting with receptor binding with subsequent microbe internalization, actin rearrangement, and phagosome maturation [17]. In that way, the microbe is getting trapped in the phagolysozyme, where the microorganisms are killed and digested by both oxygen dependent and independent mechanisms. In the oxygen dependent mechanism, NADPH-oxidase generates the toxic superoxide radicals O_2^- and hydrogen peroxidase H_2O_2 that kills the pathogen efficiently [18]. However, also non oxygen factors play a pivotal role in killing the microbe in the phagolysozyme. Among these are AMPs such as defensins, lysozyme and azurocidin [19]. The cells most efficient in phagocytosis are referred to as "professional" phagocytes and among these are macrophages, monocytes and PMNs. The membrane of both phagocytes and microbes are negatively charged, leading to an electrostatically repelling action between these cells. However, the phagocytosis of invadors can be greatly augmented by opsonization, a process when opsonins (i.e. antibodies, complement factors or AMPs) are coating the microbe. Professional phagocytes express Fc- and complement-receptors that have high affinity to these opsonins. Thus, the interaction of phagocytes to microbes is greatly enhanced bringing them in close contact and increase the phagocytic capacity [17].

1.1.2 Adaptive immunity

The adaptive immune system developed with the evolution of vertebrates and is not present in lower organisms. It is induced when the innate immunity is by-passed a pathogen. In contrast to the innate, the adaptive immunity takes 3-5 days for activation and involves clonal expansion of T and B lymphocytes with subsequent antibody production (Fig. 1). Since bacteria duplicate every 20 minutes, this delay of activation is naturally a weakness. If not the innate immunity would have control or at least slow down the bacterial growth, the invading pathogen would soon outnumber the host, indicating that the adaptive immunity is not fully efficient by its own. Instead of expressing receptors recognizing common structures on microbes, each lymphocyte bears one receptor with specificity for only one epitope. However, rearrangement of segments in the gene encoding these receptors generates lymphocytes with millions of different specificities. Antigen presenting cells (APCs) are abundant in the lymph nodes

where they present microbial epitopes to lymphocytes. Only the lymphocyte that encounters the corresponding epitope will be activated, multiplies and produces antibodies, recognizing the specific microbe. Ultimately, this response leads to an efficient and direct elimination of the microbe (Fig. 1). The adaptive immunity also differs from innate immunity by possessing an immunological memory. This is especially beneficial for organisms with a long life span such as humans, who might get in contact with the same pathogen several times. This means that a lymphocyte clone, that once has been activated, very rapidly can expand a second time upon stimulation with the same antigen. Thus, immunological memory prohibits reinfection and makes us "immune" against several diseases that we previously have suffered from. Since newborn babies have not yet been exposed to many antigens they lack memory lymphocytes and are therefore at high risk of getting infections [2].

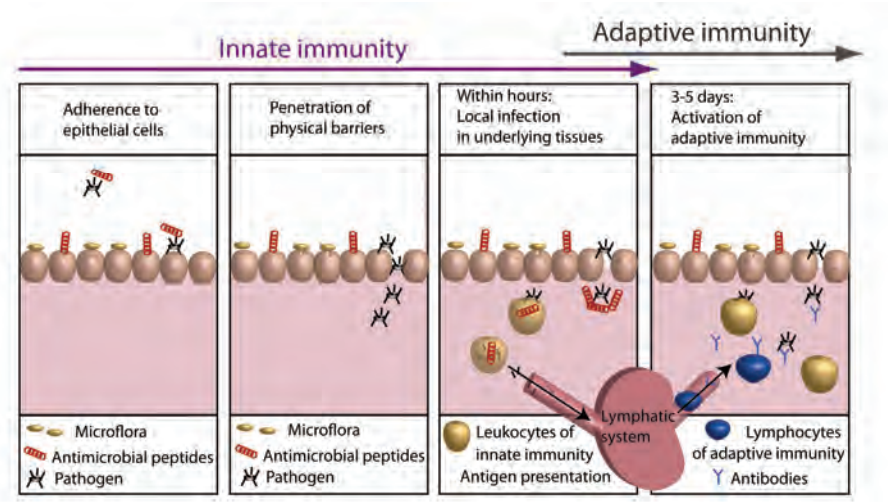

Figure 1. A summary of innate and adaptive immunity. Upon contact with a pathogen, we are first protected by the innate immunity, where the epithelial cells serve as an important barrier. On the epithelial surface, colonizing bacteria of the normal flora are present, which can prevent attachment of pathogenic microbes. The epithelial cells can also produce AMPs that can kill several pathogenic strains. If this barrier is weakened, the pathogen might breach the epithelium and reach underlying tissues, where it encounters leukocytes of innate immunity. These cells can kill the microbe with AMPs, either outside (after granule release) or inside the cell; during phagocytosis. After antigen presentation in the lymphatic system, the adaptive immune system will be activated with clonal expansion of specific lymphocytes.

1.2 ANTIMICROBIAL PEPTIDES

Antimicrobial peptides (AMPs) are gene-encoded effector molecules of the innate immunity that are able to kill bacteria, viruses and fungi. They constitute the antimicrobial arsenal in virtually all kinds of living microorganisms including vertebrates, invertebrates, bacteria and plants. This defense system is one of the most ancient strategies to ward off unwanted microbes and evolved long before the adaptive immune system [20] The oxygen-dependent killing mechanisms of granulocytes were thoroughly studied by various research groups in the middle of the 20th century. In 1975, it was demonstrated that neutrohil granules contain basic protein that were able to kill bacteria [21]. A few years later, Hans Boman and colleagues isolated and characterized two AMPs from the moth of *Hyalophora cecropia* [22]. These peptides killed bacteria efficiently and were referred to as cecropins because of their species origin. Lehrer *et al* presented the discovery of alpha-defensins as important microbial killers of the non-oxidative mechanism in human PMN granules [23]. Other pioneering discoveries in this field were the characterization of magainins in frog skin [24] and the isolation and characterization of the cathelicidin LL-37 in human PMNs [25]. Since then, many novel AMPs from different organisms and tissues have been characterized. Today, three decades later after the first identified AMP, we have a better understanding of the reportoir of AMPs and their distribution in species and tissues. However, less clear is still the physiological role of these peptides during an infection.

So far, more than 1000 AMPs have been described or predicted from gene sequences and are collected in a database [26]. Although AMPs exhibit high diversity in primary, secondary and tertiary structure, they share some common features, generating an optimal design to kill microbes. AMPs are small molecules with a size between 30-45 amino acid residues [27]. They are positively charged, making them attracted to the negatively charged membrane of microbes. Further they have an amphipatic character, with one hydrophobic and one cationic side, a structure that is beneficial for the interaction and disruption of bacterial membranes [28]. These peptides are produced at microbial exposed sites, such as at epithelial linings and in granulocytes. The expression of AMPs in epithelial cells can be induced by bacterial components [12], vitamins [29] and digestive products [30], or downregulated by certain bacteria such as *Shigella* [31]. AMPs are synthesized as prepropeptides, where the prepart function as a signal peptide, directing the translocation of the protein to endoplasmatic reticulum. After removal of the prepart, the remaining proform is subject

to proteolyic cleavage, generating the mature peptide and the remaining propart [32]. Cationic AMPs have been subgrouped based of their structure: i) peptides that forms alpha-helical conformation in various biological solutions, ii) AMPs that are rich in certain amino acids, i.e proline, arginine and phenylalanine, iii) AMPs containing several cysteines forming flat betasheet, which are cross linked and stabilized by disulfide bonds [33]. In addition to the subgrouping, AMPs are also sorted into families. The mammalian repertoire of AMPs are the defensins [34] and the cathelicidins [35]. The defensins have a typical beta-sheet fold with a framework of six disulfide linked cysteines, while the cathelicidin family is characterized by a conserved anionic N-terminal propart (cathelin) to which a C-terminal antimicrobial domain is connected. The defensins are further subdivided into α, β and θ–defensins. α- and β–defensins differ in amino acid sequence and in the positions of the disulfide bridges [34]. The θ–defensins, which have been identified only in rhesus macaque, are small and circle-formed peptides stabilized with disulfide bonds [36]. Apart from the direct antimicrobial activity of AMPs, accumulating data demonstrate that these peptides also exhibit additional immunumodulatory activities, such as chemotactic, antiendotoxin, and mitogenic activities [34, 37]. These additional activities suggest a role of AMPs in the interplay between innate and adaptive immunity.

1.2.1 Mechanisms of action

Several methods, including microscopy, model membranes, fluorescent dies, circular dichroism and NMR, have been applied to study the mechanism of action of AMPs. This process is rapid for some peptides making it challenging to characterize the steps prior to the microbial killing. It has been reported that several alpha-helical peptides kill the microbe within minutes [27], whereas most other peptides, such as magainin 2 [24] and PR-39 [38] require at least 15-90 min until the microbes are killed. The initial binding of a peptide to the microbe occurs via electrostatic interaction between negatively charged components (i.e. LPS and teichoic acid) in the bacterial membrane and the cationic peptides. When the peptide is in close contact with the microbe, it has to traverse the polysaccharides and fiber structures before they can interact with the membrane. In the case of gram-positive bacteria, the peptides have to migrate through the thick cell wall to reach the membrane. When in contact with the membrane, the peptide at low concentrations interacts in parallel to the lipid bilayer. With increasing concentrations, peptide molecules are oriented at 90° to the membrane and are inserted

into the bilayer [39]. For most peptides, this interaction leads to disruption of the membrane integrity, resulting in lysis of the microbe.

Each peptide utilizes different strategies to disrupt the microbial membranes and hence several mechanistic models have been developed, describing how this disruption occurs. The most established models are the barrel-stave pore, thoroidal pore and the carpet models [40]. In the barrel-stave pore model, peptides form a bundle in the membrane with the hydrophobic surfaces oriented towards the lipid core and the hydrophilic interphase directed inwards form the interior region of the pore (Fig. 2A) [41]. This type of pore, formed by many alpha helical peptides [39], can contain 3-11 parallel helical molecules and have an inner diameter of 1.8 nm [42]. In the toroidal pore model, the AMPs are inserted and bend the lipid monolayer continuously through the pore in a manner that causes the lipid head groups to be lined towards the water core (Fig. 2B). These pores are often larger than the barrel stave pores and are also induced by alpha helical peptides such as LL-37 [43] and magainins [44].

