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EXPRESSION AND REGULATION OF ANTIMICROBIAL PEPTIDES IN MUCOSAL IMMUNITY

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ABSTRACT

Antimicrobial polypeptides (AMPs) are effector molecules of the innate immune defense. AMPs are mainly expressed in epithelial cells and immune cells, providing the first line of defense to infection as direct antimicrobials. In addition, many AMPs display immunomodulatory functions in both the adaptive and innate immune system. Thus, a tight control of AMP-expression is necessary for a functional immune response.

In this thesis the antimicrobial polypeptide armament of neutrophils (PMNs) was evaluated for its activity against four human pathogens *S. aureus*, *H. influenzae*, *M. catarrhalis* and *C. albicans*. We observed a high degree of redundancy in antimicrobial activity for a majority of the AMPs. Still, some polypeptides exhibited a more specific activity against individual pathogens. This suggests that PMNs are equipped with a repertoire of antimicrobial peptides and proteins with broad activities, underscoring the importance of PMNs in the host response.

In a clinical study the expressions of cathelicidin LL-37 and α-defensins HNP1-3 were quantified in nasal fluids of patients with primary immunodeficiencies (PIDs). Healthy controls and most PID patients responded to pathogens with increased levels of AMPs in their nasal fluid. Interestingly, in patients with common variable immune deficiency (CVID) and Hyper IgE syndrome (HIES), the levels of AMPs did not increase in response to pathogens. Thus, there is a dysregulation in AMP-release in CVID and HIES patients, which may explain why these patients suffer from frequent respiratory tract infections.

Furthermore, we have detected an induced expression of AMPs by human breast milk in colonic epithelial cell lines. We isolated and characterized the inducing compound as lactose and noted that the inducing effect of the gene encoding LL-37 (*CAMP*) was dependent on intact p38 mitogen-activated protein kinase and c-Jun N-terminal kinase signaling. A strong synergistic effect on *CAMP* expression in HT-29 cells was observed in stimulations with lactose and phenylbutyrate or butyrate. This synergistic effect was further dissected by a proteomic approach. The subsequent pathway analysis of the proteomic results indicated that eleven pathways were activated. By using the novel

CAMP gene reporter system we confirmed that the pathways of thyroid hormone receptor and retinoid X receptor (TR/RXR) activation, eicosanoid signaling and steroid biosynthesis were associated with the regulation of *CAMP*.

In summary, AMPs exhibit both a large redundancy and strict specificity with regards to microbial killing. This may be relevant for certain disease conditions, where AMPs are lacking or dysregulated. Endogenous molecules, such as lactose and thyroid hormones are inducers of AMPs. In light of the wide-spread antibiotic resistance, attempts to strengthen epithelial barriers are highly warranted and the data presented here provide a concrete rationale for such studies.

LIST OF PUBLICATIONS

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

I. **Cederlund A**, Agerberth B, Bergman P. *Specificity in killing pathogens is mediated by distinct repertoires of human neutrophil peptides.* J Innate Immun, 2010, 2(6), 508-21.

II. **Cederlund A**, Olliver M, Rekha RS, Lindh M, Lindbom L, Normark S, Henriques-Normark B, Andersson J, Agerberth B, Bergman P. *Impaired Release of Antimicrobial Peptides into Nasal Fluid of Hyper-IgE and CVID Patients*. PLoS One, 2011, 6(12), e29316.

III. **Cederlund A***, Kai-Larsen Y*****, Printz G, Yoshio H, Alvelius G, Lagercrantz H, Strömberg R, Jörnvall H, Gudmundsson GH, Agerberth B. *Lactose in human breast milk: an inducer of infant innate immunity with implications for a role in intestinal homeostasis.* Manuscript under revision, PLoS One. *****contributed equally.

IV. **Cederlund A**, Nylén F, Miraglia E, Bergman P, Gudmundsson GH, Agerberth B. *Characterization of signaling pathways in the synergistic induction of LL-37 by phenylbutyrate and lactose.* Manuscript.

Related articles not included in the thesis.

1. Termén S, Tollin M, Rodriguez E, Sveinsdóttir SH, Jóhannesson B, **Cederlund A**, Sjövall J, Agerberth B, Gudmundsson GH. *PU.1 and bacterial metabolites regulate the human gene CAMP encoding antimicrobial peptide LL-37 in colon epithelial cells.* Mol Immunol. 2008, 45(15), 3947-55.
2. **Cederlund A**, Gudmundsson GH, Agerberth B. *Antimicrobial peptides important in innate immunity.* FEBS J. 2011, 278(20), 3942-51.
3. Nylén F, Miraglia E, **Cederlund A**, Strömberg R, Gudmundsson GH, Agerberth B. *Boosting innate immunity: development of a high-throughput cell-based screening assay to identify LL-37 inducers*. Manuscript

CONTENTS

LIST OF ABBREVIATIONS

AMP	Antimicrobial peptide
APC	Antigen presenting cell
ATRA	All-trans-retinoic acid
BA	Butyric acid
BLT1	Leukotriene B4 receptor 1
Btk	Bruton's tyrosine kinase
C/EBP	CCAAT enhancer binding protein
CAMP	Cathelicidin antimicrobial peptide
COX	Cyclooxygenase
CREB	cAMP-response element-binding
CVID	Common variable immunodeficiency
DAMP	Damage-associated molecular patterns
FPRL	Formyl peptide receptor-like
HAT	Histone acetyltransferases
HBD	Human beta-defensin
HBP	Heparin-binding protein
HD	Human α-defensin
HDACi	Histone deacetylase inhibitor
HIES	Hyper-IgE syndrome
HMGB1	High-mobility group protein B1
HMG-CoA	3-hydroxy-3-methylglutaryl-coenzyme A
HNP	Human neutrophil peptide (defensin)
Ig	Immunoglobulin
IL	Interleukin
LCA	Lithocholic acid
LPS	Lipopolysaccharide
LTB4	Leukotriene B4
LTF	Lactotransferrin
MAPK	Mitogen-activated protein kinase
mCRAMP	Mouse cathelicidin-related antimicrobial peptide
MEC	Minimum effective concentration
NAD	Nicotinamide adenine dinucleotide
NADP	Nicotinamide adenine dinucleotide phosphate
NET	Neutrophil extracellular trap
NF-κB	Nuclear factor κ-light-chain-enhancer of activated B-cells
NK-cell	Natural killer-cell
NLR	NOD-like receptor
PAMP	Pathogen-associated molecular pattern
PBA	Phenylbutyric acid
PBMC	Peripheral blood mononuclear cells
PGE2	Prostaglandin E2
PID	Primary immunodeficiency

PMN	Polymorphonuclear neutrophil
PRR	Pattern recognition receptor
RhoA	Ras homolog gene family member A
RTI	Respiratory tract infection
RXR	Retinoid X receptor
SGD	Specific granule deficiency
SILAC	Stable isotope labeling by amino acids in cell culture
SLE	Systemic lupus erythematosus
SRC3	Steroid receptor coactivator 3
STAT	Signal transducer and activator of transcription
T3	Triiodothyronine
T4	Tyroxine
TGF	Transforming growth factor
Th	T helper
TLR	Toll-like receptor
TR	Thyroid hormone receptor
TRAM	Thyroid hormone receptor activator molecule
VDR	Vitamin D receptor
XLA	X-linked agammaglobulinaemia

1 BACKGROUND

1.1 HOST-MICROBE INTERACTIONS

From the sterile environment of the womb we are delivered into a world teeming with microbes. From then on our bodies will be either transiently or permanently inhabited by microbes outnumbering our cells by ten to one [1]. Most of these inhabitants are bacteria, although also Archaeans and Eukaryans are colonizers [2, 3]. The resident microbes are divided into several sub-populations formed in response to different epithelial niches, and hence differing evolutionary pressures. All these niche-specific microbes are referred to as the microbiome.

The effect the microbiome exerts on us is dependent on the adaptation strategy chosen by its microbes. In a normal setting we co-exist in a mutualistic and beneficial relationship, where the microbiome can expand our genome and provide us with an evolutionary plasticity and traits that humans have not evolved. For instance, the microbiome provides us with several vitamins and essential amino acids. It metabolizes harmful xenobiotics and digests otherwise inaccessible polysaccharides into utilizable short-chain fatty acids [2, 4]. In addition, the microbiome inhibits infections by strengthening our immune response and by producing an inhospitable and energetically arid environment for invading pathogens [5]. However, in the case of pathogens, the microbes may choose a colonization strategy that is detrimental to us and may lead to disease. The net effect of how pathogenic colonization affects us is highly dependent on our immune status. An impaired immune response is a prime target for both classical and opportunistic pathogens, whereas hyperactivation of immune responses in sepsis or during severe influenza may also be detrimental to health [6]. Furthermore, microbial factors may also dictate the outcome of microbial infiltration, exemplified by the virulence factors of pathogens. These factors aid in the adaptation of the microbe to the host environment, inhibit host defenses and promote transmission between hosts. In addition, the delicate host-microbe balance can be disturbed by host genetic or environmental factors such as diet, lifestyle, or antibiotic use. This can result in an altered composition of the microbiome with implications for several diseases [3, 7]. For example, mice deficient in Toll-like receptor 5, a microbial sensor of innate immunity, show deviations in the composition of the gut microflora and symptoms of metabolic syndrome [8]. Interestingly, these symptoms are transferrable to wild type germ-free

mice by inoculating them with the altered gut flora from the Toll-like receptor 5 knockout mouse [8]. Conversely, by transferring the gut microflora from lean human donors to patients suffering from metabolic syndrome can result in the alleviation of several symptoms [9]. Also inflammatory bowel disease and diabetes are associated with a dysregulation of the microbiota and is attributed to immunological alterations or diet [10-13]. This further supports the notion that maintaining an optimal microbiota is a major contributor to health and defense against infection.

1.2 INNATE IMMUNITY

Background

In the host the regulation of the microbiota and the inhibition of pathogens access commonly functions without clinical symptoms, although there are instances when a strong defense response is required and mounted. In acute inflammation the body reacts with broad immune activation to potentially harmful stimulus from pathogens or endogenous detrimental conditions, *e.g.*, cancer or tissue damage. The non-specific, or innate, defense does not need prior recognition of the threat to function, rather it responds in a preprogrammed manner to broad classes of threats. The innate responses are fast acting (~1.5 h for the recruitment of neutrophils to a wound [14]) in comparison to the adaptive or antigen-specific response which is mounted days later. Still, the innate responses will lag behind a replicating microbe with a generation time of 20 minutes or less. To maintain the defenses during this time and support infiltrating immune cells, preformed defense mechanisms are already present at the site of infection. Typically, these defenses may clear an infection even without the requisition of the innate effector cells or the adaptive immune response.

Evolutionary aspects of the immune system

The innate immune system is an evolutionary ancient system of a defense born from the constant arms race between host and microbe for the same resources. Already unicellular organisms developed defense systems to detect and fend off invading pathogens [15]. However, with the advent of multicellular organisms and their associate microbiomes innate immunity evolved mechanisms to distinguish self from non-self. It also became important to distinguish commensal microbes from pathogens and to distinguish a healthy host cell from a damaged one [16, 17]. Because of the large

effective population of competing microbes with their propensity to evolve, one can even go as far as to say that an efficient immune system is a prerequisite for the survival of a multicellular organism. The ancient origin and the efficacy of innate immunity explain its presence in most life forms. It is the sole contributor to the immune defense of plants, fungi and invertebrates, and it acts as the first line of defense in vertebrates [17].

How does the innate immune system function?

The innate immune response acts at the host-microbial interface, where it is maintained by surface epithelia with support from resident or infiltrating phagocytic cells such as monocytes, macrophages and neutrophils. At this interface anatomical, mechanical, chemical, microbiological and immunological factors provide a barrier to infection (Figure 1).

