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

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# Enhancement of host defense against pathogens by antimicrobial peptides

***A new approach to combat microbial drug resistance***

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A field of tulips represents the flourishing state of antimicrobial peptide research

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*“One sometimes finds what one is not looking for”*

***Sir Alexander Fleming (1881-1955)***

*Voor Pap, Mam en Fenna*

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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 20$^{th}$ 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

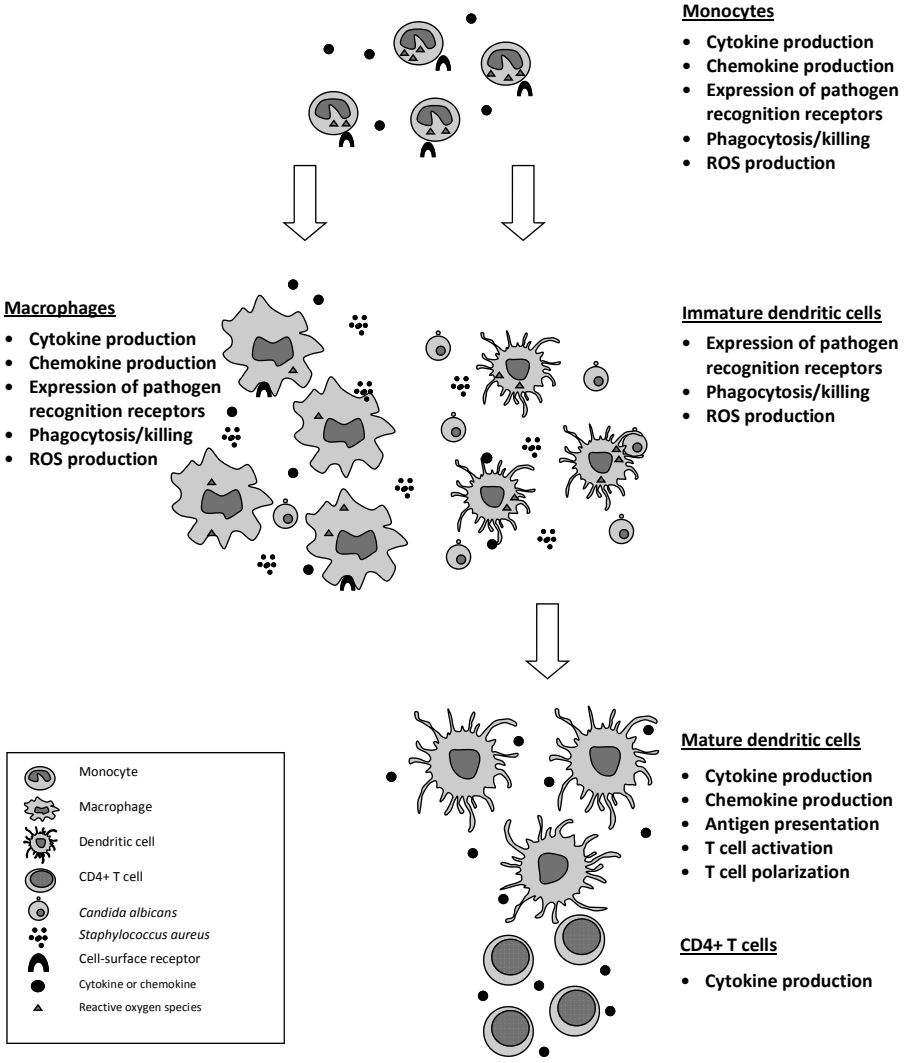


**Fig. 1 Simplified representation of the major characteristics of monocytes and monocyte-derived cells *in vitro***
Activated monocytes produce cytokines and chemokines upon encounter of pathogens, followed by phagocytosis and intracellular killling. Monocytes can differentiate into either macrophages 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 20$^{th}$ 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 20$^{th}$ 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

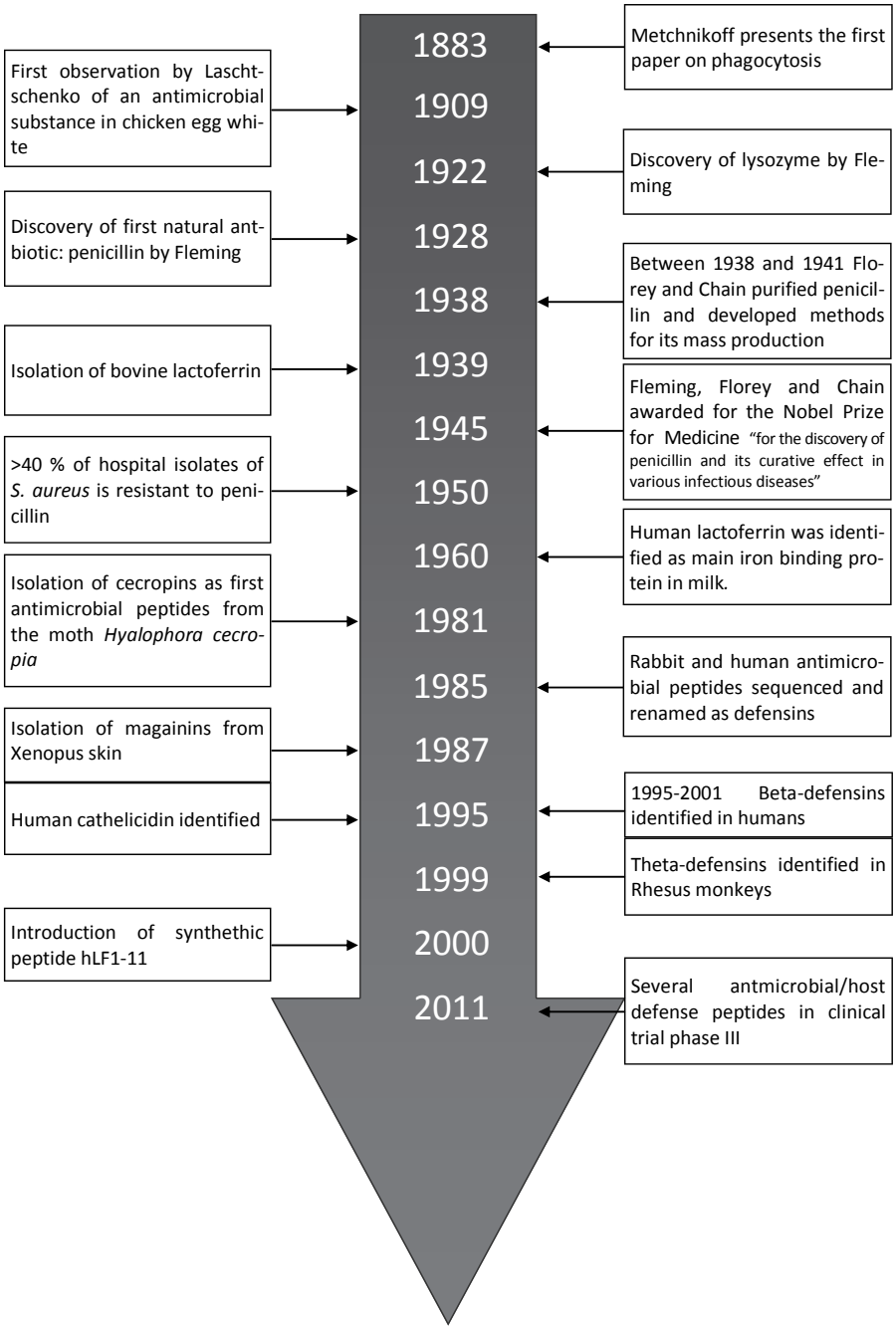


**Fig. 2 Historical events that motivated research into antimicrobial proteins and peptides** (Modified from Lehrer) (65)

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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# The human lactoferrin-derived peptide hLF1-11 primes monocytes for an enhanced TLR-mediated immune response

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# Chapter 2

**Abstract** Earlier we reported that the peptide corresponding to the first eleven N-terminal amino acids of human lactoferrin (hLF1-11) is active against multi-drug resistant pathogens in mice. The mechanisms underlying this anti-infective activity remain unclear. Since hLF1-11 is ineffective against pathogens at physiological salt concentrations and hLF1-11 directs differentiation of monocytes toward a macrophage subset with enhanced effector functions, we investigated the effects of hLF1-11 on human and murine monocytes. Results revealed that human and murine monocytes exposed for 1 h to hLF1-11 and then stimulated with the Toll-like receptor (TLR)-ligand LPS for 18 h, displayed enhanced cytokine and chemokine production as compared to control (peptide-treated) monocytes. We also found that expression of mRNA, cell-surface receptor expression, and NF-κB activation by hLF1-11-exposed human monocytes were enhanced as compared to control (peptide-treated) monocytes. Furthermore, the kinetics of the cytokine production was unchanged as mRNA levels and protein levels paralleled the enhanced response of hLF1-11-exposed monocytes to LPS. The cytokine production by human monocytes in response to TLR4, TLR5, and TLR7 stimulation, but not to TLR2 stimulation, was elevated by hLF1-11. In concordance, translocation of NF-κB subunits to the nucleus was enhanced in hLF1-11-exposed monocytes after TLR stimulation, except for TLR2, as compared to control (peptide-exposed) monocytes. In conclusion, monocytes were primed by hLF1-11 for an enhanced inflammatory response upon TLR4, TLR5, and TLR7 stimulation, but not TLR2 stimulation. Such effects of hLF1-11 on monocyte reactivity should be taken into account when considering the clinical development of this peptide for a therapeutic intervention in patients.

**Introduction**

Human lactoferrin (hLF), an ~80-kDa iron-binding glycoprotein, is a member of the transferrin family. It is found predominantly in the secreted fluids of mammals such as milk, tears, saliva, bronchial mucus, and seminal plasma and is also stored in the secondary granules of polymorphonuclear leukocytes. hLF is an important contributor to host defense due to its wound healing, endotoxin binding, antimicrobial, and immunomodulatory properties (1-3). The antimicrobial activity of hLF is based on at least two mechanisms: 1) iron sequestration, thereby depriving bacteria from this nutrient, inhibiting growth of the infectious agent and 2), direct interaction with the bacterial cell membrane resulting in cell lysis. In addition, hLF displays immunomodulatory effects on cells of both the innate and acquired immune response (2,4). Several peptides representing different domains of human lactoferrin can mimick antimicrobial, immunomodulatory or other properties of lactoferrin and may even be more potent than the parent protein (5,6). In this connection we reported that the peptide corresponding to the first eleven N-terminal amino acids of human lactoferrin (hLF1-11) is more active than hLF in reducing pathogenic load in mice (7) and hLF1-11 exerts immunomodulating activity on monocyte-macrophage differentiation (8). Others have found enhanced antimicrobial activity of peptides comprising amino acids 19–31 as compared to human lactoferrin (9). In addition, Nilsson et al. (10) have developed a human lactoferrin derived peptide PXL01 that prevented postsurgical adhesion formation in rats. In search for antimicrobial agents that are effective against infections with multi-drug resistant pathogens, we reported hLF1-11 to effectively reduce microbial load in infections with methicillin-resistant *Staphylococcus aureus* (MRSA) (7), multi-drug resistant *Acinetobacter baumannii* (11) and fluconazole-resistant *Candida albicans* (12) in mice. hLF1-11 is ineffective in vitro at physiological salt concentrations and, as discussed by Lupetti *et al.*, it may exert its antimicrobial effects partly by affecting the early innate immune response of the host. Since the peptide is also effective in clearing infections in neutropenic mice, we considered the possibility that the peptide displays immunomodulatory activity by affecting the functional activities of mononuclear phagocytes, i.e., monocytes and macrophages. The mononuclear phagocyte system includes blood monocytes that have differentiated from committed progenitor cells in the bone marrow. Monocytes circulate in the blood and upon recruitment by inflammatory mediators they enter tissues to differentiate into macrophages or (immature) dendritic cells (DCs). Recognition of pathogens through their pathogen-associated molecular patterns (PAMPs) by these cells requires pattern recognition receptors, such as C-type lectins and Toll-like receptors (TLRs)

(13). TLR2 and TLR4 are major TLRs on mononuclear phagocytes and DCs involved in sensing bacterial pathogens. This interaction initiates activation and translocation of transcription factors like NF-κB. Activation of these transcription factors will initiate the transcription of many genes leading to the production of a variety of inflammatory mediators, such as chemokines and cytokines (14), thereby regulating the inflammatory process (15,16). Previous investigations showed that incubation of monocytes with hLF1-11 modulated the GM-CSF driven differentiation of these cells resulting in a macrophage subset that showed enhanced recognition and clearance of pathogens (8). These effects were already obtained after incubation of monocytes with hLF1-11 for 60 min prior to the start of the 7-day culture system. In this study we investigated whether hLF1-11 primes monocytes with respect to an enhanced inflammatory response to various PAMPs.

**Materials and methods**

**Peptides** The synthetic peptide comprising the first eleven amino acids of human lactoferrin (further referred to as hLF1-11; GRRRRSVQWCA, 1374 Da) was purchased from Peptisyntha (Torrance, CA) and the control peptide (GAARRAVQWAA, 1155 Da) from Isogen (De Meern, The Netherlands). The purity of the peptides was determined by reverse-phase high performance liquid chromatography and exceeded 97%. Both peptides are endotoxin free. Immediately before use the peptides were dissolved in phosphate buffered saline (PBS; pH 7.4; Department of Pharmacy, LUMC) to a stock concentration of 1 mg/ml.

**Stimuli** The following TLR2 ligands were used: purified lipoteichoic acid (LTA; *Staphylococcus aureus*), lipomannan (*Mycobacterium smegmatis*), and synthetic PAM2CSK4. The TLR4 ligands, i.e., purified lipopolysaccharide (LPS) and diphosphoryl lipid A (DPLA; both *E. coli*), the TLR5 ligand recombinant flagellin (*Salmonella typhimurium*) and the synthetic TLR7 ligand CL087 were also included in this study. All TLR-stimuli were purchased from Invivogen (San Diego, CA) except DPLA (Sigma-Aldrich, Zwijndrecht, The Netherlands). Stocks of LPS (5 mg/ml), LTA (5 mg/ml), PAM2CSK4 (1 mg/ml), lipomannan (1 mg/ml), CL087 (1 mg/ml), and flagellin (2 μg/ml) were prepared in distilled water, and stored at -20°C. A stock of DPLA (1 mg/ml) was prepared in DMSO and stored at 4°C.

**Mice** The animal experiment was approved by the Leiden Experimental Animal Committee and done in compliance with Dutch laws related to the conduct of animal experiments. Fresh blood was taken via heartpunction from SPF, female Swiss mice aged 9 weeks old

(Charles River, Maastricht, The Netherlands) and collected in citrate tubes (BD biosciences, Heidelberg, Germany).

**Isolation of mouse monocytes** Mouse monocytes were isolated by Ficoll amidotrizoate density centrifugation (ρ=1.077 g/ml, Department of Pharmacy, LUMC). Cells in the interphase were washed and the CD3+ cells were depleted from this cells suspension using magnetic antiCD3 coated MACS beads (Miltenyi Biotec, Bergisch Gladbach, Germany) according to manufacturer's instructions. The remaining cells were washed and the monocytes were further purified using magnetic antiCD11b coated MACS beads (Miltenyi Biotec), resulting in a suspension of ~95% monocytes.

**Isolation of human monocytes** Human monocytes were isolated from buffycoats (Sanquin, Amsterdam, The Netherlands) from healthy donors by Ficoll amidotrizoate (Department of Pharmacy, LUMC) density centrifugation. Cells in the interphase were washed and the monocytes were further purified using magnetic antiCD14 coated MACS beads (Miltenyi Biotec) according to manufacturer's instructions resulting in a suspension of >96% monocytes. The viability of this cell suspension exceeded 98% as determined by Annexin V and PI staining 2 h after isolation.

**Experimental set-up** Murine and human monocytes were resuspended in RPMI-1640 (Invitrogen, Breda, The Netherlands) supplemented with 2 mM L-glutamine (Invitrogen), 100 U/ml penicillin (PAA GmbH, Pasching, Germany), 100 mM streptamycin (PAA GmbH), and 10% inactivated fetal calf serum (Invitrogen), further referred to as standard medium. Monocytes were cultured at a concentration of $1x10^6$ cells/ml of standard medium at 37°C and 5% $CO_2$. The cells were exposed to the peptides immediately at the start of culturing and 1 h thereafter the cells were stimulated with one of the TLR ligands. 18–20 h later the supernatants were collected for assessment of cytokine and chemokine levels. The human monocytes were assessed for cell-surface receptor expression or were further processed to obtain nuclear and cytoplasmic fractions, or RNA was isolated using RNeasy microkit (Qiagen, Valencia, CA) according to manufacturer's instructions.

**Determination of cytokine and chemokine levels** Levels of murine IL-1α, IL-1β, IL-6, IL-10, IL-12p70, MCP-1, MIP-1β, RANTES, and TNF-α were determined using Bio-plex (Bio-Rad, Hercules, CA) with a lower level of detection of 10–20 pg/ml. Levels of human IL-1β, IL-1RA, IL-6, IL-8, IL-10, IL-12p70, MCP-1, MIP-1β, RANTES, and TNF-α were determined using

Bio-plex with a lower level of detection of 5–15 pg/ml. In addition, levels of the anti-inflammatory cytokine IL-10 and the pro-inflammatory cytokines IL-12p40 and TNF-α in supernatants of human monocytes were determined using ELISA (cytosets from Invitrogen; Breda, The Netherlands) with a lower level of detection of 25 pg/ml. All assays were performed according to manufacturer's instructions. Cytokine levels are expressed as concentration or as fold induction over control cells to correct for donor variation in cytokine production.

**Curve fitting analysis** To determine the effect of hLF1-11 on the responsiveness of monocytes to LPS, two characteristics (i.e., $E_{max}$ and $EC_{50}$) of the LPS-induced IL-10 release were calculated by non-linear regression with the dose–response model according to the Hill's equation: $E = E_{max} \times C/(EC_{50} + C)$. Where E is the observed IL-10 production at a given LPS concentration C, $E_{max}$ is the estimated maximal IL-10 production, and $EC_{50}$ is the estimated LPS concentration at which 50% of the maximal IL-10 response is reached (17).

**Determination of gene-expression levels** Quantative PCRs (Q-PCRs) were used to analyze samples for expression of human IL-10, IL-12p40, TNF-α, and the housekeeping genes GAPDH and RPL13A. Total RNA was extracted from cultured monocytes and RNA levels in the samples were measured on a Nanodrop 2000 spectrophotometer (Nanodrop Technologies, Wilmington, DE). Next, cDNA synthesis was performed on 0.5 µg of RNA with an Iscript cDNA synthesis kit (Bio-Rad) according to manufacturer's instructions. Q-PCRs were performed on a MyIQ (Bio-Rad). The reaction mixture consisted of SybrGreen (Roche, Indianapolis, IN), 1 mM dNTPs (Invitrogen), 400 nM primers (Isogen, De Meern, The Netherlands) and 0.5 U amplitaq gold/test in polymerase Gold buffer or—for TNF-α only—in polymerase buffer II (Applied Biosystems, Foster City, CA). The sequences of the primers are: GAPDH: (Forward, F) AAG GTC GGA GTC AAC GGA TTT and (Reverse, R) ACC AGA GTT AAA AGC AGC CCT G. RPL13A: (F) CCT GGA GGA GAA GAG GAA AGA GA and (R) TTG AGG ACC TCT GTG TAT TTG TCA A. IL-10: (F) GGT GAT GCC CCA AGC TGA and (R) TCC CCC AGG GAG TTC ACA. IL-12p40: (F) CGG TCA TCT GCC GCA AA and (R) CAA GAT GAG CTA TAG TAG CGG TCC T. TNF- a: (F) GGT GCT TGT TCC TCA GCC TC and (R) CAG GCA GAA GAG CGT GGT G. Each cDNA sample was analyzed in duplicate. The results were analyzed using Bio-Rad software. Gene-expression levels were corrected for both GAPDH and RPL13A expression.

**Determination of NF-κB activation and translocation** The levels of NF-κB in the cytoplasm and nucleus were assessed to determine transcription activation and translocation in monocytes upon hLF1-11 or control peptide stimulation. Cytoplasmic and nuclear fractions of the human monocytes were obtained using the nuclear extract kit (Active Motif, Rixensart, Belgium) and tested for NF-κB p50, p52, p65, B-Rel, and Rel-C levels using NF-κB transcription factor assay kit (Active Motif) according to manufacturer's instructions. Values are expressed as the optical density of the sample minus background.

**Flow cytometric analysis of cell-surface molecule expression by human monocytes** Monocytes were collected and resuspended in cold PBS with 0.2% BSA, washed twice and then incubated with the selected fluorescently-labeled monoclonal antibodies for 30 min on ice in the dark. The following monoclonal antibodies were used: PE-conjugated antibodies against CD11b, CD32, CD86 and CD163 and FITC-labeled antibodies against CD14, CD16, CD64 and CD80 were obtained from BD biosciences (Heidelberg, Germany). FITC-conjugated antibodies against CD282 and CD284 were obtained from HyCult (Uden, The Netherlands). Cell-surface molecule expression was assessed by flow cytometry using FACSCalibur and BD CellQuest software (BD biosciences). Results are expressed as median fluorescence intensity (MFI).

**Statistical analysis** Differences between the values for the hLF1-11-exposed and those for control peptide-exposed and control monocytes were compared with the repeated measures ANOVA and the Bonferroni's multiple comparison post-hoc tests. For experiments without the control peptide-exposed cells the differences between hLF1-11-exposed monocytes and control cells were tested for significancy with the paired t-test. In all experiments $p$ values of $< 0.05$ were considered significant.

## Results

**Effect of hLF1-11 on cytokine and chemokine production by LPS-stimulated murine monocytes** Since (i.v. administered) hLF1-11 is effective against infections in mice, we determined whether the peptide activates monocytes by comparing cyto- and chemokine production by hLF1-11-exposed and control (peptide-exposed) murine monocytes in response to LPS. Results revealed that hLF1-11-exposed cells produced significantly more IL-6, IL-10, MIP-1β, and RANTES, but not IL-1α, IL-1β, MCP-1, IL-12p70, and TNF-α after LPS activation than control or control peptide-exposed cells did (Fig. 1).

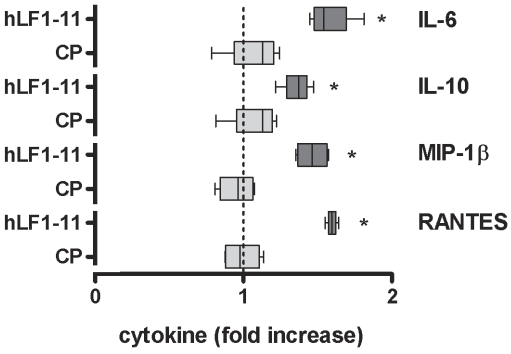



**Fig. 1 Effect of hLF1-11 on cytokine production by murine monocytes in response to LPS** Monocytes were exposed to 100 μg/ml hLF1-11 (dark gray boxes) or 100 μg/ml control peptide (CP; light gray boxes), or no peptide for 1 h and then stimulated with 100 ng/ml LPS for 18 h. Thereafter, levels of IL-1α, IL-1β, IL-6, IL-10, IL-12p70, MCP-1, MIP-1β, RANTES, and TNF-α were determined by a multiplex assay. IL-6, IL-10, MIP-1β, and RANTES production was significantly enhanced by hLF1-11-exposed monocytes as compared to control (peptide-exposed) monocytes. Values are expressed as fold increase of cytokine production compared to no peptide. Data are expressed as boxes and whiskers: boxes represent medians and second and third interquartiles, whiskers represent range of experiments within 4–7 different donors. *$p < 0.05$ for the difference between hLF1-11-exposed and control (peptide-exposed) cells

**Effect of hLF1-11 on cytokine and chemokine production by LPS-stimulated human monocytes** Since we consider administration of hLF1-11 to humans, we next determined whether exposure to hLF1-11 also affects the cyto- and chemokine production by human monocytes in response to LPS. Results showed that hLF1-11-exposed monocytes produced significantly higher levels of the various cytokines and chemokines in response to LPS than control (peptide-exposed) monocytes did, except for TNF-α (Fig. 2A). However at earlier time-points after LPS stimulation, TNF-α production was significantly enhanced by hLF1-11-exposed monocytes. Unstimulated cultured monocytes produced IL-1RA, IL-8, and MIP-1β and addition of hLF1-11 at the start of the culture resulted in cells producing significantly less of these chemokines than control (peptide-exposed) monocytes did (Fig. 2B). All further experiments were performed with human monocytes.

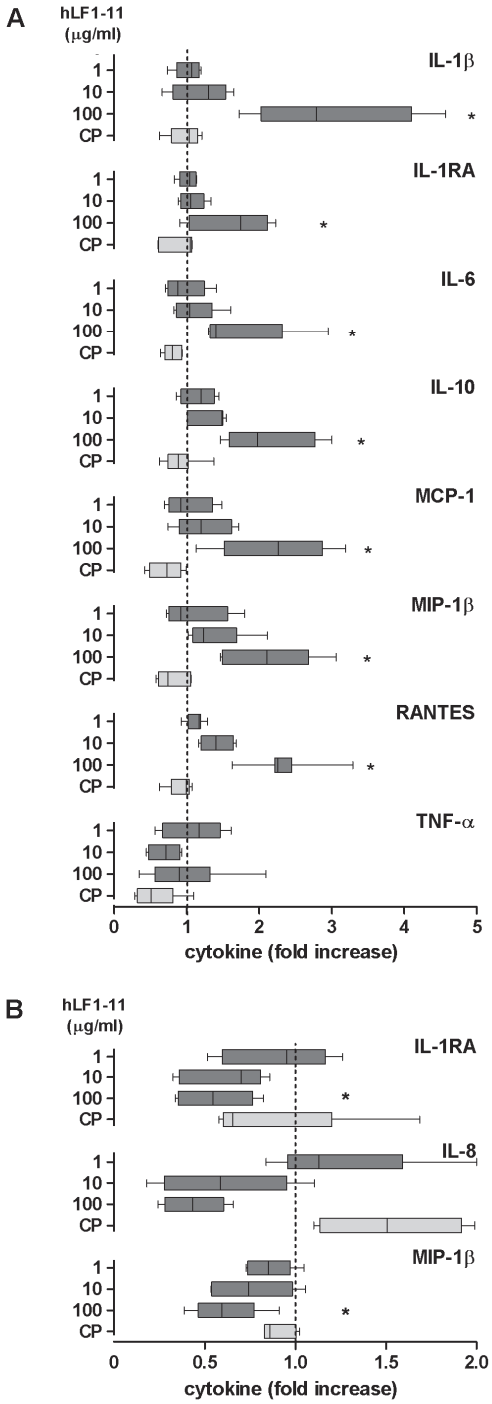



**Fig. 2 Effect of hLF1-11 on cytokine production by human monocytes in response to LPS** Monocytes were exposed to various concentrations of hLF1-11 (dark gray boxes) or 100 μg/ml control peptide (CP; light gray boxes) or vehicle for 1 h and thereafter stimulated with 100 ng/ml LPS (a) for 18 h. Levels of IL-1β, IL-1RA, IL-6, IL-8, IL-10, IL-12p70, MCP-1, MIP-1β, RANTES, and TNF-α in these cell culture supernatants were determined by multiplex assay. IL-12p70 was not detectable and IL-8 levels were above the highest values in the standard curve. In unstimulated cells (b) only IL-1RA, IL-8, and MIP-1β production was detectable and this was less in hLF1-11-exposed monocytes than in control monocytes. Values are fold increases of cytokine production compared to control monocytes. Data are expressed as boxes and whiskers: boxes represent medians and second and third interquartiles, whiskers represent range of experiments within 4–7 different donors. *$p < 0.05$ for the difference between hLF1-11-exposed and control (peptide-exposed) cells.