In the carpet model, the bacterial membrane interacts electrostatically with the AMPs at numerous positively charged sites of the peptide. Hence, accumulating peptides form a carpet lying in parallel with the membrane, leading to membrane disruption in a detergent-like manner (Fig. 2C) [41]. Apart from membrane disruption, other mechanisms of action have also been demonstrated for AMPs. For example, PR-39, which is a porcine cathelicidin, enters the cytoplasm/nuclei of the microbe and blocks protein and/or DNA synthesis, resulting in microbial death [38, 45]. Histatins use a different strategy to kill microbes. These peptides are histidine rich, mainly present in saliva and exhibit anti-fungal activity [46]. Histatins enter the cytoplasm of the fungi, causing ATP loss, disruption of the cell cycle and production of reactive oxygen species [47]. In addition to kill microbes, AMPs can destroy eukaryotic cells. This cytotoxicity has been observed *in vitro*, in particular to different blood cells, where T-cells are most sensitive [48]. However, the cytotoxic concentrations are higher than those of the bactericidal concentrations. The reason for this cell-selective mechanism is a stronger attraction of the peptides to the microbes than to eukaryotic cells, due to differences in charge of the specific membranes [49].

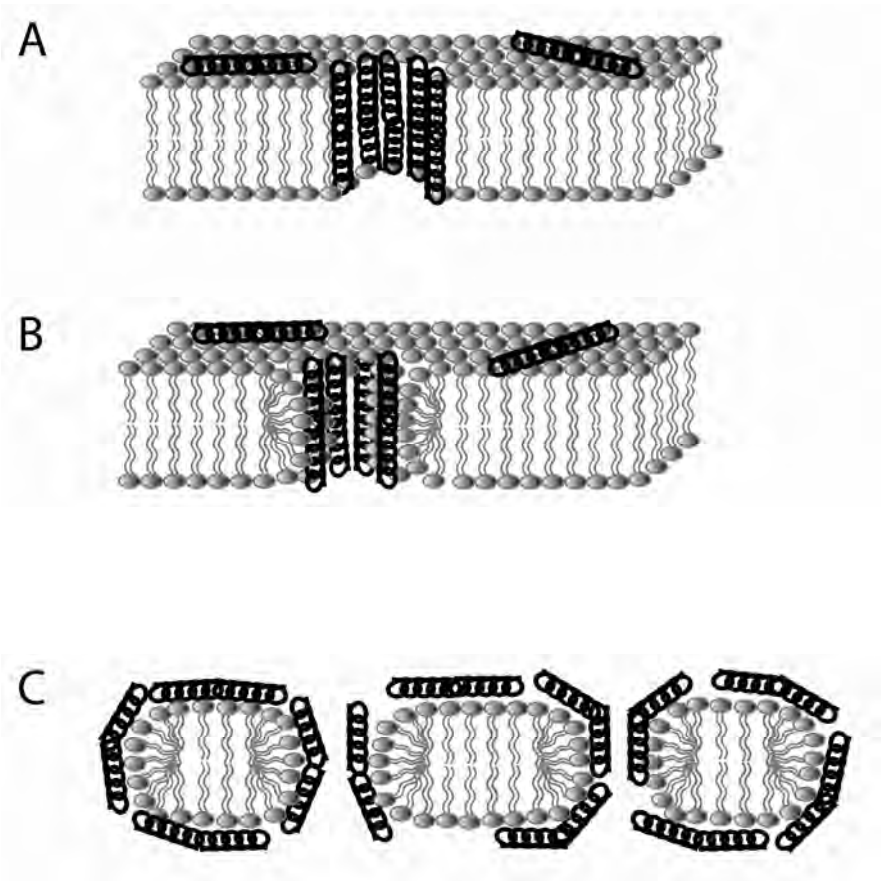

Figure 2. Different models of mechanism of action of AMPs. Three main mechanisms of action of AMPs have been proposed: the barrel stave (A), the toroidal (B) and the carpet models (C). Modified from [40].

1.3 CATHELICIDIN

Cathelicidins constitute one family of antimicrobial peptides found in mammals [35], birds [50] and fish [51]. They are all derived from a preproprotein sharing a conserved N-terminal proform (cathelin) with a variable C-terminal antimicrobial domain (cath peptide or cathelicidin). Cathelicidins are cleaved by processing enzymes to produce the cathelin protein and an antimicrobial peptide [35]. Both these parts have been found to exert biological effects related to host defense [52]. In contrast to the defensins, the structures of which are based on a common beta sheet structure, cathelicidin peptides are highly heterogeneous. LL-37 is the sole human cathelicidin peptide, abundant mainly in PMNs and at various epithelial linings [25, 53].

1.3.1 LL-37

1.3.1.1 Structure and processing

LL-37 owes it name to the first two N-terminal leucine (L) residues and the overall length of 37 amino acid residues. Originally, LL-37 was predicted from a human cDNA clone as a peptide composed of 39 amino acids residues (designated FALL-39) based on a putative processing site [54]. However, after isolation of the mature active peptide from degranulated PMNs, it was found to contain 37 amino acids residues. thus the name was corrected to LL-37 [25]. The gene encoding LL-37 is designated *CAMP* (cathelicidin antimicrobial peptide) and contains four exons. The three first exons encode the pre-and propart and the fourth exon encodes the mature LL-37 peptide (Fig. 3) [25]. The expression of *CAMP* can be both constitutive and induced by factors, i.e. vitamin D [29] and histone-deacetylase inhibitors such as butyrate and trichostatin [55, 56]. The induction of cathelicidin by butyrate has been confirmed *in vivo* in a rabbit model of shigellosis [30]. In that study, treatment with butyrate resulted in increased expression of CAP-18 (the LL-37 ortholog in rabbit) in the epithelium of the colon and rectum, thereby promoting elimination of the *Shigella* spp. The induction of LL-37 by butyrate in colonocytes has been shown to signal via the mitogen/extracellular signal protein kinase (MEK)/extracellular signal regulated protein kinase (ERK) [56], and the transcription factor PU.1 that binds to the *CAMP* gene promoter (Fig 3) [57].

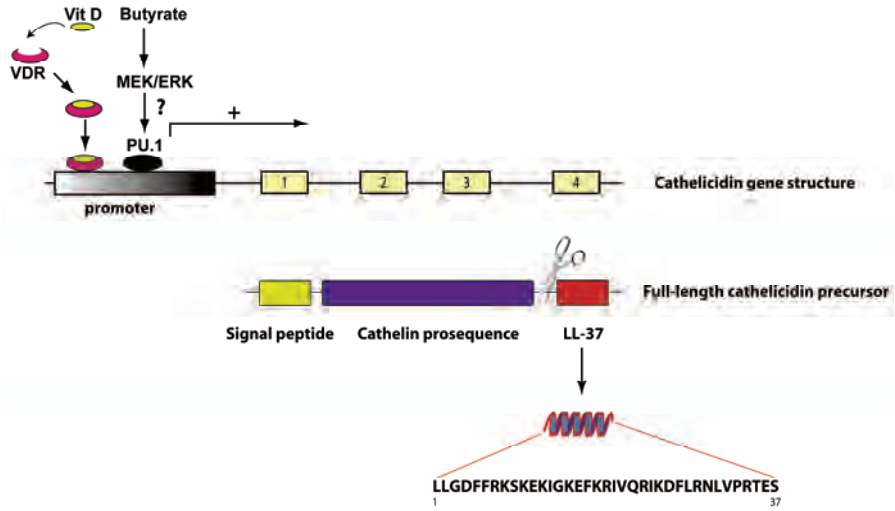

Figure 3. The structure of the *CAMP* gene encoding LL-37. The gene consists of 4 exons, where the last exon encodes the processing site and the mature LL-37 peptide. Vitamin D induces the gene expression of *CAMP* by activation of the vitamin D receptor (VDR) whereas butyrate mediates the upregulation of LL-37 via the MEK/ERK pathway and involves binding of the transcription factor PU.1 to the corresponding response element within the promoter.

The inactive proform of LL-37 is called human cationic antimicrobial peptide (hCAP)-18 and has, as its name indicates, an approximate mass of 18 kDa (Fig. 3). hCAP-18 is present in secondary granules of PMNs and has been shown to be processed by different neutrophilic proteases *in vitro*. However, only proteinase 3, present in primary granules of PMNs, has been demonstrated to liberate the active peptide LL-37 from hCAP-18 in material exocytosed from granules. Although hCAP-18 and proteinase 3 are associated in phagolysozymes formed during the phagocytic process, the processing of hCAP-18 is taken place only extracellularly upon degranulation of the PMNs, indicating that environmental conditions are crucial for the processing by proteinase 3 [58]. Interestingly, recent data demonstrated that epithelial derived proteases, i.e. kallikrein [59] and gastricsin [60] can generate peptides of different sizes of hCAP-18. The truncated forms of LL-37, i.e. RK-31, KS-30, LL-29, KS-22 and KR-20 have been identified in sweat [61] or skin [59] and

a longer form (ALL-38) [60] has been found in seminal plasma. These variant forms of LL-37 possess different antimicrobial and immunomodulatory properties, further increasing the functional spectra of one gene product.

