Enhancement of host defense against pathogens by antimicrobial peptides : a new approach to combat microbial drug resistance

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General introduction & outline of the thesis

Chapter 1

Introduction

The development of anti-infective therapies is an ancient research field that already fascinated Greek physicians, Egyptians and the Chinese thousands of years ago (1-4). However, observations within this field remained empirical until the early 20th century when researchers such as Ehrlich, Fleming, Domagk and later Waksman, discovered independently several useful agents for treatment of infections (5-7). The discovery of penicillin by Alexander Fleming in 1928, the first natural antibiotic, is a major hallmark as it was such a powerful albeit small-spectrum antibiotic (8,9). Between 1938 and 1941 Florey and Chain succeeded in the purification of benzylpenicillin and up scaling its production; a prerequisite of industrial production of benzylpenicillin. The success of benzylpenicillin initiated a surge in research and development in the anti-infectives field, and led to the commercial production of antibiotics after World War 2. For his contribution, Fleming was rewarded the 1945 Nobel Prize for medicine and physiology, together with Florey and Chain (10,11). However, already in the early 1950s, *Staphylococcus aureus* isolates from hospital patients were noted to become penicillin-resistant and this percentage has increased rapidly over the following years (12). Over the past 60 years, the usage of benzylpenicillin and other antibiotics, in both humans as well as in the veterinarian field, has resulted in the emergence of multi-drug resistance of a variety of microorganisms. Given their rapid generation time and their spontaneous mutation rate, they evolve and adapt, and in stressful environments select for useful genotypes among multiple mutants. Infections with these antibiotic-resistant microorganisms present a major problem for both the medical community as well as for society (13).

The identification of, for instance, pan-resistant *Acinetobacter baumannii,* carbapenem-resistant Gram-negative bacteria and multi-drug resistant *Staphylococcus aureus* and *Mycobacterium tuberculosis* underscores the need for reconsidering current usage of antibiotics. It also shows that novel antibiotics with a mode of action different from current anti-infectives are urgently needed (14-16). The development of new antimicrobial agents has mainly focused on ways to eliminate the pathogen, either by a direct microbicidal activity or by stopping the microorganisms in their growth, allowing the hosts' immune system to clear the invaders. Obviously, the development and spread of multi-drug resistant microorganisms nullifies these modes of elimination and development of new drugs that rely on this strategy has lagged behind because of a lack of new microbial targets to aim at. Thus, in the last decennium research has shifted toward exploring the possibility for an alternative way of coping with infections, a way that has proven its merit in human evolution: employment of naturally occurring human

antimicrobial peptides/proteins as possible alternative for current antibiotics (17,18). In a way this is a logical extension to the earlier anti-infective research, as it should be realized that antibiotics (like benzylpenicillin) were generally isolated from microorganisms that likely had evolved these compounds as a way of defense against other microorganisms competing for a niche or nutrients. Besides the direct antimicrobial activity of human antimicrobial peptides, attention has shifted toward the immune modulatory properties of some antimicrobial peptides (19). It is hypothesized that elimination of a pathogen by enhancement of the hosts' immune response will less likely result in resistance of pathogens against these peptides.

The human immune system
The human immune system has evolved over millions of years as a way to protect the host from invasive pathogens, tissue injury, toxic components, and harmful derailment of body cells. It is able to detect a wide variety of molecules that can be distinguished as self or non-self and as being either harmful or safe (20). If recognized as harmful, the immune system undertakes action to neutralize or actively fight and eliminate these causative agents. To this end, the host has developed a complex system that involves both innate and adaptive immunity. The innate immune system is directly available to react in a largely non-specific, rapid way and is effective for most problems. It recognizes invaders and starts a general immune response that, however, does not confer to long-lasting immunity to eliminate these pathogens (21). Although considered to operate in a non-specific manner, it has become clear that the recognition of pathogens by so-called pattern recognition receptors on cells elicits an immune response titrated to the specific pathogen. In this sense, also a reaction of the innate immune system carries certain specificity. Basically, the innate immune system comprises of three components: a physical barrier like skin and mucosa, the non-cellular immune response, including the complement system, and the cellular component, i.e. immune cells. Cells that are part of the innate immune response are leukocytes, for example granulocytes, monocytes, macrophages, dendritic cells and mast cells. However, the distinction of three components is somewhat arbitrarily, as there exists an intricate cross-talk between all these components in most if not all immune reactions. Within minutes after the pathogen has penetrated the host, the non-cellular and cellular response of the innate immune system is activated. This occurs by detection of typical molecular structures on the membranes of these microorganisms by pattern recognition receptors of the host cells. This detection of invading microorganisms is performed by a variety of cells including macrophages and