**Effect of hLF1-11 on LPS recognition by human monocytes** To investigate whether hLF1-11 affects the recognition of LPS, we incubated monocytes exposed to hLF1-11, control peptide or no peptide with various concentrations of LPS (0.1–1000 ng/ml) and measured IL-10 production by these cells. Results revealed that hLF1-11-exposed monocytes displayed enhanced cytokine production in response to the various LPS concentrations, i.e., $E_{max}$ for control cells amounted to 0.43 ± 0.10 ng IL-10/ml and for hLF1-11-exposed cells amounted to 0.80 ± 0.13 ng IL-10/ml, without affecting the sensitivity of these monocytes to LPS, i.e., $EC_{50}$ for control cells amounted to 0.82 ± 0.33 ng LPS/ml and for hLF1-11-exposed cells amounted to 1.22 ± 0.47 ng LPS/ml (Fig. 3).

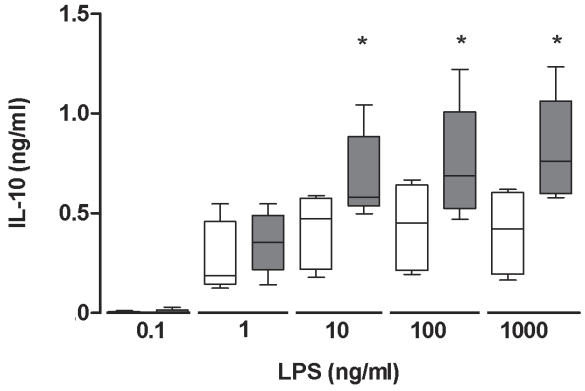



**Fig. 3 Effects of hLF1-11 on cytokine production by human monocytes stimulated with various LPS concentrations** Monocytes were exposed to hLF1-11 (dark gray boxes) or no peptide (open boxes) for 1 h and then stimulated with various concentrations LPS for 18 h. Thereafter supernatants were collected and assessed for IL-10 levels. Data are expressed as boxes and whiskers: boxes represent medians and second and third interquartiles, whiskers represent range of experiments within five different donors. *$p$ < 0.05 for the difference between hLF1-11-exposed and control cells.

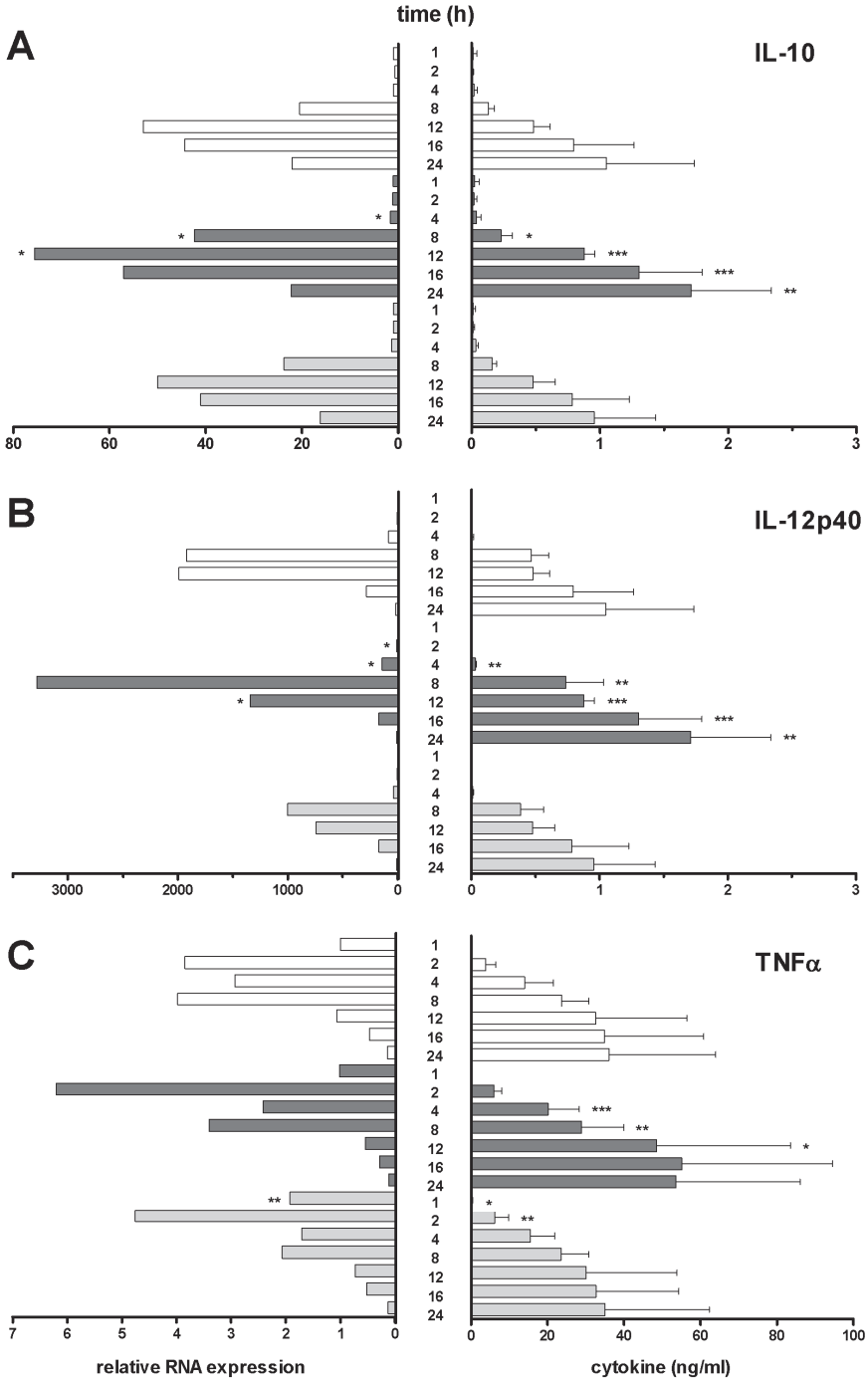
**Interaction of hLF1-11 with LPS in human monocyte cultures** To exclude the possibility that hLF1-11 exerts its effects on cytokine production by interacting with LPS, two experiments were performed. First the order of stimulation was reversed, freshly isolated monocytes were stimulated with LPS and 1 h thereafter the cells were exposed to hLF1-11 and secondly LPS and hLF1-11 were added together to the cell culture. IL-10 production by these cells was measured as readout. Results showed equal IL-10 production by these cells as compared to LPS stimulation of monocytes pre-incubated with hLF1-11 (n = 7–8, data not shown). In addition, we studied the possibility that binding of hLF1-11 to LPS in the supernatant may contribute to the hLF1-11-induced cytokine increase. Therefore, monocytes were exposed to hLF1-11 and 60 min later unbound peptide was removed by harvesting the supernatant and washing the cells once with RPMI. Immediately thereafter monocytes were stimulated with LPS and IL-10 production was measured. Results

revealed that removal of unbound hLF1-11 from the monocyte cultures did not significantly affect the hLF1-11-induced increase in IL-10 levels (n = 7–8, data not shown).

**Effect of hLF1-11 on the kinetics of cytokine production** To obtain some insight into the mechanisms underlying the effects of hLF1-11 on the cytokine production by LPS-activated monocytes, we investigated the effect of hLF1-11 on the kinetics of cytokines. Therefore, mRNA and protein levels of IL-10, IL-12p40 (both late-phase cytokines), and TNF-α (early-phase cytokine) of hLF1-11 and control (peptide-exposed) monocytes were assessed at various time-intervals after LPS stimulation. The results revealed that hLF1-11-exposed monocytes displayed increased cytokine levels, but did not show different kinetics of the cytokine-production, i.e., the start of detectable cytokine production and their peak concentrations were seen at similar time points (Fig. 4). In addition, the results for mRNA-expression of IL-10, IL-12p40 and TNF-α revealed that the peptide increased the quantity of gene expression of IL-10, IL- 12p40 and TNF-α, but did not affect the kinetics of mRNA production (Fig. 4). These results indicate that hLF1-11 enhances the LPS response but does not change its kinetics.

**Effect of hLF1-11 on the LPS-induced NF-κB activation and translocation in monocytes** To investigate whether the enhanced cytokine production by hLF1-11 in human monocytes corresponded with an enhanced NF-κB activation, we determined the levels of NF-κB subunits in the nuclei and cytoplasm of hLF1-11 and control (peptide-exposed) monocytes at 30 and 60 min after LPS stimulation. The results revealed that hLF1-11-exposed monocytes displayed significantly enhanced activation and translocation of NF-κB p50, p52 and p65 at 60 min after LPS exposure (Fig. 5). Surprisingly, hLF1-11-exposed unstimulated monocytes displayed decreased NF-κB p65 translocation in monocytes as compared to control (peptide-exposed) monocytes.

F**ig. 4 Effects of hLF1-11 on the kinetics of cytokines by LPS-stimulated human monocytes** Monocytes were exposed to hLF1-11 (dark gray boxes), control peptide (CP; light gray boxes) or no peptide (open boxes) for 1 h and then stimulated with 100 ng/ml LPS. At several intervals the supernatants were collected for determination of the IL-10 (A), IL-12p40 (B), and TNF-α (C) levels and total RNA was extracted from the monocytes in order to quantify mRNA expression of these cytokines. Cytokine levels are expressed in ng/ml (right hand panel) and gene expression as relative expression over control monocytes at 1 h (left hand panel). Data are medians and interquartile range within four different donors. *$p$ < 0.05 for the difference between hLF1-11-exposed and control (peptide-exposed) cells.►


time (h)
A
IL-10
B
IL-12p40
C
TNFα
relative RNA expression
cytokine (ng/ml)

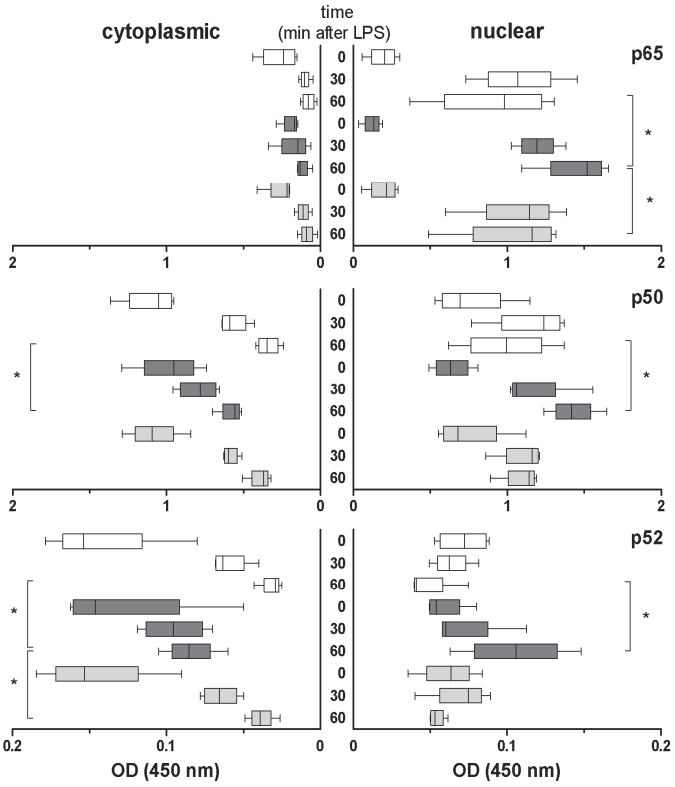



**Fig. 5 Effect of hLF1-11 on NF-κB translocation in human monocytes** Freshly isolated monocytes were exposed to hLF1-11 (dark gray boxes), control peptide (CP; light gray boxes), control peptide (open boxes) for 1 h and then stimulated with LPS for 30 or 60 min. Then cytoplasmic (left) and nuclear (right) fractions of the monocytes were prepared and assessed for their NF-κB p50, p52, and p65 levels. Values have been corrected for background. Data are expressed as boxes and whiskers: boxes represent medians and second and third interquartiles, whiskers represent range of experiments within four different donors. *$p < 0.05$ for the difference between hLF1-11-exposed and control cells.

**Effect of hLF1-11 on cytokine production by human monocytes in response to various TLR-ligands** To find out if the effects of hLF1-11 on human monocytes are specific for LPS or TLR4, monocytes were exposed to hLF1-11, control peptide or no peptide, followed by stimulation with either LTA, PAM2CSK4, lipomannan, purified LPS, DPLA, flagellin or CL087 and 18 h thereafter IL-10 production was measured in the supernatants. Results showed that hLF1-11-induced enhancement of cytokine production was observed when the monocytes were stimulated with the TLR4-ligands LPS and DPLA, the TLR5-ligand flagellin and the TLR7-ligand CL087, but not with the TLR2 ligands LTA or PAM2CSK4. Since monocytes hardly produce cytokines in response to lipomannan these data were not included in the graph (Fig. 6A). Investigations into NF-κB activation and translocation in response to these TLR-ligands revealed that hLF1-11-exposed monocytes display significantly enhanced activation and translocation of NF-κB p65 after LPS, flagellin or CL087, but not after LTA stimulation (Fig. 6B).

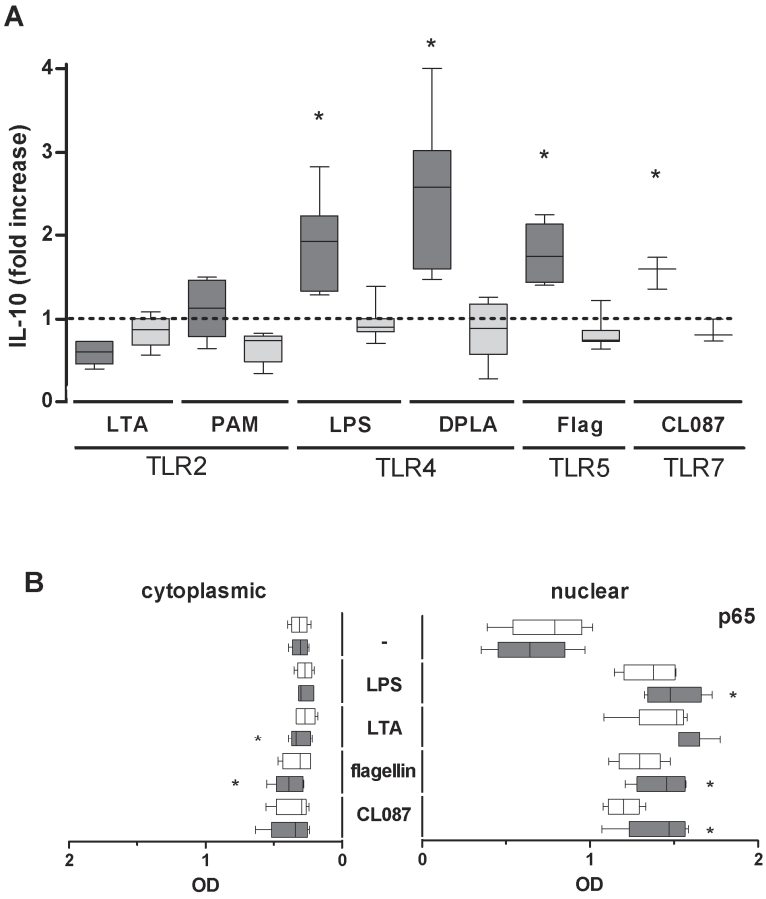



**Fig. 6 Effect of hLF1-11 on cytokine production and NF-κB translocation by human monocytes in response to different TLR-stimuli** Freshly isolated monocytes were exposed to hLF1-11 (dark gray boxes), control peptide (CP; light gray boxes), or no peptide (open boxes) for 1 h and then stimulated with LTA, PAM2CSK4 (PAM), LPS, diphosphoryl lipidA (DPLA), flagellin (Flag), and CL087. About 18 h thereafter IL-10 levels in the cell supernatants were assessed. Values are fold increases of cytokine production compared to control (no peptide) (A). In addition, monocytes were exposed for 60 min to LPS, LTA, flagellin, CL087 or not, then cytoplasmic (left panel) and nuclear (right panel) fractions of the monocytes were prepared and assessed for NF-κB p65 levels (B). Values have been corrected for background. Data are expressed as boxes and whiskers: boxes represent medians and second and third interquartiles, whiskers represent range of experiments within 4–12 different donors. *$P < 0.05$ for the difference between hLF1-11-exposed and control peptide-exposed cells

**Effect of hLF1-11 on cell-surface expression by human monocytes** To further characterize the effects of hLF1-11 on human monocytes we assessed the expression of various cell-surface receptors on human monocytes. Results showed that hLF1-11 significantly enhanced expression of CD11b, CD14, CD16, CD32, CD80, CD86, CD282 (TLR2), and CD284

(TLR4), but not CD64 and CD163 in response to LPS (Table 1). hLF1-11 had no effect on cell-surface receptor expression by unstimulated monocytes (Table 1).

**Table 1** Cell-surface receptor expression by hLF1-11-exposed human monocytes

| | | No stimulus | | | LPS (100 ng/ml) | | |
|---|---|---|---|---|---|---|---|
| | | No | hLF1-11 | CP | No | hLF1-11 | CP |
| CD11b | CR3 | **92** (51-115) | **76** (47-130) | **86** (70-137) | **52** (32-125) | **87** (74-126)** | **55** (47-108) |
| CD163 | Scavenger receptor | **5** (3-8) | **5** (3-9) | **5** (3-9) | **2** (2-3) | **2** (2-4) | **2** (2-4) |
| CD64 | FCγRI | **4** (3-6) | **4** (3-4) | **4** (3-5) | **3** (3-4) | **4** (3-5) | **3** (2-4) |
| CD32 | FCγRII | **34** (13-57) | **31** (17-53) | **34** (17-46) | **53** (34-84) | **67** (37-97)*** | **52** (33-90) |
| CD16 | FCγRIII | **5** (3-7) | **8** (7-9) | **5** (4-7) | **1** (1-2) | **2** (1-3)** | **1** (1-2) |
| CD80 | B7.1 | **1** (1-2) | **1** (1-2) | **1** (1-2) | **8** (5-14) | **12** (9-23)** | **8** (5-14) |
| CD86 | B7.2 | **14** (9-21) | **16** (9-22) | **15** (10-21) | **12** (7-19) | **21** (14-28)*** | **12** (9-20) |
| CD282 | TLR2 | **10** (7-11) | **10** (6-11) | **10** (7-12) | **5** (4-7) | **8** (5-8)*** | **5** (4-7) |
| CD284 | TLR4 | **10** (6-11) | **9** (7-11) | **10** (7-11) | **5** (5-7) | **8** (7-8)*** | **6** (5-7) |
| CD14 | LPS co-receptor | **18** (10-24) | **19** (10-26 | **18** (10-28) | **16** (11-19) | **19** (17-27)** | **17** (14-18) |

Results, expressed as fold increase compared to background fluorescence, are median and range of at least five experiments. Values are significantly different (**$p < 0.01$ and ***$p < 0.001$) from control and control peptide-exposed monocytes.

## Discussion

Earlier we reported that incubation of human monocytes with hLF1-11 directs GM-CSF-driven differentiation toward a macrophage subset that displays enhanced cytokine and chemokine production and phagocytosis and clearance of *C. albicans* and *S. aureus* (8). These findings motivated us to investigate the effects of hLF1-11 on monocytes. From the results of this study we concluded that hLF1-11 primes monocytes for enhanced cytokine/chemokine production in response to TLR4, TLR5, TLR7, but not TLR2, ligands. This conclusion is based on the following findings. First, incubation of human monocytes with the hLF1-11 peptide dose dependently increased production of an array of cytokines and chemokines as well as the expression of various cell-surface receptors in response to LPS. In the absence of a stimulus hLF1-11 did not enhance the cytokine production or cell-surface receptor expression on monocytes in fact; it even lowered the chemokine production by these cells. Apparently monocytes are altered by the peptide, but activation of the cell by a microbial stimulus is needed to reveal their increased potential. Since hLF1-11-exposed cells started to produce cytokines at similar LPS levels as control cells, we concluded that hLF1-11-exposed monocytes respond with higher capacity, but not more sensitive, toward LPS than control monocytes. We cannot offer a definitive explanation for our observation that hLF1-11-exposed monocytes discriminate between on the one hand TLR4, TLR5, and TLR7-mediated responses and on the other hand TLR2-mediated

responses. Most likely hLF1-11 interacts with components of the signal transduction route that is shared by TLR4, TLR5, and TLR7, but not by TLR2. In agreement with this suggestion we found that activation and translocation of NF-κB subunits to the nucleus of monocytes upon TLR4, TLR5, and TLR7 stimulation, but not TLR2, stimulation, was enhanced in hLF1-11-exposed monocytes as compared to control monocytes. Further research is needed to clarify these results. Secondly, hLF1-11 is also able to prime murine monocytes for enhanced cytokine/chemokine production; this is in agreement with the suggestion that the immunomodulatory properties of hLF1-11 contribute to its effects against infections (12). Third, priming of human and murine monocytes by hLF1-11 is sequence-specific as the control peptide, which lacks *in vivo* activity, did not affect the cytokine responses of monocytes to TLR-stimulation. Of note, the optimal concentration of hLF1-11 in this study (100 μg/ml) is similar to the peak concentration of hLF1-11 reached in the blood of mice injected with effective doses of hLF1-11 (12). However, it should also be realized that the concentration of the peptide at the site of infection is not known. Nevertheless, others reported that a dose of 5 mg of hLF1-11 had a favorable side-effect profile for human subjects (18) and though this is lower than we used, we have no reason to assume that a higher dose would necessarily lead to undue adverse effects. Moreover, the present concentration of hLF1-11 is similar to that of other cationic antimicrobial peptides used in *in vitro* and *in vivo* experiments, e.g. IDR-1/IDR-1002 (19,20). Several findings of this study pertain to a possible explanation for these effects of hLF1-11 on monocytes. Since the N-terminus of lactoferrin is the major binding site for LPS, lipid A, and heparin (21) and complexes of lactoferrin with LPS have been found to activate macrophages through TLR4 (22), we investigated the possibility that such complexes are responsible for the enhanced production of cytokines by monocytes. However, as washing steps in between hLF1-11 and LPS and reversing the order of these factors did not affect the priming effect of hLF1-11 on human monocytes, complexes between hLF1-11 and LPS are not likely to be responsible for the enhanced cytokine production. Although the mRNA levels for IL-12p40, IL-10 and TNF-α reflected an enhanced response of hLF1-11-exposed monocytes to LPS as compared to control (peptide-exposed) monocytes, the kinetics by which these cytokines are produced were not, indicating that the mRNA half-life for cytokines is similar in hLF1-11-exposed monocytes and control monocytes. Together, no definitive explanation for the molecular basis of priming of monocytes by hLF1-11 can be offered. Therefore, our present studies focus on the interactions of hLF1-11 with its (intra-)cellular targets. Most studies on immunomodulatory effects by lactoferrin were performed using lactoferrin from bovine origin. Since bovine and human lactoferrin differ in the amino acid

composition of the N-terminus, comparison of our results with those from studies with bovine LF is not preferred. Some studies have investigated effects of hLF on cytokine and chemokine production by mononuclear leukocytes. Interestingly, Haversen et al. reported that hLF almost completely prevented the production of various cytokines, i.e., TNF-α, IL-1β, IL-6, and IL-8, by THP-1 monocytic cells upon stimulation with LPS. In addition, mRNA levels of these cytokines were reduced by hLF as well as the LPS-induced binding of NF-κB to the TNF-α promoter (23). In agreement, Crouch et al. (24) had found that lactoferrin was able to inhibit cytokine production, like TNF-α and IL-1, by activated mononuclear cells in response to LPS. Comparison of these effects by hLF and our results found with hLF1-11 suggests that domains of human lactoferrin other than its N-terminal 11 amino acids also mediate immunomodulatory effects of lactoferrin on monocytes. Scott *et al.* (19) showed that the cationic host defense peptide IDR-1 significantly enhanced cytokine and chemokine production by human monocytes and translocation of the NF-κB p50 subunit which shows similarities with results found with hLF1-11. In contrast, Mookherjee found the cathelicidin LL-37 to significantly inhibit the proinflammatory cytokine production by LPS-stimulated monocytes (25). In addition, in the monocytic cell line THP-1, NF-κB activation by LL-37 was decreased. Together, this shows how different antimicrobial peptides can have diverse effects on human monocytes. In summary, we found that hLF1-11 primes monocytes so that upon encountering microbial stimuli the cells produce enhanced levels of proinflammatory and anti-inflammatory cytokines and chemokines. The proinflammatory cytokines and chemokines, such as IL-8, TNF-α, and RANTES, will recruit and activate other immune cells, resulting in enhanced clearance of the infection. The anti-inflammatory cytokines, such as IL-10 and IL-1RA, may contribute to the protection of the surrounding tissue against the spill-over of enzymes and radicals, thus counterbalancing the overstimulation of leukocytes by proinflammatory cytokines. Together, different antimicrobial peptides exert their effects on immune cells and their diverse actions should be taken into account when considering the clinical development of these peptides for therapeutic intervention in patients.

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# Antimicrobial peptide hLF1-11 directs GM-CSF-driven monocyte differentiation toward macrophages with enhanced recognition and clearance of pathogens

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# Chapter 3

**Abstract** The human lactoferrin-derived peptide hLF1-11 displays antimicrobial activities *in vitro* and is effective against infections with antibiotic-resistant bacteria and fluconazole-resistant *Candida albicans* in animals. However, the mechanisms underlying these activities remain largely unclear. Since hLF1-11 is ineffective *in vitro* at physiological salt concentrations, we suggested modulation of the immune system as an additional mechanism of action of the peptide. We investigated whether hLF1-11 affects human monocyte-macrophage differentiation and determined the antimicrobial activities of the resulting macrophages. Monocytes were cultured for 7 days with GM-CSF in the presence of hLF1-11, control peptide, or saline for various intervals. At day 6, the cells were stimulated with lipopolysaccharide (LPS), lipoteichoic acid (LTA), or heat-killed *C. albicans* for 24 h. Thereafter, the levels of cytokines in the culture supernatants, the expression of pathogen recognition receptors, and the antimicrobial activities of these macrophages were determined. The results showed that a short exposure of monocytes to hLF1-11 during GM-CSF-driven differentiation is sufficient to direct differentiation of monocytes toward a macrophage subset characterized by both pro and anti-inflammatory cytokine production and increased responsiveness to microbial structures. Moreover, these macrophages are highly effective against *C. albicans* and *Staphylococcus aureus*. In conclusion, hLF1-11 directs GM-CSF-driven differentiation of monocytes toward macrophages with enhanced effector functions.

**Introduction**

Antimicrobial proteins/peptides have attracted attention as candidates for the development of new agents to treat infections with drug-resistant pathogens, as their modes of action differ from those of current anti-infectives (1-4). In this connection, we reported that a peptide comprising the first 11 N-terminal residues of human lactoferrin (hLF1-11) is active in (neutropenic) mice with methicillin-resistant *Staphylococcus aureus* (MRSA) (5), multi-drug resistant *Acinetobacter baumannii* (6), and invasive fluconazole-resistant *Candida albicans* infections (7). The peptide also displays antimicrobial activity against these pathogens *in vitro* (8,9,5), but only at sub physiological salt concentrations. The antimicrobial properties of hLF1-11 *in vivo* may therefore be explained by a modulatory effect of the peptide on the hosts' immune cells. hLF1-11, which has a half-life in mice of approximately 9 min (10), is effective 24 h after intravenous (i.v.) injection, and considering that the actions of macrophages are crucial in the defense against these pathogens, we hypothesized that hLF1-11 modulates monocyte-macrophage differentiation. The mononuclear phagocyte system includes blood monocytes that have differentiated from committed myeloid progenitor cells. Monocytes circulate in the blood and, upon entering tissues, undergo further differentiation to become macrophages. The phenotype and functional activities of these macrophages are controlled by local factors, such as cytokines, chemokines, and growth factors (11-14). Most macrophages in the tissues are in an anti-inflammatory state. However, upon encountering pathogens, tissue damage, or other danger signals, granulocyte-macrophage colony-stimulating factor (GM-CSF) is rapidly produced. This directs the differentiation of monocytes toward macrophages with a proinflammatory phenotype that produce large amounts of the proinflammatory cytokines interleukin 12p40 (IL-12p40) and tumor necrosis factor alpha (TNF-α) and small amounts of the anti-inflammatory cytokine IL-10 (12, 14). Monocytes and macrophages display distinct biological functions, enabling them to undertake crucial actions in the innate immune response when pathogen associated molecular patterns (PAMPs) are detected through pathogen recognition receptors, such as toll-like receptors (TLRs) and C-type lectin receptors (15, 16). These interactions lead to activation of the macrophage, which is associated with the production of cytokines, chemokines, and growth factors, attracting more immune cells to the site of infection, thereby regulating the immune response. In addition, macrophages clear an infection by binding, phagocytosis, and intracellular killing of pathogens. To further unravel the mechanisms of action of the hLF1-11 peptide, we investigated whether hLF1-11 modulates the differentiation of monocytes into macrophages and, if so, what are the functional

characteristics of these macrophages. We found that hLF1-11 directs GM-CSF-driven monocyte differentiation toward a macrophage subset that produces both pro and anti-inflammatory cytokines, demonstrates enhanced recognition of pathogenic structures, and shows increased clearance of bacteria and fungi.