1.3.1.2 Antimicrobial activity

After the discovery of LL-37, detailed data about its direct antimicrobial properties have accumulated. *In vitro,* the antimicrobial effects of LL-37 differ in numerous reports, revealing that the activity is sensitive to the assay conditions, such as salt and pH. Nevertheless, most of the available data reveal that LL-37 exhibits a broad range of antimicrobial activities. In several reports, it has been found that some strains of *Escherichia coli, Salmonella typhimurium* and *Neisseria gonorrhoeae* are the most sensitive bacteria with minimal inhibitory concentration (MIC) values in the range of 0.2-1 μM [62, 63]. Furthermore, LL-37 displays potent activity against *Pseudomonas aeruginosa* and the group A Streptococcus (GAS), whereas low activity has been detected against the yeast *Candida albicans* [63, 64]. LL-37 has also been found to exhibit inhibitory effects against herpes simplex virus [65] and vaccinia virus [66], making it to a promising, broad-spectrum, antimicrobial molecule for potential therapeutic use. Interestingly, Johansson *et al.* found a correlation between the antimicrobial capacity and the secondary structure of LL-37, where a higher helix content improves its antibacterial effects [48]. Solutions promoting the alpha-helical structure of LL-37 are those mimicking plasma or intracellular and interstitial fluids, containing anioins such as $HCO3^-$ or $SO4^{2-}$, indicating optimal antimicrobial activity *in vivo.*

1.3.1.3 Additional activities

In addition to exhibiting a broad range of antimicrobial activities, LL-37 possesses activities related to host defense. All these activities indicate that LL-37 has a pivotal role in linking innate and adaptive immunity. LL-37 recruits monocytes, PMNs and T-cells to the site of infection via the formyl peptide receptor like-1 (FPRL-1), which is present on the surface on these cells [67, 68]. LL-37 also exerts indirect chemotaxis of inflammatory cells by stimulation of chemokine-production in epithelial cells, dendritic cells and monocytes. In epithelial cells, this effect is signaling via epidermal growth factor receptor (EGFR), which is transactivated by LL-37 via metalloproteinase-mediated cleavage of membrane anchored EGFR ligands [69]. LL-

37 is also a ligand to the purinergic receptor $P2X_7$, resulting in rapid cytokine processing and release. This receptor is predominantly expressed on monocytes, macrophages and dendritic cells [70]. Accumulating evidence demonstrate that LL-37 is able to bind and neutralize the endotoxins LPS, flagellin, lipoteichoic acid and non-capped lipoarabinomannan. By binding to these toxins, LL-37 can ameliorate the production of proinflammatory cytokines, such as TNFα and IL-6, suggesting a regulatory role in inflammatory processes [71-73]. The antiendotoxic action of LL-37 has been verified *in vivo* utilizing a rat model making it a promising pharmaceutical candidate for prevention or treatment of endotoxin shock [74]. Apart from the effects of LL-37 already mentioned, LL-37 has been demonstrated to stimulate wound closure in skin [75] and in the airways [76]. Tissue repair is also enhanced by the ability of LL-37 to induce angiogenesis as shown both *in vitro* and *in vivo* [77]. Most functions of LL-37 are associated with host defense, enhancing the chances of avoiding disease. However, it was recently found that LL-37 can drive autoimmunity in psoriasis by binding to self-DNA. This LL-37/DNA complex is recognized by TLR9 in endosomes of plasmacytoid dendritic cells as a danger signal [78].

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1.3.1.4 Physiological relevance

In human serum, the proform of LL-37 is present at 0.3 μM under normal conditions [79]. However, the levels of LL-37 can be locally dramatically higher by degranulating PMNs [58] and during inflammatory conditions, i.e. in psoriatic lesions, where concentration as high as 304 μM has been detected [80]. Thus, the MIC values of LL-37 found *in vitro* are physiologically relevant during infectious and inflammatory diseases. Rather than acting alone, LL-37 operates in synergy with other antimicrobial peptides and proteins, such as β-defensin (HBD) 2 [80], lysozyme and lactoferrin [81], to exhibit its optimal antimicrobial effect. To study the role of LL-37 in disease, mice deficient in the gene encoding mouse cathelicidin related antimicrobial peptide (mCRAMP) have been utilized. Since mCRAMP is the only mouse cathelicidin and is similar to LL-37 in secondary structure, expression pattern and antimicrobial effects, the mCRAMP null mice are a valuable tool in studying the role of cathelicidin in infections. Interestingly, the mCRAMP deficient mice are more susceptible to GAS, herpes simplex virus and vaccinia virus infections than the wildtype (wt) mice [66, 82, 83], suggesting an essential function of mCRAMP/LL-37 in host defense against these pathogens. A physiological role of LL-37 in host

defense is illustrated in patients with morbus Kostmann [84], a disease characterized by neutropenia. Although the netropenia can be treated by GM-CSF (granulocyte-macrophage colony stimulating factor) administration, the patients still suffer from recurrent periodontitis [85]. Interestingly, it was discovered by Putsep *et al*. that PMNs obtained from these patients are deficient in LL-37, which may explain their increased susceptibility to oral infections [84]. A physiological role of LL-37 is also revealed by overexpression of this peptide in various tissues. This was found in a study where an adenovirus vector was used to transfer the *CAMP* gene systemically or tracheally into of mice [86]. This vector was also inserted into human bronchial xenografts, derived from patients with cystic fibrosis (CF) [87]. The overexpression of LL-37 resulted in an increased antimicrobial activity in all these studies, which add further evidence of a protective function of LL-37.

1.4 DEFENSINS

Defensins constitute another family of AMPs in mammals. They are comprised of cysteine-rich peptides, having a characteristic antiparallel β-sheet folded infrastructure. The α–and β–defensins share a similar 3D structure but differ in the pairing of the cysteines in the disulfide bonds [34, 49]. The defensins have a broad antimicrobial spectrum at low ion concentrations [88, 89].

1.4.1 α–defensins

Human neutrophil peptides (HNP) 1-4 are α–defensins in PMNs, where they are abundant. However, they have also been detected in NK-cells, B-cells, monocytes and CD4+ T-cells [90]. In PMNs, they are stored in primary (also called azurophilic) granules at high concentrations, constituting more than 5% of the total protein content of PMNs, where they participate in the nonoxidative killing of ingested microbes [91]. HNP1-3 are all very similar in structure and differ only in one amino acid, while HNP4 differs from the other HNPs in its general amino acid sequence and by having a more hydrophobic character. HNPs are cleaved from its propart inside the PMNs by endoproteolytic enzymes [92]. The antimicrobial spectra of HNPs are broad against both gram-positive and gram-negative bacteria. Despite the structural similarity, HNP3 exhibits five times lower activity against several strains than HNP1-2 [93-95]. By utilizing HNP1-analogues, it was found that only one disulfide-bond is required for the

antimicrobial effect of HNP1 [96], indicating another physiological function of the conserved three disulfide bridges. Like LL-37, HNPs possess additional immunomudulatory functions involved in host defense, such as chemotactic activity [34].

Human defensin (HD) 5 [97] and 6 [98], are predominantly expressed in paneth cells located at the base of the crypts in the small intestine. Later, it was found that human endocervix, endometrium and chorion also express the HD5 transcript [99]. The genes encoding HD5 and 6 are located at the same cytogenetic region as the HNPs [100], suggesting that novel defensin genes have evolved by successive rounds of duplication with subsequent divergence based on evolutionary selection. In contrast to HNPs, HD5 and 6 are stored and released as propeptides at high levels (100μg/cm^2 mucosal area), and are further processed extracellularly by trypsin to mature peptides [101]. In a study by Salzman and colleagues, the physiological relevance of HD5 was investigated by generation of a transgenic mouse, expressing human HD5 in its paneth cells, matching the storage and processing observed in humans [102]. Wt mice are very susceptible to *S. typhimurium*, while the transgenic mice were resistant to oral but not intraperitoneal administration of virulent *S. typhimurium.* This finding demonstrates a role of HD5 in the defense against Salmonella infection in the gut. Moreover, knockout mice deficient in matrilysin, the enzyme responsible for the processing of murine paneth cell defensins (cryptdins), was found to be more susceptible after oral Salmonella administration than the wt mice [103]. These results contribute evidence for an important role of paneth cell defensins in the first line of defense of gut epithelium. A functional role of HD5 and 6 also in humans has been demonstrated in patients with Crohn's disease, an inflammatory bowel disease. Patients with inflammation in the ileum have a lower expression of HD5 and HD6 than controls. Most likely, the inflammatory process is a secondary effect of an impaired antimicrobial barrier. Recently it was also found that one third of the patients with Crohn's disease had a mutation in the NOD2 gene. Interestingly, the decrease of the HD expression in the ileum was even more pronounced in these patients, suggesting that the NOD2 receptor is involved in the activation of α-defensin expression in the paneth cell [104].

1.4.2 β-defensins

Although only 4 human HBDs, HBD-1-4, have been characterized at the peptide level [105-108], 28 additional HBD genes have been identified in the human genome [109]. β-defensins are mainly present in different epithelial cells, where their expressions are either constitutive (i.e. human β defensin (HBD)-1) or induced (HBD-2, -3 and -4) by cytokines or bacterial components [110, 111]. HBD-2 was originally isolated from psoriatic lesions, where it is present at very high levels [80, 107]. This peptide exhibits chemotactic activity mediated via the CCR6 receptor [112] and hence promote adaptive immune responses by recruitment of CCR6-positive cells such as dendritic cells and T-cells.

Reduced bactericidal effects of salt sensitive AMPs (such as HBD-1), present in the lung mucosa, have been suggested to be associated with frequent infections in the lung of CF patients. CF is a lethal autosomal-recessive disorder and is caused by a mutation in the gene encoding the CF transmembrane conductance regulator (CFTR) [113]. This disease is characterized by a disturbancy in the transepithelial transport of salt and fluid, leading to abnormal high levels of NaCl in the airway surface fluid (ASF) [114]. These patients suffer from chronic airway infections commonly caused by *Staphylococcus aureus* and *P. aeruginosa* [113]. It was demonstrated that ASF from CF patients exhibited lower antimicrobial activity against *P. aeruginosa* than ASF from normal individuals [114]. Interestingly, the antimicrobial deficiency in ASF of patients with CF was corrected when diluting the samples to hypotonic concentrations of NaCl, indicating that NaCl is inhibiting the action of AMPs present in the ASF [114]. However, other salt-independent mechanisms leading to an increased susceptibility of infections of CF patients have been proposed and hence the role of HBDs in CF is yet to be determined [115]. The role of HBD-1 was further investigated utilizing a KO-mouse deficient in the β-defensin 1 (mBD-1) gene. The KO-mice were more susceptible to *H. influenza* lung infections than wt mice, supporting the role of HBD-1 in the defense at lung epithelial surfaces [116].