dendritic cells that reside in the tissues. Upon encountering and subsequent recognition of a pathogen they phagocytose the pathogen and start to produce mediators as cytokines, chemokines, growth factors and antimicrobial peptides/proteins. These mediators will attract and activate immune cells from the circulation like neutrophils and monocytes. Typically, neutrophils are the first type of cells of the immune system to arrive at the site of infection. One of their main tasks is to phagocytose and intracellular kill pathogens thereby clearing the tissue of the infectious agent. Phagocytosis occurs by engulfment of the pathogen by the cell membrane. The pathogen is -while being surrounded by the membrane- then internalized. Intracellular, the phagosome which surrounds the pathogen fuses with a lysosome to become the so-called phagolysosome. Within the lysosome, a low pH is created, that together with the production of toxic reactive oxygen species, proteolytic enzymes and antimicrobial peptides, promotes death of the pathogen. Besides phagocytosis, upon activation, neutrophils also release inflammatory mediators that activate local cells and induce monocytes to exit the blood stream and recruit them into the injured tissue (22,23). Dependent on the local environment, these latter cells will differentiate into macrophages or dendritic cells; both are phagocytic cells, however where the main function of macrophages is to clear infection by phagocytosis and alarm the innate immune system upon activation by the pathogen, dendritic cells bridge between innate and adaptive immunity during infection (24). They process the internalized pathogen or foreign material and migrate toward the lymph nodes where T lymphocytes can recognize the presented components of the processed materials on their cell-surface in the context of MHC-molecules. T lymphocytes thus activated will subsequently expand and mediate an immune response specifically directed against this pathogen (25). This is the initiation phase of the adaptive immune response, which comprises of two major types of lymphocytes, the B lymphocyte and the T lymphocyte. B lymphocytes are part of the humoral component of the adaptive immune response; their main function is to produce antibodies. T lymphocytes are the main cellular component of the adaptive response, and can exist in three different stages, 1) naïve cells that have matured but have not seen an antigen yet, 2) effector cells, that have been activated by antigen and are involved in, e.g., the elimination of a pathogen and 3) memory cells, that have encountered their specific antigen in the past and now carry long-lived memory (26). T lymphocytes can be roughly divided into CD4+ T cells and CD8+ T cells. CD4+ T cells recognize exogenous antigens, while CD8+ T cells recognize endogenous antigens, i.e. antigens that are processed within the phagolysosomal pathway, or processed from within the cytoplasm of antigen presenting cells, respectively. CD4+ T cells modulate the immune
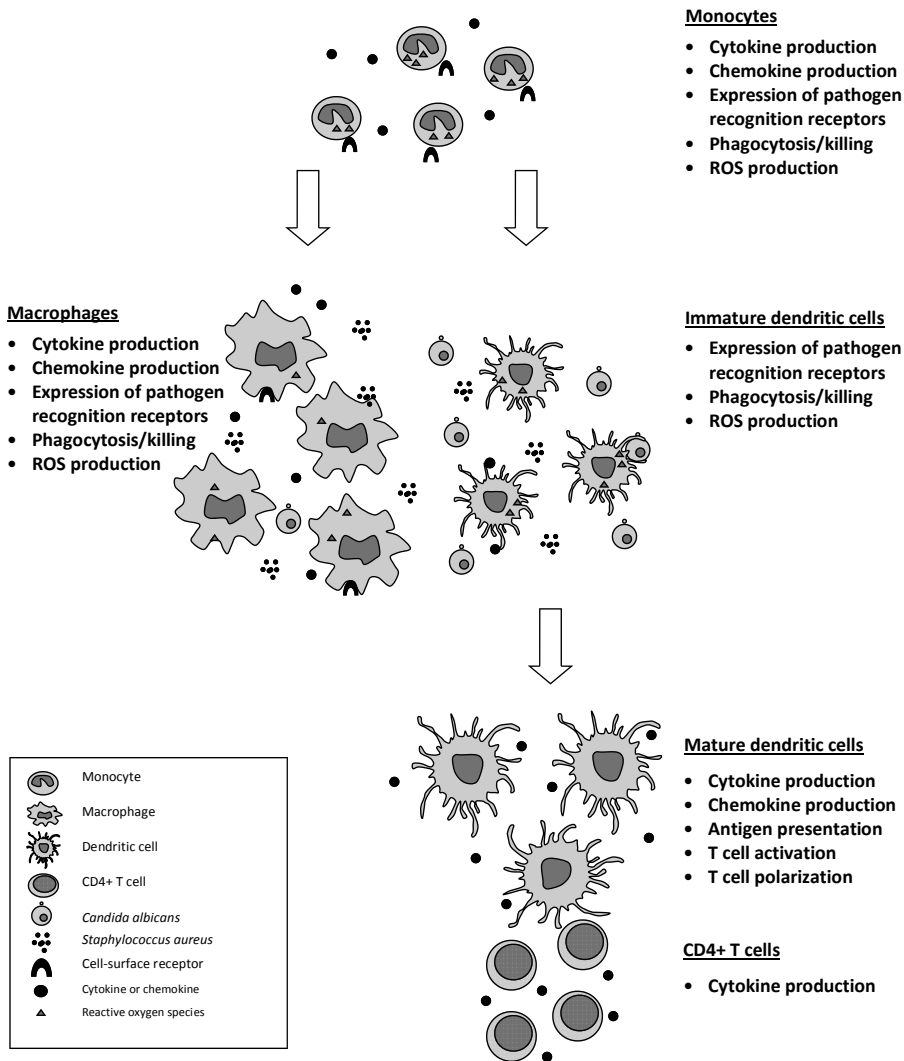
Monocytes
- **Cytokine production**
- **Chemokine production**
- **Expression of pathogen recognition receptors**
- **Phagocytosis/killing**
- **ROS production**