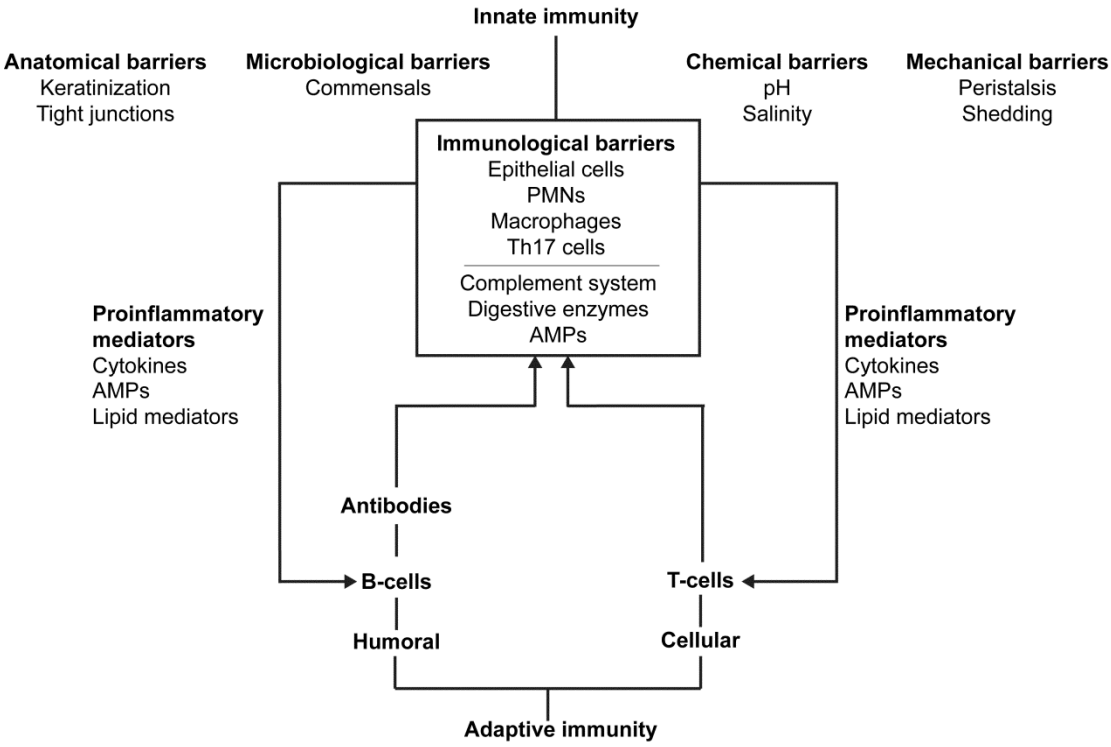

Figure 1. The human barrier defenses. *Several barrier functions cooperate in order to achieve microbial clearance. Antimicrobial polypeptides (AMPs) act directly as antimicrobials and indirectly as modulators of the immune response by recruiting and activating innate and adaptive immune cells.*

The anatomical barrier to infections is provided by the compartmentalization of sensitive tissues in efficiently sealed epithelial layers by mechanisms such as keratinization and tight junctions. The mechanical barrier is exemplified by the constant

movement of cilia, cell shedding and peristalsis that expel unwanted colonizers. The chemical barrier is provided by the secretion of fluids such as mucus, saliva, and tears that contain substances inhibiting the proliferation and spreading of microbes. The microbiological barrier is constituted by the microbes already inhabiting our bodies, providing an inhospitable environment for invading microbes. Normally microbes cannot penetrate the combined defenses of the epithelial lining, since these cells provide an additional innate immunological barrier by the secretion of AMPs.

However, tissue injury or pathogen virulence factors may allow a microbe to penetrate the pre-existing barriers of the epithelia. The microbe will at that point encounter resident macrophages, dendritic cells, neutrophils or molecules of the complement system that can neutralize the microorganism. If the pathogen evades also these obstacles it may trigger the alarm for further innate immune responses by being recognized by pattern recognition receptors (discussed below) expressed on epithelial and sentinel cells such as macrophages and dendritic cells [17].

The activation of pattern recognition receptors or signals from damaged cells will lead to a cascade of responses that ultimately result in the activation of immune genes, the secretion of antimicrobial peptides and proinflammatory cytokines. This will lead to inflammation, recruitment and activation of neutrophils followed by a rapid influx of circulating macrophages and lymphocytes, phagocytosing the pathogen. In addition, phagocytic cells can also secrete antimicrobial agents in concert with epithelial cells of the affected area for extracellular killing of the pathogens. Finally, additional adaptive immune responses will be initiated by signals from cells of the innate immune system. Although the responses of the innate immune system are germline encoded, and their reactions thus predetermined, its cells are still able to direct and tailor the adaptive immune responses to broad classes of pathogens by the secretion of specific cytokines [17].

1.2.1 Cells of innate immunity

1.2.1.1 Epithelial cells

Although not strictly immune cells, the epithelial cells at the host-microbe interface of the skin and mucosa, are the first cells an infiltrating microbe encounters. The epithelial cells must both prevent pathogen entry while functioning as a gatekeeper for molecules

transported in and out of the body. The epithelial cell contributes significantly to the immune defense and the regulation of the microbiota through the secretion of inhibiting and antimicrobial components including antimicrobial polypeptides and mucins [18-20]. In addition, epithelial cells act as sentinel cells through their expression of surface and cytoplasmic pattern recognition receptors (discussed below) [21]. The epithelial cells can also act as orchestrators of the barrier defenses. This is achieved through the interaction of epithelial cells with antigen presenting cells (APCs) or lymphocytes followed by the secretion of cytokines, recruiting cells of both the innate and adaptive immune systems [22, 23].

1.2.1.2 Granulocytes

The polymorphonuclear neutrophils (PMNs) are the most abundant leucocytes in peripheral blood, constituting 40-70 % of all leukocytes. In comparison to eosinophils and basophil granulocytes making up only a few percent of leukocytes [24]. The granulocytes diverge from a common progenitor cell and mature in a series of developmental stages in the bone marrow (granulopoiesis), during which their granules are formed. The granules contain microbicidal components, including antimicrobial polypeptides, proteases and enzymes generating reactive oxygen species [24].

The PMNs are recruited to a site of infection by chemotactic signals from epithelia and resident macrophages. They extravasate from blood to a site of infection by binding firmly to and migrating through the activated endothelium of microvessels in inflamed tissues. They are commonly the first immune cell infiltrating a site of infection [25]. During extravasation PMNs release their granules in a distinct order. Primarily, the secretory granules are released in contact with the endothelium to prepare the endothelia for the transmigration of PMNs and the tertiary/gelatinase granules are released during the transmigration through the endothelium. Finally the secondary/specific and primary/azurophil granules that contain the bulk of the microbicidal components of PMNs are exocytosed at the site of infection by degranulation [24]. The PMNs are also phagocytic cells that engulf and degrade opsonized and non-opsonized microorganisms in phagolysosomes by both oxygen-dependent and -independent mechanisms [24]. The oxygen-independent mechanisms are formed by digestive enzymes as well as antimicrobial polypeptides present in the granules. Furthermore, it was recently showed that PMNs can undergo NETosis, where the PMNs release structures called neutrophil extracellular traps, or NETs, composed of

nuclear or mitochondrial chromatin and DNA laden with microbicidal molecules that can capture and kill extracellular pathogens [26].

1.2.1.3 Macrophages

Resident macrophages are constantly present in connective tissues, the liver, the lung and the skin and are often the first immune cells an infecting microbe encounters. Additionally, there are circulating macrophages capable of migrating to a site of infection. Like other activated phagocytes the surface of macrophages is covered with pattern recognition receptors that aid in sensing microbes or cell debris [21]. Much like the PMNs, the macrophages act as phagocytes, engulfing and digesting cell debris or pathogens in their phagolysosome. They also orchestrate the innate and adaptive immune responses by antigen presentation, stimulation of lymphocytes and other immune cells through release of cytokines and lipid inflammatory mediators [17].

1.2.1.4 T-helper 17 cells

Th17 cells is a subset of T helper cells, expressing the transcription factor retinoic acid receptor (RAR)-related orphan receptor-γ (ROR-γ) and are recognized by their secretion of IL-17 and IL-22 [27]. They are distinct from both Th1 and Th2 cells and are, along with macrophages, key orchestrators of mucosal innate immunity. They recruit neutrophils and induce AMP expression in epithelia [28]. An impaired function of Th17 cells has been detected in both HIES (discussed later) [29] and in chronic mucocutaneous candidiasis [30]. The Th17 cells have recently gained much attention by their association with systemic lupus erythematosus (SLE), psoriasis, rheumatoid arthritis and other autoimmune conditions [31].

Additional cells contribute to the innate defense against infection. These include the cytotoxic natural killer- (NK-) cells, acting on virally infected or tumor cells and mast cells contributing to inflammatory responses. The antigen presenting dendritic cells are functioning as sentinel cells, sampling the environment for molecular signatures of infection by several pattern recognition receptors. Once a molecule is recognized by the receptors of the dendritic cell it migrates to the lymph nodes and present its catch to adaptive immune cells (B- and T-cells) [17].

1.2.2 Receptors of innate immunity

Pattern recognition receptors (PRRs) are involved in the sensing of microbes. PRRs include the surface and endosome/lysosomal Toll-like receptors (TLR), as well as the cytosolic NOD-like receptors (NLRs). The PRRs recognize pathogen-associated molecular patterns (PAMPs), which are structurally conserved sets of molecules that are unique and generally essential to the specific microbes [17]. The PRRs recognize a wide range of PAMPs including lipopolysaccharide (LPS), lipoteichoic acid, peptidoglycan, double-stranded RNA and unmethylated CpG DNA, structural motifs specific for different subtypes of microbes [17]. The PRRs can also detect alarm signals for aberrant tissue states in the host by the recognition of specific damage-associated molecular patterns (DAMPs) or alarmins, *e.g.*, high-mobility group protein B1 (HMGB1), self-DNA, calprotectin and heat shock proteins [32, 33].

The binding of PAMPs or DAMPs to PRRs triggers pro-inflammatory mitogen-activated protein kinase (MAPK) signaling cascades and activate nuclear factor kappa-light-chain-enhancer of activated B cells (NF-κB) or interferon regulatory factor 3 (IRF-3) [17]. This will result in increased expression of chemokines and cytokines that can attract immune cells, eliciting systemic responses such as fever [17]. Furthermore, receptor activation will lead to the expression of microbicidal molecules, *e.g.*, reactive oxygen species and AMPs [23]. The PRRs are, as expected, essential for the recognition of microbial signatures and thus the innate immune response. Mutations in PRRs result in hyporesponsiveness to a wide variety of pathogens with increased susceptibility to infections [34]. For example, mutations in NOD-2 reduce the expression of defensins and are associated with Crohn's disease [35, 36].

1.2.3 Endogenous regulators of innate immunity

Many molecules act as regulators of the innate immune response. One example is formed by cytokines, a heterogeneous group of proteins expressed and secreted by several cell types. Cytokines are part of complex signaling networks, regulating inflammatory responses both systemically and locally. Cytokines act upon specific receptors, commonly G protein-coupled, and initiate a number of responses in the target cells, *e.g.*, chemotaxis or proliferation. Cytokines can be subdivided into two types depending on what type of immune response they produce. Type 1 cytokines are associated with innate immune mechanisms, mediating inflammatory responses and are commonly produced by phagocytes, dendritic cells, endothelial or epithelial cells in

response to pathogenic stimuli. Type 2 cytokines are primarily produced by T-cells and NK-cells in response to specific antigenic stimuli and invoke mainly humoral immune responses [37]. Alarmins are yet another group of molecules capable of activating both the innate and adaptive immune systems in response to injury or infection. Examples of alarmins are HMGB1 as well as antimicrobial peptides such as defensins, cathelicidins and azurocidin [38]. Hormones can also act as immunomodulators. Several autoimmune diseases that are associated with innate immunity present at puberty and display a gender bias. This implicates sex hormones in the etiology of these diseases. Some of these diseases are lupus erythematosus, rheumatoid arthritis, Sjögren's syndrome and ankylosing spondylitis [39]. Other hormones that have been shown to modulate the immune response are insulin-like growth factor-1, prolactin, thyroid hormones and anti-inflammatory glucocorticoids [40, 41].

1.3 ANTIMICROBIAL PEPTIDES

Historical background

Already by the end of the 19th century a number of observations on antimicrobial actions of components present in tissues and secretions were presented [42, 43]. Between 1920 and 1950 several papers on the isolation and the antimicrobial activity of molecules from several different organisms were published. However, due to limitations in the isolation and characterization capabilities of the time these articles were not more than general descriptions. For example, already in 1922 Alexander Fleming described the antibacterial action of an antimicrobial polypeptide now thought to be lysozyme [43]. In 1928, the same year as the discovery of penicillin, the first lantibiotic was described as a substance produced by bacteria and capable of inhibiting the growth of other bacteria [44].

For many years investigations into the innate antimicrobial defense was in large overshadowed in favor of research on adaptive immunity and oxygen-dependent mechanisms of host defense. In organisms with an adaptive immune system the research of innate immunity was with few exceptions overlooked and was instead focused on innate defenses in invertebrates or plants [45, 46]. However, in the 1950's descriptions of proteinaceous antimicrobial compounds of rabbit leukocytes (phagocytin) showed that there was indeed antimicrobial molecules distinct from humoral immunity in animals [47]. In the early eighties seminal papers by Hans Boman on the first isolation and full characterization of invertebrate AMPs (cecropins) were

published [48, 49]. Boman's observations were followed by papers on the structure and function of several AMPs expressed also in vertebrates [50-55]. This demonstrated that AMPs were not exclusively expressed in organisms lacking an adaptive immune response, but were ubiquitous and integral molecules of the innate defense of all multicellular organisms. To date more than 2000 unique AMPs have been described and are deposited into the antimicrobial peptide database [56].