1.5 ANTIMICROBIAL PROTEINS

In addition to antimicrobial peptides, several proteins exhibit antimicrobial activities. The first such protein discovered was lysozyme in 1922 by Alexander Fleming [117]. It is a 14 kDa enzyme that breaks the bond between N-acetylglucosamine and N-acetylmuramic acid residues of the peptidoglycan of the bacterial cell wall. Lysozyme has also been reported to lyse bacteria by a non-enzymatic mechanism [118]. The antimicrobial spectrum of lysozyme is restricted to Gram-positive species. However, in the presence of certain AMPs (i.e. lactoferrin and LL-37) [119, 120], an activity against gram-negative bacteria is potentiated. Most likely, these polypeptides destroy the outer membrane of the bacteria, allowing lysozyme to have access to the cell wall. Like many other AMPs, lysozyme is mainly present in secretory granules of PMNs [121] and paneth cells [122].

Azurocidin, also called heparin binding protein (HBP) or cationic antimicrobial protein-37 (CAP-37), was isolated and characterized in 1984. The abundance of azurocidin is high in primary granules; however, a small amount is also present in secretory granules. This cysteine-rich protein is active against gram-negative bacteria and can function as an opsonin to enhance phagocytosis in monocytes [123]. Like antimicrobial peptides, azurocidin functions as a signalling molecule, activating and recruiting inflammatory cells to a site of infection [124].

The S100 proteins (21 members) are characterized by the presence of two calcium-binding sites with EF-hand motifs, defined as a helix-loop-helix structural domain [125]. Several members of the S100 protein family are bactericidal, or bacteriostatic, by chelating ions that are required for the bacterial growth [126, 127]. One example of an S100 protein is psoriasin, which is the major antimicrobial protein in human skin and is active at low concentrations against *E. coli*. It has been demonstrated that the antimicrobial activity of psoriasin is mediated by deprivation of zinc, which is required for microbial growth [127]. An additional S100 protein is calprotectin, constituting a heterodimer of calgranulin A and B. This protein is abundant in the cytosol of PMNs, however, it can also be released to the extracellular space where it exhibits high anti-microbial activity against a variety of microbes [128]. As for psorisin, the antimicrobial action of calprotectin is most likely due to deprivation of zink. In addition, calprotectin was recently found to chelate manganese [129]. The level of calprotectin in feces is elevated during intestinal infections and inflammations and is utilized as a diagnostic marker of such diseases [130].

1.6 HOST DEFENSE OF THE FETUS AND THE NEONATE

During gestation, the fetus resides in the uterus, where it is protected against infections. A sterile milieu is maintained by the physical properties of the mucous plug [131]. In addition, potent antimicrobial peptides (i.e. LL-37, lysozyme, HNP1-3) are present at high levels in amniotic fluid, *vernix caseosa* (vernix) [132], placenta and fetal membranes [133], protecting the fetus against intra-amniotic infections. Despite the antimicrobial protection of the womb, intra-amniotic infections occasionally occur and can trigger miscarriage or preterm labour [134]. At birth, the fetus undergoes a transition from the sterile environment in the womb into the outside world rich in microbes. The labour and delivery trigger several immunological processes that activate the newborn's own host defense [135]. Thereafter, the neonatal skin and gut starts to be colonized with bacteria. The first microbes to colonize the neonate are aerobic bacteria such as *E.coli* and strains of streptococci. After approximately one week, the anaerobic genera *bifidobacterium, lactobacillus* and *clostridium* are established in the gastrointestinal tract [136]. These microbes are important stimuli for the fast development of the immune system in early life. This has been demonstrated in a study where immune activation of conventional and germ free mice has been compared [137].

Due to limited antigen exposure in the uterus, the fetus has an immature adaptive immune system. Both T-cells and antigen presenting cells (APC) are naïve, producing less cytokines than in adults and requiring more costimulation to get matured into fully activated cells [138]. Also, PMNs and monocytes/macrophages have impaired chemotactic responsiveness [139]. Given the limited adaptive immune responsiveness, neonates must rely on innate immunity, where antimicrobial peptides are key players. In breast fed babies, milk components can directly confer efficient protection against pathogenic bacteria on the mucosa of the neonatal gut thus strengthening the defense system of newborn babies.

1.6.1 The skin of the fetus and the neonate

The skin of fetus/newborn is extremely sensitive and small wounds are enough to initiate an infection. A protective substance, vernix, coats the fragile skin of the fetus during the last trimester and is also present at birth. Vernix is composed of lipids from subaceous glands and corneocytes without any inter-corneocyte connection. The

specific composition reflects high water (81%), lipid (9%) and protein (10%) content [140]. The primary function of vernix is to save the fetus from skin softening caused by the amniotic fluid and to prevent waterloss. In addition, vernix can facilitate delivery by reducing friction in the birth canal. Recently, it was found that vernix also has anti-infectious properties by containing various antimicrobial peptides and proteins, such as LL-37, α-defensins (HNP1-3), lysozyme, psoriasin and ubiquitin [132, 141]. The presence of multiple antimicrobial components in vernix suggests that it offers anti-infective protection to the fetus and the neonate. AMPs, i.e. cathelicidin and HBDs, are also present in neonatal skin of mice and humans at higher levels than in the adult skin [142], revealing a robust antimicrobial shield of the skin during the first days of life. These facts are the reason why vernix should not be removed after birth.

1.6.2 The gut of the fetus and the neonate

Already at week 10-14 of gestation, meconium starts to accumulate in the fetal gut. Meconium is a sterile green-black material that is expelled within the first 24 h post partum by more than 90% of the newborn babies. It is composed of material from swallowed amniotic fluid, gland secretions and gut cell debris. Like vernix, meconium is mainly composed of water (70-80%) and the dry material consists mostly of glycoproteins (70-80%), proteins (10%) and lipids (8%) [143]. The role of meconium is poorly understood. However, its proinflammtory substances and growth hormones are thought to be important for gut maturation and cell differentiation [143].

Mallow and coworkers showed that HD5 and 6 transcripts appear in the fetal gut already at week 13 of gestation, revealing that bacterial components are not required for expression of these genes. However, the detected mRNA levels of the genes were very low. A comparison of adult versus second-trimester fetal HD5 and 6 transcripts revealed 40-250 times lower levels in the fetus [144]. Furthermore, it has been shown that the HD5 peptide in the crypt of the small intestine is present at lower levels at week 24 of gestation than at term and considerably lower than in the crypts of adults [145]. In situ hybridization demonstrated that HD5 and 6 transcripts are restricted to paneth cells and that the levels of the peptides correspond to the number of these cells. Since the fetus and the neonate have a lower number of paneth cells, it explains the relatively low level of HD transcripts in the fetal gut [144]. It has been speculated that the low expression of HD in the fetus contributes to the increased incident to necrotizing enterocolitis (NEC), a disease common among premature neonates. The etiology of NEC is still unknown but is thought to be caused by intestinal damage from

ischemia/hypoxia or infection [146]. Surprisingly, higher levels of HD transcripts were detected in NEC patients than in healthy controls, suggesting a regulatory role of HD genes by inflammatory components present in the inflamed small intestine [145].

1.6.3 Breast milk

Numerous studies demonstrate that breast-fed babies have a better protection against all kinds of infections [147] than formula-fed babies. In addition, the risk of getting inflammatory diseases, such as NEC [148] and allergies [149], is reduced in breast-fed babies. Breast milk contains high levels of immunological factors, contributing to the protection against infections in neonates. For example, breast milk contains high amounts of secretory immunoglobulin A (sIgA) and oligosaccharides, preventing the attachment of microbes to the gut mucosa [150]. These substances are present at higher levels in colostrum than in mature milk [151, 152]. In addition, several AMPs, such as HBDs [153], lactoferrin [154] and lysozyme [155], are abundant in breast milk. All these factors confer efficient defense against microbial invasion through the gut mucosa of infants. There is evidence that breast milk actively stimulates the immune system of the newborn by containing immunomodulatory components [156]. For example, lactoferrin has been shown to increase the phagocytic capacity of macrophages of *Trypanosoma cruzi* and *Listeria monocytogenesis* [157]. Furthermore, breast milk enhances TLR-4 and TLR-5 responses in several human adult and fetal cells [158]. In the same study, it was found that the activation of TLR-4 was stimulated by a proteinaceous milk component larger than 80 kDa. Most likely, these modulatory effects of milk components help the infant to respond to the presence of microbes.

1.7 BACTERIAL BIOFILM

Bacteria seldom grow as non-differentiated planktonic cells. They instead grow as multicellular communities, generating biofilm. Biofilm is characterized by production of extracellular matrix, water channels and adherence of bacteria to a surface or to each other [159]. This multicellular community of bacteria offers beneficial effects in comparison to one single cell in the protection from attacks of the host immune system and exogenous antibiotics [160]. The extracellular matrix components curli fimbriae and cellulose are two main components promoting biofilm produced by *Enterobacteriacae* [161]. Curli are nonbranching, beta-sheet rich fibers that share

several structural properties with eukaryotic amyloid fibers. Curli consist of five accessory proteins required for assembly. CsgA is the major subunit that together with CsgB constitutes the curli fiber, whereas CsgG is a lipoprotein, providing stability and transport of CsgA and B to the extracellular space. The role of CsgE and F are not yet defined. Utilizing computer modeling, CsgA is predicted to form five repeating strand-loop-strand motives. Like for many other amyloids, the amino acid sequence is rich in serine, glutamines and asparagine residues. These residues are thought to form a hydrogen network, making the structure resistant to protease degradation [162]. Curli interact with several host proteins, most likely facilitating the bacterial invasion into host cells. A good example is the binding of curli to both plasminogen and tissue type plasminogen activator (t-PA), bringing these two proteins into close contact. Hence, t-PA cleaves plasminogen to active plasmin, which degrades soft tissues and thereby allows bacteria to have access to deeper tissues [163, 164].