Macrophages
- **Cytokine production**
- **Chemokine production**
- **Expression of pathogen recognition receptors**
- **Phagocytosis/killing**
- **ROS production**

Immature dendritic cells
- **Expression of pathogen recognition receptors**
- **Phagocytosis/killing**
- **ROS production**

Mature dendritic cells
- **Cytokine production**
- **Chemokine production**
- **Antigen presentation**
- **T cell activation**
- **T cell polarization**

CD4+ T cells
- **Cytokine production**

Monocyte
Macrophage
Dendritic cell
CD4+ T cell
Candida albicans
Staphylococcus aureus
Cell-surface receptor
Cytokine or chemokine
Reactive oxygen species

Fig. 1 Simplified representation of the major characteristics of monocytes and monocyte-derived cells *in vitro*
Activated monocytes produce cytokines and chemokines upon encounter of pathogens, followed by phagocytosis and intracellular killing. Monocytes can differentiate into either macrophages or immature dendritic cells, depending on the presence of growth factors and cytokines. Upon recognition of a pathogen by their pathogen recognition receptors, macrophages will start to produce cytokines and chemokines and also phagocytose and intracellular kill pathogens. Monocytes, macrophages and dendritic cells use reactive oxygen species among others for killing of the pathogen. The processing of pathogens will mature the immature dendritic cells. Mature dendritic cells produce cytokines and chemokines and present pathogenic structures on their cell-surface using their MHC-molecules. When brought into co-culture with CD4+ T cells, these T cells may recognize the antigen that is presented by the mature dendritic cells and respond by producing cytokines that can direct innate immune cells.