**Materials and methods**

**Peptides and stimuli** The human lactoferrin-derived peptide (hLF1-11; GRRRRSVQWCA) was obtained from Peptisyntha (Torrance, CA), and the control peptide (GAARRAVQWAA) lacking *in vitro* and *in vivo* antimicrobial activity (7) was obtained from Isogen (De Meern, The Netherlands). The purity of both peptides was >97% as determined by high-performance liquid chromatography (HPLC). Stocks of the peptides were made in phosphate-buffered saline (PBS) (Department of Pharmacy, LUMC, Leiden, The Netherlands) and stored at -20°C. Endotoxin concentrations were below detection level. The following stimuli were used: lipopolysaccharide (LPS) from *Escherichia coli* (Sigma-Aldrich, Zwijndrecht, The Netherlands), ultrapure lipoteichoic acid (LTA) from *Staphylococcus aureus* (Invivogen, San Diego, CA), and heat-killed (30 min at 100°C) *Candida albicans* (strain Y01-19; Pfizer Inc., Groton, CT).

**Mononuclear phagocyte cultures** Peripheral blood mononuclear cells (PBMCs) were isolated from buffy coats of healthy donors using Ficoll-Hypaque density gradient centrifugation (ρ=1.077 g/ml). Monocytes were further purified by CD14-positive selection using magnetic microbeads (Miltenyi Biotec, Bergisch Gladbach, Germany) according to the manufacturer's protocol (>95% pure; viability, >96%). Macrophages were prepared by culturing monocytes with recombinant human GM-CSF (rhGM-CSF) according to the protocol of Verreck et al. (16). In short, monocytes were incubated for 7 days in RPMI 1640 (Gibco Invitrogen, Breda, The Netherlands) containing 10% heat-inactivated fetal bovine serum (FBSi) (Greiner Bio-one), 2 mM penicillin, 2 mM streptomycin (both PAA GmbH, Pasching, Germany), 2 mM L-glutamine (Gibco Invitrogen), and 5 ng/ml rhGM-CSF (Biosource, Camarillo, CA), referred to below as culture medium.

**Experimental setup** To investigate the effects of hLF1-11 on GM-CSF-driven monocyte-macrophage differentiation, monocytes were incubated in culture medium with hLF1-11, control peptide, or saline. On day 6, the cells were stimulated with LPS (up to 100 ng/ml), LTA (up to 1 μg/ml), or heat-killed *C. albicans* (up to $1x10^7$ CFU/ml) for 20 h; thereafter, the supernatants were collected for assessment of the production of cytokines, i.e., IL-10,

IL-12p40, and TNF-α levels, using commercially available enzyme-linked immunosorbent assay (ELISA) cytosets (Biosource) and MCP-1 using a single-plex assay (Bio-Rad Laboratories, Hercules, CA) according to the manufacturers' instructions. The surface molecule expression and antimicrobial activities of macrophages were determined on day 7 without stimulation on day 6. Where indicated, monocytes were exposed to hLF1-11 for 10 or 60 min, washed, reincubated in culture medium, and stimulated with LPS or not on day 6. hLF1-11 did not affect the viability of GM-CSF-incubated monocytes after 24 h, as determined by annexin V/propidium iodide (PI) staining.

**Flow cytometric analysis of cell surface molecule expression by macrophages** Macrophages were collected and resuspended in cold PBS with 0.2% bovine serum albumin (BSA), washed twice, and incubated with the selected fluorescently labeled monoclonal antibodies for 30 min on ice in the dark. The following monoclonal antibodies were used: phycoerythrin (PE)-conjugated antibodies against CD11b and CD163 and fluorescein isothiocyanate (FITC)-labeled antibodies against CD14 and CD206 were obtained from BD Biosciences (Heidelberg, Germany). PE-conjugated antibody against Dectin-1 was obtained from R&D Systems. FITC-conjugated antibody against CD282, and CD284 were from HyCult (Uden, The Netherlands). Cell surface molecule expression was assessed by flow cytometry using a FACSCalibur and BD CellQuest software (BD Biosciences).

**Assays for the phagocytosis and clearance of *C. albicans* by macrophages** Phagocytosis of *C. albicans* by macrophages was assessed by fluorescence activated cell sorter (FACS) analysis, as described for the phagocytosis of apoptotic cells by macrophages (17), with modifications. In short, overnight cultured *C. albicans* cells were washed twice in PBS and then labeled with 0.5 µM carboxy fluorescein succinimidyl ester (CFSE) (Invitrogen) for 30 min at 37°C in the dark, centrifuged, and resuspended in RPMI 1640 supplemented with 20% human serum (HuS). The macrophages were collected, washed twice in PBS/0.2% BSA, and labeled with PE-conjugated antibody against CD11b for 30 min (on ice in the dark). Next, the macrophages were mixed with *C. albicans* in a 1:1 ratio and incubated for various intervals at 37°C under slow rotation in the dark. The percentage of macrophages associated with *C. albicans* was assessed by determining the percentage of double-positive (CD11b+ CFSE+) macrophages by two-color flow cytometry. Furthermore, experiments were performed at 4°C to quantify the binding of *C. albicans* to the macrophages. In addition, the percentage decrease in the number of free *C. albicans* cells

over 60 min, as determined by FACS, was taken as a measure of the capacity of the macrophages to clear *C. albicans*.

**Assays for the phagocytosis and killing of *S. aureus* by macrophages** For phagocytosis of *S. aureus*, pHrodo-labeled *S. aureus* was obtained from Invitrogen and resuspended according to the manufacturer's protocol. pHrodo is a dye that is nonfluorescent at neutral pH and bright red in acidic environments (e.g., phagolysosomes). In short, equal volumes of macrophages ($1x10^5$) with 5-times-diluted pHrodo-labeled *S. aureus* stock were incubated for several intervals at 37°C or as a control at 4°C. Thereafter, the pHrodo fluorescence of the macrophages was assessed by FACS analysis. The percentage of pHrodo-positive macrophages and the median of the pHrodo fluorescence intensity of this population were obtained. In addition, equal numbers of *S. aureus* LUH2141 (LUMC, The Netherlands) and macrophages were incubated at 37°C under rotation. At several intervals, macrophages were lysed using ice-cold water with BSA (0.01%) and vortexed for 30 s. Thereafter, the lysates were serially diluted and plated onto agar plates. The next day, the viable bacteria were counted, and the percentage of killed bacteria was determined.

**Statistics** The Friedman nonparametric test, followed by Dunn's multiple comparison post test and, where indicated, by the Wilcoxon signed-rank test, was used to determine the differences between the various groups. Data are expressed as median and range. Two-sided *p* values are reported, and the level of significance was set at $p < 0.05$.

**Results**

**hLF1-11 promotes the development of macrophages with an altered cytokine profile** To find out if hLF1-11 modulates GM-CSF-driven macrophage differentiation, we first assessed the cytokine production of macrophages differentiated in the absence or presence of hLF1-11 (referred to below as hLF1-11 macrophages) in response to LPS or heat-killed *C. albicans* using a multiplex cytokine assay (data not shown). Since the production of a variety of cytokines and chemokines by hLF1-11 macrophages was enhanced, we selected for convenience three cytokines to read out pro and anti-inflammatory cytokine production. Our results showed that hLF1-11 macrophages produced significantly ($p< 0.05$) larger amounts of IL-10 (Fig. 1A), but not IL-12p40 (Fig. 1B) and TNF-α (Fig. 1C), in response to LPS than control macrophages and control peptide-macrophages did. When stimulated with LTA, the production of both IL-10 ($p< 0.05$) and

IL-12p40 ($p$< 0.001), but not TNF-α was significantly upregulated by hLF1-11 macrophages compared to control macrophages (Fig. 1D to F). In addition, hLF1-11 macrophages produced larger amounts of IL-10 ($p$< 0.01) and TNF-α ($p$< 0.001) in response to heat-killed *C. albicans* than control macrophages did, while IL-12p40 ($p$< 0.05) was upregulated only at 10 µg/ml hLF1-11 (Fig. 1G to I).

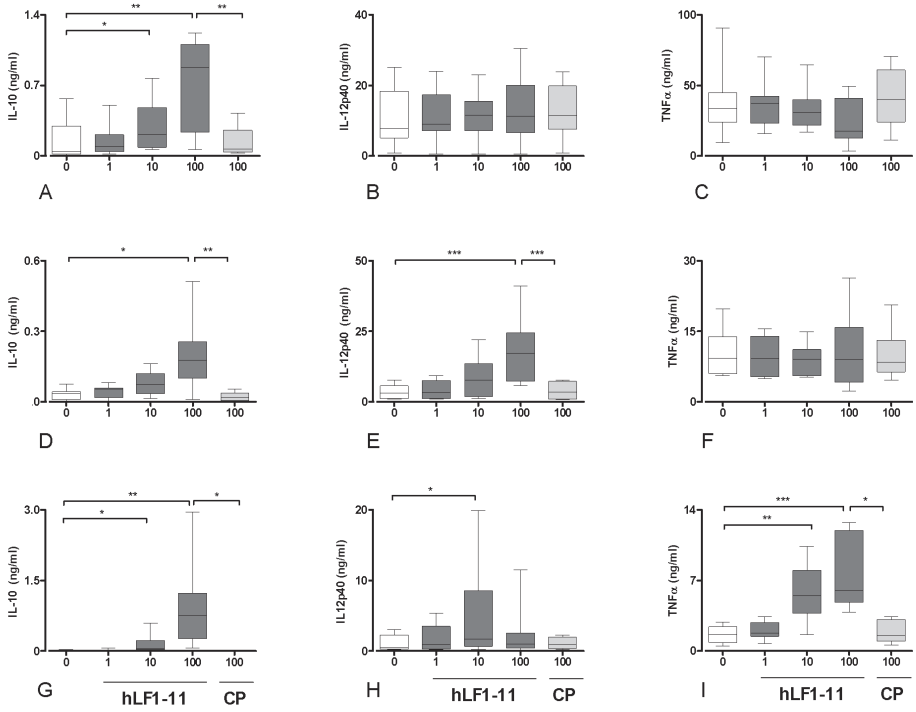


**Fig. 1 Cytokine profiles of hLF1-11-exposed GM-CSF-driven macrophages in response to LPS, LTA, and heat-killed *C. albicans*** Monocytes were cultured with rhGM-CSF in the presence of hLF1-11 (1, 10, and 100 µg/ml) (light-gray bars), control peptide (CP; 100 µg/ml) (dark-gray bars), or saline (open bars) for 6 days. Thereafter, the cells were stimulated with 100 ng/ml LPS (A to C), 1 µg/ml LTA (D to F), or 1x$10^7$ heat-killed *C. albicans* cells (G to I) for 20 h, and then the supernatants were collected and assessed for IL-10 (A, D, and G), IL-12p40 (B, E, and H), and TNF-α (C, F, and I) levels. The data are expressed as boxes and whiskers; the boxes represent medians and second and third interquartiles, and the whiskers represent the range within experiments with 8 or 9 different donors. *****$p$<0.05; ******$p$<0.01; *******$p$<0.001.

**hLF1-11 promotes the development of macrophages with enhanced recognition of various microbial stimuli** To gain insight into the responsiveness of hLF1-11 macrophages to LPS, we measured the production of IL-10 in response to increasing concentrations of LPS. Our results revealed that 0.1 ng/ml of LPS was sufficient to significantly ($p$<0.01) en-

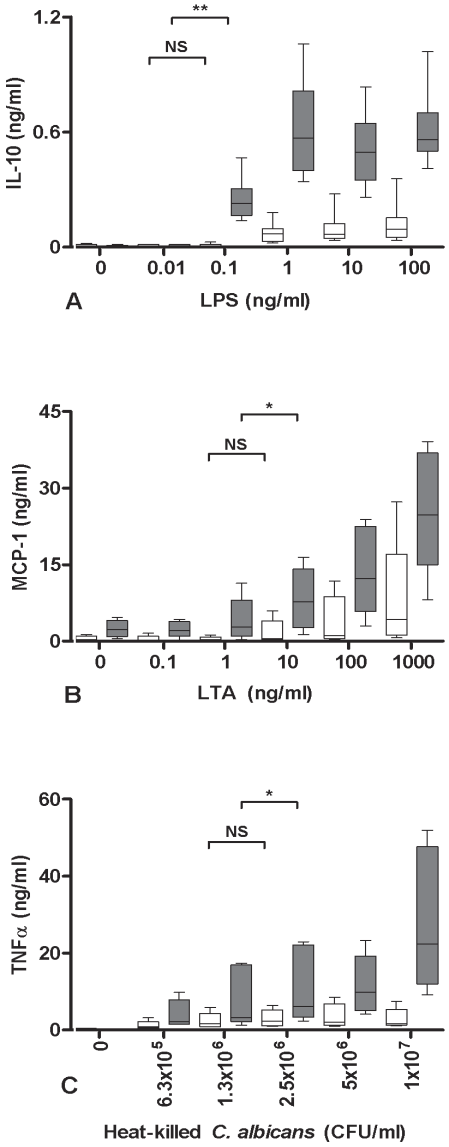


**Fig. 2 Responsiveness of hLF1-11-incubated GM-CSF-driven macrophages to LPS, LTA, or heat-killed *C. albicans*** Monocytes were incubated with rhGM-CSF and either with (gray bars) or without (open bars) hLF1-11 (100 µg/ml) and stimulated on day 6 with various concentrations of LPS (A), ultrapure LTA (B), or heat-killed *C. albicans* (C). After 20 h, the supernatants were collected and assessed for the indicated cytokine. The lowest dose of the various stimuli that induced a significant response of hLF1-11 macrophages was compared with the responses of the control macrophages at the same dose. NS, not significant. The data are expressed as boxes and whiskers; the boxes represent medians and second and third interquartiles, and the whiskers represent the range within experiments with 6 to 9 different donors. *****$p$<0.05.

hance IL-10 production by hLF1-11 macrophages, but not by control macrophages (Fig. 2A). Interestingly, expression of CD14 and TLR4, which are involved in the recognition of LPS, was significantly ($p$< 0.05) upregulated by hLF1-11 macrophages compared to control (peptide)-macrophages (Fig. 3A and B). Next, we investigated the responsiveness of hLF1-11 and control macrophages to LTA. Since macrophages hardly produce cytokines in response to lower concentrations of LTA, we chose the chemokine monocyte chemotactic protein 1 (MCP-1), based on the multiplex results, as a readout. The results revealed that upon stimulation with 10 ng/ml LTA, hLF1-11 macrophages, but not control macrophages, produced significantly ($p$< 0.05) more MCP-1 than upon stimulation with 1 ng/ml (Fig. 2B). hLF1-11 macrophages and control macrophages did not differ in expression of TLR2 (Fig. 3C). Additionally, we investigated the responsiveness of the macrophages to heat-killed *C. albicans* using TNF-α production as readout. The results showed that hLF1-11

macrophages, but not control macrophages, produced significantly ($p< 0.05$) more TNF-α when stimulated with $1x10^6$ heat-killed *C. albicans* cells (Fig. 2C). Interestingly, receptors involved in *C. albicans* recognition, such as the C-type lectin receptor Dectin-1 and the complement receptor CD11b, but not the macrophage mannose receptor CD206, were significantly ($p< 0.05$) upregulated on hLF1-11 macrophages (Fig. 3D and F). Together, these data indicate that hLF1-11 promotes the development of macrophages with increased responsiveness to different microbial stimuli.

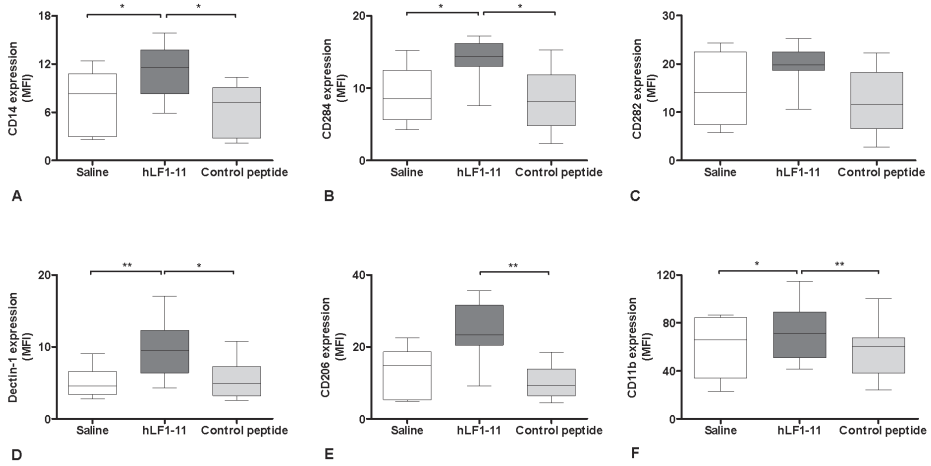


**Fig. 3 Comparison of surface molecule expression by hLF1-11 or control peptide-exposed GM-CSF-driven macrophages** Monocytes were cultured in the presence of rhGM-CSF and hLF1-11 (100 µg/ml; dark gray boxes), control peptide (100 µg/ml; light gray boxes), or no peptide (saline; open boxes). On day 7, macrophages were harvested and the expression of CD14 (A), TLR4 (CD284) (B), TLR2 (CD282) (C), Dectin-1 (D), mannose receptor CD206 (E), and complement receptor 3 CD11b (F) by these cells was assessed using flow cytometry. The results are expressed as the median fluorescence intensity corrected for background fluorescence. The boxes represent medians and second and third interquartiles, and the whiskers represent the range within experiments with 8 or 9 different donors. **p* < 0.05; ***p*< 0.01.

**hLF1-11 promotes the development of macrophages that are highly effective against *C. albicans* and *S. aureus*** Since hLF1-11 macrophages showed increased responsiveness to several pathogenic structures, we next determined whether the antimicrobial activities of hLF1-11 macrophages were also enhanced compared to control macrophages. Our results revealed that after 15 and 30 min, significantly ($p< 0.05$) more hLF1-11 macrophages than control macrophages had taken up *C. albicans*. At 60 min, no difference was observed in the percentages of hLF1-11 and control macrophages associated with *C. albicans*.

Experiments performed at 4°C revealed that less than 5% of the macrophages bound *C. albicans* at all intervals. Moreover, at 60 min, significantly ($p< 0.05$) fewer free *C. albicans* cells were present in samples containing hLF1-11 macrophages than in those containing control macrophages, indicating that hLF1-11 macrophages had cleared more *C. albicans* within 1 h than control macrophages (Table 1). In addition, we determined whether hLF1-11 macrophages displayed enhanced antimicrobial activities against *S. aureus* compared to control macrophages. The results revealed that at 60 and 120 min of incubation, the percentage of *S. aureus* phagocytosing hLF1-11 macrophages was significantly ($p< 0.05$) higher than that of control macrophages (Table 1). However, fluorescence levels within hLF1-11 macrophages and control macrophages did not differ; indicating that the phagocytosis per macrophage was not enhanced (Table 1). When these experiments were performed at 4°C, no bacterial fluorescence could be detected in macrophages. Notably, after 60 min of incubation, hLF1-11 macrophages killed *S. aureus* significantly better than control macrophages; however, after 120 min, the cells were equally effective (Table 1).

**Table 1** Antimicrobial activities of hLF1-11-macrophages

| Peptide | *C. albicans* | | | | *S. aureus* | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|
| | % Phagocytosing macrophages | | | % Cleared *C. albicans* | % Phagocytosing macrophages | | Bacteria per cell (MFI)# | | % Killing | |
| | $t$ =15 | $t$ = 30 | $t$ = 60 | $t$ = 60 | $t$ = 60 | $t$ = 120 | $t$ = 60 | $t$ = 120 | $t$ = 60 | $t$ = 120 |
| None | 13 (3-34) | 38 (11-48) | 53 (30-61) | 52 (9-77) | 35 (19–57) | 63 (27–81) | 123 (82–198) | 218 (157–256) | 9 (0–34) | 27 (7–48) |
| hLF1-11 | 12 (14-34)** | 41 (27-57)* | 54 (45-66) | 71 (49-82)* | 49 (36–81)* | 72 (56–91)* | 138 (123–200) | 231 (181–328) | 21 (14–40)* | 30 (6–51) |
| Control | 13 (2-34) | 28 (12-49) | 47 (28-60) | 56 (4–79) | 30 (24–58) | 66 (26–74) | 138 (78–205) | 250 (176–276) | 9 (0–27) | 17 (8–44) |

Results are expressed as median (range) for at least five experiments. *$p$ < 0.05 , **$p$< 0.01 is significantly different from control macrophages. # MFI, median fluorescence intensity.

**Time-dependent effect of the presence of hLF1-11 during macrophage differentiation**

Since our *in vivo* results suggested the possibility of a priming effect by hLF1-11 on mononuclear phagocytes (13), we investigated whether a short exposure of monocytes to the hLF1-11 peptide during differentiation could induce effects similar to those of the continuous presence of this peptide during culture. Our results showed that the presence of hLF1-11 for 60 min, but not 10 min, at the start of differentiation was sufficient to induce a significant ($p< 0.05$) increase in LPS-induced IL-10 production similar to cells that were exposed to hLF1-11 during the whole period of differentiation (Fig. 4A). In agreement with this, the presence of hLF1-11 for 60 min, but not 10 min during differentiation was sufficient to significantly ($p< 0.05$) enhance the phagocytosis of *C. albicans* by the resulting macrophages (Fig. 4B). These data indicate that a short exposure

of monocytes to hLF1-11 is sufficient to direct GM-CSF-driven differentiation of monocytes toward macrophages with an altered phenotype.

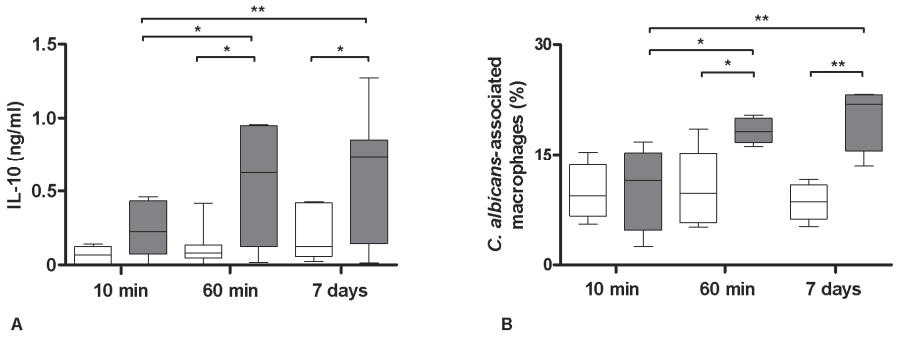


**Fig. 4 Effect of the presence of hLF1-11 on the GM-CSF-driven differentiation of monocytes into macrophages**
(A) Monocytes were cultured in the presence of rhGM-CSF. At the start of the culture, hLF1-11 was added (100 µg/ml, filled bars) for 10 min or 60 min or for constant exposure for 7 days. Saline was used as a control (open bars). On day 6, the cells were stimulated with 100 ng/ml LPS, and 20 h thereafter, the supernatants were collected and assessed for IL-10 concentrations. The results are expressed as boxes and whiskers. The boxes represent medians and second and third interquartiles, and the whiskers represent the range of experiments with 7 different donors. *****, $p < 0.05$; ******, $p < 0.01$. (B) The potential for the macrophages co-incubated for various intervals with hLF1-11 to phagocytose *C. albicans* was assayed using flow cytometry. In short, cells were harvested on day 7, labeled with antiCD11b, and co-incubated with CFSE-labeled *C. albicans* for 15 min to determine the percentage of macrophages associated with *C. albicans*. The data are medians and second and third interquartiles, and the whiskers represent the range within experiments with 7 different donors. *****, $p < 0.05$; ******, $p < 0.01$.

## Discussion

The main conclusion to be drawn from the present results is that hLF1-11 directs GM-CSF-driven monocyte differentiation toward macrophages demonstrating enhanced effector functions. This conclusion is based on the following observations. First, hLF1-11 was able to modulate the production of a variety of cytokines and chemokines by macrophages in response to three different microbial stimuli. Interestingly, IL-10 production was significantly enhanced by hLF1-11 macrophages in response to all these stimuli; proinflammatory cytokine production was either enhanced or unchanged. This indicates a general enhancement of the inflammatory response by hLF1-11 rather than a shift toward an anti-inflammatory phenotype. No definitive conclusion can be drawn as to whether the additional IL-10 production is beneficial for the control of infection (18). In this

connection, Scott et al. (19) suggested that increased IL-10 levels (induced by the IDR-1 peptide) could help control inflammation, while immune responses were enhanced. Second, hLF1-11 macrophages can detect lower concentrations of the microbial stimuli than control macrophages, suggesting that these cells respond to an infection more adequately than control cells. Third, the results of the phagocytosis and killing experiments showed that hLF1-11 directs GM-CSF-driven differentiation toward a subset of macrophages that are highly effective against *C. albicans* and *S. aureus*. The fourth main finding of this study is that the effects of hLF1-11 on monocyte-macrophage differentiation were already achieved by 60 min of exposure of the monocytes to the peptide at the start of a 7-day culture system. These actions may contribute to the anti-infective effects of the peptide against infections in mice, as reported earlier (7). However, further investigations are required to discover if this mechanism could play a role *in vivo*. The optimal concentration of hLF1-11 in this study (100 µg/ml) is within the therapeutic range of hLF1-11 used in animals. A dose of 5 mg had a favorable side-effect profile (20) and though this is lower than what we used, we do have good reasons to suppose that the present dose does not lead to undue adverse effects. Moreover, the present concentration of hLF1-11 is similar to that of other cationic antimicrobial peptides in *in vitro* experiments (e.g., IDR-1 [19]). Unfortunately as our attempts to unravel the signal transduction in monocytes after incubation with hLF1-11 have not been successful, we cannot offer a mechanistic explanation for the ability of hLF1-11 to affect human monocyte-macrophage differentiation. Interestingly, the functional properties of hLF1-11 macrophages show striking similarities to a set of responses of macrophages resulting from stimulation with foreign or nonself molecular patterns, characterized as the adaptive component of innate immunity as described by Bowdish et al. (11). This set includes upregulation of certain receptors on macrophages, resulting in a general enhancement of host immunity, such as the ability to clear bacteria and to produce cytokines. Our results indicate that the hLF1-11 peptide induces such properties during GM-CSF-driven differentiation of monocytes into macrophages, creating a subset with increased affinity for (a broader class of) pathogens, thereby possibly strengthening the innate immune response of the host to a subsequent infectious challenge. Furthermore, the present results are important for the further development of this peptide as a therapeutic agent for treatment of infections in patients with compromised immune systems.

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# LL-37 directs macrophage differentiation toward macrophages with a proinflammatory signature

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# Chapter 4

**Abstract** The human cathelicidin LL-37 has broad-spectrum antimicrobial activity. It also participates at the interface of innate and adaptive immunity by chemoattracting immune effector cells, modulating the production of a variety of inflammatory mediators by different cell types, and regulating the differentiation of monocytes into dendritic cells. In this study, we investigated the effects of LL-37 on the differentiation of human monocytes into anti-inflammatory macrophages (Mϕ-2; driven by M-CSF) versus proinflammatory macrophages (Mϕ-1; driven by GM-CSF) as well as on fully differentiated Mϕ-1 and Mϕ-2. Results revealed that monocytes cultured with M-CSF in the presence of LL-37 resulted in macrophages displaying a proinflammatory signature, namely, low expression of CD163 and little IL-10 and profound IL-12p40 production on LPS stimulation. The effects of LL-37 on M-CSF-driven macrophage differentiation were dose- and time-dependent with maximal effects observed at 10 mg/ml when the peptide was present from the start of the cultures. The peptide enhanced the GM-CSF–driven macrophage differentiation. Exposure of fully differentiated Mϕ-2 to LL-37 for 6 d resulted in macrophages that produced less IL-10 and more IL-12p40 on LPS stimulation than control Mϕ-2. In contrast, LL-37 had no effect on fully differentiated Mϕ-1. Peptide mapping using a set of 16 overlapping 22-mer peptides covering the complete LL-37 sequence revealed that the C-terminal portion of LL-37 is responsible for directing macrophage differentiation. Our results furthermore indicate that the effects of LL-37 on macrophage differentiation required internalization of the peptide. Together, we conclude that LL-37 directs macrophage differentiation toward macrophages with a proinflammatory signature.