1.3.1 Structure

AMPs are a diverse group of molecules that display a great interspecies variability in structure, amino acid composition and tissue distribution. For example, mouse PMNs lack defensins [57], as opposed to human PMNs where defensins are a major constituent. This interspecies variability of structure and distribution probably reflects the differential selective pressures that microbes assert upon their host. Collectively, AMPs display a broad spectrum of antimicrobial activities against bacteria, fungi, protozoa and certain viruses [58, 59]. However, defined AMPs may display a more specific microbicidal activity to subsets of microbes [20]. This specificity is reflective of what microbes the AMP encounters in the host niche and of what microbe is to be present there [46, 60].

Although AMPs are a heterogeneous group of molecules, one can make some generalizations about their structural characteristics. AMPs are between 5-60 amino acid residues in length and commonly ~30 residues long [56]. They are often generated by proteolytic cleavage from larger precursor proteins with or without antimicrobial activity [61]. AMPs carry a high proportion of cationic amino acid residues interspersed with hydrophobic residues, adopting a cationic amphipathic secondary structure [62]. Notably, there are also reports of anionic AMPs, *e.g.*, dermcidin, that are proposed to be membrane active through interactions with receptors, by ionic bridges or by acting on internal targets [63].

The cationic AMPs can be subdivided based on their primary and secondary structures. The predominant secondary structures of AMPs are antiparallel β-sheets (frequently in peptides rich in disulfide bonds exemplified by defensins and protegrins), α-helical folded peptides (magainins and many cathelicidins) and peptides enriched in specific amino acids such as arginine, glycine, proline or phenylalanine (PR-39) [53, 61, 64].

The cationic properties of AMPs also increases their solubility in aqueous solutions, *e.g.*, blood, urine or saliva, hence increasing their bioavailability [62].

1.3.2 Expression and regulation

The majority of AMPs are expressed by cells that are exposed to microbes such as epithelial cells and immune cells. Alternatively, as in the case of granulocytes, AMPs are stored in intracellular granules ready to be secreted in response to stimuli [65]. The distribution within a specific tissue and expression levels of AMPs are highly niche-specific [66]. For example, the human α-defensins are expressed mainly in PMNs or by Paneth cells, whereas the human β-defensins are expressed mainly by epithelial cells [67]. This cell-specificity is most likely selected for by the inherent differences in the microenvironment, pH, salinity, etc. and the niche-specific composition of microbes the AMPs are acting upon [68]. Commonly AMPs are not secreted as individual peptides, but rather as a cocktail of several co-regulated or co-localized AMPs in a tissue- and context-dependent manner, acting in synergy to achieve optimal microbial killing. The expression of AMPs can be either constitutive or induced by several factors such as microbial components, pro-inflammatory stimuli, hypoxia or tissue injury [65]. Moreover, also exogenous factors, such as sodium butyrate and vitamin D, are capable of either inducing or repressing the expression and release of AMPs, some of these modulators may be suitable for future therapeutic use (discussed later and reviewed in [69]).

1.3.3 Mode of microbicidal action

The proposed model of microbicidal interaction of AMPs is through electrostatic attraction of AMPs to the electronegative components of the microbial surface. The microbial membrane is generally negatively charged as a result of exposed anionic phospholipids and molecules such as LPS, teichoic or lipoteichoic acids. This is in contrast to the eukaryotic cell membranes, carrying a more neutral charge state by the incorporation of zwitterionic phospholipids and cholesterol. After electrostatic attraction of an AMP to the membrane lipids it is integrated into the lipid bilayer, destabilizing the microbial membrane (Figure 2) [61, 62, 70]. This destroys the ionic gradient of the membrane, the osmotic potential and may halt the microbe's respiration [61]. Since AMPs are structurally diverse there are also differences in how a specific AMP is integrated into the microbial membrane. Generally, at low concentrations the AMP is oriented in parallel to the membrane with resulting membrane thinning and as

the concentration increases the AMP is inserted into the membrane orthogonally, forming transmembrane pores. Presently there are three proposed mechanisms for the membrane-dependent antimicrobial action of AMPs. Specific membrane active AMPs may use one or a combination of these mechanisms to assert their direct microbicidal activities. In the barrel-stave model (Figure 2) the AMP, *e.g.*, alamethicin, forms a multimeric helical bundle much like the staves of a barrel. Thus, the AMP cuts through the membrane by an interaction between the hydrophobic residues of the AMP and the acyl chains of the membrane phospholipids [61]. In the carpet model (Figure 2) the AMP, *e.g.*, cecropin and ovispirin, aggregates in parallel to the membrane surface, coating the lipid bilayer much like a carpet [61]. In the toroidial pore model (Figure 2) the AMP, *e.g.*, LL-37 and magainin, thins and bends the membrane into a membrane pore, resembling a torus and interacts with the anionic head groups of the membrane phospholipids [61, 71].

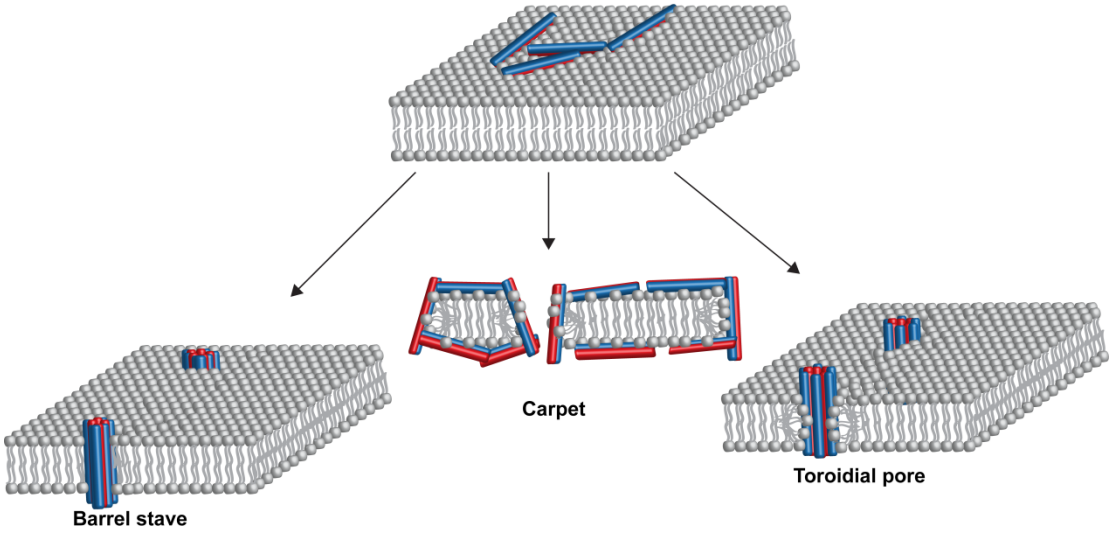

Figure 2. ***The current models of interaction of membrane active AMPs with microbial cell membranes****. An electrostatic attraction of the AMP is followed by a barrel stave, carpet or toroidial pore type of integration into the microbial membrane. Hydrophilic and hydrophobic regions of the AMP are indicated in red and blue, respectively.*

For a number of AMPs the disrupting activity lags behind its actual antimicrobial activity, indicating that these peptides have additional targets of microbial inhibition [61, 72]. Some of these intracellular targets are nucleic acids or essential enzymes of the microbial metabolism [62, 73, 74].

1.3.4 Additional activities

Throughout evolutionary history AMPs have been the main mode of defense against infections. However, with the increasing complexity of the immune defense, AMPs have adopted additional roles in the immune system. A multitude of *in vitro* results indicate that specific AMPs are directly microbicidal and *in vivo* gene deletions of specific AMPs increase the host's susceptibility to infections [75]. However, recent indications suggest that AMPs should be regarded as more broad-acting host defense peptides rather than merely direct antimicrobials [65]. In support of this view are observations that the antimicrobial activities of a number of AMPs are inhibited at physiological concentrations of salts, divalent cations and serum lipoproteins [65, 76] or are expressed at too low concentrations to be directly microbicidal. Yet, still these peptides have been proven essential *in vivo* in the inhibition of microbial infections [75]. One complementary opinion to this notion suggest that, since AMPs are generally not secreted as isolated entities, synergistic effects on the microbicidal activity will occur [77]. Moreover, removal of one AMP from this system will result in an impaired synergism and therefore an increased susceptibility to infection. However, in favor of AMPs as host defense peptides, several observations indicate that AMPs are not acting exclusively as direct antimicrobials, rather AMPs are modulators of immune responses. AMPs can modulate responses of immune cells of both adaptive and innate origin. Several AMPs have been shown to act as modulators of pro-inflammatory responses [78-82], or as modulators of cell differentiation and proliferation [83-85]. Other AMPs have been demonstrated as chemoattractants for immune cells [86-90], as inducers of cytokine expression [91-93], or as regulators of T- and dendritic cell responses [65]. Notably, AMPs have also been shown to attenuate pro-inflammatory responses in a tissue and context-dependent manner by either binding to key innate receptor ligands, thus inhibiting receptor mediated signaling, or by interacting directly with its cognate receptor [65, 94]

These additional functions implicate that AMPs are important immunomodulators for both the innate and adaptive immune responses, as mitogenic, anti-inflammatory and pro-angiogenic molecules outside of their capacity as direct microbicidals. Hence, the proposition that AMPs should be regarded as host defense peptides rather than only antimicrobials does not seem too far-fetched [65].

1.4 CATHELICIDINS

The cathelicidins are one of the major classes of AMPs in mammals. However, cathelicidins are also expressed in as distant species as humans, hagfish and snakes [54, 95, 96]. Cathelicidins are translated as antimicrobially inactive pre-pro-proteins containing an N-terminal signal peptide (Figure 3). The signal peptide is connected to a highly conserved cathelin domain, sharing structural homology with the cathepsin L inhibitor cathelin. The cathelin domain that displays only limited antimicrobial activities [97] is connected in its C-terminus to a structurally variable cationic AMP [98]. The C-terminal AMP is evolutionary divergent both in sequence and in length. Structurally, the cathelicidin peptides are either α-helical, β-sheets stabilized by disulfide bounds or peptides enriched in proline or arginine residues [99]. Commonly, the mature AMPs of cathelicidins are 23-40 residues in length, adopting amphipathic α-helical conformations in lipid bilayers [100].

The interspecies distribution of cathelicidins is great; all mammals examined have at least one copy of the cathelicidin gene. In organisms with only one copy, *e.g.*, humans, rabbits, mice and rats, the peptide are generally α-helical. In examples of mammals with several copies of cathelicidin genes, *e.g.*, cattle, sheep and pigs, the secondary structure is highly variable [100].