response by the production of mediators, like cytokines that direct several innate immune cells. There are several subtypes of CD4+ T cells that have a phenotype that is important for the elimination of different types of pathogens (27). Most well-known are the T helper (Th)1, Th2, Th17 and regulatory T cells (Treg) (28,29). The Th1 response is characterized by the production of IFN-γ, which results in an enhanced cellular immune response (30). The Th2 response is characterized by the production of IL-4 among others, which results in an enhanced humoral immune response (30). The Th17 response is characterized by the production of IL-17, which induces the release of inflammatory mediators that affect innate immune cells thereby linking innate and adaptive immunity (31). And lastly Tregs suppresses ongoing immune responses thereby regulating the homeostasis of the immune system (32). In Fig. 1, a simplified representation of immune processes that are relevant to the work presented in this thesis is displayed.

Antimicrobial peptides in general

After the first reports of an antimicrobial substance in chicken egg white in 1909 by Laschtschenko, observations on interaction of nasal mucus with bacteria led to the discovery of the first human antimicrobial protein, lysozyme, in the early 20th century by Fleming (33,34). The discovery of this antimicrobial protein initiated research leading to the characterization of a variety of antimicrobial proteins like lactoferrin (35-37). While antimicrobial protein discovery was already developing from the early 20th century, it needed several decades more before the first antimicrobial peptide was discovered. This discovery resulted from research on host defense mechanisms of insects, and led to the isolation of cecropins from silk moth pupae about three decades ago by Hans Boman and co-workers (38). Further research showed that expression of antimicrobial peptides was not unique to insects. Over the following years Robert Lehrer and co-workers discovered defensins in human neutrophils (39,40) shortly after followed by Michael Zasloff who reported in the late '80s a potent antimicrobial peptide called magainin in the skin of frogs (41). These studies established the basis of antimicrobial peptide research which has culminated at present-day in the identification of far over a thousand antimicrobial peptides. An online database that is reporting all antimicrobial peptides from natural sources can be consulted at http://aps.unmc.edu/AP/main.php. Due to their diversity, several classes of antimicrobial peptides are distinguished, based on their structure, origin and amino acid composition. Antimicrobial peptides can be naturally occurring or are derived from enzymatic cleavage of naturally occurring antimicrobial proteins. Also, new peptides can be designed *in silico* (42-44). In general, antimicrobial peptides are cationic

and active against many types of microorganisms, often including the presently emerging multi-drug resistant microorganisms. The mechanism of action of the peptides is divers, but generally the mechanism involves direct action on the membrane of microorganisms, inducing leakage of intracellular content followed by cell death (45). In addition, the peptides can be internalized and induce cell death by acting on intracellular components (46). It was long thought that antimicrobial peptides possess antibacterial activity only (47,48); however research has shown that their activity extends to antifungal (49,50), antiviral (51), antiparasitic (52) and antitumor (53-55) activity. Some of them were shown to exert modulatory effects on cells of the human immune system (56,57). The latter is now increasingly recognized as being an important contribution to the clearance of infections. For possible therapeutic application of antimicrobial peptides, it will thus be important to understand the interactions of these peptides with the hosts' immune cells.