**Introduction**

Macrophages (and dendritic cells [DCs]) in tissues continuously monitor their microenvironment for invading pathogens and other danger signals. On pathogen encounter, they trigger the migration of neutrophils and monocytes into the affected site and regulate an adequate innate immune response. In this regulatory process, macrophages may serve a dual purpose. Initially, they contribute to the elimination of pathogens and the elicitation of an inflammatory reaction. When the infection recedes due to removal of pathogens and cell debris, however, their function may shift toward resolution of inflammation and tissue repair (1). Macrophages can thus exhibit pro and anti-inflammatory properties to a degree that is determined by stimuli from their local microenvironment, such as pathogens, inflammatory mediators as well as other factors. In line with this notion, two clearly distinct types of human macrophages, designated as Mϕ-1, namely, macrophages with a proinflammatory signature, and Mϕ-2, which are macrophages having an anti-inflammatory/proangiogenic signature, have been derived from cultured human blood monocytes by incubation with GM-CSF and M-CSF, respectively (2). Mϕ-1s are fried egg-shaped macrophages that profoundly produce IL-12p40 and little IL-10 on stimulation by LPS and support Th cell type 1 (Th1) responses. Mϕ-2 display a stretched, spindle-like morphology and are characterized by the marked production of IL-10 and little IL-12p40 on LPS stimulation (2), poor Ag-presenting capacities, and promotion of T regulatory cell responses (3). A hallmark of Mϕ-2 is the cell-surface expression of CD163, the scavenger receptor involved in the clearance of free hemoglobin from the circulation (4) and supposedly functionally associated with anti-inflammatory processes (5). Cationic antimicrobial peptides, such as cathelicidins (6), play important roles in the defense against infections by eliminating a wide range of pathogens (7, 8). hCAP-18/LL-37 is the only cathelicidin identified in humans and it is produced by neutrophils, monocytes, mast cells, and epithelial cells. It is stored in these cells as a propeptide, which can be cleaved extracellular by enzymes like proteinase 3, resulting in the formation of LL-37 and a cathelin part. LL-37 is an amphipathic α-helical peptide that can affect both planktonic bacteria and those residing in biofilms (9, 10), viruses such as HIV (11) and fungi (12), and it can neutralize LPS and lipoteichoic acid (LTA) (13). In addition to its antimicrobial actions, LL-37 participates at the interface of innate and adaptive immunity by modulating cytokine and chemokine production by a range of cell types, chemoattracting various immune effector cells (14) and mesenchymal stem cells (15), regulating autophagy in conjunction with vitamin D (16), and stimulating angiogenesis and wound healing (17). Others reported that LL-37 enhances the GM-

CSF/IL-4–driven differentiation of blood monocytes to immature DCs (18), whereas inhibiting the maturation of immature DCs by TLR ligands (19). Furthermore, this peptide enhances the responses of monocytes (and macrophages) to GM-CSF and IL-1β (20, 21), but suppresses those to IFN-γ (22), indicating that LL-37 affects the responses of mononuclear phagocytes to cytokines differentially. All effects of LL-37 on human cells may be mediated through specific cell-surface receptors (14, 23–26) or intracellular receptors (27). The effects of LL-37 on macrophage differentiation are not known. As LL-37 can be abundantly present at sites of inflammation/infection (28–30) and monocyte–macrophage differentiation depends on factors from the local microenvironment, in this study, we investigated the effects of LL-37 on the differentiation of monocytes to pro and anti-inflammatory macrophages.

**Materials and Methods**

**LL-37 and derived peptides** Human LL-37, control scrambled peptide (LG-37), as well as the set of 16 overlapping 22mer peptides covering the complete amino acid sequence of LL-37 were prepared by solid phase synthesis on an automated peptide synthesizer (SyroII, Multisyntech, Witten, Germany) as described (31). The sequences of the various peptides are summarized in Table I. The purity of the various peptides was at least 87%, as determined by reverse-phase high performance chromatography, and their molecular mass was confirmed by Maldi-Tof mass spectrometry. Stocks (50 mg/ml) of the peptides prepared in DMSO were kept at -80°C until use.

**Isolation of human monocytes** PBMCs from healthy donors were isolated from buffy coats (Sanquin Blood Bank, Leiden, The Netherlands) by Ficoll Amidotrizoate (ρ = 1.077 g/ml; Department of Clinical Pharmacy and Toxicology, Leiden University Medical Center, Leiden, The Netherlands) density centrifugation at 700 x g for 20 min. Cells in the interphase were washed three times with PBS (pH 7.4), and monocytes were isolated using antiCD14–coated MACS microbeads (Miltenyi Biotec, Bergisch Gladbach, Germany) according to manufacturer's instructions. Next, monocytes (puritiy >96%, viability >98% as determined by annexin V and PI staining 2 h after isolation) were centrifuged and resuspended in RPMI 1640 supplemented with 2 mM glutamine, 2 mM penicillin and streptomycin, and 10% (weight/volume) heat-inactivated FCS (all from Life Technologies, Invitrogen, Breda, The Netherlands), further referred to as standard medium.

**Macrophages** Mϕ-1 were obtained by culturing 1 x $10^6$ monocytes/ml in 75 $cm^2$ Cell-Star tissue culture flask (Greiner Bio-One, Frickenhausen, Germany) or 1 x $10^6$/ml in 12-well tissue culture plates (Costar, Corning B.V. Life Sciences, Schiphol-Rijk, The Netherlands) in standard medium containing 10 ng/ml rhGM-CSF (Invitrogen, Carlsbad, CA) for 6 d in a 5% $CO_2$ incubator at 37°C. To generate Mϕ-2, 50 ng/ml rhM-CSF (R&D Systems, Abingdon, U.K.) was added to standard medium instead of rhGM-CSF (2). At day 3 of culture and at day 6, Mϕ-1 and Mϕ-2 were either harvested with trypsin/EDTA and used in the experiments or washed and stimulated with LPS from *Escherichia coli* (1–100 ng/ml; Sigma-Aldrich, St. Louis, MO), a sonicate of *Mycobacterium tuberculosis* [lysate of heat-inactivated *M. tuberculosis* H37Rv; 5 μg/ml, (32)], LTA (1 μg/ml; Invivogen, San Diego, CA), zymosan A from *Saccharomyces cerevisiae* (ZymA; 10 μg/ml; Sigma-Aldrich), or PAM3CSK4 (PAM3; 100 ng/ml; Invitrogen) for an additional 24 h and then harvested. Where indicated the modulating effect of LL-37(-derived peptides) and the scrambled peptide LG-37 on macrophage differentiation was studied by addition of the peptides to the culture medium at various intervals during the cell culture.

**Measurement of cell-surface molecule expression by macrophages** To verify the differentiation of monocytes into Mϕ-1 or Mϕ-2, the expression of various cell-surface markers by macrophages was assessed using PE-conjugated mAbs directed against CD163 (BD BioSciences, Heidelberg, Germany) and FITC- or PE-conjugated mAbs directed against CD1a, CD14, CD80, and CD86 (BD Pharmingen). Cultured macrophages were incubated with these mAbs diluted in PBS containing 0.2% (w/v) BSA for 30 min on ice. Samples were measured on a FACSCalibur (Becton and Dickinson, La Jolla, CA) and analyzed with CellQuest Pro 4.0.2 software.

**Measurement of cytokine production by macrophages** IL-10 and IL-12p40 levels in the supernatants of the cell cultures were assessed using CytoSets ELISA kits (Invitrogen) according to manufacturer's instructions. The lower limits of detection in these ELISA were 5 pg/ml.

**Detection of cell viability** Monocytes were cultured in standard medium supplemented with rh(G)M-CSF and 50 μg/ml LL-37 or LG-37, equimolar concentrations of LL-37-derived peptides, or vehicle (diluted DMSO) for 24 h and then harvested. Thereafter, the cells were stained with 1 μg/ml FITC-annexinV (Sigma-Aldrich) and 1 μg/ml propidium iodide

(PI; Sigma-Aldrich) in 10 mM HEPES (pH 7.4) and the mean fluorescence intensity (MFI) determined on the FACSCalibur. Results are the percentage of PI-negative monocytes.

**Involvement of cell-surface receptors in the effects of LL-37 on macrophage differentiation** Inhibitors/activators of the G-protein–coupled fMLP receptor FMLR-1, the epidermal growth factor receptor (EGFR), and the ATP-gated purinergic receptor P2X7 were used to determine whether one of these three cell-surface receptors mediated the effects of LL-37 on M-CSF-driven macrophage differentiation. For this purpose, the FPRL-1 agonist WKYMV(DMet)-NH2 (10 μM; Phoenix Pharmaceuticals, Belmont, CA) and the FPRL-1 antagonist WRWWWW-NH2 (10 μM; Phoenix Pharmaceuticals) were used. The possible involvement of Gi-proteins in the modulation of M-CSF–driven macrophage differentiation by LL-37 was determined in mononuclear phagocytes preincubated with 1–10 μg/ml pertussis toxin from *Bordetella pertussis* (Sigma-Aldrich) for 30 min at 37°C. The EGFR tyrosine kinase-inhibitor AG1478 [4-(3-chloroanilino)-6,7-dimethomethyl-benzylamine; Merck Biosciences, Nottingham, U.K.] at a concentration of 1 μM and the nonselective P2X7-antagonist suramin (naphthalene sulfonic acid derivate; Sigma-Aldrich) at a concentration of 30 μM were used to investigate whether EGFR or the P2X7 on monocytes mediates the modulatory effects of LL-37 on the M-CSF–driven macrophage differentiation.

**Involvement of intracellular uptake of LL-37 in its effects on macrophage differentiation** To investigate whether LL-37 needs to be taken up by monocytes for its effects on the M-CSF–driven macrophage differentiation, monocytes were preincubated for 30 min with 5 μg/ml cytochalasin-D (Sigma-Aldrich, stock 5 mg/ml DMSO). Next, the cells were washed to remove unbound cytochalasin-D and thereafter the cells were incubated for 6–7 d in M-CSF–containing medium supplemented with 10 μg/ml LL-37 or not.

**Statistical analysis** Statistical analyses were performed by Wilcoxon's matched pairs test or Friedman repeated measures test, as appropriate, to determine the difference between groups using GraphPad Prism 4.0 (San Diego, CA) software. Differences were considered significant when $p < 0.05$.

**Results**

**Effects of LL-37 on the differentiation of monocytes to Mϕ-1 and Mϕ-2** To determine the effects of LL-37 on the differentiation of monocytes to Mϕ-2 or Mϕ-1, we incubated

monocytes for 6 d with M-CSF or GM-CSF in the presence of either LL-37, the scrambled peptide LG-37 (Table I), or no peptide. Thereafter, we studied the morphology and quantified the expression of the cell-surface molecules CD163 and CD14 as well as the LPS-stimulated production of IL-10 and IL-12p40 by the resulting macrophages. Light microscopy revealed Mϕ-2 to display an elongated, spindle-like morphology, whereas the presence of LL-37, but not LG-37, during differentiation by M-CSF led to fried egg-shaped macrophages, which is the typical morphology of Mϕ-1 (Fig. 1A). In line with this, the presence of LL-37 during M-CSF–driven macrophage differentiation led to macrophages expressing significantly less CD163 and CD14 than control macrophages did (Fig. 1B). Furthermore, macrophages differentiated by M-CSF in the presence of LL-37 produced significantly less IL-10 and more IL-12p40 on LPS stimulation than macrophages differentiated by M-CSF in the presence of LG-37 (results not shown) or no peptide (Fig. 2A); without LPS stimulation no effect of LL-37 on the production of these cytokines was seen (Table II). These effects were already observed with 5 μg/ml LL-37 and maximal at ≥10 mg/ml LL-37 (Figs. 1A, 1B, 2A). The most profound effects of LL-37 on the cytokine profile of the resulting macrophages were observed when the peptide was present from the start of the monocyte cultures with M-CSF; addition of LL-37 at later intervals during the 6 d culture of monocytes with M-CSF resulted in macrophages displaying a less distinct proinflammatory cytokine profile (Fig. 2B). Of note, inwell crystal violet staining indicated comparable numbers of macrophages in the different wells within experiments (results not shown).

**Table 1** LL-37 derived synthetic peptides used in the study

| Peptide | $NH_2$ Sequence COOH |
|---|---|
| LL-37 | LLGDFFRKSKEKIGKEFKRIVQRIKDFLRNLVPRTES |
| LG-37 | LGFRSEIKFRVRKFRLPTSLDFKKKGEKIQIDLNVRE |
| 1 | LLGDFFRKSKEKIGKEFKRIVQ |
| 2 | LGDFFRKSKEKIGKEFKRIVQR |
| 3 | GDFFRKSKEKIGKEFKRIVQRI |
| 4 | DFFRKSKEKIGKEFKRIVQRIK |
| 5 | FFRKSKEKIGKEFKRIVQRIKD |
| 6 | FRKSKEKIGKEFKRIVQRIKDF |
| 7 | RKSKEKIGKEFKRIVQRIKDFL |
| 8 | KSKEKIGKEFKRIVQRIKDFLR |
| 9 | SKEKIGKEFKRIVQRIKDFLRN |
| 10 | KEKIGKEFKRIVQRIKDFLRNL |
| 11 | EKIGKEFKRIVQRIKDFLRNLV |
| 12 | KIGKEFKRIVQRIKDFLRNLVP |
| 13 | IGKEFKRIVQRIKDFLRNLVPR |
| 14 | GKEFKRIVQRIKDFLRNLVPRT |
| 15 | KEFKRIVQRIKDFLRNLVPRTE |
| 16 | EFKRIVQRIKDFLRNLVPRTES |

**Cytokine production by LL-37–differentiated macrophages in response to various bacterial stimuli** To determine whether the modulatory effect of LL-37 on the M-CSF–driven macrophage differentiation with respect to the cytokine profile was restricted to LPS/TLR4, we used other bacterial stimuli, such as *M. tuberculosis*, LTA, PAM3, and ZymA.

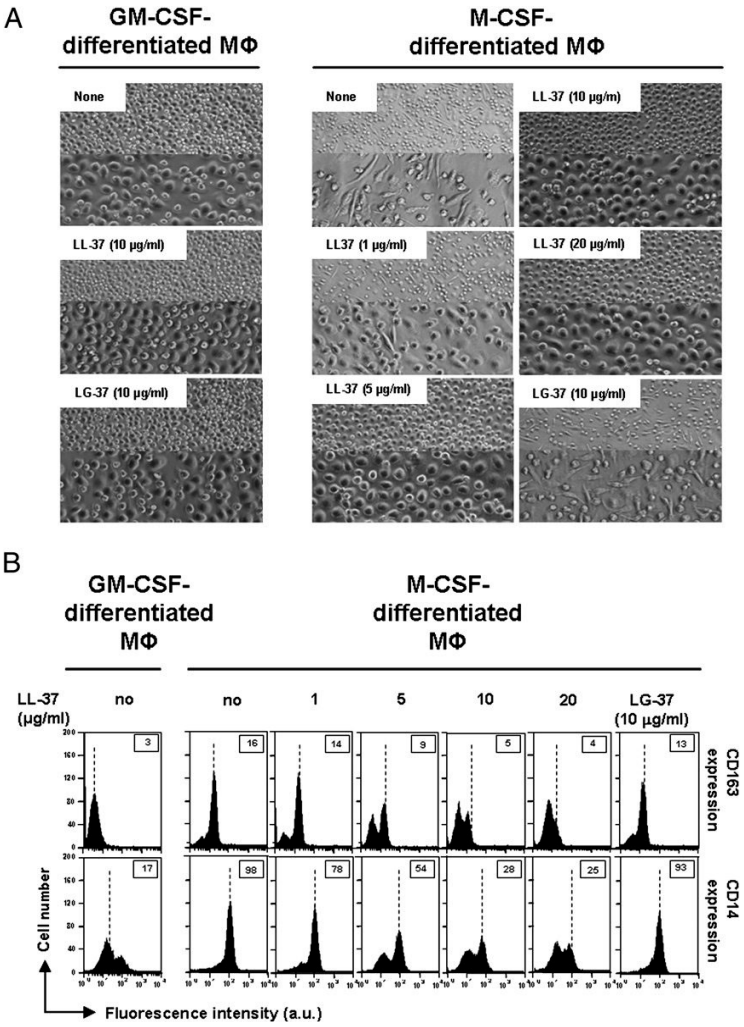
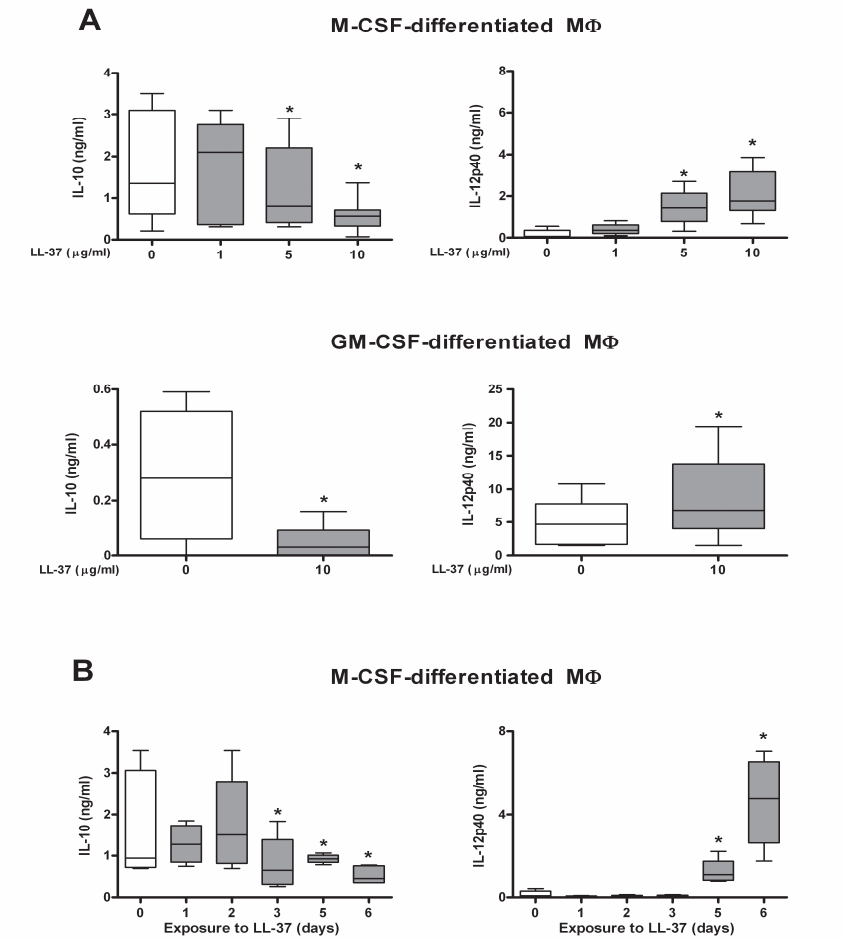



**Fig. 1 Effects of LL-37 on the M-CSF–driven and GM-CSF–driven monocyte–macrophage differentiation regarding morphology and cell-surface molecule expression** Monocytes were cultured for 6 d in medium containing GM-CSF (to generate Mϕ-1) or M-CSF (to generate Mϕ-2) in the presence of LL-37, the scrambled peptide (LG-37), or no peptide (none), and then either subjected to microscopic analysis of the morphology or harvested for assessment of CD163 and CD14 expression. Representative phase contrast microscopic images revealing the effects of LL-37 on the morphology of the resulting macrophages after 6 d of culture (A). Magnification 310 (upper half of image) and 320 (bottom half of image). FACS profiles of a representative experiment (of five individual experiments) demonstrating the LL-37-induced changes in membrane expression of CD163 and CD14 by macrophages at day 6 of the culture of monocytes with M-CSF (B). The vertical dotted lines represent the MFI. Boxed numbers represent the MFI (expressed in arbitrary units) of the analyzed cells. Mean background fluorescence of cells incubated with an isotype-matched control mAb ranged from two to four arbitrary units.

The results revealed that exposure of macrophages differentiated by M-CSF in the presence of LL-37 to these stimuli resulted in macrophages displaying significantly decreased IL-10 production (*M. tuberculosis* lysate and LPS [i.e., stimuli signaling through TLR4 and TLR2] but not the stimuli signaling through TLR2, TLR2/1, and TLR2/6) and more IL-12p40 production than macrophages differentiated by M-CSF alone (Table II). Interestingly, LL-37 did not influence the GM-CSF–driven macrophage differentiation as judged by cell morphology (Fig. 1A) and expression of CD163 and CD14 (results not shown) by the resulting macrophages. However, these LL-37–exposed macrophages produced significantly less IL-10, and more IL-12p40, on stimulation with the various stimuli than control macrophages did (Fig. 2A, Table II).



◀**Fig. 2 Effects of LL-37 on the M-CSF–driven and GM-CSF–driven monocyte–macrophage differentiation regarding LPS-stimulated IL-10 and IL-12p40 production** Monocytes were cultured for 6 d in medium containing M-CSF (to generate Mϕ-2) or GM-CSF (to generate Mϕ-1) in the presence of LL-37 (gray boxes) or no peptide (white boxes). At day 6, these cultures were stimulated for 24 h with 100 ng/ml LPS and the levels for IL-10 and IL-12p40 in the culture supernatants were measured by ELISA (A). Box plots display the median, the second and third interquartiles, and the lowest and highest cytokine concentrations. n = 5 independent experiments. *p < 0.05. Monocytes were cultured for 6 d with M-CSF together—for the indicated intervals—with LL-37. At day 6, macrophages were washed and then stimulated with LPS for 24 h. Thereafter, levels of IL-10 and IL-12p40 in the culture supernatants were measured by ELISA (B). Box plots display the median, the second and third interquartile, and the lowest and highest cytokine concentrations. *n* = 5 independent experiments. *p < 0.05.

**Table 2** Cytokine production by GM-CSF– and M-CSF–differentiated macrophages in response to different microbial stimuli

| | | | Cytokine production (pg/ml) by M-CSF -differentiated macrophages | | Cytokine production (pg/ml) by GM-CSF -differentiated macrophages | |
|---|---|---|---|---|---|---|
| Peptide | Stimulus | *n* | IL-10 | IL-12p40 | IL-10 | IL-12p40 |
| None | None | 11 | **0** (0-61) | **5** (0-14) | **0** (0-2) | **3** (0-21) |
| LL-37 | None | 11 | **3** (0-47) | **4** (0-109) | **0** (0-7) | **0** (0-22) |
| None | *M. tuberculosis* | 7 | **1254** (492-5403) | **41** (0-570) | **293** (19-2609) | **4950** (2409-13835) |
| LL-37 | *M. tuberculosis* | 7 | **397*** (291-560) | **690*** (144-1679) | **117*** (0-699) | **5760** (99-18449) |
| None | LTA | 5 | **478** (297-504) | **36** (0-41) | **79** (31-126) | **1856** (486-4561) |
| LL-37 | LTA | 5 | **186*** (76-533) | **633*** (476-886) | **0*** (0-38) | **1649** (445-5194) |
| None | $PAM_3$ | 5 | **153** (15-271) | **130** (0-294) | **66** (14-226) | **1960** (1102-2563) |
| LL-37 | $PAM_3$ | 5 | **142** (57-366) | **1185*** (299-3449) | **8*** (0-38) | **1620** (1095-2256) |
| None | ZymA | 7 | **102** (0-1409) | **12** (0-334) | **32** (0-832) | **610** (19-4139) |
| LL-37 | ZymA | 7 | **104** (0-321) | **36** (0-113) | **4*** (0-237) | **307*** (6-4835) |
| None | LPS | 11 | **1848** (450-3839) | **67** (0-542) | **282** (0-587) | **4740** (1489-10801) |
| LL-37 | LPS | 11 | **561*** (74-2969) | **2133*** (669-9228) | **29*** (0-156) | **4878** (1478-19384) |

Monocytes were cultured for 6 d in medium containing M-CSF or GM-CSF in the presence of 10 μg/ml LL-37 or no peptide (none). At day 6, the cells were incubated with medium supplemented with 5 μg/ml *M. tuberculosis* lysate, 1 μg/ml LTA, 100 ng/ml $PAM_3$, 10 μg/ml ZymA, 100 ng/ml LPS, or no stimulus (none) for 24 h. The concentrations of the indicated cytokines in the supernatants were quantified by ELISA. Values are medians and range. *n* = number of different donors. *p < 0.05, compared with similar macrophages not incubated with LL-37 but exposed to the same stimulus.

**LL-37 modulates fully differentiated Mϕ-2** To investigate the ability of LL-37 to redirect fully differentiated macrophages we added this peptide (10 μg/ml) or scrambled peptide LG-37 to fully differentiated Mϕ-2 and Mϕ-1 and maintained the cells for an additional 6 d in culture medium. At the end of this second culture period, macrophages were washed and then stimulated for 24 h with LPS. Results revealed that culturing of fully differentiated Mϕ-2 with LL-37 for 6 d resulted in macrophages that produced significantly less IL-10 and more IL-12p40 on LPS stimulation than Mϕ-2 cultured with scrambled

peptide or no peptide (Fig. 3). In contrast, culturing of fully differentiated Mϕ-1 with LL-37 did not affect IL-10 and IL-12p40 production by the resulting macrophages in response to LPS (Fig. 3).

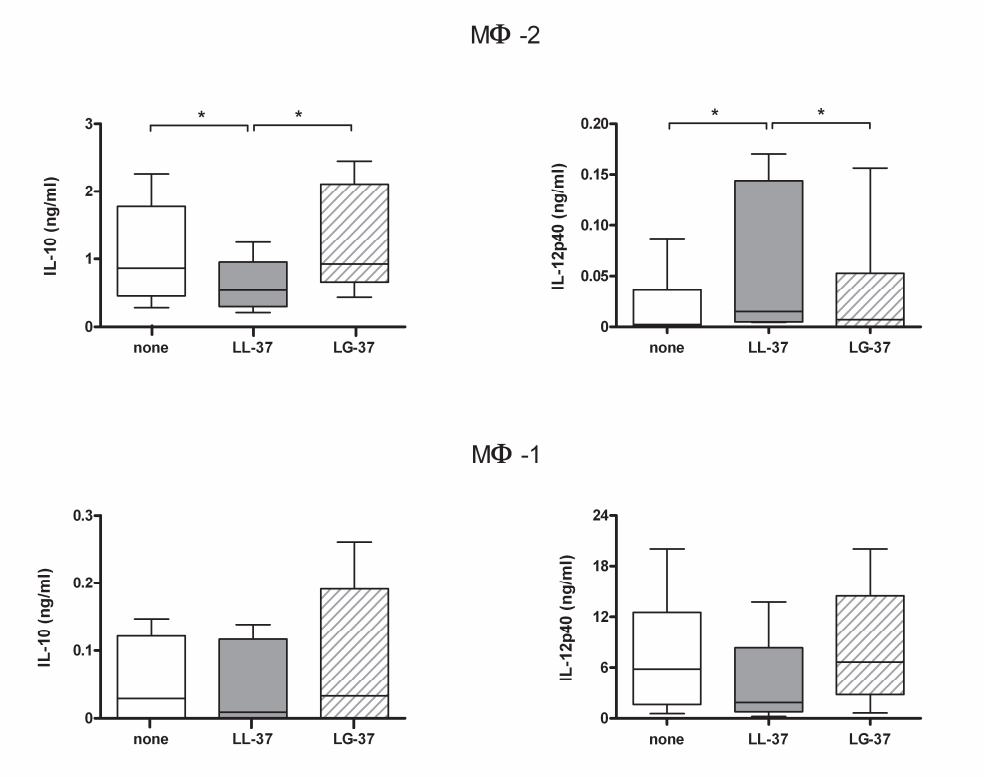



**Fig. 3 Effects of LL-37 on fully differentiated macrophages** Fully differentiated Mϕ-2 and Mϕ-1 were cultured for an additional 6 d in medium supplemented with LL-37 (10 µg/ml; gray boxes), LG-37 (10 µg/ml; hatched boxes), or no peptide (white boxes) and then stimulated with LPS (100 ng/ml) for 24 h. Thereafter, the levels of the indicated cytokines in the culture supernatants were assessed by ELISA. Box plots display the median, the second and third interquartile, and the lowest and highest cytokine concentrations. *n* = 7–8 independent experiments. *p < 0.05.