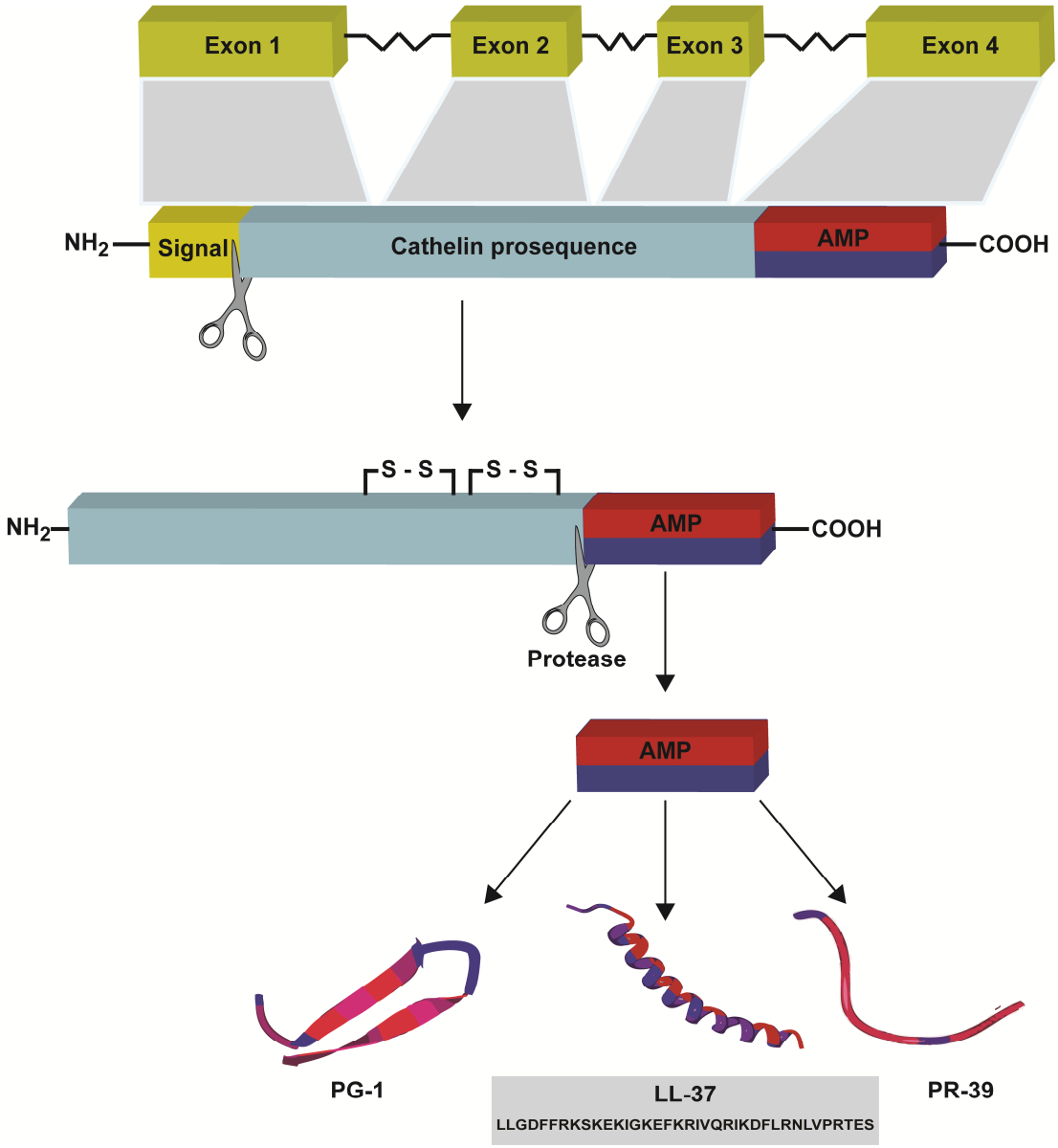

Figure 3. ***A schematic representation of the gene and protein structure of cathelicidins.*** *The mature antimicrobial peptide (AMP) may include tertiary structures such as α-helical, β-sheets or peptides enriched in proline or arginine residues. Red and blue colors indicate hydrophobic and hydrophilic amino acid residues, respectively. The primary sequence of the human cathelicidin LL-37 is shown in the gray box. The 3D-structures of LL-37 and the porcine protegrin-1 (PG-1) and PR-39 are shown to highlight the large variability cathelicidin tertiary structure.*

1.4.1 LL-37

1.4.1.1 Structure and processing

The gene *CAMP* (Cathelicidin AntiMicrobial Peptide) encodes the sole human cathelicidin LL-37. From four exons a pre-pro-protein is translated and after cleavage of the signal peptide the inactive pro-protein hCAP-18 (human cationic antimicrobial protein 18 kDa) is produced. Exons 1-3 encode the cathelin prodomain and the signal peptide, whereas exon 4 encodes the C-terminal AMP LL-37 [101]. Once expressed, hCAP-18 can be stored or exported from the cell and be subjected to proteolytic cleavage, thus liberating LL-37. hCAP-18 has been shown to be cleaved by proteinase 3 secreted from neutrophils [102], by kallikrein from keratinocytes [103] or by gastricsin present in seminal plasma [104]. In addition, other niche-specific proteases may further process LL-37 into fragments with altered activities [97, 103]. LL-37 is a 37 residue arginine- and lysine-rich cationic peptide (+6 at physiological pH) adopting an amphipathic α-helical structure at physiological pH or in lipid bilayers [71, 105]. Furthermore, the antimicrobial activity of LL-37 is increased by divalent anions, increasing the α-helicity of LL-37 [106].

1.4.1.2 Expression and regulation

LL-37 is constitutively expressed in epithelial cells, monocytes, macrophages, mast- natural killer- (NK-), B-, γδT-cells and is stored as its precursor hCAP-18 in large quantities in the specific granules of neutrophils [90, 107, 108]. The expression of LL-37 can also be modulated by both host and pathogenic factors in a tissue- and cell-specific manner. The expression of LL-37 is induced in wounds [109], hypoxia [110] inflammation [111], and by growth factors [112]. In contrast, glucocorticoids [113] and microbial virulence factors from *Neisseria* and *Shigella* [114, 115] have been shown to down-regulate the expression of LL-37.

Also extrinsic factors have been shown to induce the expression of LL-37. For example, nicotinamide (vitamin B3) [116], histone deacetylase inhibitors (HDACis) such as trichostatin, phenylbutyrate (PBA) or the colonic fermentation product butyrate (BA) can induce the expression of LL-37 in colonic and lung epithelial cells [117, 118]. HDACis increase decondensation of the nucleosomes by increasing the degree of acetylation of histones, thus facilitating transcription. The effect of PBA and BA on LL-37 expression appears to be mediated via mitogen-activated protein kinase kinase-1 and -2 (MEK1/2) and p38 mitogen-activated protein kinase (MAPK) pathways [66,

118]. The inducing effect of BA is associated with an inhibition of NF-κB signaling and a recruitment of the transcription factors AP-1, PU.1, vitamin D receptor (VDR), steroid receptor coactivator 3 (SRC3) and cAMP-response element-binding protein (CREB) to the *CAMP* promoter. [66, 117, 119-122]

Several studies have also shown that the hormonal form of vitamin D, 1,25-dihydroxyvitamin D3, is able to act as an inducer of LL-37 expression in keratinocytes and monocytes. The inducing effect of vitamin D is directly mediated by VDR and is associated with a recruitment of PU.1 to the promoter [122-124]. Also the secondary bile acid lithocholic acid, a ligand to VDR has been shown to recruit PU.1 and VDR to the *CAMP* promoter and induce the expression of LL-37 [122]. An overview of factors that are known to induce the expression of LL-37 is shown in figure 4.

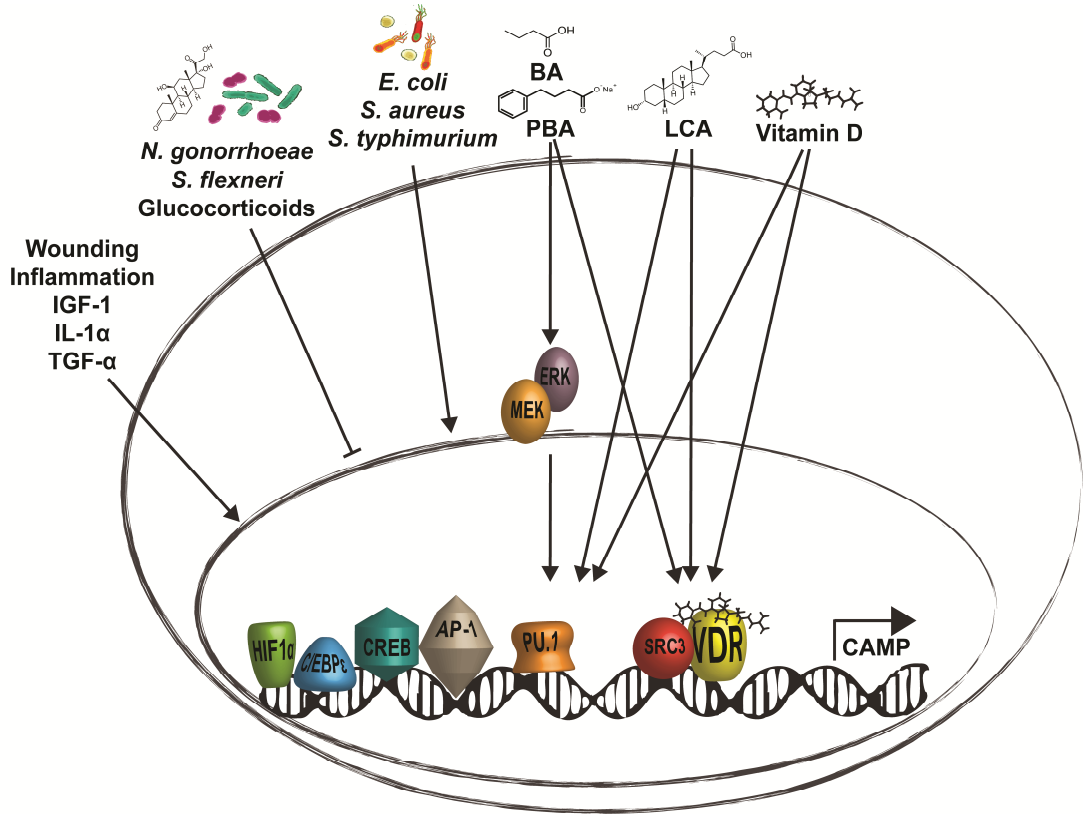

Figure 4. Schematic representation of factors known to modulate the expression of CAMP. *Activating pathways are indicated by arrows, whereas inhibiting pathways are indicated by solid lines. The order here of transcription factor binding is arbitrary. Phenylbutyric acid (PBA), butyric acid (BA) and lithocholic acid (LCA).*

Interestingly, much like in disease or inflammation there are several instances of additional AMPs being co-regulated with LL-37 in response to stimulation with extrinsic molecules. For example, vitamin D is capable of inducing the expression of

both *CAMP* and the gene encoding β-defensin HBD-2 in keratinocytes and subsets of epithelial cells [123]. Furthermore, in response to treatment with phenylbutyrate the gene encoding β-defensin HBD-1 is induced together with *CAMP* in lung epithelial cells, but downregulated in monocytes [118].

1.4.1.3 Antimicrobial activity

LL-37 is an AMP with potent antimicrobial activities against both $Gram^+$ and $Gram^-$ bacteria [58]. In addition, LL-37 has also been shown to act as an antifungal and antiviral AMP [58, 125, 126]. Most of the studies pertaining to the microbicidal activity of LL-37 have been performed *in vitro*. There are only limited data on the *in vivo* situation of the antimicrobial functions of LL-37. One example is the chronic congenital neutropenia, morbus Kostmann, signified by a lack of LL-37 in PMNs and in saliva and characterized by recurrent infections and chronic periodontal disease [127]. Thus, LL-37 is implicated in the defense against infection. In neutrophil-specific granule deficiency (SGD), mutations of the transcription factor CCAAT enhancer binding protein ε (C/EBPε) have been detected [128]. These mutations result in a lack of secondary and tertiary granule proteins in PMNs and an observed reduction in the expression of LL-37 [128]. SGD is associated with an increased susceptibility to infection of *Staphylococcus aureus*, *Pseudomonas aeruginosa* and *Candida albicans* [128, 129]. However, one confounding factor is that the genetic mutations resulting in morbus Kostmann and SGD also reduces the expression of additional AMPs present in neutrophils, *e.g.*, α-defensins HNP1-3 [127, 128], making it hard, if not impossible, to distinguish the contribution of a specific lack of LL-37 in the etiology of these diseases.

In the $Cnlp^{-/-}$ mouse, in which the gene encoding the mouse LL-37 homologue mCRAMP has been knocked out, further information of the functional role of cathelicidins can be found. The $Cnlp^{-/-}$ mouse exhibits an increased susceptibility to infection by Group A *Streptococcus* [75], *Pseudomonas aeruginosa* [130], Vaccinia virus [125] and Herpes simplex virus [131]. However, caution is as always advised when extrapolating results from animal models into functional information in man. There are also disease conditions in which the local expression of LL-37 is altered. In atopic dermatitis that is signified by a low to no induction of LL-37 in keratinocytes, in contrast to a dramatic induction of LL-37 in the skin of patients suffering from rosacea and psoriasis [132-134]. In psoriasis, Lande *et al* have shown that self-DNA in complex with LL-37 can activate plasmacytoid dendritic cells by triggering Toll-like receptor 9.

This activation results in an increased interferon-α production and can drive the inflammation associated with psoriasis [134].

1.4.1.4 Additional activities

LL-37 has been shown to be a proinflammatory orchestrator of the immune defense as a chemotactic agent for monocytes, neutrophils, and T-cells, mediated by the FPRL1 receptor [89, 90]. LL-37 is also an agonist of the P2X7 receptor, thereby recruiting immune cells through the induction of chemokine production [91]. LL-37 has been demonstrated to induce chemokine production in epithelial cells and to induce degranulation of mast cells [135, 136]. Our group has reported that the lipid mediator leukotriene B4 (LTB4) can induce the release of LL-37 from PMNs by triggering the BLT1 receptor [82]. Conversely, LL-37 can stimulate the synthesis and release of LTB4 in PMNs through binding to the FPR2/ALX receptor [81]. Furthermore, LL-37 also functions as a suppressor of immune responses by binding endotoxins such as LPS, lipoteichoic acid and lipoarabinomannan, thus dampening inflammatory responses [137-139]. Also wound healing or the resolution after infection can be augmented by LL-37 through the induction of re-epithelialization and pro-angiogenic effects [85, 140]. Recently, our group and others have shown that LL-37 can inhibit the formation of biofilm even at sub-microbicidal concentrations through several distinct mechanisms [141, 142].