2 AIMS OF THIS THESIS

There have been three main aims with this thesis. The first aim was to determine levels and to identity AMPs in materials originated from fetuses and neonates. The material analyzed was:

- Vernix
- Meconium
- Neonatal feces

The second aim was to investigate how the expression/release of some AMPs is altered during the first weeks of life upon colonization and breast feeding by:

- Comparing levels of AMPs in meconium to neonatal feces
- Analyzing the effect of breast milk on LL-37 expression in colon epithelium

The last aim was to explore new functions of AMPs that may be important during an infection or inflammation. The specific aims were to:

- Study the effect of LL-37 on biofilm formation
- Identify neutrophilic proteins as enhancer of macrophage phagocytosis

3 EXPERIMENTAL PROCEDURES

3.1 EXTRACTIONS

3.1.1 Protein extraction/enrichment

The material was homogenized in 60% (v/v) aqueous acetonitrile (AcN) containing 1% trifluoroacetic acid (TFA) and extracted by shaking overnight at 4°C. The different homogenates were centrifuged and the supernatants were lyophilized and redissolved in 0.1% (v/v) TFA. The dissolved material was then loaded onto AcN activated Oasis HLB cartridges, which were equilibrated in 0.1% (v/v) TFA. The cartridges were washed with 0.1% (v/v) TFA, and in some cases, with 10% (v/v) AcN in 0.1% TFA. Bound material was eluted with 80 or 100% (v/v) AcN in 0.1% (v/v) TFA, and the eluates were lyophilized. The concentrations of protein were determined with the Bradford method [165].

3.1.2 Folch's extraction

Breast milk was mixed with chloroform and methanol in a ratio of (1:2:1). The upper, hydrophilic, phase was collected and washed once more with chloroform. Both the hydrophilic and hydrophobic phases were lyophilized and were reconstituted in water and isopropanol, respectively.

3.2 ANTIMICROBIAL ASSAYS

3.2.1 Inhibition zone assay

The antimicrobial activity was analyzed in an inhibition zone assay with four microbial strains, i.e. *Bacillus megaterium* (strain Bm11), *Escherichia coli* (strain D21), group B streptococci, a clinical isolate verified by a Phadebact Strep B test, and *Candida albicans* (strain ATCC 1443). Agarose in Luria-Bertani broth, with or without the salt medium E [166], was mixed with bacterial culture to achieve a final density of 6×10^4 bacterial cells/ml. This mixture was poured into Petri-dishes to create a 1 mm layer of agarose. Wells, 3 mm in diameter, were punched into the agarose layer and samples of 3 µl were loaded in each well. After incubation overnight at 30°C, the diameters of inhibition zones were recorded.

3.2.2 Broth microdilution method

The susceptibility of uropathogenic *E. coli* strains (wt and isogenic mutants deficient in biofilm production) to synthetic LL-37 and mCRAMP peptides was determined using a broth microdilution method. Bacteria were grown overnight at 37°C on LB agar plates with NaCl (inhibiting biofilm formation) or without NaCl (promoting biofilm formation). Bacteria were then suspended in PBS, diluted in LB broth without salt to a concentration of 10^5 CFU/ ml, and 90 µl of this solution was added to each well in a 96-well plate together with 10 µl synthetic LL-37 in a 2-fold dilution of a concentration ranging from 0.6 µM to 20 µM. The next day, bacterial viability was quantified by reduction of Alamar blue for 1 h at 37°C. The IC_{50} was determined as the concentration of peptide giving 50% reduction of the absorbance at 570 nm relative to bacteria grown without peptide.

3.3 BACTERIAL ADHESION AND BIOFILM THICKNESS

Plates (96-wells) were filled with bacterial cultures in LB without salt together with LL-37 in the same concentrations as in the susceptibility assay. As control peptides, scrambled LL-37 (sLL-37) and vasointestinal peptide (VIP) with similar structural properties as LL-37 were utilized. The plates were incubated without shaking at 37°C for 20 h, unbound bacteria were removed by washing with PBS and the biofilm was stained with crystal violet (3%). After removing excessive crystal violet with milliQ water, the dye was solubilized with ethanol (95%) and the optical density was measured at 570 nm.

3.4 HPLC

An ÄKTA purifier system was employed in all high performance liquid chromatography (HPLC) steps. Several different principles of separation were used in the specific projects. For reverse phase chromatography, C18 columns were used and fractions were eluted with linear gradients of acetonitrile with 0.1% TFA. Also, weak and strong cationic exchange chromatographies were employed with S-Sepharose or Ultropac columns. These columns were equilibrated in 0.2 M acetic acid, and fractions were eluted with linear gradients of ammonium acetate in 0.2 M acetic acid.

3.5 WESTERN BLOT AND DOT BLOT ANALYSES

Western blot or dot blot analyses were conducted to detect AMPs. In the Western blot analyses, polypeptides were first separated on SDS-PAGE (10-20% tricine gels) or NU-PAGE (4-12% Bis-tris gels). The polypeptides were thereafter transferred onto polyvinylidene difluoro (PVDF) membranes. After blocking of non-specific binding in 5% fat free milk and 0.25% Tween in PBS, the membranes were incubated with primary antibodies over night, i.e. monoclonal anti-LL-37 (0.6 µg/ml), polyclonal anti-LL-37 (2.5 µg/ml), monoclonal anti-HNP (0.2 µg/ml) or polyclonal anti-azurocidin (2 µg/ml). The secondary antibodies were anti-mouse IgG for monoclonal antibodies and anti-rabbit IgG for polyclonal antibodies. Immunoreactive protein bands were visualized with ECL-plus after exposure on ECL Hyperfilm.

3.6 REVERSE TRANSCRIPTASE PCR AND REAL TIME PCR

To measure LL-37/hCAP-18 transcript in cells stimulated with breast milk components, total RNA from each sample was isolated using RNeasy Mini Kit. Utilizing reversed transcriptase (M-Moloney Murine Leukemia Virus) PCR, cDNA was produced. Absolute quantification of LL-37/hCAP-18 transcript was conducted with a 7300 Real-Time PCR system using a fluorescent probe and specific primers for the *CAMP* gene. After normalizing to total RNA quantity, results were presented as relative expression of LL-37/hCAP-18 in treated cells compared to untreated cells.

3.7 SURFACE PLASMON RESONANCE

Biacore analyses were conducted to study the interactions of LL-37 to both monomeric and polymeric CsgA protein. The surface of the CM5 sensor chip was normalized in 70% glycerol and the lanes were activated by injection of 0.05 M *N*-hydroxysuccinimide (NHS)/0.2 M *N*-ethyl-*N*′-[3-dimethylamino) propyl]carbodiimide (EDC). Both monomeric and polymeric CsgA were then immobilized on the surface by amine coupling. After immobilization, remaining active carboxylic groups on the lanes were deactivated with 60 µL ethanolamine (1 M, pH 8.5). One lane, activated and deactivated without protein immobilization, was used as negative control. Standard biacore hepes-buffered saline (HBS-EP) was utilized as running buffer, and 0.1 µM of either LL-37, sLL-37 or VIP were injected at a flowrate of 20 µl/min for 3 min. The surface was regenerated after each cycle with 100 mM HCl.

3.8 CIRCULAR DICHROISM SPECTROSCOPY

The structure of polymerized CsgA in the presence or absence of LL-37 was determined with a Jasco J-810 spectropolarimeter. The samples were assayed from 190 to 250 nm in a quartz cell with a 1-mm path length. The spectra for buffer and LL-37 in buffer were subtracted from CsgA and for CsgA together with LL-37, respectively.

3.9 MALDI-MS

The samples were mixed with matrix (saturated α-cyano-4-hydroxy-cinnamic-acid in AcN containing 0.1% TFA) on a target plate and left to dry. The mass values of the peptides were analyzed with matrix-assisted laser desorption/ionization mass spectrometry (MALDI-MS) using an Applied Biosystems Voyager DE-PRO instrument.

3.10 DETECTION OF CURLI FIBERS WITH THIOFLAVIN T

3.10.1 Tecan plate reader

Monomeric CsgA, with or without LL-37 or control peptides, was mixed with 10 μM of the fiber-specific fluorescent probe thioflavin T (ThT). Fluorescence was assayed with a Tecan infinite M200 reader and excitation/emission wavelength was 430/490 nm. Measurements were conducted every 10 min at 37°C.

3.10.2 Confocal microscopy

Monomeric CsgA was mixed with equimolar LL-37 or the control peptides, sLL-37 and VIP, and 20 μM ThT, with subsequent incubation over night at 37°C. Fibers formed were washed twice and suspended in a minimum volume (10 μL) of 10 mM Kpi, pH 7.2 before mounting in ProLong Gold. Images were acquired on a Leica TCS SP5 confocal microscope and fluorescence intensity was quantified using ImageJ software.