The human cathelicidin LL-37

An example of a natural occurring antimicrobial peptide is LL-37. This peptide is the single member of the cathelicidin family identified in humans. It is stored in neutrophils, monocytes, mast and epithelial cells as an (inactive) pro-peptide called hCAP18/LL-37, which is cleaved extracellular by enzymes to its active form. LL-37 is an amphiphatic alpha-helical peptide that has a wide range of biological activities (58). It is able to affect a variety of organisms, like bacteria including those residing in biofilms (59,60), viruses like HIV (61) and fungi (62). Moreover, it is able to neutralize bacterial endotoxins (63,64). In addition to its antimicrobial properties LL-37 also exerts activities on a variety of cells of the human immune system. These effects are mediated through an array of cell-surface receptors (66-70) and an intracellular target (71). Since LL-37 is naturally occurring in humans and is expressed by a variety of immune cells, its main actions are directly on or synergistically with the immune system. For example, LL-37 can act as a chemoattractant by recruiting neutrophils, monocytes and T cells (72). In addition, it suppresses the Toll-like receptor induced cytokine production by human peripheral blood mononuclear cells (73). It is also able to modulate the differentiation of dendritic cells by enhancement of their endocytic capacities and promotion of Th1 responses *in vitro* (74). However, when dendritic cells were incubated with LL-37 it inhibited their maturation and the toll-like receptor-induced cytokine response (75). LL-37 can also induce secondary necrosis in apoptotic neutrophils and can induce apoptosis in infected airway epithelium, thereby promoting the clearance of respiratory pathogens (76). These are all just examples of the
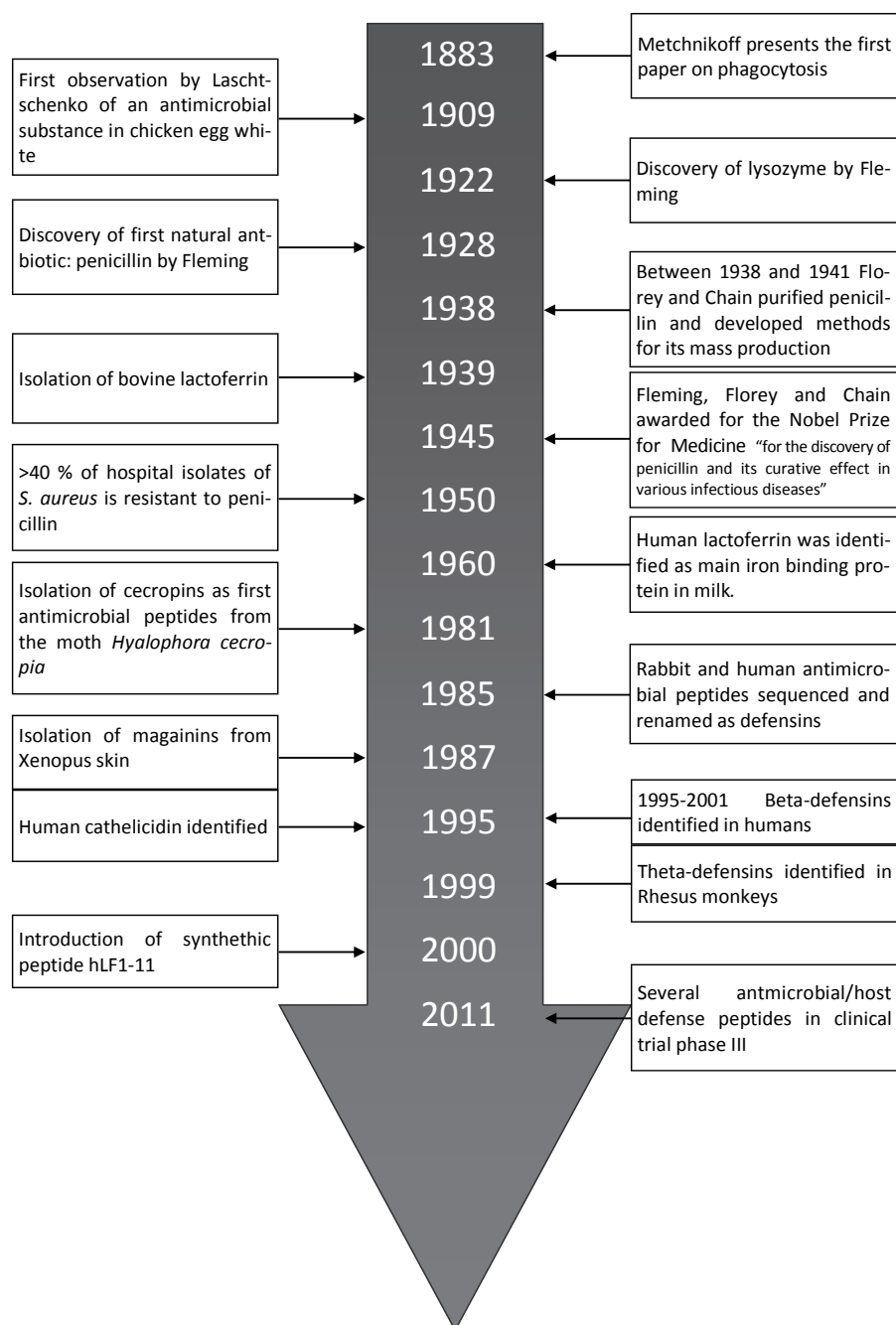
First observation by Lascht-schenko of an antimicrobial substance in chicken egg white