In vitro LL-37 has also been shown to be cytotoxic to a number of cells, although these concentrations far exceed those of antimicrobial activity [106]. LL-37 is also capable of inducing or inhibiting apoptosis in a cell specific manner. While it is pro-apoptotic in subsets of T-cells, smooth muscle and epithelial cells [143-145] LL-37 is an anti-apoptotic mediator of neutrophils [144].

1.5 DEFENSINS

The defensins constitute an evolutionary ancient family of AMPs present in animals, plants, fungi and myxobacteria [67, 146, 147]. The defensins have been shown to have a broad and potent antimicrobial activity against Gram$^+$ and Gram$^-$ bacteria, fungi and subsets of enveloped viruses [59]. They exert their antimicrobial activity mainly through the disruption of microbial cell membranes, but have also been shown to interfere with RNA and DNA synthesis [59]. The defensins are processed from preproproteins into 18-45 residue long peptides containing six conserved cysteine residues forming three disulphide bonds, stabilizing a cationic amphipathic β-sheet conformation [59, 148]. Based on the distribution of cysteines and the pairing of cystine bonds defensins can be subdivided into α-, β- and θ-defensins, of which humans only express the α- and β-peptides [59](Figure 5). In contrast to the single cathelicidin gene, the human genome encodes more than thirty variants of α- and β-defensins.

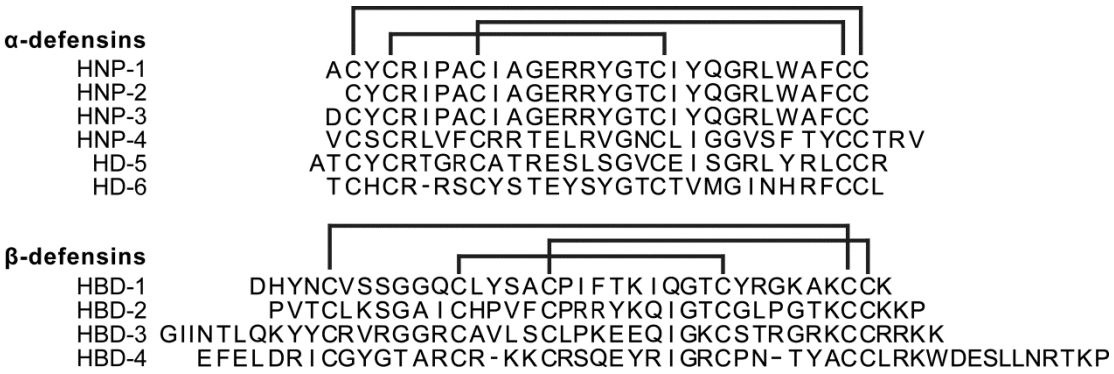

Figure 5. ***Sequences of the human α- and β-defensins****. The cystine linkages are indicated by black lines. Please note that HNP1-3 differ in only one amino acid residue and how the disulphide bonds are organized in α- and β-defensins.*

1.5.1 α-defensins

Expression and regulation

Six α-defensins are expressed in humans, of which human neutrophil peptide-1 to -3 (HNP-1 to -3) differ in only one residue and are particularly abundant in the primary (azurophilic) granules of PMNs (Figure 5). HNP-4 is not as similar as the other HNPs and is present in PMNs at a much lower concentration [149]. In addition to neutrophils the HNP-1 to -4 are also expressed in monocytes, NK-, B- and γδT-cells [90]. The microbicidal activity of α-defensins is salt-sensitive and can be inhibited by physiological concentrations of cations, *e.g.*, 2 mM divalent or 100 mM monovalent cations [65]. The neutrophilic α-defensins are generally not regulated in PMNs, instead

they are released by degranulation together with other AMPs in response to specific stimuli.

The human defensins-5 and -6 (HD-5 and -6) (figure 5) are constitutively expressed in the small intestinal crypts by Paneth cells [19, 150] and are released in response to bacterial or cholinergic stimuli, or in some diseases, *e.g.*, coeliac sprue [59, 151]. Interestingly, mice expressing the human defensin HD-5 are less susceptible to infection caused by oral administration of virulent *Salmonella typhimurium*. Furthermore, human HD-6 produces nanonets that inhibit the motility of infiltrating pathogens. These observations implicate enteric α-defensins as key effectors in the defense against enteric pathogens [152-154] and as essential in the maintenance of the gut microflora [19]. In addition to their direct microbicidal activity the α-defensins can act as mitogens for epithelial cells and as chemoattractants for monocytes, naïve T-cells and immature dendritic cells. The α-defensins are also pro-inflammatory by inhibiting glucocorticoid production and by induction of cytokine expression. [86, 155-158]

1.5.2 β-defensins

The human genome contains more than 30 β-defensin genes, however only the peptide products of four genes (HBD-1 to -4) have been identified (Figure 5) [159]. The β-defensins are expressed by keratinocytes or mucosal epithelial cells [46, 59]. The β-defensins can either be expressed constitutively (HBD-1), or be induced (HBD-2-4) by TLR ligands, cytokine stimuli, injury or in psoriasis and ulcerative colitis [59, 160, 161].

Interestingly, the reducing environment of the colon affects HBD-1 by reduction of its disulphide bonds, resulting in an increase in antimicrobial activity of the peptide against opportunistic fungi and commensal bacteria [162]. This suggests a further role of defensin disulfide bonds as regulators of microbicidal activity.

1.6 ADDITIONAL ANTIMICROBIAL POLYPEPTIDES

1.6.1 Azurocidin

Azurocidin, or heparin-binding protein (HBP), is a glycosylated 26 kDa protein present in the azurophilic granules of PMNs [25]. In addition, it is also stored in secretory vesicles of PMNs and facilitates the transmigration of PMNs and monocytes through endothelia [163, 164]. Structurally HBP is an enzymatically inactive member of the serpocidin serine protease superfamily together with cathepsin G, proteinase 3 and elastase. It is an AMP with antimicrobial activity against fungi, $Gram^+$ and $Gram^-$ bacteria [165]. In addition, HBP can function as an opsonin and a chemoattractant for monocytes and macrophages [166].

1.6.2 Lysozyme

Lysozyme was early recognized as a muramidase, an enzyme capable of hydrolyzing the glycosidic bonds of peptidoglycans in cell walls of $Gram^+$ bacteria. Lysozyme was later found to exhibit antimicrobial activities independent of its enzymatic activity [167, 168] against fungi and, some $Gram^-$ bacteria [169, 170]. Lysozyme is present in the granules of PMNs and in several secretions, *e.g.*, tears, saliva and breast milk [24, 171].

1.6.3 Calprotectin

Calprotectin is part of the large structural family of S100 calcium binding proteins. Calprotectin consists of a heterodimer of the proteins S100A8 and S100A9. It is present in high concentrations in PMNs, and is also expressed in the cytosol of monocytes, macrophages and endothelial cells [172, 173]. It has been found in high concentrations in NETs and acts as a chelator of divalent manganese and zinc ions, thereby depriving microbes these ions from their intended use as essential minerals or in the defense against reactive oxygen species [174, 175]. In addition to its microbicidal activity calprotectin has also been shown to act as an alarmin, alerting the immune response of cellular damage or infection [33]. Also other S100 proteins have been shown to display antimicrobial activities. Psoriasin (or S100-A7), a polypeptide first isolated from psoriatic scales is capable of efficiently killing *Escherichia coli*, while *Staphylococcus epidermidis*, a commensal of the skin, remains unscathed [60].

1.6.4 Lactotransferrin

Lactotransferrin (lactoferrin or LTF) is an 80 kDa iron sequestering glycoprotein present in secretions such as tears, breast milk and seminal plasma and in the secondary

granules of PMNs [176]. Primarily, it was regarded as a transporter of iron in breast milk and blood but is now also considered a powerful microbicidal polypeptide. It has been demonstrated to be antimicrobially active against bacteria, fungi and viruses. One mode of microbicidal action is by sequestering iron from its environment, thereby depriving microbes of essential iron. Secondly, it can interact with microbial cell membranes, much like an AMP, resulting in microbial lysis [177]. Furthermore, lactotransferrin can be proteolytically cleaved into several AMPs with extended antimicrobial activities [178, 179].

1.6.5 Histones

The histones H1, H2A, H2B, H3 and H4 are the main structural components of the nucleosome that are utilized in the packing of DNA in the nucleus. The posttranslational modifications of histones by methylation or acetylation result in either a decrease or increase accessibility of DNA for transcription factors, respectively. Thus, histones constitute key factors in the regulation of gene transcription. However, histones are not exclusively located in the nucleus and have since long been associated with antimicrobial activities against $Gram^+$ and $Gram^-$ bacteria as well as fungi [180]. Their importance as antimicrobials has also been highlighted as constituents of the NET structures of PMNs. Also peptide fragments of histones are functional AMPs, for example the N-terminal segment of H2A corresponds to the toad AMP buforin I [181].

1.7 ADAPTIVE IMMUNITY

The constant evolution of pathogens, a long lifespan and the sheer numbers of cells in larger multicellular organisms led to an evolutionary benefit of allocating precious resources for an additional adaptive immune response. The adaptive immune response evolved out of the innate immune system and is dependent on several innate immune molecules and cells, such as cytokines and macrophages for proper function [17, 182]. The adaptive immune system is also dependent on the innate immune system to recognize and hold the line against invading microbes until 3-5 days post-infection when the adaptive immune system is activated and operational [17]. The adaptive immune system is overall dependent on the B- and T-cells, whose activation and selective expansion is driven by antigen-presenting cells such as dendritic cells and macrophages [17].

An important difference between the innate and the adaptive immune system is the form of microbial recognition receptor each system is utilizing. Whereas the innate system relies on a limited number of germline encoded receptors with broad specificities (PRRs), the adaptive immune system generates a vast number of antigen receptors tailored for the recognition of highly specific epitopes (B- and T-cell receptors) [17, 183].

The adaptive immune receptors are, in contrast to the innate and thus somatically invariant PRRs, rapidly adapted to the fast evolving pathogens by somatic mutation. Gene segments of the adaptive immune receptors are permutated by gene splicing known as somatic recombination. This recombination yields a large and diverse cell population of immune cells with clonally distributed immune receptors [17, 182]. This pool of cells is then purged of cells carrying auto-reactive receptors by the process of clonal deletion. The surviving immune cells are released into circulation and are allowed to clonally expand in response to antigen recognition [183]. Recognition of an antigen may additionally lead to the production of memory cells that confer an immunological memory of past infections. These cells will in turn mount a quicker as well as stronger defense in the event of re-infection [183].

1.8 IMMUNODEFICIENCIES

Immunodeficiency is a disease state when the immune system's ability to fight infectious diseases is reduced or absent, resulting in an increased susceptibility to infections. Commonly the immunodeficiency is secondary, *i.e.*, acquired as a result of malnutrition, young/old age, immunosuppressant drug treatment or disease such as AIDS [184]. The rarer genetically acquired primary immunodeficiencies (PIDs) are a heterogeneous collection of disorders. The PIDs affect both the adaptive and innate immune system, leading to a dysregulation of the immune response [185]. Over 120 different genetic mutations affecting PMNs, macrophages, dendritic cells, the complement system, NK, T- and B- cells have been discovered, resulting in more than 150 different PIDs, of which antibody-related PIDs account for 65% of all PIDs [185, 186]. PIDs characterized by lack of antibodies include syndromes such as common variable immunodeficiency (CVID), selective IgA-deficiency and IgG-deficiency. These PIDs are signified by frequent respiratory tract infections (RTIs), but also autoimmune disease [187]. In order to alleviate symptoms the patients are commonly administered IgG preparations that generally reduce the incidence of RTIs [187].

CVID is the most common clinically relevant PID and this disease may be caused by a number of different mutations, resulting in low titers of IgG, IgA and/or IgM. Symptoms of CVID includes sinusitis, otitis media and pneumonia [186]. In the PID X-linked agammaglobulinaemia (XLA), a mutation in the Bruton's tyrosine kinase (Btk)-gene leads to a halt in the maturation of B-cells and therefore a lack of all immunoglobulin classes [188]. In contrast to the other PIDs mentioned, hyper-IgE syndrome (HIES) or Job's syndrome is associated with elevated titers of IgE, aberrant neutrophil chemotaxis and eosinophilia, resulting in recurrent skin and pulmonary infections by bacteria and fungi [185]. HIES has been linked to mutations of signal transducer and activator of transcription 3 (STAT3), causing defective signaling in the STAT3 pathway that is essential for functional immune signaling and the differentiation of Th-17 cells [29].