4 RESULTS AND DISCUSSIONS

4.1 ANTIMICROBIAL PEPTIDES IN THE IMMEDIATE DEFENSE OF NEONATES (PAPERS I-III)

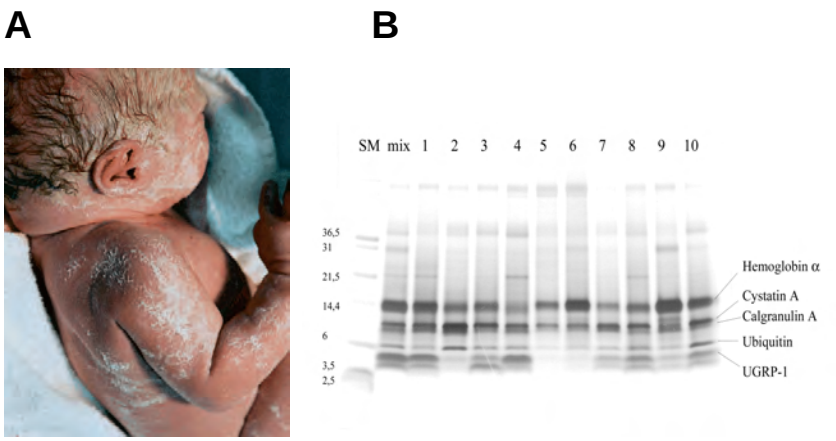
4.1.1 *Vernix caseosa* as a multi-component defense system based on polypeptides, lipids and their interactions (paper I)

The presence of LL-37 in vernix was discovered by our group in 2002 [141]. Later, several defensins and psorisain was found at high levels [132], suggesting vernix to be important in the protection against invading microbes and in regulation of the colonization of the normal flora of the neonatal skin. In this study, we aimed to further identify proteins in vernix implicated in host defense, by utilization of a proteomic approach. Proteins were extracted from 88 vernix samples derived from neonates after uncomplicated vaginal deliveries. All these extracts exhibited potent antimicrobial activity against *B. megaterium* (strain Bm11) and *C. albicans* with mean inhibition zones of 8.4 and 7.3 mm, respectively. The mean inhibition zones for *E. coli* (strain D21) and GBS were 5.6 mm and 8.4 mm, respectively, with a high variation between the samples. For characterization of proteins with immunological functions, proteins of eight vernix extracts were separated utilizing electrophoresis (Fig. 4, right). The most abundant proteins, identified by N-terminal sequence analysis, were haemoglobin α, cystatin A, calgranulin A, ubiquitin and uteroglobin related protein (UGRP-1). For further identifications, proteins in vernix extracts were separated with reverse phase chromatography and the fractions obtained were screened for anti-*E. coli* or -GBS activity. After this step, proteins in the active fractions were either directly identified by sequence analysis and MALDI-MS or further separated on SDS-PAGE. After excision of protein bands from this gel, followed by trypsin digestion and MALDI-MS, proteins were identified as psorisain, lysozyme, and calgranulin A, B and C. In total, 17 proteins were identified and almost all of these are related to host defense, suggesting that a substantial part of vernix proteins contributes to the anti-infective properties. Several of the proteins identified in this study belong to the S100 family, i.e. psorisain and calgranulin A, B and C. Calgranulin A and B form a heterodimer constituting calprotectin, which is a zink binding protein, depriving microbes of this important ion leading to microbial death. This calprotectin is mostly antifungal against *C. albicans* [126]. Also psoriasin, expressed in skin, exhibits antimicrobial activity by chelating

zink ions. However, its antimicrobial spectrum differs from calprotectin by exhibiting efficient killing activity towards *E.coli* [127]. One of the highly abundant proteins in vernix was cystatin A. This is a reversible inhibitor of cysteine proteinases, which are present in PMNs [167] and keratinocytes [168]. This protein does not kill microbes directly but is believed to be important in preventing damage caused by microbial cysteine proteases. Uteroglobulin-related protein 1 (UTRP-1), also present at high levels in vernix, is a homodimeric secretory protein expressed in the epithelial cells of the airways [169] and is therefore most likely transported from the lung via amniotic fluid to the vernix. It has been found to bind to bacteria and yeast, functioning as an opsonin that can enhance phagocytosis of macrophages [170].

Lipids in vernix were also separated, using thin-layer chromatography (TLC), giving eight main fractions. The lipid fraction active against *B. megaterium* was the fraction containg free fatty acids, where palmitoleic (C16:1) and linoleic acid (C18:2), known to be antimicrobial [171, 172], were present.

Interestingly, we also observed that the antibacterial activity of LL-37 against *B. megaterium* was enhanced in the presence of vernix lipids, indicating a peptide-lipid interaction. The activity of LL-37 is known to be augmented by anions that induces the peptide to adopt alpha helical conformation [48]. We here speculate that lipids can also stabilize a favorable structure of LL-37, enhancing its antimicrobial activity. However, this has to be confirmed at more physiological conditions.



Photograph by Lennart Nilsson

Figure 4. Vernix contains various proteins implicated in host defense. **(A)** Vernix is a creamy material covering the newborn baby. **(B)** The most abundant proteins in vernix are hemoglobin α, cystatin A, calgranulin, ubiquitin and UGRP-1. All these proteins, except hemoglobin α, are implicated in the innate immune defense.

4.1.2 Antimicrobial components of the neonatal gut affected upon colonization (paper II)

In this study, we investigated the antimicrobial activity and AMP composition in meconium, a material accumulated during gestation and expelled as the first stool. In addition, we analyzed neonatal feces, collected approximately one week after birth. These materials were chosen since they reflect a sterile and colonized environment, respectively, of the fetal/neonatal gut. Both meconium and fecal protein extracts exhibited high activity against the bacterium *B. megaterium* and low activity against *E.coli* and GBS. All these activities were reduced in the presence of the salt solution medium E, indicating the presence of salt-sensitive AMPs, i.e. defensins [88, 89, 106]. Digestion with pepsin of the proteins in the extracts abolished most of the antibacterial activity of feces, while the activity of meconium extracts were not significantly altered. This could be due to non-proteinaceous antimicrobial components in meconium, such as lipids or bile acids. However, it could also be explained by degradation of large AMPs to smaller peptide fragments that exhibit potent antibacterial activity. With chromatographic separation and various identification methods, such as MALDI-MS, N-terminal sequence analysis, Western blot analysis, we identified several AMPs in both meconium and neonatal feces. In meconium, cathelicidin LL-37, neutrophilic defensins HNP1-3, paneth cell defensin HD5, lysozyme and two histones (2A and 4), exhibiting antimicrobial activity, were identified. Many of these AMPs were also identified in neonatal feces, such as LL-37, HNP1-2, HD5 and lysozyme. Since several of the identified AMPs are expressed by several cell types, it is difficult to establish their origin. For example, LL-37, HNPs, lysozyme and azurocidin are all expressed in PMNs [91]. Furthermore, LL-37 is expressed in colonic epithelial cells [56], while HD5 [145] and lysozyme [122] are produced and secreted by paneth cells present in the crypts of the small intestine. It is also possible that some of these AMPs are derived from ingested amniotic fluid [132] or breast milk [153], both known to contain many of these components.

In this study, we also sought to study whether levels of AMPs are increased after birth when breast feeding and colonization have started. Interestingly, the levels of LL-37 and lysozyme were higher in the fecal samples than in the corresponding meconium sample of the same baby. There might be several reasons for this increased concentration of AMPs in the neonatal gut one week after birth. For example, the expression of AMPs can be induced by bacterial products, entering the neonatal gut [173]. Furthemore, components of the bacterial cell wall can induce secretion of AMPs

from paneth cells [174] and PMNs [175]. As shown in paper III, breast milk components can also induce expression of LL-37 in colonic epithelial cells, and thus strengthen the immune system of the neonatal gut.

4.1.3 Enhanced expression of LL-37 by breast milk component(s) in human colonic epithelial cells (paper III)

As described above, LL-37 is more abundant in the lumen of the neonatal gut, one week after birth, than in the lumen of the fetal gut. Based on this finding, together with the fact that breast milk is a rich source of immunomodulatory factors [156], we hypothesized that breast milk contains components that can induce the expression of the *CAMP* gene, encoding LL-37. In this study, we found that hydrophilic, but not hydrophobic, breast milk components activate the *CAMP* gene in the colonic epithelial cell line HT-29 (Fig 5). Upon stimulation with physiological concentrations (50g/l) of hydrophilic components, an induction of the *CAMP* gene was found after 24 h, and this induction is increased with prolonged incubation time up to 48 h. Ten times lower concentration of hydrophilic components (5g/l) required 48 h to give a detectable increase of *CAMP* transcripts. Detection of LL-37 peptide released in the cell supernatants revealed an induction of LL-37 by hydrophilic milk components also at the protein level. However, we found that LL-37 is present only in its inactive proform, hCAP-18. The main enzyme responsible for the processing of hCAP-18 to LL-37 and cathelin is proteinase 3. This protease is expressed and released from PMNs, which are recruited to the gastrointestinal tract by specific bacterial factors [176]. Hence, it is possible that the *in vivo* induced hCAP-18 protein in colon epithelium is processed to its active form LL-37 by incoming PMNs.

In this project, we also compared differences in the induction of LL-37 from breast milk at different lactation periods after birth, i.e. colostrum (0-3 days), transitional milk (4-10 days) and mature milk (11 days, or later). Interestingly, the median of the LL-37 induction for colostrum was 2.7 fold whereas the induction by transitional or mature milk was 5.1- or 4.2-fold, respectively. Most likely, this increase of LL-37 induction reflects the compositional change of breast milk during the first days post partum. Also, seven brands of formulas were investigated for LL-37 induction. The median induction was 2.1-fold with a high variation between the samples. Formula-fed babies possess a higher risk of getting infectious diseases

[147]. Hence, the poor induction of LL-37 of various brands of the formula might contribute to the increased susceptibility to infections for these newborn babies.

Although not yet identified, we found that the active component(s) is a small, water soluble molecule (less than 10kD), and resistant to heat and DNase treatment. It was also found to bind to a cationic column, from which it eluted already at 60 mM ammonium acetate, revealing a weak cationic character during acidic conditions. No protein was detected in the active fraction, suggesting the active component to be non-proteinaceous.

In this study, we present a novel function of human breast milk in inducing the cathelicidin LL-37 in colonic epithelial cells. Hence, this stimulatory effect may be important for the defense mechanisms of the neonatal gut.