**Effects of LL-37–derived peptides on the M-CSF–driven macrophage differentiation** To determine which domain of LL-37 is responsible for the effects on M-CSF–driven macrophage differentiation, we analyzed the effects of a set of 16 overlapping peptides covering the entire amino acid sequence of LL-37 (~6 µg/ml; equimolar concentration to 10 µg/ml LL-37) on the morphological and immunological (CD163/CD14 expression and LPS-stimulated IL-12p40 production) characteristics of the resulting macrophages. Light microscopy revealed macrophages differentiated under the influence of M-CSF in the

presence of the C-terminal peptides 10–16, but not the other peptides, to display the typical morphology of Mϕ-1 (results not shown). In agreement, the presence of the C-terminal peptides 10–16, but not the other peptides, during incubation of monocytes with M-CSF led to low/undetectable expression levels of CD163 (Fig. 4A). In line with this observation, macrophages differentiated in the presence of these peptides produced significantly more IL-12p40 on LPS than Mϕ-2 did (Fig. 4B). Analyses of the dose-effect relations for these C-terminal peptides indicated that peptides 11 and 12 were the most effective peptides in modulating M-CSF–driven macrophage differentiation (results not shown).

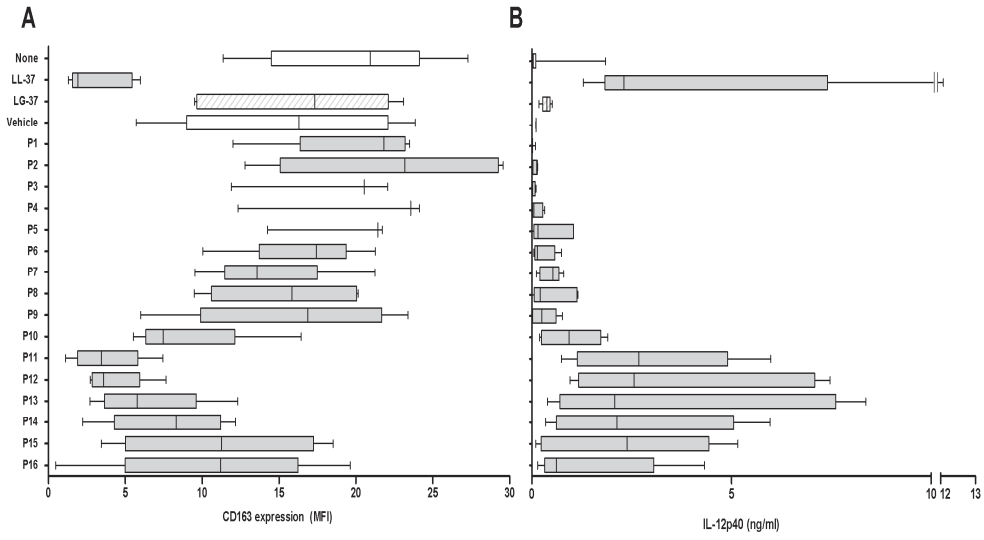



**Fig. 4 Effects of LL-37-derived peptides on the M-CSF–driven macrophage differentiation.** Monocytes were cultured for 6 d in medium containing M-CSF in the presence of LL-37–derived peptides (peptides 1–16; 6.25 μg/ml; gray boxes) or as controls LL-37 (10 μg/ml; gray boxes), LG-37 (10 μg/ml; hatched boxes), or no peptide (none; white boxes). At day 6, the macrophages were harvested and either incubated with a fluorescently labeled mAb against CD163 and FACS for assessment of the expression of this cell-surface marker (A) or reincubated for an additional 24 h in medium supplemented with LPS (100 ng/ml) for assessment of the IL-12p40 production by these cells (B). Box plots display the median, the second and third interquartile, and the lowest and highest CD163 or IL-12p40 values. *n* = 6–7 independent experiments, except peptides 3–5 where *n* = 3 independent experiments. *$p < 0.05$.

**Involvement of receptors for LL-37 in its modulatory effects on the M-CSF–driven macrophage differentiation** Next, we determined the effects of selected activators and/or inhibitors of known cell-surface receptors of LL-37 on its effects on the M-CSF–driven

macrophage differentiation using CD163 expression and IL-12p40 production as read-outs. Our results revealed that the FPRL1 agonist WKYMV(D-Met)-NH2 did not mimic LL-37 in modulating the M-CSF–driven macrophage differentiation nor did preincubation of the monocytes with the FPRL1- antagonist WRWWWW-NH2 affect the activities of LL-37 on macrophage differentiation (results not shown). In addition, preincubation of monocytes with pertussis toxin also did not affect the LL-37 activity. Furthermore, the nonselective P2X7-antagonist suramin as well as the EGFR tyrosin kinase inhibitor AG1478 failed to suppress the effects of LL-37 on M-CSF–driven macrophage differentiation (results not shown). In contrast, the LL-37–induced morphological changes (results not shown), decreased CD163/CD14 expression and increased IL-12p40 production by LPS-stimulated, M-CSF–differentiated macrophages were completely abolished by a 30 min preincubation of the monocytes with the endocytosis-inhibitor cytochalasin-D (Fig. 5).

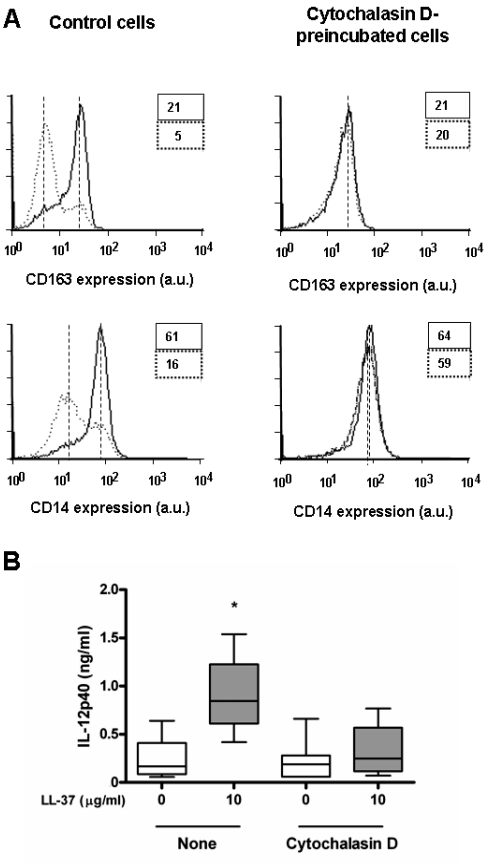



**Fig. 5 Effect of preincubation of the monocytes with cytochalasin-D on the modulation of the M-CSF–driven macrophage differentiation by LL-37** Monocytes were preincubated with cytochalasin- D for 30 min or left untreated, then washed and incubated in medium containing M-CSF and LL-37 (or as control no peptide) for 6 d. Thereafter, the macrophages were harvested and exposed to fluorescently labeled mAbs against CD163 and CD14 and assessed by FACS the expression of these cell surface markers (A). Overlays show the expression of CD163 and CD14 by cytochalasin-D–preincubated and control macrophages that were cultured in the presence of LL-37 (dotted lines) or not (solid lines). Boxed numbers represent the MFI (expressed in arbitrary units) of the cells. The vertical dotted lines represent the MFI. Data are from a representative experiment of five independent experiments. LPS-stimulated IL-12p40 production by macrophages resulting from a 6 d culture of cytochalasin-D–preincubated and control monocytes in medium with M-CSF and LL-37 (gray boxes) or not (white boxes) (B). Box plots display the median, the second and third interquartile, and the lowest and highest IL-12p40 concentrations. *n*=7 independent experiments. *$p < 0.05$.

**LL-37 does not affect monocyte viability** Monocytes were cultured in medium containing M-CSF supplemented with LL-37 (or LG-37 or no peptide) for 24 h and thereafter stained with annexin V-FITC and PI. Subsequent FACS analysis revealed that the viability of monocytes amounted to 61 ± 3% (*n* = 3) and that of monocytes treated with LL-37 to 72 ± 4% (*n* = 3) and monocytes exposed to LG-37 to 65 ± 4% (*n* = 3), indicating that LL-37 at the present concentrations is not toxic for monocytes. In addition, no effect of LL-37 on the viability of monocytes exposed to 10 ng GM-CSF/ml and 50 ng M-CSF/ml was observed (results not shown).

**Discussion**

The main conclusion from the current study is that LL-37 directs M-CSF–driven monocyte–macrophage differentiation toward macrophages with a proinflammatory signature and redirects fully differentiated Mϕ-2. In agreement with reports by others that LL-37 can enhance the effects of GM-CSF (18, 21), we found LL-37 to enforce the GM-CSF–driven macrophage differentiation. Our conclusion is based on the following findings. First, addition of LL-37 to 6 d cultures of monocytes with M-CSF resulted in macrophages with a proinflammatory instead of an anti-inflammatory signature. Our observation that LL-37 added at the start of the monocyte cultures is more effective than when added on the second or subsequent days may indicate that LL-37 either affects monocytes more effectively than macrophages or it exerts its modulatory effects only when continuously present. The actions of LL-37 on macrophage differentiation regarding the cytokine profile were independent of the stimulus used for inducing cytokine production. This was found for all stimuli used, except ZymA and $PAM_3$, which may be explained by the poor response of the macrophages to these two stimuli using IL-10 and IL-12p40 as read-outs. Furthermore, it should be noted that the effects of LL-37 on M-CSF–driven macrophage differentiation (and fully differentiated Mϕ-2) are sequence-specific as the scrambled peptide LG-37 was without effect. Secondly, culturing of fully differentiated Mϕ-2 for 6 d with LL-37 resulted in macrophages producing more IL-12p40 and less IL-10 on LPS stimulation than control Mϕ-2 did. In agreement, LL-37 promoted the development of macrophages with a fried egg-shaped appearance from fully differentiated Mϕ-2, although the peptide did not affect the CD163 expression by these macrophages. It should be realized that the doses of LL-37 used in our study (1–20 μg/ml) are within the physiological range, namely, levels of LL-37 at sites of infection, such as tracheal aspirates of newborn infants, with an airway infection [LL-37 levels ranging between 5 and 15 μg/ml; (28)] and psoriasis skin lesions [up to 1.5 mg of LL-37/ml; (29)]. The second

conclusion to be drawn from our findings is that the C-terminal part of LL-37 harbors its ability to modulate M-CSF–driven macrophage differentiation, because of all tested LL-37–derived peptides only those comprising the C-terminal part were effective. Dose response experiments revealed that the peptides covering residues 11–32 and 12–33 were the most effective in modulating macrophage differentiation. Secondary structure prediction (33) indicated that these peptides comprise the optimal amphipathic helix among the present LL-37–derived peptides examined in this study. Preliminary experiments revealed that these C-terminal peptides are more effective than LL-37 in killing of *Staphylococcus aureus* as detected by radial diffusion assays (T. Vos, A. van der Does, B. Ravensbergen, H. Beekhuizen, and P. Nibbering, unpublished results). In agreement, is has been reported that the LL-37–derived peptides covering residues 13–35 are at least as effective as LL-37 with respect to LPS neutralization (31), modulation of TLR-mediated responses (34), and inducing secondary necrosis of apoptotic neutrophils (35). These structure-function studies can be helpful in the design of new candidate peptides for the treatment of infections. Experiments with a selection of inhibitors revealed that the cell-surface receptors involved in LL-37 signaling, such as FPLR1, P2X7 and EGFR, did not mediate the effects of this peptide on the M-CSF–driven macrophage differentiation. The main disadvantages of these inhibitors concern the instability of some of them, for example, AG1478 and suramin, and their specificity. However, we used stock solutions that were adequately prepared and stored for only a limited interval to exclude possible instability problems. In previous studies, these precautions allowed us to demonstrate an inhibitory effect of AG1478 on TGF-α-induced activation of airway smooth muscle cells and an inhibitory effect of suramin on the LL-37–induced activation of these cells (36). Nevertheless, based on our results with inhibitors we cannot rule out a possible involvement of cell-surface receptors in the effects of LL-37 on M-CSF–driven macrophage differentiation. In this connection, experiments with cytochalasin-D-preincubated monocytes revealed that LL-37 needs to be internalized by monocytes to affect M-CSF–driven macrophage differentiation. The possibility that cytochalasin-D has toxic effects on monocytes and therefore affects the M-CSF–driven macrophage differentiation is unlikely as the viability of the macrophages resulting from cytochalasin-D preincubated monocytes and control monocytes did not differ. In addition, we noted that cytochalasin-D pretreated and control monocytes differentiated to type-2 macrophages equally well, for example, these macrophages express equal levels of CD163 and IL-12p40 production on LPS stimulation. The intracellular target of LL-37 in human monocytes could be GAPDH, which was recently shown to be also essential for the LL-37–induced activation of p38 MAPK

signaling in monocytes (27). Others reported that the LL-37–induced activation of p38 MAPK signaling in monocytes is enhanced in the presence of GM-CSF, but not M-CSF (21). Furthermore, LL-37 internalized by DCs was found to be responsible for the altered morphological and functional characteristics of these cells (37). Finally, in view of the observed effects of LL-37 alone or in synergy with other mediators on cytokine and chemokine secretion of monocytes and macrophages (13, 20, 21), it cannot be excluded that LL-37 directs macrophage differentiation via its effects on cytokine actions/production. What could be the relevance of the current findings? M-CSF is constitutively produced by a variety of cells and circulates at detectable levels under steady-state conditions in serum and extracellular space (1). Most macrophage populations in the tissues are exposed to levels of tissue-derived M-CSF (38, 39) that are sufficient to maintain them in a Mϕ-2–like state. However, on encountering infectious agents, tissue damage, or other danger signals, the M-CSF–driven macrophage differentiation may be counteracted by the local production of inflammatory mediators, such as GM-CSF (reviewed in Ref. 1) and LL-37 (28, 29) derived from infiltrating neutrophils and stimulated epithelial cells, resulting in macrophages with a proinflammatory signature. These macrophages may be effective in recruiting and activating other leukocytes resulting in the enhanced clearance of the infection. For example, LL-37 can activate macrophages to produce inflammatory cytokines in response to microbial stimuli and induce efficient phagocytosis of IgG-opsonized bacteria (40). The peptide can modulate the differentiation of monocytes to DCs that promote the development of a Th subset expressing high levels of IFN-γ and low/undetectable levels of IL-4/IL-5 (18), although maturation of the LL-37–differentiated DCs by several TLR ligands may be suppressed (19). In addition, it can enhance plasmacytoid DCs to produce IFN-α in response to self-DNA (41).When the infection recedes due to removal of pathogens and cellular debris by phagocytes, the levels of LL-37/GM-CSF in the local environment subside to homeostatic levels that facilitate differentiation of monocytes to macrophages with the “default” anti-inflammatory signature again. These cells suppress the inflammatory responses (42, 43) and induce regulatory T cells (3) and mediate tissue repair (44). Taken together, we demonstrated that LL-37 can (re)direct macrophage differentiation toward macrophages with a proinflammatory profile. These macrophages play important roles in the clearance of infections and tissue homeostasis.

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# The human lactoferrin-derived antimicrobial peptide hLF1-11 drives monocyte-dendritic cell differentiation toward dendritic cells that promote antifungal responses and induce Th17 polarization

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# Chapter 5

**Abstract** The hLF1-11 peptide comprising the first 11 N-terminal residues of human lactoferrin, exerts antimicrobial activity *in vivo*, enhances the inflammatory response of monocytes, and directs monocyte-macrophage differentiation toward cells with enhanced antimicrobial properties. Since its effects on mononuclear phagocytes are already seen after short incubation of monocytes with hLF1-11, we hypothesized that the differentiation of these cells toward dendritic cells (DCs) may also be affected by hLF1-11. We therefore determined the effects of hLF1-11 and a control peptide on human monocyte-dendritic cell differentiation and subsequent T-cell activation. The DCs thus obtained were assessed for their antimicrobial activity against the yeast *C. albicans*. Also, cytokine and reactive oxygen species production in response to this yeast was quantified. Results revealed that -compared to control DCs- hLF1-11-differentiated DCs displayed enhanced antimicrobial activity against *C. albicans* and produced enhanced amounts of IL-6 and IL-10 upon stimulation with *C. albicans*, whereas IL-12p40 production was reduced. Moreover, 6 day-cultured hLF1-11-differentiated DCs and control (peptide-differentiated) DCs were stimulated with a memory mix for 24 h and co-cultured with autologous CD4+ T cells for 72 h; hereafter cytokine production in supernatants as well as in T cells was assessed. The hLF1-11-differentiated DCs induced an enhanced IL-17 but reduced IFN-γ production by T cells as compared to control DCs. Collectively, the hLF1-11 peptide drives monocyte-dendritic cell differentiation toward DCs that promote antifungal responses and induce Th17 polarization. The ability of hLF1-11 to modulate the hosts' immune response may be key to its potential for treatment of infections with (multi-drug resistant) pathogens.

**Introduction**

In tissues dendritic cells (DCs) have the capacity to recognize and process pathogens and following their migration to the lymph nodes, present pathogen-derived antigens to T cells thereby activating an adaptive immune response. Dendritic cells can differentiate from a precursor cell depending on factors present in the local microenvironment. For example, when monocytes upon entering tissue encounter inflammatory mediators like cytokines, chemokines, complement components and antimicrobial peptides (AMPs), they differentiate into immature dendritic cells (iDC). These cells are known for abundant expression of pathogen recognition receptors, efficient antigen uptake and processing capacities. Upon tissue injury, microbial infection or other danger signals, iDCs mature into antigen presenting cells that produce inflammatory mediators, migrate toward lymphoid organs and instruct T lymphocytes to proliferate and differentiate into specific T cell subsets. This polarization of T cells depends greatly on the DCs and the inflammatory mediators present in their environment. Important molecules that influence differentiation of monocytes and functional properties of immune cells are antimicrobial peptides (AMPs). AMPs are cationic, relatively short and are active against a variety of microorganisms including multi-drug resistant pathogens (1). The principal mechanism of action of antimicrobial peptides was long thought to be perturbation of the microbial cell membrane. However, an increasing number of studies shows the diversity in the mechanisms of action of these peptides that have therefore been renamed as host defense peptides (HDPs) (2, 3). These mechanisms of action include direct killing of pathogens (4, 5), modulation of pathogen development (6, 7) and modulation of immune cells (8-10), the latter is now increasingly recognized as being an important contribution to clearance of infection. For possible therapeutic application of HDPs, it will be important to understand the interactions of these peptides with the hosts' immune cells. For example, cathelicidin-based peptides IDR-1 (11) and IDR1002 (12) have been developed on the basis of their ability to affect the host immune system by enhancement of chemokine production by innate immune cells. The cathelicidin LL-37 is able to direct both monocyte-macrophage differentiation (13) as well as differentiation toward dendritic cells that promote a Th1 response *in vitro* (14) through interaction with an intracellular target (15). LL-37 is also able to modulate the adaptive immune response by directly affecting the maturation of DCs (16). We recently reported that the antimicrobial peptide comprising the first 11 N-terminal residues of human lactoferrin, further referred to as hLF1-11, enhances the inflammatory response of monocytes and modulates monocyte-macrophage differentiation; an additional mechanism of action to its already established

antimicrobial effects (5,7,17-20). hLF1-11 enhances inflammatory mediator production by murine and human monocytes (21) and promotes differentiation of GM-CSF-stimulated monocytes toward a macrophage subset that shows enhanced responsiveness to microbial stimuli and demonstrates increased clearance of pathogens (8). These effects were already obtained after incubation of monocytes with hLF1-11 for 60 min, indicating that hLF1-11 can modulate monocytes at an early stage, resulting in long-term alterations. Since monocytes can also differentiate toward (immature) dendritic cells, we have here investigated the effects of hLF1-11 on monocyte-dendritic cell differentiation. We found that when hLF1-11 was present during GM-CSF and IL-4-driven differentiation of monocytes toward dendritic cells, the resulting immature DCs displayed enhanced antimicrobial properties against *C. albicans* and -upon maturation- induced IL-17 production by T cells while reducing IFN-γ production.

**Materials and methods**

**Peptides** The human lactoferrin-derived peptide hLF1-11 (GRRRRSVQWCA; 1.374 kDa) was purchased from Peptisyntha (Torrance, CA) and the control peptide (GAARRAVQWAA; 1.115 kDa) from Isogen (De Meern, The Netherlands). The control peptide shows no activity against pathogens *in vitro* and *in vivo* (7). The purity of both peptides was >97% as determined by reverse-phase high-performance liquid chromatography (HPLC). Stocks of the peptides were made in phosphate-buffered saline (PBS, Dept. of Pharmacy LUMC, Leiden, The Netherlands) and stored at -20˚C. Endotoxin concentrations were below detection level.

**Cell culture** Peripheral blood mononuclear cells (PBMCs) were isolated from buffy coats of healthy donors using Ficoll-Amidotrizoate density gradient centrifugation. Monocytes were further purified by CD14-positive selection using antiCD14-conjugated magnetic microbeads (Miltenyi Biotec, Bergisch Gladbach, Germany) according to manufacturers' protocol. Next, monocytes (>95% pure, viability >96% as determined by propidium iodide staining) were incubated for 7 days in culture medium (RPMI 1640, GIBCO Invitrogen, Breda, The Netherlands) containing 10% heat-inactivated fetal bovine serum (FCSi, Greiner Bio-One B.V., Alphen a/d Rijn, The Netherlands), 2 mM penicillin, 2 mM streptomycin (both PAA GmbH, Pasching, Germany) and 2 mM L-glutamine (GIBCO Invitrogen) supplemented with 10 ng/ml recombinant human granulocyte macrophage-colony stimulating factor (rhGM-CSF) and 10 ng/ml recombinant human IL-4 (both Biosource,

Camarillo, CA). To study the effect of hLF1-11 on differentiation of monocytes into iDCs, at the start of the culture monocytes were exposed to various concentrations of either, hLF1-11 (up to 100 μg/ml) or control peptide (100 μg/ml) or saline as a control. On day 6, the immature DCs were matured with heat-killed (30 min at 100 °C) *Candida albicans* ($1x10^7$ CFU/ml strain Y01-19, Pfizer Inc., Groton, CT) for 20 h. Thereafter supernatants were harvested for assessment of cytokine (and chemokine) levels, or immature and mature DCs were harvested at day 7 and used for analysis of their capacity to take up dextran, phagocytose *S. aureus* and *C. albicans*, produce reactive oxygen species (ROS), and polarize CD4+ T cell differentiation in co-culture experiments.

**Flow cytometric analysis of cell-surface molecule expression by dendritic cells** For measurements of the expression of a variety of cell-surface molecules, the following monoclonal antibodies were used: PE-conjugated antibodies directed against CD11b, CD40, CD54, CD80, CD83 and CD86 and FITC-labeled antibodies against HLA-DR, CD14, CD206 and CD209 were all obtained from BD Biosciences (Heidelberg, Germany). PE-conjugated antibody against Dectin-1 was purchased from R&D Systems and Alexa Fluor 647-conjugated antibody against CD197 from BD Biosciences. DCs were harvested and resuspended in ice cold 0.2% PBS/BSA, washed twice and then incubated with the selected antibodies for 30 min on ice in the dark. Cell-surface molecule expression was assessed on a FACSCalibur and analysed by BD CellQuest software (BD Biosciences). Results are expressed as MFI corrected for background measurements.

**Flow cytometric analysis of dextran-FITC uptake by dendritic cells** The endocytic property of the various iDCs was examined by incubation of the cells with 1 mg/ml FITC-labeled dextran (Invitrogen) for 2 h at 37°C in culture medium. Background measurements were performed at 4°C. After washing, dextran-FITC fluorescence was assessed using a FACSCalibur and analysed by BD CellQuest software (BD Biosciences). Results are expressed as median fluorescence intensity (MFI) corrected for background measurements.

**Assays for the phagocytosis of *S. aureus* and *C. albicans* by immature dendritic cells** Phagocytosis of pHrodo-labeled *S. aureus* was performed as described for macrophages (8). Briefly, a stock suspension of pHrodo-labeled *S. aureus* (Invitrogen) was prepared according to manufacturer's protocol. pHrodo is a dye that is non-fluorescent at neutral pH and bright red in acidic environments (e.g. phagolysosome). Equal volumes of iDCs

($1x10^6$/ml) and 5-times pre-diluted pHrodo-labeled *S. aureus* stock were mixed and then incubated for several intervals at 37˚C, or as a control, at 4˚C. Thereafter, pHrodo fluorescence of the iDCs was assessed on a FACSCalibur. Results are expressed as the percentage of pHrodo-positive iDCs.

Phagocytosis of *C. albicans* by iDCs was assessed by FACS analysis as described for macrophages (8). In short, overnight cultured *C. albicans* were washed twice in PBS and then labeled with 0.5 µM carboxy fluorescein succinimidyl ester (CFSE, Invitrogen) for 30 min at 37°C in the dark, centrifuged and resuspended in RPMI 1640 supplemented with 20% human serum (HuS). iDCs were washed twice in PBS/0.2% bovine serum albumin (BSA) and then labeled with PE-conjugated antibody against CD54 for 30 min (on ice in the dark). Next, these labeled iDCs were mixed with CFSE-labeled *C. albicans* in a 1:1 ratio and incubated for various intervals at 37°C under slow rotation in the dark. The percentage of iDCs associated with *C. albicans* was assessed by determining the percentage of double positive iDCs (CD54+/CFSE+) using a FACSCalibur and analysed by BD CellQuest software. Control experiments were performed at 4°C to correct for binding of *C. albicans* to iDCs. Results are expressed as percentage iDCs that were positive for both CD54 and CFSE.

**Flow cytometric analysis of ROS production by dendritic cells** Intracellular ROS production by iDCs was quantified using the fluorescent probe 2',7'-dichlorofluorescein-diacetate (DCFH-DA) (Invitrogen). In short, iDCs were loaded with 10 µM DCFH-DA for 20 min at 37˚C in the dark. Thereafter, iDCs were incubated with $1x10^7$ heat-killed *C. albicans* for various intervals. ROS production was measured on a FACSCalibur. Results are expressed as median fluorescence intensity (MFI) with interquartile range.

**Determination of cytokine levels by ELISA** Enzyme-linked immunosorbent assay (ELISA) cytosets were used to determine the concentrations of interleukin (IL)-6, IL-10, IL-12p40 and TNF-α (Invitrogen) according to manufacturers' instructions.