1.9 NEONATAL HOST DEFENSE

1.9.1 Neonatal immunity

From the sterile environment of the womb the neonate will after birth have to fend for itself in a world teeming with microbes. The neonate will also have to switch from a sterile source of nourishment via the umbilical cord to a non-sterile supply of nutrients from breast milk absorbed in the microbial environment of the neonatal intestine. At birth and the months following, the neonatal adaptive immune system is naïve. During the maturation of the adaptive immune system the neonates rely, with the exemption of maternal immunoglobulins, mostly on innate immune factors, *e.g.*, complement system, phagocytes, NK-cells, APCs and AMPs for the defense against infections [189, 190]. At birth the neonate is covered in a layer of *vernix caseosa*, a film composed of microbicidal lipids, antimicrobial polypeptides that protects the skin from unwarranted colonization [191]. Underneath the layer of vernix the neonatal skin is ramping up its defenses by the increased expression of AMPs such as defensins and cathelicidin [192]. Also the innate defenses of the gut are strengthened in the first weeks of life. For example, the concentration of LL-37 is higher in neonatal feces compared to fetal stools (meconium) [193]. Also Menard *et al.* observe a similar high expression of the mouse homologue of LL-37 mCRAMP in the gut epithelia of neonatal mice during the first two weeks postpartum, conferring protection against infection with *Listeria monocytogenes* [194].

1.9.2 Immunological factors of breast milk

Although the neonate is fighting its own battle against colonizing pathogenic microbes it also receives immunologic support from its mother by immune factors of breast milk. Breast milk contains molecules that can modulate the neonatal immune responses, as well as hamper microbial adherence or proliferation *e.g.*, secretory IgA, lactotransferrin, lysozyme, glycans, lipids and cytokines [190]. In addition, milk oligosaccharides have been shown to function as prebiotics for beneficial commensals such as bifidobacteria and lactobacillae [190, 195]. Moreover, breastfeeding is associated with several health benefits for the neonate. These include a reduced risk of contracting otitis media, respiratory tract infections, inflammatory bowel disease, atopic dermatitis and childhood asthma [190, 196].

2 AIMS OF THIS THESIS

The general aims of the present thesis are to study the regulation and expression of selected human antimicrobial polypeptides. An emphasis has been put on the expression and the regulation of the human cathelicidin LL-37. The aims are divided into two separate parts where part I focuses on how the AMP armament of PMNs is directed to common human pathogens (paper I) and how the innate immune system is affected in PID patients (paper II). Part II focuses on how human breast milk can contribute to the innate defenses of human colonic epithelial cells by modulating the expression of mucosal epithelial AMPs (paper III). Furthermore, part II focuses on how lactose can synergize with phenylbutyrate to induce the expression of the gene *CAMP* encoding LL-37 and what pathways that are affected during this synergy (paper IV).

Part I*: "antimicrobial polypeptides in the defense against respiratory tract infections*".
The aim of paper I was to determine the specific antimicrobial pattern of antimicrobial polypeptides present in PMNs and how these inhibit the proliferation of the common human pathogens *Moraxella catarrhalis, Staphylococcus aureus, Haemophilus influenzae* and *Candida albicans*.

The aim of paper II was to evaluate if PID patients exhibit an impaired expression of AMPs in nasal fluid. The nasal fluids of PID patients were assessed for their capacity to invoke chemotaxis in PMNs of healthy donors. In addition, the IL-17A responses of PID patients were investigated to determine if PID patients exhibit functional Th17-cell responses.

Part II "*Regulation of LL-37 by lactose*"
The aim of paper III was to search for compounds in breast milk capable of inducing the expression of the genes encoding LL-37 and the β-defensins HBD-1-3 in colon epithelial cells.

The aim of paper IV was to elucidate the signaling pathways contributing to the synergistic effect on the expression of the gene encoding LL-37 in colonic epithelial cells after stimulation with phenylbutyrate and lactose.

3 METHODOLOGY

Most of the methodology utilized in paper I-IV has for a long time been established in our laboratory for the extraction, purification and characterization of antimicrobial polypeptides. These methods, *e.g.*, Western and dot blot analyses, real-time and reverse-transcription PCR, have been described by our group in several papers [193, 197]. Detailed descriptions of the methods utilized in paper I-IV can be found in the papers as referred below.

Method	**Paper**
Isolation of PMNs	I and II
Extractions and enrichment of polypeptides	I, II and III
Extraction and purification of PMN components	I and II
Inhibition zone assay	I
Chromatography	I and III
Western and dot blot analyses	I, II, III and IV
Real-time and reverse-transcription PCR	II and IV
Mass spectrometry	I, III and IV
Luciferase reporter assay	IV
Cell culture	I, II, III and IV
Bacterial culture	I
Image analysis	II and III
Enzyme-linked immunosorbent assay, ELISA	II and III
Nuclear magnetic resonance spectroscopy	III
Pathway analysis	IV

An evaluation of two novel methodologies

One novel method used in paper IV is label-free quantitative mass spectrometry (MS) proteomics. Although the datasets generated typically are smaller than for microarrays, proteomics will, in contrast to transcript-based methodology, reveal information of the actual protein products, rather than inferring expression information from transcript abundances. Thereby proteomics removes artifacts associated with translational regulation and transcript stability. Specifically, using a label-free approach in proteomics will provide several benefits compared to the alternative and established proteomic techniques such as two-dimensional difference gel electrophoresis (2D-

DIGE), stable isotope labeling by amino acids in cell culture (SILAC) or isotope tags for relative and absolute quantification (iTRAQ). Advantages of label-free MS proteomics over the gel based 2D-DIGE is a lower sample requirement and a shorter turnaround time from sample to quantification of a given protein [198]. Label-free quantification reduces the number of experimental steps and costs associated with the label-based techniques. In addition, the dynamic range of the label-free methodology is greater than label-based methods and there are no limitations of how many samples that may be compared at any given time [198]. By pipelining the proteomic results through pathway analysis software, the information of detected protein abundances are organized into comprehensive regulatory models of how cells respond to specific stimuli in a fast and extensive manner. These regulatory models may then be tested using complementary methods with the aim to further elucidate how these regulatory models affect your gene of interest.

A second method utilized in paper IV is the *CAMP* gene reporter system. There are several benefits for utilizing a reporter system for screening a large number of compounds. The system is, compared to conventional methodology including Western blot analysis and ELISA, fast, convenient and shows a wide linearity over several logs of concentrations [199]. However, it is important to note the limitations of this method. For example, the cloned section of the promoter may not entirely reflect how the native gene is regulated. Additional regulatory elements may have been omitted and the chromatin environment of the construct may be different from that present in the parental cell-line. This may result in an altered regulation of the reporter protein compared to the native target gene. Since the introduction of a reporter construct may also alter the phenotype of the cells, it is important to confirm obtained results in the parental cell-line using orthogonal methods such as Western blot analysis or chromatin immunoprecipitation.

4 RESULTS AND DISCUSSION

4.1 AMPS IN THE DEFENSE AGAINST RESPIRATORY TRACT INFECTIONS

The respiratory tract mucosa is an interesting niche of the human body in regards to its host-microbe interactions. The upper respiratory tract is inhabited by commensal and opportunistic microbes, whereas the lower tract is kept sterile. It is a difficult task to uphold the defenses due to the sheer volume of air flowing over the mucosa and depositing potentially harmful microbes. In the respiratory tract, epithelial cells and PMNs are at the forefront in the defense against pathogenic infestation. The defense is to a great extent dependent on AMPs released by these cells. In a normal setting the epithelia and infiltrating PMNs release AMPs to the extracellular environment to incapacitate microbes by a host of different mechanisms. An aberrant expression or release of these polypeptides may result in an increased susceptibility to infection.

4.1.1 Specificity in killing pathogens is mediated by distinct repertoires of human neutrophil peptides

Protein and peptide-enriched PMNs extracts from healthy human donors were assayed for their antimicrobial activity using the inhibition zone assay against four clinical isolates of common human pathogens *Staphylococcus aureus*, *Haemophilus influenzae*, *Moraxella catarrhalis* and *Candida albicans*. Thus, $Gram^-$ (*H. influenzae* and *M. catarrhalis*), $Gram^+$ (*S. aureus*) and fungal (*C. albicans*) pathogens were assayed for their sensitivity to neutrophil peptides and proteins. The sensitivity of the pathogens to PMN extracts were ordered as followed: *M. catarrhalis* > *C. albicans* > *S. aureus* >*H. influenzae*. Although *H. influenzae* is a pathogen that is difficult to culture due to its fastidious nature requiring NAD^+ and hemin (X and V-factor), it was the microbe most resistant to microbicidal insults from the antimicrobial polypeptides of PMN extracts.

Interestingly, Lysenko *et al.* show that when *H. influenzae* is co-inoculated with *Streptococcus pneumoniae* into the nasal cavity of mice it leads to an influx of PMNs, resulting in clearance only of *S. pneumoniae* from the nasopharynx [200]. This suggests *H. influenzae* may use an environment rich in PMNs as a survival strategy in the competition with other microorganisms and that it therefore is adapted to the microbicidal effects of PMNs.

Despite the similarity of *H. influenzae* and *M. catarrhalis* in their surface structure (both $Gram^-$) and host habitat (nasopharynx) *M. catarrhalis* is significantly more sensitive to PMN extracts than *H. influenzae* (11 vs. 7 mm in the zone assay). Despite this shortcoming, *M. catarrhalis* is still a common and successful pathogen, especially in otitis media. This may be attributed to other defensive mechanisms present in *M. catarrhalis* such as a failure to invoke a PMN response as observed by *H. influenzae* in mucosa [200]. Another explanation may be co-operation of *M. catarrhalis* with other microbes co-infiltrating or already present, as shown by Armbruster *et al.* [201].

In order to elucidate what antimicrobial polypeptides that are the main contributors to the observed microbicidal effect of PMNs, the material in the extracts was fractionated using reversed-phase chromatography. Samples from the resulting fractions were assayed against the four pathogens in the zone assay. Polypeptides present in the antimicrobially active fractions were subjected to mass spectrometric analysis and were identified by peptide mass fingerprinting. The identified antimicrobial polypeptides were HNP1–3, azurocidin, lysozyme, cathepsin G, S100A8, LL-37 and LTF. To evaluate the contribution of each polypeptide to the antimicrobial activity asserted by the fractions, antibodies specific to these antimicrobial polypeptides or non-specific control antibodies were added to the fractions. Antibody-depleted fractions were then assayed for their antimicrobial activity in the zone assay against the four pathogens. In addition to the blocking experiments synthetic or purified antimicrobial polypeptides were utilized in order to establish minimum effective concentration (MEC) values for these polypeptides against the four pathogens.

Collectively, the blocking experiments and MEC values of the specific antimicrobial polypeptides against the four pathogens indicated that LTF is a major contributor to the observed antimicrobial effect against *S. aureus.* In line with observations of Aquila et al. we showed that LTF is a powerful inhibitor of *S. aureus* proliferation and that the effect was dependent on iron-chelation [202]. Moreover, it has been observed that LTF knockout mice display an increased susceptibility to spontaneous abscess formation (most often caused by *S. aureus*) compared to wild type mice [203]. We further demonstrated that lysozyme is not antimicrobially active against *S. aureus* or any other of the bacteria assayed. Instead, lysozyme was mainly active against *C. albicans*. Lysozyme was first regarded as an antimicrobial enzyme capable of hydrolyzing the peptidoglycans of $Gram^+$ bacteria such as staphylococci. Nevertheless, many

pathogenic strains of *S. aureus* are resistant to lysozyme since the membranes of these strains contain O-acetylated peptidoglycans that cannot be degraded by lysozyme [204]. However, now the antifungal activities of lysozyme have been recognized and lysozyme is now regarded as one of the key effectors of the anti-candidal defense in immunocompromised patients [205].

The most potent and broad-acting antimicrobial polypeptides of the PMN extracts were HNP1-3 and calprotectin. These polypeptides exhibited antimicrobial activity against all pathogens analyzed. Thus, it is probable that the major antimicrobial constituents of neutrophils, *i.e.*, HNP1-3 and calprotectin, are present in such large quantities due to their potency and versatility [59, 172]. In our blocking experiments we observed that by removing azurocidin from the antimicrobial fraction a significant reduction of the antimicrobial activity was observed against *H. influenzae*, *M. catarrhalis* and *C. albicans*. This together with previous reports suggests that azurocidin, apart from acting as an alarmin, is also a potent antimicrobial polypeptide against Gram$^-$ bacteria and fungi [206]. In our assay we found that several of the antimicrobial fractions contained histones. By estimating the MEC values of histone extracts we noticed that histones were mainly active against *C. albicans* and to a lesser extent against *M. catarrhalis.* Histones exhibit antimicrobial activities, although a nuclear proteins acting as an AMPs may be regarded as of limited relevance due to their low extracellular bioavailability [180]. Nevertheless, recently it was demonstrated that neutrophils undergo NETosis and secrete extracellular structures containing nuclear histones and cytosolic calprotectin providing histones and additional antimicrobial polypeptides not normally secreted with a possibility to interact with and inhibit the growth of microbes [26].