Metchnikoff presents the first paper on phagocytosis

Discovery of lysozyme by Fleming

Discovery of first natural antbiotic: penicillin by Fleming

Between 1938 and 1941 Florey and Chain purified penicillin and developed methods for its mass production

Isolation of bovine lactoferrin

Fleming, Florey and Chain awarded for the Nobel Prize for Medicine *"for the discovery of penicillin and its curative effect in various infectious diseases"*

>40 % of hospital isolates of *S. aureus* is resistant to penicillin

Human lactoferrin was identified as main iron binding protein in milk.

Isolation of cecropins as first antimicrobial peptides from the moth *Hyalophora cecropia*

Rabbit and human antimicrobial peptides sequenced and renamed as defensins

Isolation of magainins from Xenopus skin

Human cathelicidin identified

1995-2001 Beta-defensins identified in humans

Theta-defensins identified in Rhesus monkeys

Introduction of synthethic peptide hLF1-11

Several antmicrobial/host defense peptides in clinical trial phase III

1883
1909
1922
1928
1938
1939
1945
1950
1960
1981
1985
1987
1995
1999
2000
2011

Fig. 2 Historical events that motivated research into antimicrobial proteins and peptides (Modified from Lehrer) (65)

involvement of LL-37 in immune processes that will lead to recruitment of leukocytes to the site of infection, the promotion of wound healing and to modulate adaptive immunity.

Immunomodulatory effects of other antimicrobial peptides

Human defensins are natural occurring antimicrobial peptides that display a triple-stranded beta-sheet structure. Three groups have been identified based on their disulfide bonds, firstly, alpha-defensins that are produced by neutrophils, natural killer cells, T cells, epithelial cells and paneth cells. Secondly, beta-defensins that are produced by leukocytes and epithelial cells and, thirdly, theta-defensins that are found in leukocytes, but have only been identified in rhesus macaque, baboons and orangutans (65,77). Defensins have shown to act as chemoattractants for neutrophils, mast cells, dendritic cells and T cells and are able to enhance keratinocyte migration and proliferation (56). In addition they play an important role in gut and in skin diseases (78-80). Besides natural occurring peptides, also other antimicrobial peptides that were developed based on the structure of natural occurring peptides/proteins have shown to display immunomodulatory activities. Immune defense regulator-1 (IDR-1) and IDR-1002 are both designed peptides that display immune enhancing properties. IDR-1 lacks direct antimicrobial activity but is effective against infections. In the absence of monocytes and macrophages this peptide lost its activity against these infections, indicating that it displays immunomodulatory properties. Further research showed that it was able to enhance the LPS-stimulated chemokine and IL-10 production by peripheral blood mononuclear cells thereby controlling inflammation (43). IDR-1002 was able to induce similar effects as IDR-1 but much stronger (81). Since more and more antimicrobial peptides are linked to effects on the immune system, antimicrobial peptide research is shifting toward the development of new anti-infective agents based on these immunomodulating peptides.