**T cell isolation and DC-T cell co-cultures** CD4+ T cells (>95% purity) were obtained from the CD14-negative fraction after monocyte isolation (stored at -80˚C) by CD4-positive selection using anti-CD4-conjugated magnetic microbeads (Miltenyi Biotec) according to manufacturers' protocol. They were resuspended in IMDM (Lonza, Verviers, Belgium) containing 10% heat-inactivated fetal bovine serum (FBSi, Gibco), antibiotics and 2 mM L-glutamine and left overnight to recover. Immature DCs were reseeded in a 96-wells plate ($1x10^5$/well). Three hours thereafter cells were stimulated with a mix of LPS (100 ng/ml;

Sigma-Aldrich, Zwijndrecht, The Netherlands), purified protein derivative of *Mycobacterium tuberculosis* (PPD, Statens Serum Institute, Copenhagen, Denmark; 5 µg/ml), tetanus toxoid (TT; 1% of the stock, 80 IE/ml) and heat-killed *C. albicans* (1 x $10^6$/ml) for 24 h. Thereafter, DC's were co-cultured with 1 x $10^6$ autologous T cells for 72 h in the presence of TT (1% of the stock, 80 IE/ml) and supernatants were collected and assessed for IL-17, IL-10, IFN-γ, IL-2 and IL-4 levels using a custom-made multiplex bead-array (Bio-Rad, Hercules, CA), according to manufacturers' protocol. For intracellular cytokine staining, cells in the co-culture were incubated with Brefeldin A (BFA, 3 µg/ml, Sigma) during the last 18 hours of co-culture, replicates (n = 10) pooled and T-cells were labeled extracellular using anti-CD3-AMCyan, anti-CD4-PECy7 and anti-CD25-FITC (all BD Biosciences) antibodies. Next, cells were fixed and intracellular labeled using Intrastain reagents (DakoCytomation, Heverlee, Belgium) with antibodies directed against IL-17-PE, (Ebioscience, San Diego, CA), IFN-γ-Alexa700 (BD Biosciences) and IL-10-APC (Miltenyi Biotec) before acquisition on a LSRII flowcytometer (BD Biosciences) and analysed using FlowJo software version 8.7.3 (Tree Star Inc, Ashland, Oregon). The core population of live $CD3^+$ T cells was analyzed for IL-17 production, while activated and $CD3^{dim}$ T cells were also included in IFN-γ production analysis.

**Statistical analysis** Friedman followed by Dunn's multiple comparisons post-hoc test or, where indicated, Wilcoxon's test was used to determine the differences between the results for hLF1-11-differentiated and control (peptide-differentiated) DCs. Data are expressed as median and range. Two sided p-values are reported and the level of significance was set at $p< 0.05$.

## Results

**Morphology and cell-surface molecule expression by hLF1-11-differentiated and control (peptide-differentiated) iDCs and mature DCs** First, we inspected the morphology of the dendritic cells that had been differentiated in the presence of hLF1-11 or the control peptide. The morphology, density and attachment of the cells to the wells did not differ between hLF1-11 and control (peptide-differentiated) DCs. Next, we compared cell-surface molecule expression between hLF1-11-differentiated and control (peptide-differentiated) iDCs. Results revealed that hLF1-11-differentiated iDCs expressed significantly higher levels of CD14, CD80, HLA-DR and Dectin-1, a receptor involved in *C. albicans* recognition, on their cell-surface as compared to control (peptide-differentiated) iDCs (Table I). Upon maturation by *C. albicans*, the expression of the maturation marker CD83 as well as the co-stimulatory molecule CD86 by hLF1-11-differentiated DCs was significantly decreased as compared to control (peptide-differentiated) DCs (Table I).

**Table 1** Cell-surface molecule expression by hLF1-11 and control (peptide-differentiated) DCs

| | | Immature DCs | | | *C. albicans*-matured DCs | | |
|---|---|---|---|---|---|---|---|
| | | Control | hLF1-11 | Control peptide | Control | hLF1-11 | Control peptide |
| CD14 | LPS co-receptor | **2** (1-3) | **4*** (2-7) | **2** (1-3) | **3** (2-7) | **2** (1-6) | **2** (0-6) |
| CD83 | Maturation marker | **1** (0-4) | **2** (0-3) | **1** (0-3) | **32** (21-54) | **22*** (12-42) | **36** (26-74) |
| CD197 | CCR7 | **8** (5-17) | **12** (7-19) | **10** (7-16) | **29** (26-34) | **27** (21-42) | **32** (24-38) |
| CD54 | ICAM-1 | **199** (123-241) | **138** (92-245) | **183** (109-256) | **881** (512-1190) | **1025** (500-1434) | **850** (356-1155) |
| CD40 | Co-stimulatory | **52** (30-81) | **57** (31-89) | **56** (20-68) | **86** (52-127) | **77** (42-127) | **70** (26-104) |
| CD80 | B7.1 | **5** (0-9) | **7*** (0-19) | **4** (0-7) | **32** (24-83) | **29** (18-90) | **30** (29-99) |
| CD86 | B7.2 | **14** (0-26) | **7** (2-48) | **11** (4-25) | **407** (233-459) | **247*** (217-375) | **382** (317-466) |
| HLA-DR | MHC class II | **46** (24-100) | **63*** (36-252) | **45** (21-73) | **170** (97-399) | **138** (86-517) | **147** (68-365) |
| CD11b | CRIII | **294** (131-382) | **261** (104-299) | **288** (201-379) | **278** (183-320) | **224** (189-304) | **269** (226-396) |
| CD206 | Mannose receptor | **45** (32-71) | **33** (22-152) | **54** (27-75) | **30** (17-45) | **31** (16-44) | **33** (19-53) |
| CD209 | DC-SIGN | **44** (29-66) | **44** (19-74) | **42** (23-54) | **41** (23-49) | **41** (26-51) | **33** (23-50) |
| Dectin-1 | β-glucan receptor | **13** (9-23) | **19*** (13-39) | **12** (7-19) | **20** (10-25) | **19** (11-28) | **21** (14-34) |

Results are expressed as median fluorescence intensity (MFI) and corrected for background fluorescence. Data are expressed as median and range within experiments with at least six different donors. *, $p < 0.05$

**Endocytic and phagocytic properties of hLF1-11-differentiated and control (peptide-differentiated) DCs** To investigate whether hLF1-11-differentiated DCs also displayed differences in their functional activities, we compared endocytic and phagocytic capacities of iDCs differentiated in the presence of hLF1-11 with those of control (peptide-differentiated) iDCs. No significant difference in dextran uptake between hLF1-11-

differentiated iDCs and control (peptide-differentiated) iDCs was observed (Fig. 1A). In addition, phagocytosis of *S. aureus* by hLF1-11-differentiated iDCs did not differ from that by control (peptide-differentiated) iDCs (Fig. 1B). However, the percentage of *C. albicans*-phagocytosing hLF1-11-differentiated iDCs was significantly higher than that of control (peptide-differentiated) iDCs (Fig. 1C). Control experiments at 4˚C revealed that approximately 10% of the iDCs bound *C. albicans* at all intervals, independent of the presence of hLF1-11 or control peptide during differentiation (data not shown).

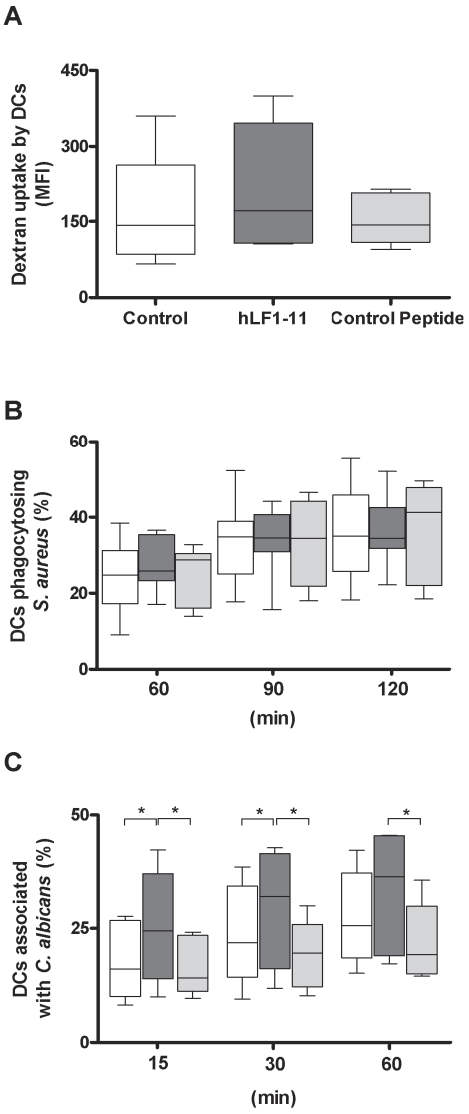


**Fig. 1 Endocytosis of dextran-FITC and uptake of *C. albicans* and *S. aureus* by hLF1-11- or control (peptide-differentiated) immature dendritic cells**
Monocytes were cultured with rhGM-CSF and IL-4 in the presence of hLF1-11 (100 µg/ml, dark gray bars), control peptide (100 µg/ml, light gray bars) or no peptide (open bars). At day 7, iDCs were harvested, washed and cultured for 2 h in the presence of dextran-FITC, thereafter the amount of dextran uptake by these cells was assessed using flow cytometry (A). Furthermore, after 60, 90 or 120 min co-incubation of the iDCs with pHrodo-labeled *S. aureus*, the percentage of pHrodo-positive iDCs was determined using flow cytometry (B). Lastly, iDCs were co-incubated with CFSE-labeled *C. albicans* and after 15, 30 and 60 min co-incubation, the percentage of iDCs associated with *C. albicans* was determined using flow cytometry (C). Boxes represent medians and second and third interquartiles, whiskers represent range within experiments with 6-10 different donors. *, $p<0.05$

**ROS production by hLF1-11-differentiated and control (peptide-differentiated) iDCs in response to *C. albicans*** Since hLF1-11-differentiated iDCs displayed enhanced phagocytosis of *C. albicans*, we considered the possibility that more antimicrobial functions of these cells were enhanced in response to *C. albicans*. We therefore determined the production of reactive oxygen species by the various iDCs in response to this yeast. Results revealed that hLF1-11-differentiated iDCs produced significantly more ROS 30 and 60 min after stimulation with *C. albicans* than control (peptide-differentiated) iDCs (Fig. 2). ROS levels decreased 90 min after stimulation with *C. albicans*, however hLF1-11-differentiated DCs still produced at that point significantly more ROS than control peptide-differentiated DCs.

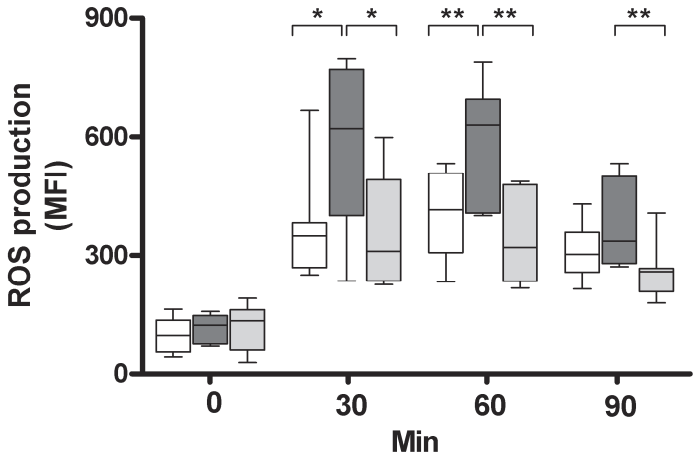


**Fig. 2 Intracellular ROS production by hLF1-11 and control (peptide-differentiated) immature dendritic cells** Monocytes were cultured with rhGM-CSF and IL-4 in the presence of hLF1-11 (100 µg/ml, dark gray bars), control peptide (100 µg/ml, light gray bars) or no peptide (open bars) and labeled at day 7 with DCFH-DA. Next, the labeled iDCs were stimulated with heat-killed *C. albicans* and the MFI was assessed directly and after 30, 60 and 90 min as a measure of ROS production. Boxes represent medians and second and third interquartiles, whiskers represent range within experiments with 8 different donors. *, $p<0.05$; ** $p<0.01$

**Differential cytokine production patterns in hLF1-11-differentiated DCs and control (peptide-differentiated) DCs** Another functional property of DCs is the production of inflammatory mediators. We therefore assessed pro- and anti-inflammatory cytokine production by the various DC groups in response to *C. albicans*. Results showed that heat-killed *C. albicans*-matured hLF1-11-differentiated DCs produced significantly more IL-6 and IL-10 and less IL-12p40 than control (peptide-differentiated) DCs (Fig. 3). The production of TNF-α did not differ between the various groups of DCs (Fig. 3).

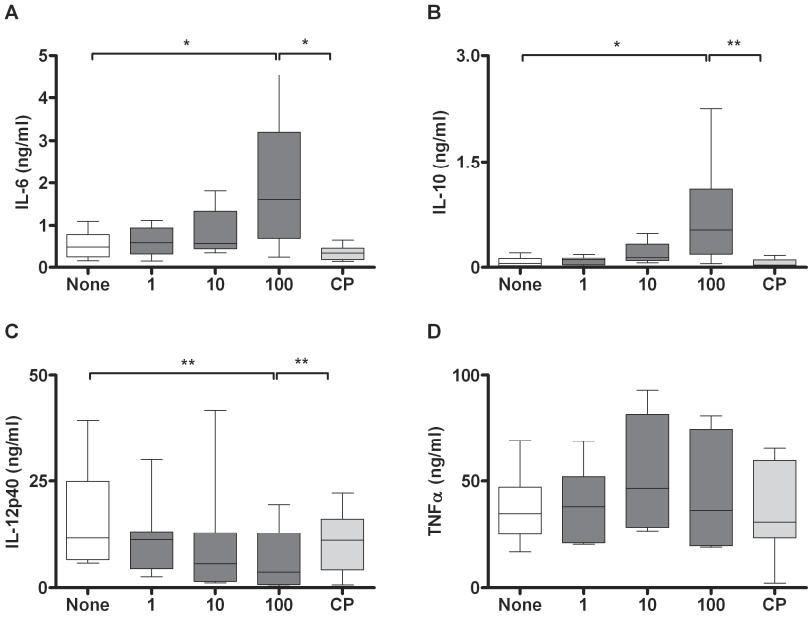


**Fig. 3 Cytokine profiles of hLF1-11- and control (peptide-differentiated) dendritic cells in response to heat-killed *C. albicans*** Monocytes were cultured with rhGM-CSF and IL-4 in the presence of hLF1-11 (1, 10, 100 µg/ml; dark gray bars) or control peptide (CP, 100 µg/ml; light gray bars) for 6 days. Thereafter, cells were stimulated with $1x10^7$ heat-killed *C. albicans* for 20 h and then supernatants were collected and assessed for IL-6 (A), IL-10 (B), IL-12p40 (C) and TNF-α (D) levels. Values are expressed as fold increase of cytokine production compared to control DCs (none, no peptide; open bars). Data are expressed as boxes and whiskers, boxes represent medians and second and third interquartiles, whiskers represent range within experiments with 7-8 different donors. *, $p<0.05$; **, $p<0.01$ as compared to control and control peptide-differentiated DCs

**Polarization of CD4+ T cells by hLF1-11-differentiated or control (peptide-differentiated) DCs** Since the innate activities of hLF1-11-differentiated DCs differed from that of control (peptide-differentiated) DCs, we compared their T cell activating capacities. First, the supernatants of the co-cultures of hLF1-11-differentiated DCs -matured by a memory mix- with autologous CD4+ T cells, were found to contain significantly more IL-17 ($p<0.05$) and more IL-10 ($p=0.06$), but less IFN-γ (Fig 4A) than co-cultures with control (peptide-differentiated) DCs. In these cultures, IL-2 was out of range and no IL-4 was detected (data not shown). Next we determined the percentage of T-cells expressing the various cytokines (IL-17, IL-10 and IFN-γ) by performing intracellular cytokine staining. Results revealed that enhanced percentages of IL-17-producing T cells and reduced percentages of IFN-γ producing T cells were found in the co-cultures with hLF1-11-differentiated DCs as compared to control (peptide-differentiated) DCs (Fig. 4B). IL-10 producing T cells were present at very low frequencies and no difference in percentages of IL-10-producing T cells

were found between the different groups (Fig. 4B), suggesting that IL-10 in the supernatants of the co-culture (Fig 4A) is mainly derived from the DCs.

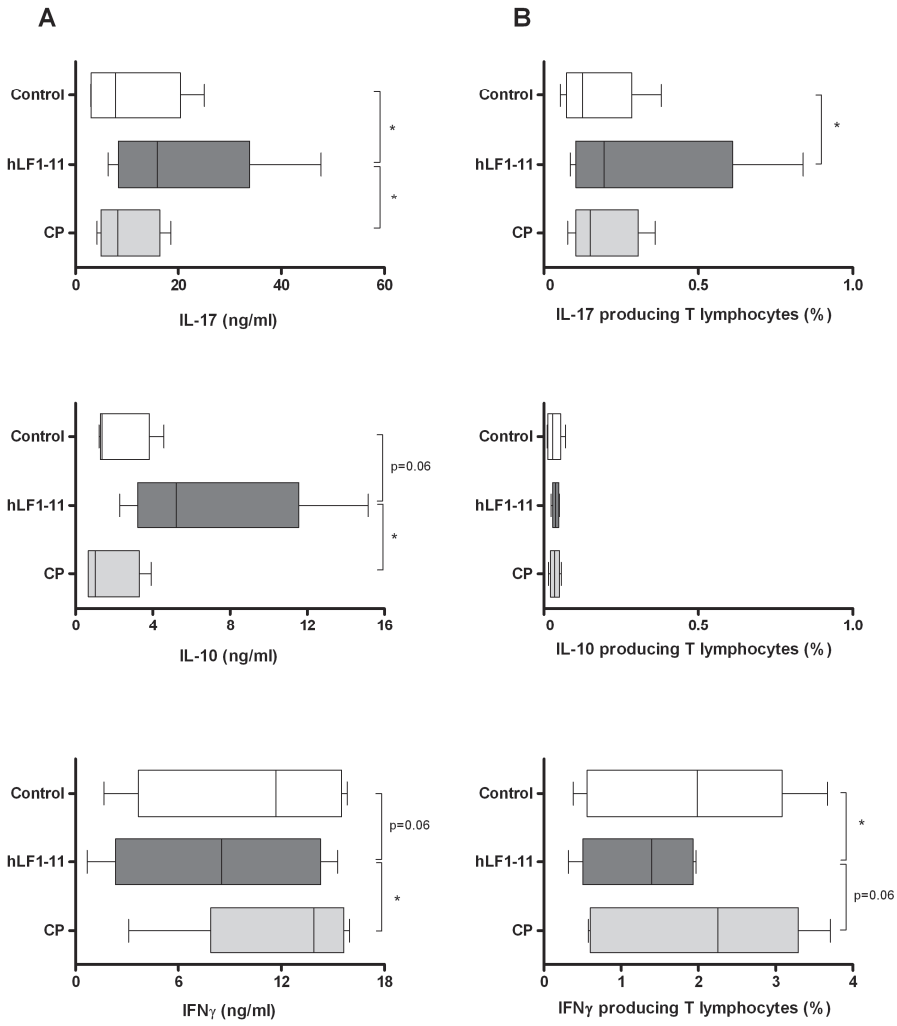


**Fig. 4 Cytokine profile by CD4+ T cells after co-culture with hLF1-11 and control (peptide-differentiated) dendritic cells** Monocytes were cultured with rhGM-CSF and IL-4 in the presence of hLF1-11 (100 µg/ml; dark gray bars), control peptide (CP, 100 µg/ml; light gray bars), or saline (open bars) for 6 days. Thereafter, cells were stimulated with heat-killed *C. albicans* ($5x10^5$/ml), purified protein derivative of *M. tuberculosis* (PPD; 5 µg/ml), tetanus toxoid (TT; 150 lf/ml) and LPS (100 ng/ml) for 24 h. Next, cells were washed and CD4+ T cells from the same donor and TT were added to the culture. 72 h later, supernatants were harvested and assessed for IL-17, IL-10 and IFN-γ (A) and IL-4, IL-2 levels (data not shown). Next, intracellular cytokine production by the T►

►lymphocytes in the co-culture was assessed by addition of brefeldin A for the last 16 hours of the co-culture (3 µg/ml). T cells were stained for intracellular IL-17, IL-10 and IFN-γ (B) Data are expressed as boxes and whiskers, boxes represent medians and second and third interquartiles, whiskers represent range within experiments with 6-8 different donors. *, $p<0.05$

**Discussion**

In this study we demonstrate that the presence of hLF1-11 during differentiation of monocytes into dendritic cells results in DCs that promote antifungal responses and induce Th17 polarization. This conclusion is based on the following findings. First, phagocytosis of *C. albicans* and ROS production in response to this yeast were elevated in hLF1-11-differentiated DCs as compared to the control (peptide-differentiated) DCs, while phagocytosis of *S. aureus* by hLF1-11-differentiated DCs was not enhanced. Second, the production of IL-6 and IL-10 by hLF1-11-differentiated mDCs in response to *C. albicans* was significantly enhanced, while IL-12p40 production was reduced as compared to control (peptide-differentiated) DCs. Incubation of hLF1-11-differentiated DCs with bacterial stimuli like LPS or LTA resulted in a significant ($p< 0.05$) reduction of TNF-α and IL-12p40 (without affecting the IL-6 and IL-10 levels) as compared to control (peptide-differentiated) DCs (data not shown). These data indicate that the presence of hLF1-11 during monocyte-DC differentiation has differential effects on the cytokine profile of the resulting mDC upon maturation by fungal or bacterial stimulation. Interestingly, we reported earlier that the presence of hLF1-11 during GM-CSF-driven differentiation of monocytes into macrophages, results in macrophages that also display enhanced production of IL-10 but not IL-12p40 or TNF-α in response to *C. albicans* (8). In addition, these macrophages displayed enhanced antimicrobial properties against *C. albicans*, but also *S. aureus* and displayed enhanced expression of pathogen recognition receptors like dectin-1. One possible explanation for these partial overlapping effects of hLF1-11 on monocyte-macrophage and monocyte-DC differentiation is the following. GM-CSF is known to be involved in regulation and enhancement of myeloperoxidase -the intracellular target of hLF1-11 (van der Does *et al*, submitted)- during monocyte-macrophage differentiation, whereas GM-CSF and IL-4 or with IL-4 alone diminished myeloperoxidase expression as compared to freshly isolated monocytes. As dendritic cells are obtained *in vitro* by incubation of monocytes with a combination of GM-CSF and IL-4, possible hLF1-11 is less able to affect monocyte-DC differentiation than monocyte-macrophage differentiation. Some properties of hLF1-11-differentiated dendritic cells and macrophages might therefore be overlapping between these cell types, while others are

not. Interestingly, as Dectin-1 expression was also significantly enhanced by hLF1-11-differentiated iDCs, it is tempting to speculate that myeloperoxidase might be involved in signaling leading to dectin-1 expression.

The most striking finding of this study is that hLF1-11 drives differentiation of monocytes toward DCs that promote Th17 polarization. The development of Th17 cells is linked to activation of several receptors including dectin-1 and CD206 (24,25,26), as recognition of *C. albicans* by these receptors is associated with Th17 responses. Also cytokines like IL-6, IL-1β and IL-23 can facilitate Th17 polarization. Th17 responses are thought to be important in host defense against fungi and *S. aureus* (27,28,29,30) especially at epithelia and mucosa. In addition, IL-17 is involved in the influx of neutrophils and can induce production of cytokines/chemokines and antimicrobial peptides by epithelial cells (31). Although Th17 cells are part of the adaptive immune response, they serve mainly to regulate innate immune responses (32). Besides promoting Th17 polarization, hLF1-11-differentiated DCs also reduced IFN-γ production by CD4+ T cells. It could be that this reduction of Th1 polarization is the consequence of the enhanced IL-10 production by the hLF1-11-differentiated DCs in the co-cultures (33). Interestingly, the effects of hLF1-11 on monocyte-DC differentiation differ from those reported for other AMPs/HDPs, such as the human cathelicidin LL-37 and α- and β-defensins. Davidson *et al*. (14) showed that the presence of LL-37 during monocyte-DC differentiation resulted in DCs promoting IFN-γ-producing T cells. Kandler *et al.* (16) have shown that this peptide inhibited the response iDCs toward microbial stimuli such as LPS, thereby indicating that LL-37 reduced maturation of these cells. Human defensins HNP-1 and hBD-1 promoted the activation and maturation of DCs and stimulated the production of TNF-α, IL-6, and IL-12p70 but not IL-10. Clearly, AMPs/HDPs differentially affect the differentiation and subsequent maturation of DCs (34). The concentration of hLF1-11 in the present study (100 μg/ml) is determined by dose response experiments with monocytes, macrophages and dendritic cells. hLF1-11 has been shown to be effective in mice up to concentrations of 4 mg/kg body weight, which corresponds to injection of 100 μg/mice. However, it should be realized that extrapolation of *in vitro* to *in vivo* levels is difficult since the local concentration of the peptide at the site of infection remains unknown. Together, these data show that hLF1-11 is able to modulate monocyte-dendritic cell differentiation, resulting in DCs displaying enhanced antimicrobial activities against *C. albicans* and promoting Th17 polarization after co-culture with CD4+ T cells. The immunomodulatory properties of hLF1-11 may aid in balancing the immune responses of the host leading to the resolution of infections with (multi-drug) resistant pathogens.

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# The human lactoferrin-derived peptide hLF1-11 exerts immunomodulatory effects by specific inhibition of myeloperoxidase activity

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# Chapter 6

**Abstract** Due to their abilities to eliminate pathogens and to modulate the hosts' immune response, antimicrobial peptides are considered as potential alternative for classical antibiotics in the treatment of infection. We recently reported that hLF1-11; an eleven amino acid antimicrobial peptide derived from the N-terminus of human lactoferrin, modulates the LPS-induced inflammatory response of human monocytes and directs the GM-CSF-driven monocyte-macrophage differentiation toward macrophages with enhanced antimicrobial effector functions. In this study we focused on the identification of the cellular target of hLF1-11 that mediates these effects and found that hLF1-11 binds to and subsequently enters human monocytes. Pull-down assays using monocyte lysates with hLF1-11-coated columns resulted in the isolation of a single protein of approximately 110 kDa, identified by mass spectrometric analysis as human myeloperoxidase (MPO). The hLF1-11 peptide inhibited chlorination and peroxidation activity of MPO, whereas ABAH, an inhibitor of the enzymatic activity of MPO, mimicked the effects of hLF1-11 on the inflammatory response by monocytes, i.e., it reduced production of reactive oxygen species and enhanced production of IL-10 in response to LPS. Similarly to hLF1-11, the MPO-inhibitor affected monocyte-macrophage differentiation as assayed by IL-10 production upon LPS stimulation and phagocytosis of *Staphylococcus aureus.* Experiments with a set of hLF1-11 peptides with a single amino acid substituted by alanine, identified the cysteine at position 10 as pivotal in hLF1-11 to enable it to inhibit myeloperoxidase activity and affect monocyte function.

We conclude that hLF1-11 exerts modulatory effects on monocytes by specific inhibition of myeloperoxidase activity. These findings merit further research regarding the development of hLF1-11 as possible therapeutic agent in disease states in which myeloperoxidase activity plays an unfavorable role.

**Introduction**

Over the past 60 years, the usage of 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 (1-3). This was helped by the ability of the latter to rapidly evolve and adapt, and in stressful environments to select for useful genotypes among multiple mutants. Nowadays, antibiotic-resistant microorganisms present a major problem for both the medical community as well as for society. The emergence of multi-drug resistant pathogens underscores the need for reconsidering current usage of antibiotics, and shows that novel antibiotics with a mode of action different from current anti-infectives are urgently needed. In the past decennia, the development of 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' phagocytic cells to ingest and kill the invaders. Recently, research has shifted toward exploring the possibility for an alternative way of coping with infections, through modulation of the hosts' immune system, thereby enhancing its ability to reduce pathogenic load.

Promising candidates in this respect may be found in the class of antimicrobial peptides (4-6). Antimicrobial peptides are cationic, relatively short and active against a variety of microorganisms, including many multi-drug resistant pathogens. In addition to their ability to directly kill pathogens, many antimicrobial peptides -also called host defense peptides (HDPs)- have immunomodulatory properties. This quality makes them possible candidates to serve as an alternative for current anti-infectives. In addition, identification of the (intra)cellular target(s) of AMPs in immune cells could provide the basis for further development of agents that modulate the (innate) immune response. Extracellular receptors for AMPs on immune cells have been reported to mediate for example chemotaxis of neutrophils and monocytes (7). However, uptake of AMPs like LL-37 and IDR-1 by immune cells was found to be essential for their ability to induce the production of cytokines/chemokines by immune cells and to exert antimicrobial functions (8,9).

The lactoferrin-derived peptide hLF1-11, a synthetic peptide comprising the first 11 N-terminal residues of human lactoferrin, displays in addition to its antimicrobial effects (10-14) also immunomodulatory properties by enhancement of cyto- and chemokine production by murine and human monocytes (15). Moreover, it directs the GM-CSF-driven monocyte-macrophage differentiation toward an IL-10-producing macrophage subset that shows increased responsiveness toward microbial stimuli and enhanced phagocytosis and intracellular killing of pathogens (16). As this peptide displays potential for development

as a therapeutic agent, the aim of the present study was to identify the cellular target(s) of hLF1-11 that mediate(s) these immunomodulatory effects.