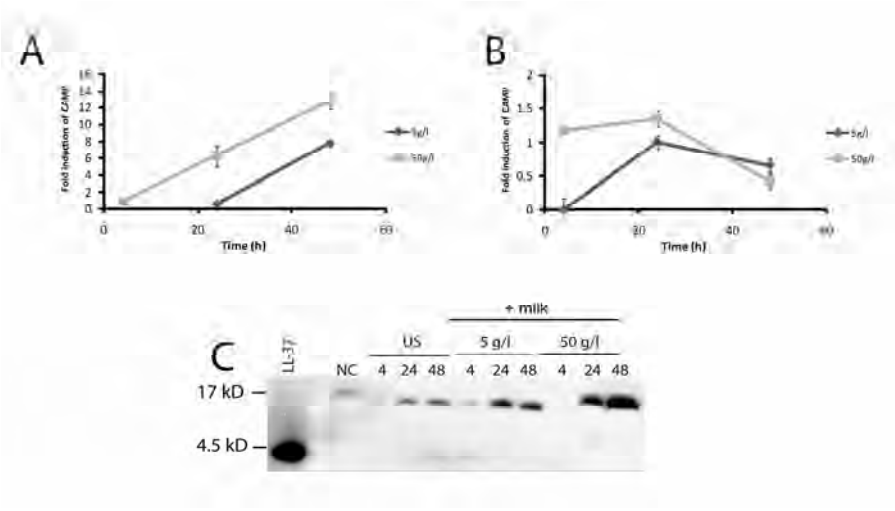


Figure 5. **Induction of LL-37 in HT-29 cells is time- and dose-dependent.** Only hydrophilic (A), but not hydrophobic components of breast milk (B) induced the *CAMP* gene. **(A)** The induction was most pronounced at 50g/l extracts of hydrophilic components after stimulation for 48 h. **(C)** A similar pattern of LL-37 induction was observed also at the protein level. Only the proform hCAP-18, corresponding to a band of ~17 kDa, was detected.

4.2 NOVEL FUNCTIONS OF AMPS IMPLICATED IN HOST DEFENSE (PAPER IV-V)

4.2.1 Uropathogenic *Escherichia coli* Modulates Immune Responses and its Curli Fimbriae Interact with the Antimicrobial Peptide LL-37 (paper IV)

Bacteria rarely grow as single cells (planktonic cells), instead they aggregate into multicellular communities, called biofilm [159]. A characteristic of biofilm is production of extracellular matrix [159], creating a higher resistance to antimicrobial agents [160]. In this study, we found that uropathogenic strains of *E.coli* produce more biofilm than commensal bacteria, indicating that biofilm formation is an important virulence factor of bacterial strains. We also studied the role of the two major biofilm promoting components of *E.coli*, curli and cellulose, during urinary tract infection. For this purpose, we used a uropathogenic *E.coli* strain from which three isogenic mutants were generated, deficient in curli and/or cellulose. We found that curli promote adherence and stimulation of proinflammtory cytokines (i.e IL-8 and MIP-2) in early stage of infection. In contrast, cellulose reduces this immune induction, likely by masking immunostimulatory structures present on the bacterial surface. Previously, it has been demonstrated that LL-37 is implicated in the defense against pathogenic bacteria in the urinary tract [177]. We here show that the curliated bacteria are more resistant to cathelicidins that may play a role for the survival of the bacteria in the urinary tract.

In this study, we demonstrated a novel function of LL-37 by preventing biofilm formation of *E.coli*. This function could play a physiological role in making *E. coli* more vulnerable to be killed by antimicrobial components. The biofilm inhibition was pronounced at 2.5 μM LL-37, a concentration that is physiologically relevant [80] and is far below the MIC value of the same *E.coli* strain [80]. To elucidate the mechanism involved in the inhibition, the effect of LL-37 on curli formation was investigated by utilization of the major subunit of curli, CsgA, which polymerizes spontaneously. Two approaches were employed for this purpose: in the first approach we detected curli fibers formed in the presence and absence of LL-37. This was conducted by using the fiber specific fluorescence probe thioflavin T (ThT), for which fluorescence intensity correlates with the amount of CsgA fibers present in the sample (Fig 6 A+B). In the second approach we instead determined remaining levels of monomeric, unpolymerized CsgA with or without LL-37 (Fig 6C). Both approaches demonstrated

that LL-37 inhibits CsgA polymerization efficiently at a molar ratio of 1:1 (LL-37: CsgA). This result could be confirmed with CD spectrometry, revealing an unordered random coil structure of CsgA, typical for monomeric CsgA [178], in the presence, but not absence of LL-37. The inhibition of CsgA is most likely mediated via a direct interaction of the LL-37 peptide and CsgA that was demonstrated with surface plasmon resonance. The binding of LL-37 to CsgA monomer may block reactive regions of the CsgA molecule that are crucial for CsgA elongation. Inhibition of biofilm formation by LL-37 has previously been described for *P. aeriginosa* by alteration of expression of genes encoding proteins involved in twitching and motility [179]. In our study, we propose a novel mechanism for biofilm inhibition by a direct interaction of LL-37 with the extracellular matrix of *E. coli*. This mechanism may also be relevant for other strains of bacteria, keeping them more sensitive to antimicrobial components at several sites of the body.

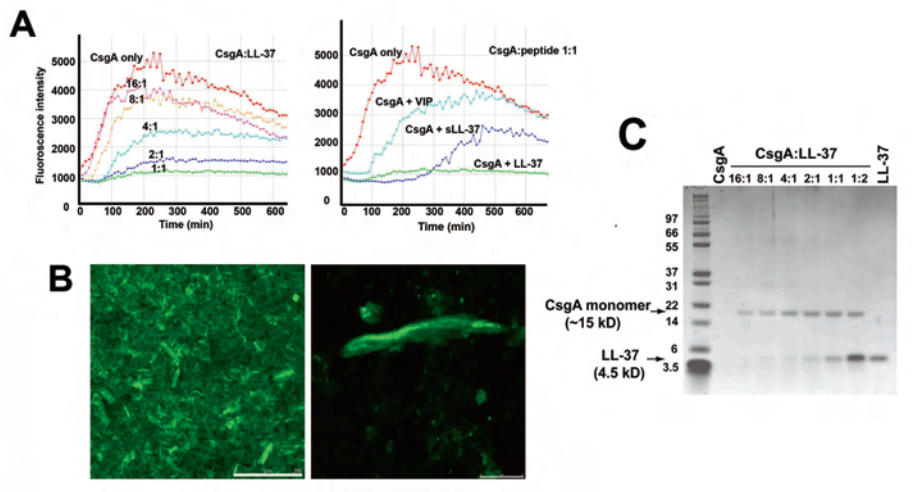


Figure 6. LL-37 inhibits CsgA polymerization. (A) Monomeric CsgA was incubated alone or together with LL-37 (left) or control peptides (right). The amount of formed CsgA polymers correlates with flurorescence intensity of ThT. The inhibition of CsgA polymerization by LL-37 is dose dependent with complete inhibition at a molar ratio of 1:1 (CsgA:LL-37). **(B)** Confocal visualization of CsgA fibers stained with ThT. **(C)** In the presence of LL-37, the CsgA is kept in its monomeric form.

4.2.2 Neutrophil primary granule proteins HBP and HNP1-3 boost bacterial phagocytosis by human and murine macrophages (paper V)

The first cells to reach a site of infection are the PMNs, preceded by a second wave of recruited monocytes/macrophages. During the migration from the circulation to the tissues, PMNs release granule proteins, affecting neighbouring cells [4]. In this study, we investigated the cross-talk between PMNs and macrophages. Our initial finding was that pretreatment of macrophages with secreted products of PMN enhanced their phagocytic capacity. This was observed for IgG-opsonized *S. aureus*, but not for complement-opsonized or non-opsonized *S. aureus.* The aim of this study was to resolve mechanisms involved in this activation.

Our results show that the enhanced phagocytosis of macrophages was due to upregulation of the high affinity Fcγ–receptors CD32 and CD64. Pepsin treatment of the secreted material of PMNs abolished the enhanced phagocytic capacity, demonstrating that the activity was mediated by proteins of PMNs. In an attempt to identify the protein(s) responsible for the enhanced phagocytosis, two approaches were employed. In the first approach, subcellular fractionation of human PMN granules was performed. After incubation of macrophages with components of the respective PMN granule subsets, it was found that proteins from the primary granules enhanced phagocytosis. Subsequently, recombinant or isolated variants of known proteins abundant in these granules were tested for activity. Interestingly, only the AMPs HNP1-3 and HBP enhanced phagocytosis, suggesting a critical role of these polypeptides in PMN–mediated bacterial phagocytosis by macrophages. To confirm these results, we sought to characterize the polypeptides in degranulated secretions of PMNs. We first separated proteins with reverse phase chromatography and screened the obtained fractions for phagocytosis enhancing activity. Fractions eluting at 25 % and 35 % acetonitrile (fractions 45-47 and 60-62, respectively) were found to be active and proteins in these fractions were identified with mass fingerprinting together with Western blot and dot blot analyses. We found that HNP1-3 and HBP were present in the active fractions 45-47 and 60-62, respectively, supporting the results obtained from the first approach. To determine the relative contribution of these proteins to the enhanced phagocytosis, HNPs and/or HBP were immunodepleted from the secreted material of PMNs. Our results showed that depletion of HBP or HNP1-3 reduced the activity with 65% and 25 %, respectively, whereas depletion of all these four proteins abolished the activity with more than 80%, attributing an exclusive role to HBP and

HNP1-3 in this process. This novel function of these polypeptides was confirmed *in vivo*, by utilization of a peritonitis model in mouse and rat. Removal of PMNs in these animals significantly reduced the ability of the murine/rat macrophages to phagocytose opsonized bacteria. Human HNP1-3 and/or HBP were then injected intraperitoneally in mouse and rat, respectively, which restored the phagocytic capacity in a pattern similar to that observed in the *in vitro* experiments. These findings suggest that the observed function of HNPs and HBP is physiologically important and seems not to be species-specific. The enhanced phagocytosis caused by HBP was blocked with monoclonal antibodies to CD18, revealing that β2-integrins constitute the receptors mediating the HBP activation.