The capacity of PMNs to kill microbes or inhibit their growth has long been recognized and novel mechanisms and effector polypeptides, contributing to the antimicrobial activity of PMNs are still being discovered. Here we show that the antimicrobial activity of PMNs against common respiratory tract pathogens is achieved by antimicrobial polypeptides. However, these polypeptides are microbicidal by several distinct mechanisms such as enzymatic, ion-chelating and membrane-active. The polypeptides that are most potent in the growth inhibition of our assayed pathogens display also a broad spectrum of antimicrobial activity, *i.e.* active against all or several classes of the pathogens tested. However, some of the antimicrobial polypeptides, *e.g.*, LTF and lysozyme appear to be more specific in their antimicrobial repertoire,

inhibiting only one or two of the pathogens assayed. Thus, the antimicrobial polypeptide armament of PMNs is both specific and redundant in its capacity to eliminate microbes.

The present antimicrobial polypeptide armament of humans is a snapshot in time of a system subjected to continuous change. The constant evolution of pathogens to adapt to the microbicidal environment provided by the antimicrobial polypeptides give rise to the evolution of defense mechanisms of the pathogens [207]. In turn, the host counteracts pathogenic defense mechanisms by a constant evolution of defense polypeptides directed at components essential for the proliferation or virulence of the microbe [208].

The main mechanistic characteristics of pathogen defense aim to counteract several of the specific modes of action of host antimicrobial polypeptides. For example, several pathogens have modified their envelope in order to reduce its negative charge, the main attractant for several AMPs [207]. Other pathogens modify their envelope in order to reduce the capacity for select host digestive enzymes to degrade components of the microbial membrane [204]. The invading microbes may also produce chelating molecules capable of harvesting essential factors such as iron (siderophores), zinc and manganese, used as nutrients or in the defense against reactive oxygen species produced by the innate defenses of the host [175, 176]. Other microbes such as *H. influenzae*, *Neisseria* and *Shigella* evades defenses of the host by either not evoking the host immune response, or by down-regulating it [114, 115, 200].

Although the development of resistance to antimicrobial polypeptides is rare, it does occur. The scarcity of AMP resistance is likely due to the non-specificity of AMPs microbicidal actions and possible multiple targets within a microbe [209]. Even though a pathogen at a given moment may have acquired resistance to one or a distinct class of antimicrobial polypeptides, there are still several more antimicrobial polypeptides that the host can rely on to achieve microbial clearance. Forcing a pathogen to modify factors essential for the survival and virulence comes with a tremendous cost on the fitness or on the virulence of the microbe. Thus, if several of these microbial factors are targeted, there is a reduced risk of microbial multi-resistance.

4.1.2 Impaired release of antimicrobial peptides into nasal fluid of hyper-IgE and CVID patients

A comparison of AMP responses, PMN chemotaxis, nasopharyngeal (NPH) culture and the capacity to express proinflammatory cytokines was made between healthy controls and PID patients. The PID patients were diagnosed with either IgA-deficiency, IgG-deficiency, common variable immunodeficiency (CVID), X-linked agammaglobulinaemia (XLA), hyper-IgE syndrome (HIES), or as patients without an immunological diagnosis but with increased numbers of respiratory tract infections (Not Defined, ND-group).

From PID patients and healthy controls nasal fluid was collected and assayed for AMP expression, IL-8 levels and the capacity to induce neutrophil chemotaxis of healthy donor PMNs. The expression of the cytokine IL-17A was monitored in culture supernatants from peripheral blood mononuclear cells (PBMCs) of PID patients and healthy controls after challenge with heat killed *C. albicans* and Staphylococcal Enterotoxin B. In addition patients and controls disclosed their health status in regard to symptoms of infection.

There were no significant differences in the clinical score between groups nor was there any correlation between the clinical score and the presence of pathogenic bacteria in NPH cultures. We detected a significant increase in the levels of LL-37 and HNP1-3 in nasal fluid of patients and healthy controls colonized by a primary pathogen compared to individuals culture-positive for commensal bacteria or a negative bacterial culture. In individuals with a bacterial culture positive for *H. influenzae*, *S. aureus* or *S. pneumoniae* a higher induction of AMPs was observed, whereas a bacterial culture positive for *Enterobacteriacae spp*. or *M. catarrhalis* were not associated with a significant increase in the level of AMPs. In addition, we could observe an increase in HNP1-3 in individuals that were culture positive for commensals in comparison to individuals with a negative bacterial culture.

As opposed to CVID patients, a significant increase in the levels of LL-37 and HNP1-3 in nasal fluid in response to colonization by pathogens was observed in the groups of controls, ND, XLA and IgA/IgG. The HIES-group showed basal expression of LL-37 but with no significant increase in response to pathogenic colonization. Moreover, no expression of HNP1-3 was observed in nasal fluids of culture-negative HIES patients

or those HIES patients colonized by pathogens. This observation may be explained by the fact that in a normal setting LL-37 is expressed both by epithelial cells and by infiltrating immune cells. However, HIES patients suffer from mutations of the CXCR1 receptor, resulting in an impaired chemotactic response of their PMNs to IL-8 [210]. The capacity to induce the expression of IL-17A in PBMCs was significantly lower in patients diagnosed with HIES and CVID compared to all other groups. In addition, nasal fluids of CVID and IgA/IgG-patients showed an impaired capacity to induce chemotaxis in healthy donor PMNs compared to controls.

Thus, subsets of PID patients, specifically CVID and HIES patients, are suffering from a immunological condition that in addition to affecting their adaptive immune system also perturbs their innate immune responses. These patients may benefit from treatments that restore their impaired innate immune function. A representation of our main observations coupled to our proposed model is presented in figure 6.

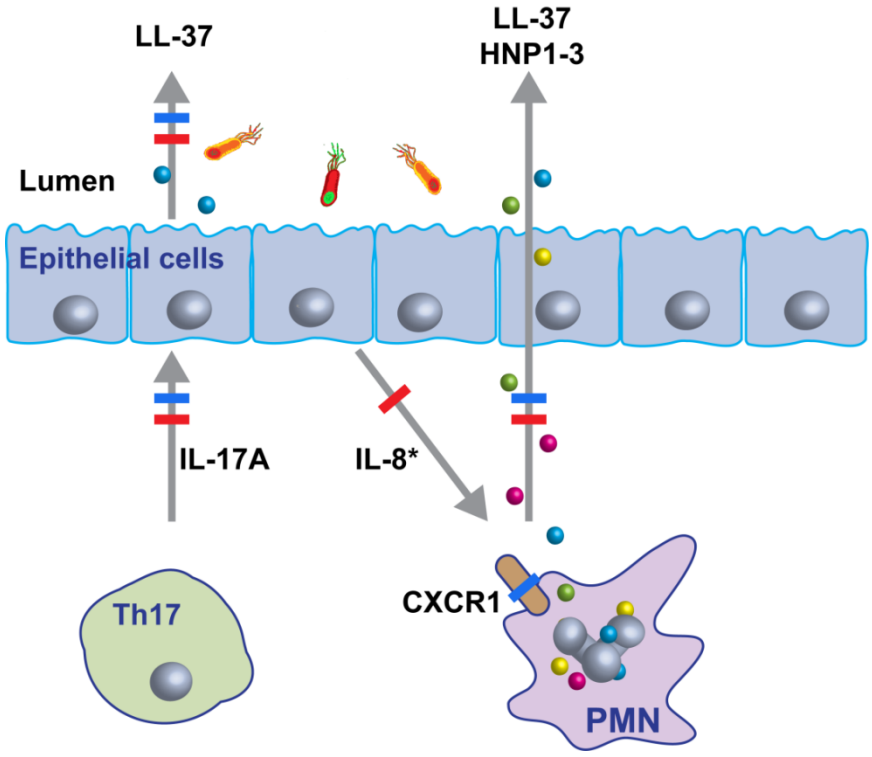

Figure 6. The proposed model explaining the observed differences in HIES and CVID patients*. HIES patients (blue line) show impaired release of IL-17A and low levels of LL-37 and HNP1–3, despite normal IL-8 levels and functional chemotaxis of nasal aspirates against healthy donor PMNs. This may be due to CXCR1 dysfunction. CVID patients (red line) show reduced release of AMPs (spheres), probably due to an impaired Th17 response or the reduced chemotaxis observed by nasal aspirates by IL-8 or alternative inducers of chemotaxis (*). The picture is adapted from paper II.*

4.2 REGULATION OF LL-37 BY LACTOSE

A reduced expression of AMPs may lead to, or is the result of, infectious disease [114, 115, 127, 131]. Excessive expression of AMPs is, on the other hand, associated with inflammatory conditions and autoimmunity [132, 134]. Enhancing AMP expression would be a valuable therapeutic strategy in infectious diseases signified by a reduced AMP expression and may result in an increased bacterial clearance [211]. Hence, discovering modulators that are able to correct the expression of AMPs may be an alternative or a complement to traditional antibiotics in anti-infective and prophylactic therapies.

4.2.1 Lactose in human breast milk: an inducer of infant innate immunity with implications for a role in intestinal homeostasis

Our group has reported on an increase in the expression of LL-37 in stools of neonates in comparison to fetal stool (meconium) [193]. This result led us to further examine the mechanism behind this observation. The increase in LL-37 expression may be a result of the maturation of the infant gut, of microbial colonization or of dietary factors. Since breast milk is rich in immunomodulatory factors we examined if breast milk also can induce the expression of LL-37.

In stimulations of several human cell lines with fractions of breast milk we could observe an increased expression of both *CAMP* transcript and LL-37 peptide. We showed that the inducing effect was only present in a hydrophilic fraction containing low-molecular weight components of breast milk. Moreover, the induction of LL-37 was detected after 24 h of stimulation and was further increased at 48 h. The hydrophilic fraction of breast milk was capable of inducing LL-37 in the colonic epithelial cell lines HT-29, Caco-2 and T-84. The expression was also induced in THP-1 monocytes and macrophages. No induction of *CAMP* was observed in the bronchial epithelial cell line VA-10. This indicates that the inducing compound in breast milk is to some extent cell-specific, much like what has been observed with other *CAMP* inducers, *e.g.*, butyrate, phenylbutyrate and vitamin D [68, 197]. This suggests that, although innate immunity is not a highly tailored response to stimuli, the regulation of innate immunity is tightly regulated select tissues in response to specific stimuli.

Hydrophilic fractions of breast milk from different lactation periods were assayed for their capacity to induce *CAMP* expression. Notably, breast milk from all postpartum

time points was able to induce the expression of *CAMP* in HT-29 cells. However, colostrum (collected 0-3 days postpartum) and transitional milk (4-11 days postpartum) were not as efficient as late milk (11 or more days postpartum) to induce *CAMP* expression. This indicates a compositional change in milk or in the efficacy of the *CAMP* inducing components over time.

The main inducing compound of *CAMP* present in breast milk was characterized as lactose by mass spectrometric analysis and nuclear magnetic resonance spectroscopy. Furthermore, the inducing effect of commercially available lactose could be corroborated in HT-29, T-84 and in THP-1 monocytes/macrophages in a time- and dose-dependent manner. Similarly to the induction of *CAMP* by breast milk, the inducing effect of lactose was most prominent in THP-1 monocytes. This suggests that lactose is the major contributor to the induction of *CAMP* by breast milk. However, the induction of *CAMP* in T84 cells was 6-fold increased in stimulations with lactose compared to breast milk, suggesting compounds in milk capable of inhibiting *CAMP* gene expression. By inhibition of signaling pathways in cells stimulated with lactose we could demonstrate that the lactose-mediated induction was dependent on an intact p38 MAPK and JNK signaling. MAPK signaling is also associated with the induction of *CAMP* in butyrate and phenylbutyrate treated cells [66, 119, 212]. However, in experiments stimulating cells with both lactose and butyrate/phenylbutyrate a synergistic effect on *CAMP* expression was observed. This suggests alternative pathways of induction for lactose and butyrate/phenylbutyrate.

In experiments using additional di- and mono-saccharides we observed an induction of *CAMP* in stimulations with the lactose moieties glucose and galactose in combination. Furthermore, we show that also maltose and trehalose were capable of inducing the expression of *CAMP*. This supports a more general effect of saccharides as inducers of *CAMP* transcription. Also β-defensin genes were induced in HT-29 and T84 cells by both breast milk and lactose. In HT-29 cells HBD-1 was induced in response to breast milk, whereas the concentration of HBD-2 and -3 transcripts were increased in stimulations lactose. The expression of HBD-2 returned to the basal level after 24h, whereas HBD-3 expression returned to the basal level already after 4h. Combined, these results suggest that there are shared regulatory elements present in both the β-defensin and cathelicidin genes. Moreover, these observations suggest that there are compounds other than lactose present in breast milk capable of modulating the

expression of β-defensins. Since the initial isolation of lactose from breast milk utilized *CAMP* expression as the readout, it is not unlikely that there are additional components in breast milk capable of modulating the expression of additional AMPs.