**Materials and Methods**

**Peptides** The synthetic peptide comprising the first eleven amino acids of human lactoferrin (further referred to as hLF1-11; GRRRRSVQWCA) was purchased from Peptisyntha (Torrance, CA). The control peptide: GAARRAVQWAA, N-terminal biotinylated hLF1-11 (hLF1-11-biotin), N-terminal biotinylated control peptide (control peptide-biotin) and a set of alanine-substituted peptides were from Isogen (De Meern, The Netherlands). The purity of the peptides was determined by reverse-phase high-performance liquid chromatography and exceeded 97%. All peptides were endotoxin free. Immediately before use the peptides were dissolved in phosphate buffered saline (PBS; pH 7.4; Department of Pharmacy, LUMC).

**Isolation of human monocytes** Human monocytes were isolated from buffycoats from healthy donors by Ficoll Amidotrizoate (Department of Pharmacy, LUMC) density gradient centrifugation. Monocytes were further purified by CD14-positive selection using anti-CD14-conjugated magnetic microbeads (Miltenyi Biotec, Bergisch Gladbach, Germany) according to the manufacturers' protocol. The resulting suspension comprised of approximately 96% monocytes with a viability exceeding 98% as determined by annexin V and propidium iodide staining 2 h after isolation.

**Interaction of hLF1-11 with human monocytes** Monocytes were incubated with 10 µg/ml hLF1-11-biotin at 37˚C and 4˚C for several intervals up to 70 min, then washed with ice-cold PBS and incubated with phycoerythrin-labeled streptavidin (Invitrogen, Breda, The Netherlands) for 15 min on ice. Hereafter, the monocytes were washed and the mean fluorescence intensity (MFI) of the cells was assessed by flow cytometry on a FACSCalibur and analysed using BD CellQuest software (BD biosciences, Heidelberg, Germany). To find out whether the hLF1-11 peptide enters cells we incubated monocytes with 100 µg/ml of hLF1-11-biotin or saline for 15 or 60 min at 37˚C or for 60 min at 4˚C. Thereafter, the cells were fixed with 4% paraformaldehyde for 10 min, washed three times with PBS and adhered on labtek II $cc^2$ slides (Nunc, Rochester, NY) by 15 min of incubation. Next, the cells were carefully washed with PBS supplemented with 0.05% Tween20 and permeabilized with 90% methanol for 10 min. Finally, these cells were washed with PBS and incubated with Alexa 647-labeled streptavidin (Invitrogen) for 30 min. After rinsing,

the cells were incubated with rhodamine-labeled wheat germ agglutinin (WGA, Invitrogen) for 20 min to stain the membranes. Then, cells were washed and mounted with Vectashield containing DAPI (Vector Laboratories, Amsterdam, The Netherlands). High quality, 3D stacks of optical sections were obtained with a Marianas$^{TM}$ digital imaging microscopy work station (Zeiss 200M, Zeiss, Göttingen, Germany) using a 63x oil-immersion objective and a Z-stepsize of 0.25 µm. Finally, 3D image acquisition, deconvolution and automated image analysis were performed under full software control (SlideBook version 5.0, Intelligent Imaging Innovations, Denver, CO).

**Isolation of the cellular target(s) of hLF1-11 in human monocytes** Monocytes (3 x $10^7$/ml) were lysed by keeping them on ice for 30 min in TNE-buffer [10 mM Tris-HCl (pH 7.5), 150 mM NaCl, and 2 mM EDTA] containing a protease inhibitor mixture (Pierce Biotechnology, Rockford, MA) and 1% Nonidet P-40. Lysates were centrifuged for 10 min at 10.000 x g and supernatants were stored at -80˚C. Next, hLF1-11 target proteins were isolated from these supernatants using the biotinylated protein interaction pull-down kit from Pierce Biotechnology according to the manufacturers' protocol. In short, hLF1-11-biotin or control peptide-biotin was immobilized on a streptavidin column. Next, these columns were incubated for 2 h with monocyte lysate supernatant and after several washings, proteins were eluted with the manufacturers' elution buffer. Eluted samples were dried in a centrifugal vacuum concentrator (Eppendorf, Hamburg, Germany) and taken up in a small volume of elution buffer. Next, 20 µl of this sample was mixed with 5 µl of 5x concentrated non-reducing loadingbuffer (100 mM Tris-HCL, 4% w/v SDS, 50% glycerol and 0.05% bromophenolblue) and then subjected to SDS-PAGE on a 10% or 15% SDS-polyacrylamide gel. After electrophoresis, the gel was fixed overnight in 50% methanol, 12% acetic acid, 0.05% formaldehyde and then washed three times for 20 min in 35% ethanol. Next, the gel was sensitized for 2 min in 0.02% sodium thiosulfate and washed three times for 5 min in water, followed by staining of the proteins for 20 min in 0.2% (wt/v) silver nitrate and 0.076% formaldehyde. After washing, the gel was developed using 6% (wt/v) $Na_2CO_3$, 0.05% formaldehyde, 0.0004% (wt/v) $Na_2S_2O_3$ for maximal 5 min, after which the reaction was stopped with 50% methanol and 12% acetic acid.

**In-gel tryptic digestion and mass spectrometry** Protein bands were excised from the gel, cut into small pieces, and washed with 25 mM $NH_4HCO_3$ followed by dehydration with 100% acetonitrile (ACN) for 10 min. For reduction and alkylation, dried gel particles were first incubated with 10 mM dithiothreitol for 30 min at 56°C. Following dehydration with

ACN, gel plugs were subsequently incubated in 55 mM iodoacetamide for 20 min at room temperature. After two rounds of washing with 25 mM $NH_4HCO_3$ and dehydration with 100% ACN, the gel particles were completely dried in a centrifugal vacuum concentrator (Eppendorf). The dried gel particles were re-swollen for 15 min on ice after the addition of 15 µl of a trypsin solution (Sequencing Grade Modified Trypsin, Promega, Madison, WI; 5 ng/µl in 25 mM $NH_4HCO_3$). Following this, 20 µl of 25 mM $NH_4HCO_3$ was added and the samples were kept on ice for an additional 30 min. Tryptic digestion was subsequently performed overnight at 37 °C. The overlaying digestion-solution containing the tryptic peptides was collected (extract 1). One additional round of extraction with 20 µl 0.1 % TFA was used to extract peptides from the gel plugs and this extract was pooled with extract 1. MALDI-ToF(-ToF) analyses was performed on an Ultraflex II mass spectrometer (Bruker Daltonics, Bremen, Germany) using dihydroxybenzoic acid (5 mg/ml in 50% ACN/0.1% trifluoroacetic acid) as a matrix. The mass spectrometer was used in the positive ion reflector ion mode. Spectra were imported in Flexanalysis 3.0 (Bruker) for smoothing, baseline subtraction and peak picking. Peak lists were searched against the human IPI database (Date of release 23-08-2010, 89486 sequences) using the Mascot search algorithm (Mascot 2.2, Matrix Science, London, UK). Trypsin was selected as the enzyme and one missed cleavage was allowed. Carbamidomethylcysteine was selected as a fixed modification and oxidation of methionine as a variable modification. The MS tolerance was set to 50 ppm.

**Assays for MPO enzymatic activity** The effect of hLF1-11 and the control peptide on myeloperoxidase enzymatic activities was assessed using a myeloperoxidase inhibitor screening assay kit of Cayman Europe (Tallinn, Estonia). Both the chlorination activity and the peroxidation activity were assessed following manufactures' instructions. Several concentrations of hLF1-11 or control peptide were mixed with MPO in the presence of a reactivity mixture containing $H_2O_2$ and substrate. Both chlorination and peroxidation activity were measured at an excitation wavelength of 485 nm and an emission wavelength of 535 nm.

**Measurement of intracellular reactive oxygen species in human monocytes** Intracellular ROS production by human monocytes was assayed using the fluorescent probe 2',7'-dichlorofluorescein-diacetate (DCFH-DA; Invitrogen). In short, monocytes were incubated with hLF1-11, control peptide, no peptide or the myeloperoxidase inhibitor ABAH (Merck

Chemicals Ltd. Nottingham, UK) for 1 h at 37˚C. Next, 10 µM DCFH-DA were added to the culture and incubated for 20 min at 37˚C in the dark. Thereafter, monocytes were stimulated with 100 ng/ml LPS for various intervals. ROS production was measured on a FACSCalibur and analysed with BD CellQuest software. Results are expressed as median fluorescence intensity (MFI) with interquartile range.

**Cell cultures** Monocytes were resuspended in RPMI-1640 (Invitrogen, Breda, The Netherlands) supplemented with 2 mM L-glutamine (Invitrogen), 100 U/ml penicillin (PAA GmbH, Pasching, Germany), 100 mM streptamycin (PAA GmbH), and 10% inactivated fetal bovine serum (Invitrogen), further referred to as standard medium. Monocytes were cultured at a concentration of $1x10^6$ cells/ml of standard medium at 37˚C and 5% $CO_2$. Monocytes were exposed to the different peptides or ABAH immediately at the start of the culture and 1 h thereafter these cells were stimulated with 100 ng/ml LPS (Sigma-Aldrich). About 18–20 h thereafter the supernatants were collected for assessment of cytokine levels. To investigate the effects of hLF1-11 on GM-CSF-driven monocyte-macrophage differentiation, monocytes were incubated in culture medium supplemented with GM-CSF (5 ng/ml) in the presence of hLF1-11, control peptide, ABAH or solvent control. On day 6, the cells were stimulated with LPS (100 ng/ml) or heat-killed *C. albicans* ($1x10^7$ CFU/ml) for 20 h; thereafter supernatants were collected for assessment of the production of IL-10 using a commercially available ELISA cytoset. Where indicated, macrophages were harvested, washed and resuspended in RPMI for assessment of their ability to phagocytose bacteria. For this purpose a stock suspension of pHrodo-labeled *Staphylococcus aureus* (Invitrogen) was prepared according to manufacturers' protocol. pHrodo is a dye that is non-fluorescent at neutral pH and bright red in acidic environments (e.g., the phagolysosome). Equal volumes of macrophages ($1x10^6$/ml of medium) and 5-times pre-diluted pHrodo-labeled *S. aureus* stock were mixed and then incubated for several intervals at 37˚C, or as a control experiment, at 4˚C. Thereafter, pHrodo fluorescence by the macrophages was assessed on a FACSCalibur. Results are expressed as the percentage of pHrodo-positive macrophages.

**Statistical analyses** Friedman followed by Dunn's multiple comparisons post-hoc test or, where indicated, Wilcoxon's test was used to determine the differences between the results for hLF1-11-differentiated, ABAH and control (peptide-differentiated) DCs. Data are expressed as median and range. Two sided p-values are reported and the level of significance was set at $p<0.05$.

## Results

**Binding and uptake of hLF1-11 by human monocytes** To find out whether hLF1-11 binds to and enters cells we incubated monocytes for various time intervals with biotin-hLF1-11 and assessed fluorescence of these cells by FACS. Results revealed that hLF1-11 binds rapidly to monocytes (Fig. 1A) after which the peptide either detaches or enters the cells as the fluorescence decreased over time. To discriminate between these two possibilities, monocytes were incubated with hLF1-11-biotin for 15 or 60 min at 37°C or 4°C and analyzed using fluorescence microscopy. Results showed that hLF1-11-biotin mainly associated with the cell membrane of monocytes incubated with hLF1-11-biotin for 15 min (data not shown). After 60 min of incubation, hLF1-11-biotin was also localized intracellular (Fig. 1B). Similar results were obtained after incubation at 4°C (Fig. 1C), suggesting that hLF1-11 passively enters the cells.

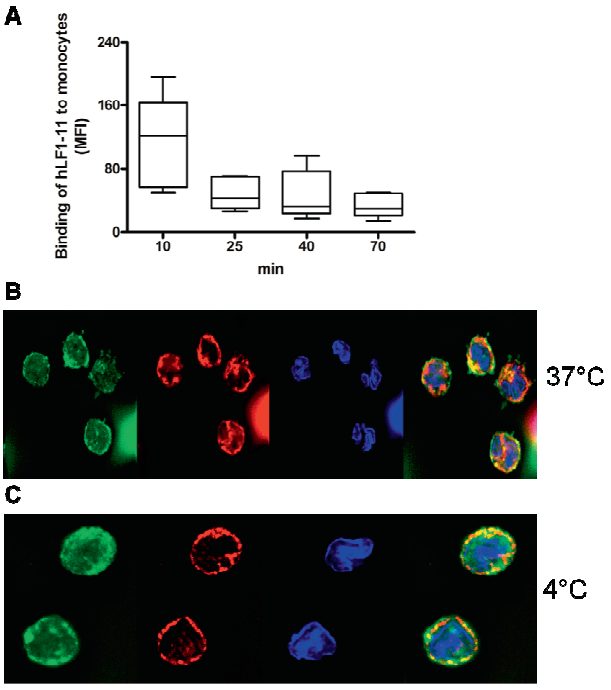


**Fig. 1 Binding and uptake of hLF1-11 by human monocytes** To assess whether hLF1-11 binds to monocytes, human monocytes were incubated with biotinylated hLF1-11 for several intervals. Thereafter, the cells were washed, incubated with streptavidin-PE for 15 min, washed again and then the mean fluorescence intensity (MFI) of the monocytes was assessed by flow cytometry. Results are expressed as boxes and whiskers, boxes represent medians and second and third interquartiles, whiskers represent range within 4 independent experiments (A). To find out if hLF1-11 enters human monocytes or detaches from the cells, monocytes were incubated with biotinylated hLF1-11 for 60 min at 37˚C (B) and 4˚C (C), fixed with 4% paraformaldehyde, permeabilized with 90% methanol, and washed with PBS. Thereafter, the presence of this peptide in cells was visualized with Alexa 647-labeled streptavidin (left), the cells were stained with rhodamine-conjugated wheat germ agglutinin (WGA, a membrane marker; second left) and DAPI (to stain the nucleus; second right). Thereafter, cells were analyzed on a Marianas digital imaging microscope using a 63x oil-immersion objective and a Z-stepsize of 0.25 µm, scale: 10 pixels is 1 µm. An overlay of the three images is displayed in the right panel.

**Identification of the intracellular target of hLF1-11** Next, we sought to isolate the intracellular binding partner(s) of hLF1-11, using monocyte lysates and hLF1-11-coated columns. After elution with PBS, we separated the bound proteins by SDS-PAGE. Results revealed that a single protein with an apparent molecular weight of approximately 110 kDa had bound to hLF1-11, whereas no protein was detected within the eluate from the control peptide-coated column (Fig. 2, insert). The hLF1-11 binding protein was subjected to in-gel tryptic digestion and identified as human myeloperoxidase (MPO) using MALDI-ToF mass spectrometry followed by database searching (Fig. 2). MPO was identified as the principal binding partner of hLF1-11 in three independent experiments.

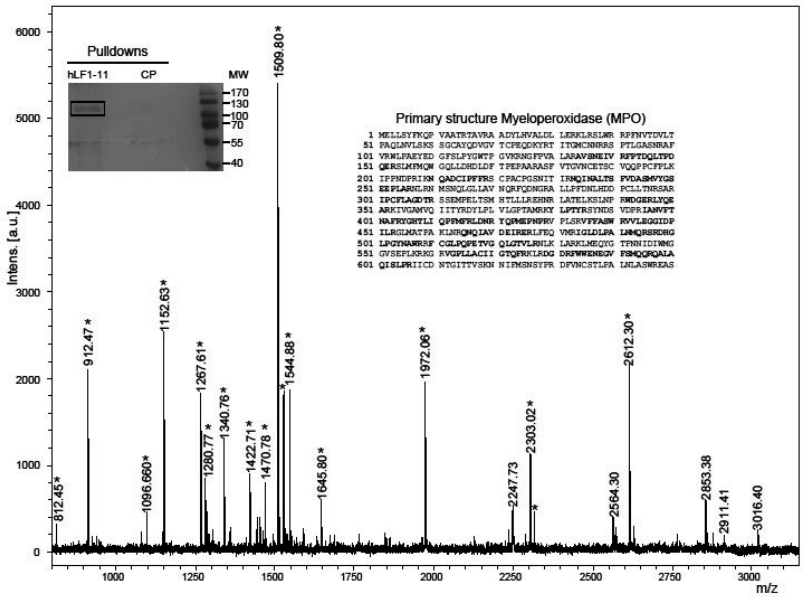


**Fig. 2 Identification of myeloperoxidase as the principal binding partner of hLF1-11 in monocytes** To investigate the intracellular binding partner of hLF1-11, biotinylated hLF1-11- and control peptide (CP)-coupled streptavidin columns were used in pull-down assays using monocytic cell lysates as input. After washing, bound proteins were eluted and subjected to SDS-PAGE under non-reducing conditions. hLF1-11 specifically bound to a protein with an apparent molecular weight of 110 kDa (insert). Tryptic digestion followed by MALDI-ToF MS analysis and database searches using the Mascot search algorithm showed that this protein corresponds to human myeloperoxidase (MPO, IPI00236554, Mascot score 241). Fragments indicated with an asterix correspond to matched tryptic peptides of MPO and are shown in bold within the primary structure of MPO (total sequence coverage 35%).

**Effects of hLF1-11 on the enzymatic activities of human myeloperoxidase** To investigate whether hLF1-11 affects the enzymatic activities of myeloperoxidase, we determined both the peroxidation as well as the chlorination activity of myeloperoxidase in the presence of hLF1-11 or the control peptide. hLF1-11 inhibited both activities of myeloperoxidase in a dose dependent fashion (Fig. 3A and B). The control peptide was not able to inhibit peroxidation activity of myeloperoxidase. Surprisingly, the control peptide did inhibit the chlorination activity of myeloperoxidase although approximately 25 times less efficiently than hLF1-11.

**Effect of hLF1-11 on the production of reactive oxygen species by monocytes in response to LPS** Since myeloperoxidase enforces the formation of ROS after initiation of the oxidative burst we considered the possibility that binding of hLF1-11 to myeloperoxidase and its subsequent loss of enzymatic activity would result in reduced ROS production by human monocytes. Therefore, we measured the ROS production by hLF1-11-or control peptide-treated monocytes in response to LPS. Results showed that hLF1-11-treated monocytes produced significantly less ROS after LPS than control (peptide-treated) monocytes (Fig. 4A). In addition, ABAH, a known inhibitor of the enzymatic activities of MPO reduced the LPS-stimulated ROS production by human monocytes (Fig. 4B), indicating that inhibition of MPO by ABAH mimics the effect of hLF1-11 on ROS production.

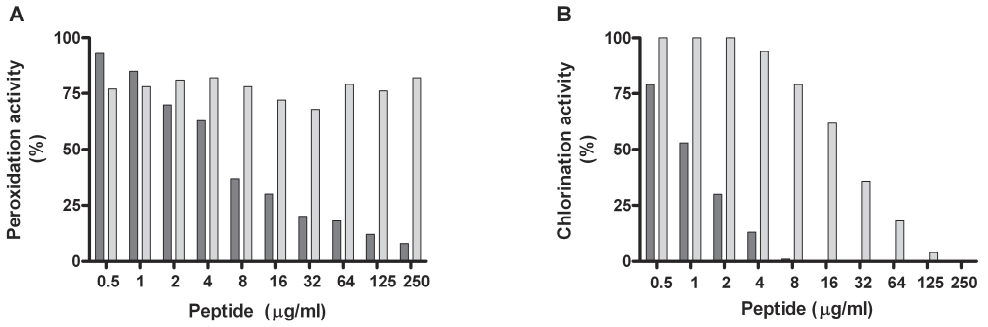


**Fig. 3 Myeloperoxidase activity in the presence of hLF1-11 and control peptide** The peroxidation (A) and the chlorination (B) activity by myeloperoxidase were assessed in the presence of several concentrations of hLF1-11 (dark gray) or control peptide (light gray). The peroxidation assay utilizes the reaction between hydrogen peroxide and ADHP (10-acetyl-3,7-dihydroxyphenoxazine), which produces the highly fluorescent compound resorufin. The chlorination assay utilizes the non-fluorescent 2-[6-(4-aminophenoxy)-3-oxo-3H-xanthen-9-yl]-benzoic acid (APF), which is selectively cleaved by hypochlorite (-OCl) to yield the highly fluorescent compound fluorescein. The results of a representative experiment (out of three independent experiments) are displayed.

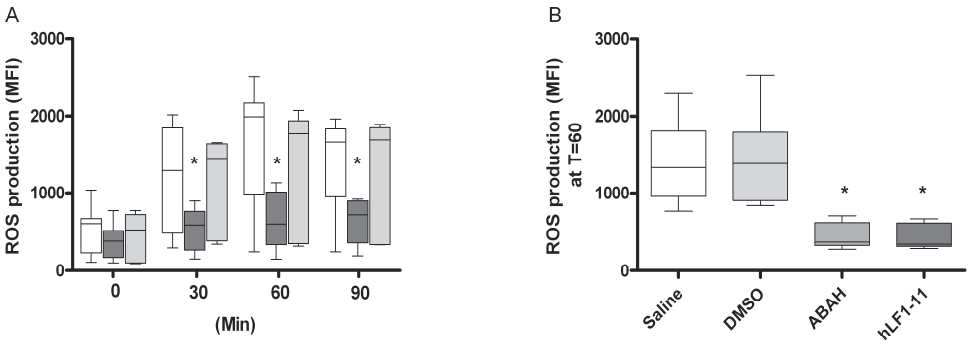


**Fig. 4 Intracellular ROS production by hLF1-11- or control peptide-incubated monocytes in response to LPS** Monocytes were cultured in the presence of hLF1-11 (100 µg/ml, dark gray), control peptide (100 µg/ml, light gray) or no peptide (open) for 1 h and then labeled with DCFH-DA. Next, the labeled monocytes were stimulated with LPS (100 ng/ml) and the MFI was assessed directly by flow cytometry and after 30, 60 and 90 min as a measure of ROS production. Data are expressed as boxes and whiskers, boxes represent medians and second and third interquartiles, whiskers represent range within experiments with 8 different donors (A). In addition, monocytes were cultured in the presence of ABAH (100 µM) or hLF1-11 (100 µg/ml which represents 72 µM) and then labeled with DCFH-DA. Next, the labeled monocytes were stimulated with LPS for 60 min. Thereafter, intracellular ROS production was assessed by flow cytometry. Data are expressed as boxes and whiskers, boxes represent medians and second and third interquartiles, whiskers represent range within experiments with 7 different donors (B). *, $p<0.05$ as compared to control monocytes and control peptide-incubated monocytes. Wilcoxon's test was used to determine the significance shown in Fig. 4B

**Comparison of the effects of the myeloperoxidase inhibitor ABAH and hLF1-11 on LPS-induced IL-10 production by monocytes and on monocyte-macrophage differentiation**

To investigate whether the inhibition of myeloperoxidase by hLF1-11 is sufficient for the immunomodulatory effects of hLF1-11 we compared the effects of ABAH and hLF1-11 on IL-10 production by LPS-stimulated human monocytes (8) and on monocyte-macrophage differentiation (9). Results showed that ABAH-incubated and hLF1-11-incubated monocytes produced significantly higher levels of IL-10 upon stimulation with LPS than control monocytes. The effect of 100 µM ABAH on LPS-induced IL-10 production by human monocytes was similar to that of 72 µM hLF1-11 (Fig. 5A). Furthermore, macrophages differentiated by GM-CSF in the presence of ABAH displayed significantly enhanced levels of IL-10 in response to LPS similar to macrophages differentiated in the presence of hLF1-11 (Fig. 5B). The percentage of *S. aureus*-phagocytosing ABAH-differentiated macrophages was significantly enhanced in comparison to saline-incubated cells but not to DMSO-incubated cells (data not shown).

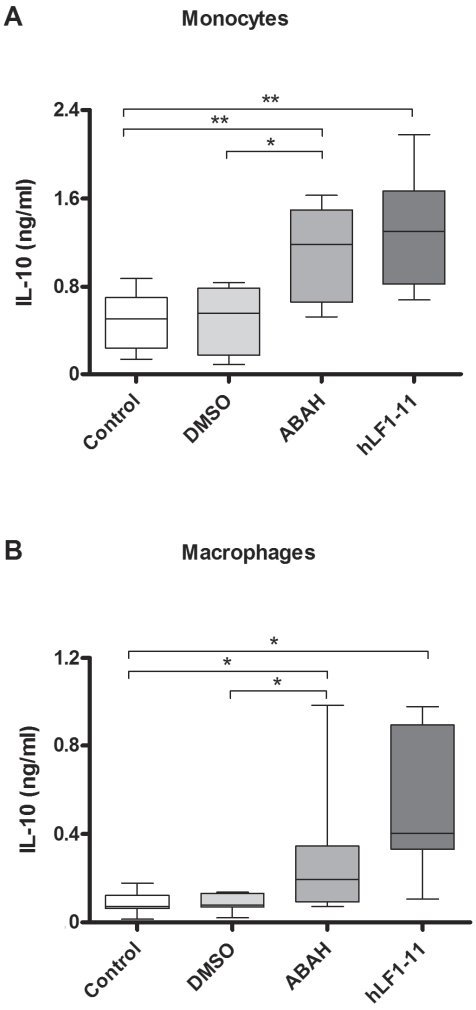


**Fig. 5 Comparison of activities of ABAH- and hLF1-11-incubated monocytes and GM-CSF-differentiated macrophages** Monocytes were cultured in the presence of ABAH (100 μM), hLF1-11 (72 μM) or solvent control for 1 h and then stimulated for 24 h with LPS (100 ng/ml). Thereafter, supernatants were collected and assessed for IL-10 production (A). In addition, monocytes were cultured with rhGM-CSF in the presence of ABAH (100 μM) or hLF1-11 (72 μM) for 7 days. Thereafter, IL-10 production by the resulting macrophages after LPS stimulation was assessed by ELISA (B) Data are expressed as boxes and whiskers, boxes represent medians and second and third interquartiles, whiskers represent range within experiments with 6-9 different donors. *, $p<0.05$ as compared to control monocytes or macrophages.

**Identification of amino acids within hLF1-11 primarily responsible for hLF1-11 activity** To determine which amino acid(s) in hLF1-11 is (are) essential for its MPO-inhibiting activity, we assessed for a set of peptides with a single amino acid substitution to alanine, the effect on IL-10 production by LPS-stimulated monocytes and the chlorination activity of MPO. Results revealed that the cysteine at position 10 was the most important amino acid for the enhanced IL-10 production by hLF1-11-incubated monocytes upon LPS stimulation (Fig. 6A). In agreement, this amino acid was found to be most important for the hLF1-11-mediated inhibition of the chlorinating activity of MPO (Fig. 6B).

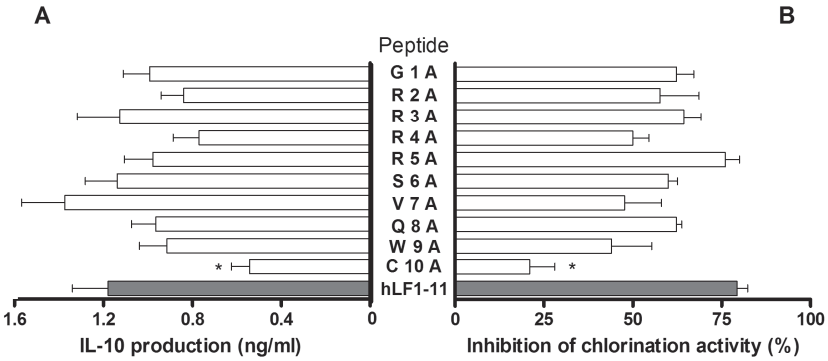


**Fig. 6 Effects of hLF1-11 and single alanine substituted analogs on myeloperoxidase activity and on IL-10 production by monocytes in response to LPS** Human monocytes were incubated with hLF1-11 or derived peptides in which one amino acid within the hLF1-11 sequence was replaced by an alanine (all 100 μg/ml) for 1 h, thereafter the monocytes were stimulated with LPS (100 ng/ml) for 24 h after which the supernatants were collected for assessment of the IL-10 levels. Data are expressed as medians and SEM of experiments with 8 different donors (A). In addition, hLF1-11 and derived peptides in which one amino acid is replaced by an alanine (all at a concentration of 8 μg/ml) were compared for their effect on the chlorination activity of myeloperoxidase using the myeloperoxidase inhibitor screening kit according manufacturers' instructions. Data are expressed as medians and SEM of 3 independent experiments (B). *, $p<0.05$ as compared to hLF1-11 as determined by Wilcoxon's statistical test.