The lactoferrin-derived antimicrobial peptide hLF1-11

The synthetic peptide hLF1-11 is one promising antimicrobial peptide developed as a potential therapeutical candidate with activity against several multi-drug resistant microorganisms, including fluconazole-resistant *Candida albicans*, multi-drug resistant *Acinetobacter baumannii* and methicillin-resistant *Staphylococcus aureus* (MRSA). hLF1-11 comprises the first 11 N-terminal amino acids (GRRRRSVQWCA) of human lactoferrin and shows antimicrobial activity. Lactoferrin is an 80 kDa iron-binding glycoprotein and member of the transferrin family (82). Lactoferrin is provided to newborns by breast-feeding and it is a major component of neutrophil-specific granules. It is present in high

concentrations (1-10 mg/ml) in many mucosal secretions, being synthesized by mucosal gland epithelial cells (83). Lactoferrin is part of the innate defense system and has a large diversity of mechanisms of action. For example, lactoferrin plays a role in the host defense by sequestering environmental iron through its two high-affinity ferric iron binding sites, thereby inhibiting microbial growth (84). It displays direct antimicrobial activity and can interact with the bacterial endotoxin (i.e., lipopolysaccharide, LPS). In addition, it reduces the negative charge of Gram-positive bacteria, thereby favoring contact between lysozyme and the underlying peptidoglycan (82) and displays anti-inflammatory activities by reducing pro-inflammatory cytokines and induction of IL-10 production (85). Small amounts of lactoferrin are expressed on the surface of resting neutrophils, mediating the binding of these cells to various structures (86). More importantly, lactoferrin is the source of peptides with antimicrobial activity, since by acid-pepsinolysis it generates the antimicrobial peptide lactoferricin H (residues 1 to 47). This region contains two cationic domains; four arginines at residues 2-5 and two arginines, a lysine and valine at residues 28-31. Both domains show antimicrobial activity, though hLF1-11 (comprising the first cationic domain) was over 10 times more efficient against various multi-drug resistant bacterial strains than hLF21-31 (comprising the second cationic domain) (87,88).

Antibacterial activities of hLF1-11

Direct antibacterial activity of hLF1-11 has been demonstrated by a number of studies using a variety of microorganisms including several multi-drug resistant pathogens. Its antibacterial activity was first tested in *in vitro* killing experiments using the Gram-positive *Listeria monocytogenes* and MRSA and the Gram-negative *Escherichia coli* and *Klebsiella pneumoniae*, in which the peptide killed these pathogens at relatively low concentrations (88). These experiments were later successfully reproduced using various Gram-negative multi-drug resistant *A. baumannii* strains by Dijkshoorn *et al.* demonstrating the activity of hLF1-11 against some clinically relevant multi-drug resistant strains (89). However, as the antimicrobial activity of hLF1-11 is salt-sensitive, these experiments were all performed *in vitro* at low salt conditions (~10 mM NaCl). At physiological salt concentrations (~155 mM NaCl) this peptide is ineffective *in vitro* at similar concentrations against all bacterial strains. Remarkably, it proved to be very active against a variety of bacterial strains when tested in animal models. The peptide was tested in a thigh-muscle infection mice model against several multi-drug resistant *A. baumannii* strains and against MRSA. In this infection model the relevant microorganism is injected in the right thigh muscle, followed by an intravenous injection of the peptide 24 hours later. The next day, the thigh muscle is

removed and the number of surviving microorganisms is determined microbiologically. hLF1-11 was highly active against the various *A. baumannii* strains in this *in vivo* infection model, as it reduced bacterial counts in a dose-dependent manner (89). When tested against MRSA the peptide showed a dose-dependent bactericidal effect, with a maximum effect of 2-3 log reduction using a concentration of 0.4-40 µg of peptide/kg of body weight. Moreover, when the peptide was administered to animals on a daily basis for four consecutive days, the infection was almost completely cleared ((90) and own observations).