Since HNPs and HBP activated the macrophages in a classical manner with upregulation of Fc-receptors, we sought to study whether these polypeptides stimulated the release of the classical cytokines interferon γ (IFNγ) and TNFα. After incubation of macrophages with both HNPs and HBP, we observed a clear increase of these cytokines in the cell supernatant. Treatment with neutralizing antibodies directed towards IFNγ and TNFα resulted in depletion of the enhanced phagocytosis and the induced Fc-receptor expression, demonstrating that these cytokines are central molecules in mediating the signals from HNPs and HBP.

In this study, we have demonstrated a novel function of the AMPs HNP1-3 and HBP in augmenting the phagocytic capacity of macrophages. In addition to the direct antimicrobial action, most AMPs contribute to bacterial elimination by several further mechanisms (i.e. by chemotaxis) [34, 37], hence the physiological role of these host defense molecules is under debate. Our findings support the fact that AMPs are crucial in modulating the immune system by activation of defense mechanisms of cells in the immune system.

5 CONCLUSIONS

5.1 ANTIMICROBIAL PEPTIDES IN THE IMMEDIATE DEFENSE OF NEONATES

- *Vernix caseosa* contains numerous proteins and lipids exhibiting host defense properties, protecting the fetus and the newborn baby against microbial invasion. Furthermore, lipids and proteins in vernix cooperate in a synergistic manner to enhance the antimicrobial action. (Paper I)

- Meconium and neonatal feces exhibit antimicrobial activity against both gram-positive and gram-negative bacteria. In both meconium and neonatal feces, we demonstrate the presence of AMPs, which may act as a defense barrier and as regulators of the composition of the normal flora in the neonatal gut. (Paper II)

- Higher levels of AMPs are present in neonatal feces than in the corresponding meconium, suggesting that this defense system is induced upon colonization and introduction of breast milk into the neonatal gut. A part of this induction is likely due to breast milk components that upregulate cathelicidin LL-37 in colonic epithelial cells. (Paper II and III)

5.2 NOVEL FUNCTIONS OF AMPS IMPLICATED IN HOST DEFENSE

- LL-37 inhibits biofilm formation of uropathogenic *E. coli* at sub-inhibitory concentrations, making the bacteria more vulnerable to killing by antimicrobial components. The mechanism of this inhibition may be prevention of curli fiber formation by an interaction of LL-37 and CsgA, the major subunit of curli. (paper IV)

- The neutrophil defensins HNP1-3 and the antimicrobial protein HBP contribute to bacterial clearance by enhancing the phagocytic capacity of macrophages. This antimicrobial mechanism reveals a novel physiological role of AMPs that might be crucial in the immediate host defense. (Paper V)

6 ACKNOWLEDGMENTS

This study was mainly carried out at the division of Chemistry 1, Department of Medical Biochemistry and Biophysics, Karolinska Institutet. I wish to express my sincere gratitude to many people for all kinds of help and support, in particular to:

My main supervisor Professor **Birgitta Agerberth,** thank you for accepting me as a PhD student. I feel extremely lucky to have been given the opportunity to work with you during these years. You have guided me through this journey by teaching me scientific thinking, writing and presentations. With your honesty, trust, humanity and open mind, you have been a great support for me, almost like an "extra mother". I also want to thank you for making it possible for me to go to the exciting conferences in Japan and Italy. I really enjoyed your company, we had so much fun: a lot of good Japanese beer, delicious Italian wine and great sightseeing! I can't thank you enough for everything, I will remember these five years as something very positive for the rest of my life.

Professor **Hans Jörnvall**, thank you for providing excellent working facilities, for teaching me how to write scientific English and proof reading my manuscripts/thesis, for presenting exciting scientific news at Monday morning meetings, and last but not least; for the great trip to Reykjavík!

Former and present members of the group of Birgitta Agerberth: **Andreas Cederlund**, THANK YOU! I have learned so much from you, and you have helped me solving so many problems, in particular technical and computational. It has been great fun to work with you. **Monica Lindh,** for being such an excellent "mother of the AMP-lab". I can't imagine it without you! Monica, you are such a nice person, you spread love, harmony and laughter around you, thank you! **Protim Sarker,** my present office mate, I am happy that you came to our group, you are a very kind person! **Maria Tollin,** for the great support when I was new in the group. Thank you also for laughing at my silly jokes☺, I still remember us having lots of fun in the lab! **Gudmundur "Gummi" Bergsson,** for introducing me to the field and teaching me several methods during my master thesis project, you inspired me to persue a doctoral degree! **Peter Bergman,** for sharing your great scientific knowledge, and for your outstanding sense of humor!

Professor emeritus **Jan Sjövall**, thank you for always taking your time to discuss scientific problems.

I also want to thank past and present colleagues in HEJLAB who have meant a lot to me during the time at chemistry 1: **Theres Jägerbrink,** for lots of fun when sharing office and for dragging me to the gym some days a week. **Emma Lindahl,** thanks for bringing good party spirit to chemistry 1 and for understanding my urgent need of "baby-talk" in the lunch room. **Claudia Staab,** for encouragement of playing/writing music to dissertations parties, and organizing after beer and "girl-sauna"☺. **Essam Refai**; you are an original! You always make me laugh, independent of my current mood. **Mikko Hellgren,** thanks for help regarding the dissertations process. I made it finally! **Susanne Vollmer,** for all deep talks regarding life in general. **Linus Östberg**,

for help with technical things, **Micheal Fitzen,** for bringing young spirit to the lab, **Åke Norberg,** for being a nice colleague and for valuable help in the lab.

I also want to thank all my friends who have supported me to 110%. **Rebecca Ceder**, what would the time at KI be without you!!? You have been a constant source of happiness, humor and love. **Daniel Johansson,** for all fun memories and your extreme curiosity for my research. **Roza Ghotbi**, for your great friendship and support! **Sofia Lundgren,** I'm so happy I got to know you. I have never met a person seeking the truth as much as you. You would be an excellent researcher! I also want to thank my friends **Malin Eriksson** and **Elias Arnestrand** for support during my time as a PhD student.

The HEJLAB technicians, which must be the absolutely nicest and best at the whole KI: **Monica Lindh, Margareta Brandt, Gunvor Alvelius**, **Marie Ståhlberg**, **Ella Cederlund**, **Carina Palmberg,** and the secretary **Ingegerd Nylander.** Thanks for all nice lunches. I will miss you all!

Further past and present members of HEJ-Lab: **Jan-Olov Höög, Tomas Bergman, Juan Astorga-Wells, Charlotte Nerelius, Uzma Naseeb**, **Magnus Gustafsson**, **Andreas Almlén**, **Karl Bodin**, **Erik Brandt**, **Haneen Hamada**, **Mikael Henriksson**, **Daniel Hirschberg**, **Moin Ishrat**, **Ermias Melles**, **Jawed Shafqat**, **Rannar Sillard**, **Johnny Söderlund**, **Sam Tryggvason**, **Jan Wiberg**, **Xiaoqiu Wu.**

Present members of chemistry 2 at MBB: **Min Wan,** it has been great to have you around in our lab! Past members of MTC: **Charlotte Linde** and **Jenny Sjöberg**

MBB teaching staff: **Susanne Rothstein**, **Susie Björkholm**, **Agneta Nordberg** and **Birgitta Berthold,** for nice company at the coffee-breaks, **Åke Rökaeus, Tomas Cronholm, Mats Hamberg**. Thanks to all of you for help with things related to teaching. With your planning and excellent organization, I discovered teaching to be extremely fun and educating, both at a professional level but also at a personal!

For technical support and other assistance at MBB, **Angelica Arribada**, **Svante Backlund**, **Sören Lundmark**, **Anders Lundsjö** and **Chad Tunell**

Thanks also to the players of the **MBB football team** and other members of MBB for kind gifts of chemicals, etc.

Thanks for collaborations:

At the Dept. of Microbiology, Tumor and Cell biology, KI: **Annelie Brauner, Petra Luthje, Åsa Holm, Milan Chromek, Lavinia Kádas, Ute Römling and Xiaoda Wang.** At the Dept. of Anatomy, Physiology and Biochemistry in Uppsala: **Jan Johansson** and **Kerstin Nordling.** At the Dept. of Woman and Child health, Neonatal Unit, at KS: **Giovanna Marchini, Annika Nelson,** thanks for being a nice company during the trip to Siena in Italy, and **Gordana Printz,** thanks for new exciting ideas! At the Dept. of Physiology and Pharmacology, at KI: **Oliver Söhnlein and Lennart Lindbom,** thanks for our nice collaboration, it was a success! University of Iceland: Thank you **Gudmundur Gudmundsson** for your enthusiasm and for good input to

manuscripts. Thanks also to **Gudrun Skuladottir** for introducing me to the lab at the Dept. of Physiology at the University of Island and for teaching me new methods. **Gudrun and Logi:** I will never forget how friendly you were to me when I worked in Reykjavik and stayed at your house, thank you!! Thanks also to **Hiroyuki Yoshio** at the Dept. of Neonatology, st. Marianna University in Japan for showing us around in Okayama, it was a great trip!

Mamma och pappa, för att ni alltid finns där för mig och har stöttat mig genom min doktorandtid. När självförtorendet sviktat så har ni fått mig på fötter igen genom att tro att jag klarar allt. Jag strävar efter och hoppas att jag kommer bli en lika bra förälder som ni är och har varit.

Mina älskade bröder **Niklas och Roland** som också varit ett enormt stöd. Ni är så viktiga för mig! Tack också **Anne** och **Anette** för att ni gör mina bröder lyckliga och för att ni är så trevliga svägerskor. Tack också lilla **Tove,** som givit mig så mycket livsglädje under dessa år.

Tack familjen **Widén Holmqvist: Lotta**, **Bosse**, **Marie**, **Dag**, **Kristin**, **Rickard**, **Petter** och **Sussi**, för att ni med stor kärlek har visat att jag blivit en del av er familj. Tack också **Lotta** för alla tips och råd jag fått under resans gång.

Sist men inte minst, min älskade familj **Karin** och **Love**. Vi har rott i land det här projektet, tillsammans! Inga ord kan tillräckligt beskriva min tacksamhet över att få vara en del av era liv. Jag älskar er.

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