There is a large variability in the concentration of lactose in milk between mammals, with human breast milk containing the highest concentrations of lactose [213]. This suggests that humans have evolved a greater dependency on lactose during nursing than other mammals. Although the reasons for this increase may be several, *e.g.*, nutritional, prebiotic etc., we have provided an additional function of lactose in the defense against infection that may account for the high concentration of lactose in human breast milk. The induction of AMP expression has previously most been associated with cytokines, lipid mediators, PAMPs and DAMPs [17, 59, 214]. However, now several exogenous and endogenous factors not strictly associated with immune function have been shown to induce the expression of AMPs. The short-chain fatty acid butyrate is a bacterial metabolite produced in the colon after dietary fermentation of starches. Butyrate is regarded as a key nutrient for colonic epithelial cells and is tightly associated with the colonic tissue homeostasis [215]. Butyrate has been shown to be a key modulator of the colonic inflammatory response by inhibiting IFN-γ mediated STAT-1 activation [216]. Notably, now the important colonic nutrient butyrate has shown promise as an inducer of AMPs, suggesting that AMPs are regulated in association with the nutrient state of the colon. Lactose is also tightly associated with butyrate in that unhydrolyzed lactose is converted into butyrate by colonic fermentation [217], indicated as one causative agent in symptoms of lactose intolerance [217].

4.2.2 Characterization of signaling pathways in the synergistic induction of LL-37 by phenylbutyrate and lactose

In paper III we demonstrated that HT-29 cells stimulated with lactose and PBA in combination (PBA/lactose) for 24 h responded with a robust synergistic increase of the expression of *CAMP*. In order to elucidate what mechanisms are responsible for this observation we performed a proteomic screen of cell lysates from HT-29 cells stimulated with vehicle, lactose, PBA or PBA/lactose. From these treatments more than 1300 proteins were identified and quantified by mass spectrometric analyses. The protein identities and their associate abundances were subsequently used for pathway analysis. Several metabolic and regulatory pathways in cells treated with lactose, PBA or PBA/lactose were activated in comparison to vehicle treated cells. Out of these

pathways, eleven were further investigated for their contribution to *CAMP* expression using the *CAMP* promoter-reporter cell line MN8CampLuc (Nylén et al. Manuscript in preparation).

Three pathways showed a differential expression of the reporter protein pro-LL-37Luc when challenged with specific cognate inhibitors or inducers. These pathways were thyroid hormone receptor/retinoid X receptor (TR/RXR) activation, eicosanoid signaling and steroid biosynthesis. In MN8CampLuc cells stimulated with the TR ligands triiodothyronine (T3) and thyroxine (T4) or the RXR ligand all-trans-retinoic acid (ATRA) the expression of reporter protein was significantly increased (Figure 7). In addition, the expression of the reporter protein was additively enhanced in stimulations of the cells with a combination of lactose and thyroid hormone. Moreover, by combining PBA with triiodothyronine the expression of *CAMP* was synergistically enhanced. In line with our results it has previously been established that ATRA induces the expression of the *CAMP* promoter in a human monocytic cell line [218]. Furthermore, retinoic acid can also induce the gene encoding the porcine cathelicidin PR-39 [219].

Interestingly Schauber *et al.* may have provided a link between the inducer of LL-37 1,25-dihydroxyvitamin D3 and thyroid hormones. They demonstrated that the induction of LL-37 in keratinocytes by vitamin D is influenced by histone acetyltransferases (HATs) [120]. HATs are in turn dependent on the nuclear receptor steroid receptor coactivator 3 (SRC3), also known as thyroid hormone receptor activator molecule 1 (TRAM-1). SRC3 has been demonstrated to interact with several nuclear receptors including, as the name suggests, the thyroid hormone receptor. By interacting with nuclear receptors SRC3 potentiates the induction of transcription by the nuclear receptors in a hormone-dependent manner [120, 220]. Additionally, there are putative binding sites for TRs in the promoter of *CAMP*, warranting further experiments to elucidate if this nuclear factor is recruited to the promoter.

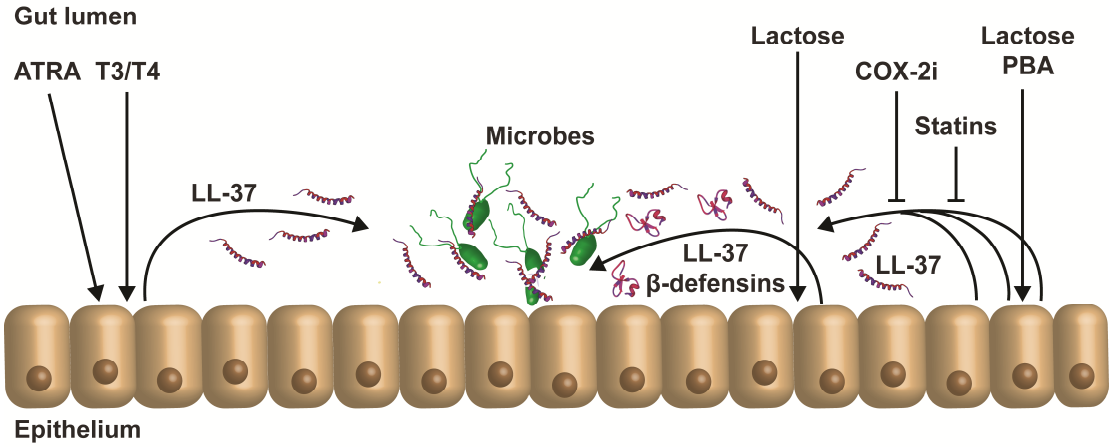

Figure 7. Schematic representation of the actions of known and novel regulators of CAMP in colonic epithelial cell lines*. Lactose induce the expression of both LL-37 and β-defensin genes. A synergistic effect is observed when combining lactose with PBA. This synergism can be inhibited by COX-2 inhibitors (COX-2i) and statins. Additionally, cells stimulated with all-trans retinoic acid or thyroid hormones T3 and T4 display induced expression of CAMP.*

LL-37 is induced by bacterial metabolites not strictly regarded as PAMPs such as lithocholic acid and butyrate or in response to nutrients (saccharides) and to metabolic hormones, *e.g.*, parathyroid hormone [221], thyroid hormones, insulin-like growth factor I and transforming growth factor-α (TGF-α) [112]. These observations suggest that LL-37 is not exclusively expressed in response to pathogenic stimuli or wounding, but also in a normal situation, to obtain homeostasis in the gut between the mucosa and the microflora. The activation of TR/RXR signaling indicates that nuclear receptors other than VDR are involved in the activation of *CAMP*.

The hypothalamic–pituitary–thyroid hormone axis in the control of inflammation has for a long time been investigated regarding the anti-inflammatory effects of the glucocorticoids [40]. However, thyroid hormone dysregulation is associated with proinflammatory states such as irritable bowel syndrome, SLE, rheumatoid arthritis and Sjögren's syndrome [40]. The duality of the anti-inflammatory effects of butyrate and vitamin D together with their capacity of inducing the expression of LL-37 can also be observed by the thyroid hormones. Commonly the effect of enhanced levels of thyroid hormones is associated with a decrease in inflammation. However, there are instances when thyroid hormones act in a proinflammatory manner by the induction of specific

cytokines [222]. In line with these results, we also observed an enhanced expression of *CAMP* with increasing levels of thyroid hormones in the colonic epithelial cell line HT-29.

Eicosanoid signaling was investigated for its involvement in the expression of *CAMP* in either lactose and/or PBA stimulations. Several inhibitors to eicosanoid biosynthesis were employed in stimulation experiments using MN8CampLuc cells. By inhibiting cytosolic phospholipase-A2 and cyclooxygenase-2 (COX-2) in cells treated with PBA/lactose, the expression of the reporter protein was significantly reduced. However, this reduction could not be rescued by exogenous prostaglandin E2 (PGE2). Moreover, no reduction of the reporter protein expression was observed in cells incubated with a 5-lipoxygenase inhibitor in combination with PBA/lactose compared to cells stimulated with PBA and lactose, separately. Thus, the induction of *CAMP* is dependent on functional eicosanoid biosynthesis in general and on a functional COX-2 pathway in particular (Figure 7). Nevertheless, we have not yet been able to identify the specific lipid mediator responsible for the induction of *CAMP*. Interestingly, Chamorro *et al.* observed that keratinocytes subjected to LL-37 stimulation responded with an increased expression of COX-2 and production of PGE2, suggesting that eicosanoid signaling may be a secondary feed-forward effect of an increased expression of LL-37 [223]. Also our group has reported on the cross-talk between eicosanoids and LL-37, suggesting a tight interaction of specific lipid mediators and LL-37 [81, 82].

Furthermore, we evaluated if steroid biosynthesis contributed to the induction of *CAMP* in the reporter system after stimulation with lactose and/or PBA. The reporter cells were additionally incubated with statins, thus inhibiting the production of 3-hydroxy-3-methyl-glutaryl-CoA, the precursor molecule of cholesterol and other steroids. All statins used in incubations were able to reduce the expression of the reporter protein that was induced by incubation with PBA/lactose. No rescue of this inhibition could be observed when adding exogenous cholesterol or mevalonate. In reporter cells treated with lactose or PBA, only PBA induction was significantly affected by addition of statins. This suggests that the effect of statins on the PBA/lactose-mediated synergism is specifically acting on the PBA-mediated signaling (Figure 7). Interestingly, Roy *et al.* has reported that PBA can lower the level of serum cholesterol, inhibiting the activation of NF-κB through the depletion of intermediates in the mevalonate pathway [224], thus acting to reduce inflammatory responses. Statins are also capable of

dampening pro-inflammatory responses of TLR2 by inhibition of the Ras homolog gene family member A (RhoA) [225]. These two observations indicate that statins act as anti-inflammatory mediators by inhibition of key regulatory pathways of innate immunity.

In conclusion, coupling proteomics to pathway analyses appears to be a valuable method in generating information on how AMPs are regulated. By corroborating the generated information using a promoter-reporter cell line the validation can be performed in a high-throughput manner. Moreover, this methodology is a rational complement to large drug screens of compound libraries without a detailed *a priori* knowledge of inherent signaling mechanisms of the target cell.

5 CONCLUSIONS

5.1 ANTIMICROBIAL POLYPEPTIDES IN THE DEFENSE AGAINST RESPIRATORY TRACT INFECTIONS

- We demonstrate that although several antimicrobial polypeptides of PMNs (*i.e.*, lysozyme, lactotransferrin and histones) were specific in their antimicrobial repertoire against the four human pathogens *M. catarrhalis*, *C. albicans*, *S. aureus*, *H. influenzae*, many revealed a broad antimicrobial spectrum (*i.e.*, LL-37, azurocidin, HNP1-3 and calprotectin). This suggests that the armament of PMNs is selected for a broad spectrum of antimicrobial activity, reflecting the broad spectrum of microbes the PMNs encounter.
- We observe an impaired release of LL-37 and HNP1-3 in the nasal fluids of patients diagnosed with the primary immunodeficiencies HIES and CVID. In addition, PBMCs of HIES and CVID patients display an aberrant IL-17 response to antigenic stimuli. Moreover, the nasal fluids of CVID patients exhibited a reduced capacity to induce chemotaxis in PMNs of healthy donors. Patients diagnosed with HIES and CVID suffer from deficiencies pertaining to the adaptive arm of the immune system. However, here we have now shown that these patients also suffer from deficiencies in the innate immune defense.

5.2 REGULATION OF LL-37 BY LACTOSE

- We demonstrate that lactose in human breast milk induces the expression of the genes encoding LL-37 and HBD1-3 in human colonic epithelial cell lines and LL-37 in a monocytic cell line. We also observe a strong synergistic effect on the induction of LL-37 with lactose and phenylbutyrate or butyrate in the colonic epithelial cell line HT-29. This indicates that endogenous molecules can synergize to achieve AMP induction and thus a strengthening of epithelial barrier function.
- We observe that thyroid hormones T3 and T4 can induce the expression of the reporter-protein in a *CAMP* gene reporter system. This induction is additively or synergistically enhanced with lactose or phenylbutyrate, respectively. We further observe that the synergistic induction of reporter-protein by lactose and phenylbutyrate is sensitive to inhibition of HMG-CoA reductase, as well as to inhibition of the COX-2 branch of eicosanoid biosynthesis. These results reveal novel regulatory circuits capable of modulating the expression of AMPs.

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