Antifungal activities of hLF1-11

Invasive fungal infections, such as those caused by *Aspergillus fumigatus* and *Candida albicans* are recognized as an important cause of morbidity and mortality, in the immuno-compromised host in particular. Treatment of these infections is frequently hampered by the limitations of current anti-fungals, either due to intrinsic resistance, interactions with other drugs, adverse effects, etc. Therefore, the antifungal effect of the hLF1-11 peptide has been tested against these pathogens as well. *In vitro* hLF1-11 was more active than hLF21-31 in killing *A. fumigates* and *C. albicans*, as 10- and 5-times higher concentrations of hLF21-31 than hLF1-11 were necessary to obtain similar antifungal activity (87,91). *In vivo* hLF1-11 was tested in a *C. albicans* neutropenic mice model in which a maximum of 1.5 log reduction was obtained when using 0.4 µg of hLF1-11/kg of body weight. This effect was even more extreme (up to 4 log reduction) when IL-10 was neutralized by injection of IL-10 neutralizing monoclonal antibodies. This result was in agreement with the observation that IL-10 serum levels increased upon injection with high doses of hLF1-11 (49). In addition, hLF1-11 was found to act synergistically with fluconazol against fluconazole-resistant *C. albicans in vitro* (92). Besides direct killing of *C. albicans*, the peptide is also capable of reducing the virulence of *C. albicans* by inhibition of the morphological transition of this yeast. *C. albicans* is able to switch morphology from a round-shaped conidial form to a more virulent elongated hyphal shape. hLF1-11 was able to reduce the morphology switch of *C. albicans in vivo* to the hyphal state, thereby reducing its virulence (49). The inhibition of conidia to hyphae transition could be reproduced *in vitro* at physiological salt concentrations, indicating that besides its antimicrobial properties, hLF1-11 also displays pathogen modulatory properties that could contribute to its overall antimicrobial effects.

Safety of hLF1-11

In vivo toxicity studies in mice and rats have shown that hLF1-11 can be safely administered at a 100 fold higher dose than appears therapeutically effective in these animals. hLF1-11 has also been tested for safety in two phase I studies in healthy human male volunteers. In both studies the subjects received an i.v. dose of hLF1-11 or placebo; concentrations were different in the studies. Both showed that the peptide was safe and well tolerated; any adverse events were graded mild. Phase IIa safety has also been established using a first patient population (hematopoietic stem cell recipients) receiving a single (high) dosis of hLF1-11 (93). The next phase II trial will focus on treatment of MRSA and systemic fungal infections.

Hypothesis

As hLF1-11 has proven safe in the first small phase I studies in humans and will now be further tested for a potential therapeutic application, we wanted to establish the mechanism(s) of action of this candidate peptide. Since hLF1-11 is able to reduce the infectious load in mice within 48 hours, the time span of innate immunity, but is unable to kill these pathogens *in vitro* at physiological salt concentrations, we hypothesized that hLF1-11 displays immunomodulatory properties on cells of the innate immune system.

Outline of the thesis

In this thesis we investigate the interactions of hLF1-11 and LL-37 with immune cells, in order to gain insight in their mechanism of antimicrobial action. In **chapter 1** the history and current knowledge on antimicrobial proteins and peptides and in particular LL-37 and hLF1-11, is described. Also, the background on basic immunological responses and processes has been provided and some insight on current knowledge on interactions of antimicrobial peptides with the human immune system is given. As put forward in our hypothesis, we expect immunomodulatory effects of hLF1-11 on cells of the innate immune system and therefore we first investigated effects of hLF1-11 on murine and human monocytes of which the results are described in **chapter 2.** Upon entering the tissue, monocytes can differentiate toward macrophages or dendritic cells. The effect of hLF1-11 on monocytes prompted us to investigate whether the interaction between monocytes and hLF1-11 has consequences for the functions of the resulting macrophages. Described in **chapter 3** are the results regarding the effect of hLF1-11 on monocyte-macrophage differentiation. As the effects of LL-37 on monocytes differ from those of hLF1-11, we determined whether the differentiation of monocytes into macrophages was also differentially modulated by LL-37 as compared to hLF1-11. The results of this investigation are described in **chapter 4.** Furthermore, as monocytes can also differentiate into dendritic cells, in **chapter 5** we report the results of our study into the effect of hLF1-11 on monocyte-dendritic cell differentiation. After establishing several immuno-modulatory effects on mononuclear phagocytes, we investigated the possible cellular target of hLF1-11 on human monocytes that likely mediates the modulatory actions of hLF1-11 on these cells. Results of this investigation are described in **chapter 6**. Finally, the main findings of this thesis are summarized and discussed in **chapter 7** and a Dutch summary can be found in **chapter 8**.

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