## Discussion

Recently, we reported that hLF1-11 has an immunomodulatory activity on monocytes, by modifying the LPS-induced inflammatory response and by directing the GM-CSF-driven monocyte differentiation toward macrophages displaying enhanced antimicrobial activities (15,16). The present findings show that hLF1-11 exerts these effects by specific inhibition of MPO activity in monocytes. This conclusion is based on the following observations. First, hLF1-11 binds to and is subsequently internalized by monocytes. Second, hLF1-11, but not a control peptide, bound to a single protein in lysates of monocyte cells, which was subsequently identified as myeloperoxidase. Third, hLF1-11 inhibits the peroxidation and chlorination activity of human myeloperoxidase. Lastly, the myeloperoxidase inhibitor ABAH mimicked the modulatory effects of hLF1-11 on LPS-induced IL-10 release and reactive oxygen species (ROS) production by monocytes. In addition, ABAH mimicked hLF1-11 in the LPS-induced enhanced release of IL-10 by macrophages when present during cell differentiation. ABAH also mimicked hLF1-11 in the

enhanced phagocytosis of *S. aureus* by these cells, although the effect of ABAH on monocyte-macrophage differentiation seems to be less potent than hLF1-11. Taken together, these observations indicate that myeloperoxidase is the prime target of hLF1-11 regarding mediation of the assessed immunomodulatory activities. Other findings of this study pertain to the specific amino acid(s) in hLF1-11 that are pivotal to its effects. The alanine scanning experiments revealed that cysteine at position 10 is very important for the ability of hLF1-11 to inhibit myeloperoxidase activity. We suggest that hLF1-11 comprises two regions that are important for its activity, i.e., arginines that most likely mediate cell binding and entry (17-20), and cysteine that mediates the inhibition of myeloperoxidase enzymatic activity. As four arginines are present in the hLF1-11 peptide, it was not surprising that substituting only one of these arginines in hLF1-11 was without effect. Since the control peptide lacks two of the four arginines and the cysteine as compared to hLF1-11, it most likely lacks the ability to internalize and inhibit MPO activity. This would explain why the control peptide did not affect the inflammatory responses of monocytes (15), GM-CSF monocyte-macrophage differentiation (16) and why it is not active against infections in mice (12).

The present findings show that myeloperoxidase is a negative regulator of the production of IL-10 (and some other cytokines) by LPS-stimulated monocytes. In agreement with this finding, others have reported that neutrophils from myeloperoxidase deficient mice express higher levels of IL-10 in response to LPS than cells isolated from wild-type mice (21). Furthermore, we previously described that hLF1-11 enhanced the mRNA expression of IL-10 as well as the NF-κB activation and translocation in LPS-stimulated monocytes (15). Apparently, myeloperoxidase negatively affects (a) signal transduction pathway(s) regulating the production of IL-10 and possibly more cytokines, because ABAH, an inhibitor of myeloperoxidase mimicked these actions of hLF1-11 on IL-6, IL-12p40 and TNF-α production by these monocytes (data not shown). Several studies have shown the involvement of myeloperoxidase and its products in multiple intracellular signaling pathways (22,23). Still, it remains unclear which pathway is related to the effects observed in this study and how ROS derived from myeloperoxidase are involved in this phenomenon. Clearly, these aspects warrant further investigation.

Interestingly, there are a few clinical presentations which support the notion that myeloperoxidase might be involved as a negative regulator of cytokine production and immune responses. Patients with Wegener's granulomatosis disease, for example, express high levels of anti-neutrophil cytoplasmic autoantibodies (ANCA) with specificity for myeloperoxidase. It has been shown that the presence of these autoantibodies enhance

the phagocytic activitiy, IL-8 production and glucose uptake by neutrophils (24). Also, human monocytes that were incubated with IgG from patients with ANCA-positive Wegener's granulomatosis disease, displayed enhanced expression of CD14 and CD18 on their cell-surface (25).

The identification of hLF1-11 as an inhibitor of myeloperoxidase activity raises the question about its possible therapeutic potential against pathologies in which myeloperoxidase may play an unfavorable role (26). In this context, Liu *et al* have shown that rabbits pre-treated with ABAH and subsequently submitted to myocardial ischaemia and reperfusion displayed significantly reduced cardiac caspase-3 activity, suggesting that myeloperoxidase is a significant contributor to post-ischaemic cardiomyocyte apoptosis (27). Consistent with this notion, myeloperoxidase deficiency appeared to be associated with lower incidence of cardiovascular heart diseases (28).

In conclusion, hLF1-11 modulates the inflammatory response of monocytes after exposure to inflammatory stimuli and modulates the GM-CSF-driven monocyte-macrophage differentiation by inhibiting myeloperoxidase enzymatic activity. Given preliminary reports on the safety of hLF1-11 in human healthy volunteers (29), the present findings merit research regarding the further development of hLF1-11 as possible antimicrobial drug and therapeutic agent in disease states in which myeloperoxidase plays an unfavourable role.

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Summary and general discussion

# Chapter 7

**Introduction**

The availability of successful anti-infective drugs has taken away much of the prior threat of infectious diseases in society in the last century. Overconfidence in the accomplishments of antibiotics 'the miracle drugs', however, has now been replaced by apprehension, as antibiotic usage in the human and veterinary field has boomed out of control and boomerangs back at us with the emergence of multi-drug resistant pathogens. Bacteria have developed resistance to most of the existing drugs, making infections difficult and sometimes even impossible to treat. A multi-facetted approach will be needed to constrain the impact of antibiotic resistance on treatment options in infectious diseases. Efforts should include proper education and appropriate antibiotic usage, to both physicians and patients and applying strict infection control measures to prevent transmission. Furthermore, alternatives to antibiotic usage in veterinary practice and agriculture should be identified. In addition to all these measures, it is recognized that there is an urgent need for research into and development of new anti-infective drugs as the pharmaceutical pipeline of new types of antibiotics has dried up and new anti-infective drugs will not become available for some time. Infectious diseases societies have taken up these issues and advocate major investments in research and development of new antibiotics. The development of new antimicrobial agents has mainly focused on variants of current anti-infectives. A recognized disadvantage of this approach is the experience that sooner or later, these new drugs suffer the same limitations as the parental drugs, i.e., in the end emergence of multi-drug resistant pathogens. New long-lasting anti-infective agents are yet to be successfully developed and therefore research into alternative agents is essential (1). Antimicrobial peptides are naturally occurring antimicrobial agents of all living beings and these peptides have been pointed out as potential candidates for drug development (2-4).

In the past, research into these peptides has focused on their antimicrobial activity. Soon it was recognized that some of these peptides, further referred to as host defense peptides, have the potential to strengthen or modulate the hosts' immune response resulting in an enhanced resolution of infection (5,6). As host defense peptides act indirectly, via the very broad armamentarium of the hosts' immune systems, development of drug-resistance against such peptides is unlikely. This makes host defense peptides a promising alternative to current anti-infective agents. Among these peptides, one interesting candidate comprises the first eleven amino acids of the N-terminus of human lactoferrin therefore named hLF1-11. This peptide appears active against a variety of

(multi-drug resistant) pathogens (7-9). However, *in vitro* the antimicrobial activity of this peptide is dependent on factors in its environment, such as salt concentration, e.g. the peptide is highly active against pathogens in low (10 mM) salt concentrations, but hardly active at physiological salt concentrations. Given the natural occurrence of lactoferrin and its degradation product lactoferricin, we hypothesized that in addition to its direct antimicrobial effects the hLF1-11 peptide displays immunomodulatory activities. Since the time-span in the *in vivo* assays was usually set at 48 h maximum, we focused on possible effects of hLF1-11 on cells of the innate immune system, more specifically on monocytes and cells derived thereof such as macrophages and dendritic cells.

**Effects of hLF1-11 on murine and human monocytes** The antimicrobial activity of hLF1-11 was established in mice first. Therefore, we focused our attention on murine cells and first compared the lipopolysaccharide (LPS)-induced cytokine production of hLF1-11-incubated murine monocytes to that of control monocytes. The findings showed that hLF1-11 enhanced the production of IL-6, IL-10, IL-1β and RANTES by monocytes in response to the immunoreactive component of the Gram-negative bacterial cell wall, LPS (chapter 2). After thus establishing that hLF1-11 has the ability to modulate murine monocyte activity, we next investigated the effect of hLF1-11 on human cells, for two reasons. First, we strive to reduce the number of animal experiments if an alternative approach to the problem is possible, e.g., human blood-derived cells. Secondly, since the ultimate goal would be to assess the feasibility and the development of hLF1-11 as potential anti-infective in humans, next we focused on the interaction of hLF1-11 with human immune cells. We observed that hLF1-11 enhanced the production of almost all cytokines tested and of various chemokines by monocytes in response to LPS (chapter 2). As the N-terminus of lactoferrin is the major binding site in this molecule for a microbial structure like LPS and lipid A (10), we next excluded the possibility that binding of hLF1-11 to LPS was responsible for the enhanced cytokine production by hLF1-11-monocytes. In addition, we found that in the absence of microbial stimuli hLF1-11 did not exert any action on monocytes besides reducing the production of some of the chemokines released by these cells. Taken together, these findings indicate that the actions of hLF1-11 are observed mainly after exposure of the hLF1-11-incubated cells to a second stimulus. We next established that the enhancement of the inflammatory response of monocytes by hLF1-11 was not limited to LPS and also included other microbial structures, like diphosphoryl lipid A, flagellin and CL087. These microbial components are recognized by human monocytes and macrophages through specific cell-surface receptors, collectively designated toll-like

receptors (TLR), respectively TLR4, TLR5 and TLR7/8. Interestingly, hLF1-11 hardly affected the inflammatory response of human monocytes upon stimulation with lipoteichoic acid (LTA) or PAM2CSK4, both TLR2 ligands. This was confirmed by measuring downstream activation of the transcription factor NF-kB and mRNA production in response to these toll-like receptor ligands (chapter 2). Based on our observation that hLF1-11 inhibits myeloperoxidase (MPO) (chapter 6) in human monocytes we can offer an explanation for the lack of effect of hLF1-11 on TLR2-mediated activation of monocytes. In short, activation of monocytes via the various toll-like receptors triggers an oxidative burst and induces the release of cytokines/chemokines. However, TLR2 activation by LTA and PAM2CSK4 induced a weak cytokine response by human monocytes (chapter 2). It is hypothesized that the oxidative burst induced by TLR2 ligands is only weak and therefore the role of myeloperoxidase may be small. In such cases, inhibition of myeloperoxidase by hLF1-11 might not have enough consequences to result in differences on cytokine level. Unfortunately it is not known at what level of the TLR-signal transduction route toward cytokine production myeloperoxidase is involved.

**Effects of hLF1-11 on monocytes are mediated by inhibition of myeloperoxidase activity**

After establishing the effect of hLF1-11 on human monocytes, we were intrigued to identify the target of hLF1-11 on/in monocytes that mediates these effects. We first investigated whether hLF1-11 binds to monocytes. Results showed that hLF1-11 binds to monocytes within minutes and that binding is followed by internalization within 60 min after addition of the peptide. Next, we identified myeloperoxidase as the intracellular binding target of hLF1-11 (chapter 6). In addition, we observed that hLF1-11 has the ability to inhibit the enzymatic activity of myeloperoxidase. By use of ABAH, a specific inhibitor of myeloperoxidase activity, we could establish that the inhibition of myeloperoxidase can result in immunomodulating effects, e.g. altered cytokine production, that are comparable to those induced by hLF1-11. The involvement of myeloperoxidase in signal transduction routes leading to cytokine production has not been described previously. There are, however, reports describing the involvement of reactive oxygen species in signal transduction pathways leading to cytokine production. In these publications, enhanced levels of reactive oxygen species (ROS) are necessary to induce cytokine production (11,12). However, based on our data myeloperoxidase appears to be a negative regulator of the production of some cytokines (e.g. IL-6 and IL-10) and chemokines; inhibition of myeloperoxidase enhances the LPS-stimulated cytokine production by monocytes. Either, myeloperoxidase shifts the balance of reactive oxygen species into products less active in

intracellular signaling. Or, MPO is - independently of its ROS-producing activities - involved in a different signaling pathway that leads to cytokine production. Clearly the intracellular pathway involving myeloperoxidase needs to be further deciphered. Interestingly, there are a few diseases involving myeloperoxidase that seem to already hint to its participation in cytokine production and other immune activities. For instance, Wegener's granulomatosis disease comprises the production of anti-neutrophil cytoplasmic autoantibodies (ANCA) with specificity for myeloperoxidase. It has been shown that ANCA enhance phagocytosis, IL-8 production and glucose uptake by neutrophils (13). Also, human monocytes that were incubated with IgG from patients with ANCA-positive, active Wegener's granulomatosis, displayed enhanced expression of CD14 and CD18 on their cell-surface (14).

To establish which amino acids are essential for inhibition of myeloperoxidase enzyme activity we investigated the enzymatic activity of myeloperoxidase in the presence of a set of alanine-substituted hLF1-11 peptides, i.e. peptides that had one (different) amino acid replaced by an alanine. We thus established that the cysteine in the sequence of hLF1-11 is essential for the inhibition of myeloperoxidase activity. Consistent with this notion, the same amino acid was found to be important for enhanced IL-10 induction by hLF1-11-incubated human monocytes in response to LPS (chapter 6). As arginine-rich peptides easily penetrate cellular membranes (15-17), we suggest that the four arginines in hLF1-11 are necessary for cell penetration. Thus, hLF1-11 has two important regions: the arginine-rich penetration site and the cysteine that is responsible for the inhibition of myeloperoxidase activity. The results from this study can be used to facilitate the design of antimicrobial peptides with the optimal qualities for inhibition of myeloperoxidase.

**Modulation of the differentiation of monocytes toward macrophages and dendritic cells by hLF1-11**

After thus establishing an action of hLF1-11 on monocytes, we next hypothesized how this would affect the differentiation of monocytes into macrophages or dendritic cells. These antigen-presenting cells play a major role in the linking of the innate to the adaptive components of the immune system. We studied whether monocytes that differentiated in the presence of hLF1-11 *in vitro* displayed a different phenotype and if so, whether such an altered phenotype would be potentially beneficial to help clear infection. Results revealed effects of hLF1-11 on GM-CSF-driven macrophages differentiation but not on M-CSF-driven macrophage differentiation (chapter 3). Of interest, GM-CSF was previously reported to be involved in the regulation and enhancement of myeloperoxidase activity in

monocytes and macrophages, while M-CSF was not (18). In view of the above described interaction of hLF1-11 with myeloperoxidase, this might help to explain why the action of hLF1-11 was limited to GM-CSF driven macrophages. Moreover, the presence of hLF1-11 during GM-CSF-driven monocyte-macrophage differentiation resulted in macrophages that displayed enhanced IL-10 production in response to several microbial stimuli and were more responsive toward these microbial stimuli than unstimulated control cells. Also, expression of pathogen recognition receptors like Dectin-1, the mannose receptor and others by hLF1-11-differentiated macrophages as well as the phagocytosis of both *C. albicans* and *S. aureus* by these cells were enhanced as compared to control cells. Strikingly, when we incubated monocytes for 60 min with hLF1-11, washed the peptide away and then induced differentiation toward macrophages for 7 days, these macrophages had a similar phenotype as macrophages that differentiated in the continuous presence of hLF1-11 (chapter 3). Taken together, these findings indicate that hLF1-11 can prime monocytes in such a manner that upon encountering microbial components, these cells differentiate into macrophages with a phenotype that appears to be beneficial for resolution of infection (19).

As hLF1-11 was able to prime these monocytes, we also investigated the effect of this peptide on monocyte-dendritic cell (DC) differentiation. Matured hLF1-11-differentiated dendritic cells displayed enhanced production of IL-10 in response to heat-killed *C. albicans*. In addition, IL-6 production by these cells was enhanced in response to this yeast. These dendritic cells also displayed enhanced expression of Dectin-1, and phagocytosis of *C. albicans* and ROS production in response to *C. albicans* was also elevated (chapter 4). It was noted that hLF1-11-macrophages displayed enhanced activities to several pathogens, while for hLF1-11-dendritic cells this change was restricted to *C. albicans*. What could be the explanation for this difference? At present, this is not clear. One possible explanation touched upon above could be that GM-CSF is involved in regulation and enhancement of myeloperoxidase during monocyte-macrophage differentiation (18). Roy *et al* reported that monocytes incubated with GM-CSF and IL-4 or with IL-4 alone, display diminished myeloperoxidase expression as compared to freshly isolated monocytes (20). As dendritic cells are obtained *in vitro* by incubation of monocytes with both GM-CSF and IL-4, it could be that IL-4 diminished the myeloperoxidase enhancement, thus preventing some of the actions of hLF1-11. Some properties of hLF1-11-differentiated dendritic cells and macrophages might therefore be overlapping between these cell types while others are not.

**Differential effects of hLF1-11 and LL-37 on the differentiation of monocytes toward macrophages and dendritic cells**

When studying the effects of hLF1-11 on monocyte-macrophage differentiation, we considered the possibility that another antimicrobial peptide i.e. LL-37, similarly affects monocyte-macrophage differentiation. LL-37 also modulates monocyte-macrophage differentiation, although incubation of the cells with this peptide resulted in a completely different macrophage subset as obtained after differentiation in the presence of hLF1-11. While hLF1-11 modulates GM-CSF-driven monocyte-macrophage differentiation, LL-37 modulates M-CSF-driven monocyte-macrophage differentiation resulting in macrophages that displayed striking similarities to the GM-CSF-macrophages (chapter 4). As M-CSF-macrophages display an anti-inflammatory phenotype and GM-CSF-macrophages display a proinflammatory phenotype, LL-37 is thus able to completely redirect the phenotype of M-CSF-differentiated macrophages toward a proinflammatory phenotype. LL-37 is able to induce these effects during differentiation and also upon incubation with already differentiated macrophages.

Investigations on dendritic cell differentiation revealed that in addition to enhanced antimicrobial activities against *C. albicans*, hLF1-11-differentiated dendritic cells were able to induce Th17 polarization upon co-culture with CD4+ T cells (chapter 5). T cells co-cultured with hLF1-11-differentiated dendritic cells produced enhanced levels of IL-17 and diminished Th1 polarization as indicated by reduced levels of IFN-γ. IL-17 is associated with an enhanced anti-fungal and anti-bacterial response (21,22). In addition, IL-17 is involved in the host defense against extracellular pathogens by mediating the recruitment of neutrophils and macrophages to the infected site. Also, IL-17 can induce the production of cytokines/chemokines and antimicrobial peptides by mucosal epithelial cells (23). Others have reported that LL-37 and β-defensins (24) can also modulate monocyte differentiation toward dendritic cells. For example, when CD4+ T cells were co-cultured with LL-37-differentiated dendritic cells these T cells produced enhanced levels of IFN-γ, thereby inducing Th1 polarization (25). Th1 cells drive the protective immune response against intracellular pathogens such as *Mycobacterium tuberculosis*. These data indicate that antimicrobial peptides can modulate immune processes, thereby 'fine tuning' the immune response upon activation. This suggests a possible therapeutic opportunity for employment of antimicrobial peptides, i.e., to (re)direct immune responses to enhance resolution of infection. Figure 1 has depicted the effects that are described in this thesis of hLF1-11 on human immune cells.

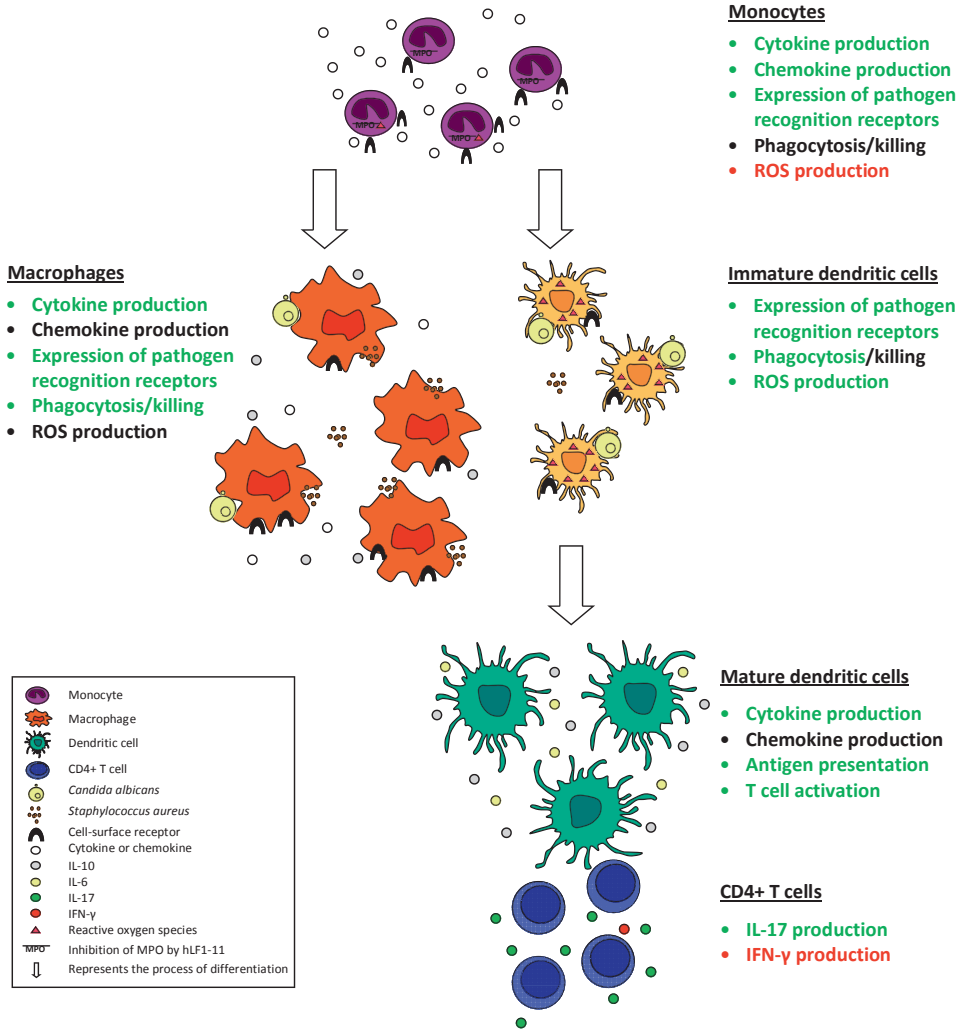


**Fig. 1 Simplified representation of the effects of hLF1-11 on the major characteristics of monocytes and monocyte-derived cells *in vitro*** When monocytes are incubated with hLF1-11, this peptide is internalized where it binds to and inactivates the enzymatic activities of myeloperoxidase. The hLF1-11-incubated monocytes produce upon activation reduced levels of reactive oxygen species and enhanced levels of cytokines and chemokines as compared to control monocytes. Monocytes differentiated in the presence of both GM-CSF and hLF1-11 become macrophages that display enhanced pathogen recognition receptor expression and phagocytosis and killing of *S. aureus* and *C. albicans* as compared to control (peptide-differentiated) macrophages. In addition, in response LPS, LTA and heat-killed *C. albicans* these macrophages produce enhanced levels of IL-10 as compared to control macrophages. Monocytes differentiated in the presence of GM-CSF, IL-4 and hLF1-11, become immature dendritic cells that display enhanced expression of Dectin-1 and enhanced►

►phagocytosis of *C. albicans*. These hLF1-11-differentiated dendritic cells also produce enhanced levels of reactive oxygen species in response to this pathogen as compared to control (peptide-differentiated) immature dendritic cells. Moreover, upon maturation hLF1-11-differentiated dendritic cells produce enhanced levels of IL-10 and are able to induce Th17 polarization upon co-culture with CD4+ T cells. These T cells produce enhanced amounts of IL-17 and reduced levels of IFN-γ. Represented in green: enhancement, red: reduction, black: untested.

**Future research**

The research laid down in this thesis concerns *in vitro* experiments only. It should be realized that this is an important limitation because extrapolation of the present findings to the *in vivo* situation clearly is not possible without further studies. Thus, future research in animal models should shed further light on the contribution of the immunomodulatory effect of hLF1-11 to its antimicrobial effects previously described *in vitro*. For instance, experiments in myeloperoxidase knock-out mice could be instrumental in investigating whether myeloperoxidase is the main intracellular target mediating the actions of hLF1-11 in monocytes and monocyte-macrophage differentiation. In addition, cytokine and chemokine responses could be measured in the blood of these mice. An alternative approach would be to assess that action of hLF1-11 on monocytes of individuals with myeloperoxidase-deficiency or monocytes in which myeloperoxidase is silenced. Recently, it has been reported that enhanced levels of IL-10 were present in infected mice that were treated with hLF1-11 (7). Moreover, based on our *in vitro* findings, it is expected that in mice that are depleted of monocytes the anti-infective activity of hLF1-11 is reduced (26). In addition, a longer *in vivo* infection set-up could be instrumental in investigating the subsequent involvement of components of the adaptive immune system that hLF1-11 is known to modulate *in vitro*. Again, it should be realized that our experiments mainly concerned human blood-derived mononuclear cells, and findings may not necessarily be identical in the murine model.

As safety of hLF1-11 in humans has been established (27), the goal of the studies in patients should be to obtain proof of principle for the usefulness of hLF1-11 as an anti-infective agent in a human patient population. Besides experiments that would further elucidate the mechanism of action of hLF1-11, one could also research the involvement of myeloperoxidase in signal transduction routes that lead to cytokine production. Currently, it is not known whether these effects are mediated through the reactive oxygen species production route or that myeloperoxidase displays an additional mechanism of action by being a component of another signaling pathway.

**Therapeutical applications**

As a future perspective, we suggest that antimicrobial/host defense peptides like hLF1-11 and LL-37 might prove useful in several distinct therapeutic applications. Obviously such applications are at the end of a long road of development still to come, and some general comments and thoughts on possible applications should suffice here. First, LL-37, as well as hLF1-11, could act as a single anti-infective agent; either to prevent infection when used in a prophylactic approach or to treat infection. As LL-37 and hLF1-11 displayed differential effects on the immune system (chapters 3-5), several infectious diseases might be treated with different antimicrobial/host defense peptides. For example, transplantation recipients are susceptible for infections and need an alternative when current antibiotics can no longer perform adequately. hLF1-11 could be involved in an alternative treatment for these infections. Host defense peptides like LL-37 and hLF1-11 can also be applied locally, for example like Stallmann *et al.* have shown (28). They used hLF1-11 as a prophylactic agent in treatment of osteomyelitis (29-31). hLF1-11 in cement was injected into the femoral canal of rabbits earlier infected with MRSA. The continuous release of the peptide by the cement significantly reduced the bacterial load as compared to the control group. In over 75% of the hLF1-11 treated rabbits no growth at all of bacteria was detected (32). Also, hLF1-11 could be used in combination with conventional antibiotics making use of a synergistic antimicrobial activity. Lupetti *et al* showed *in vitro* that hLF1-11 at non-candidacidal concentrations exerted synergistical effects with fluconazole, results showed this combination to be highly effective against fluconazole-resistant *C. albicans* (33). hLF1-11 was necessary to initiate this effect as the observed synergistic effect was only induced when *C. albicans* were pre-incubated with (non-candidacidal concentrations of) hLF1-11 followed by exposure to fluconazole in comparison to no killing effect when the experiment was performed in the opposite order. Second, an alternative approach would be to focus on ways to induce endogenous antimicrobial peptide synthesis, thereby modulating the host response, and such an approach could be employed to treat infections of any kind (34). This could also be induced locally resulting in specific targeting of the infection. For instance, experiments with butyrate -an inducer of endogenous LL-37 synthesis- have shown that it promoted elimination of *Shigella* (35). Moreover, it is known that vitamin D metabolites regulate the expression of LL-37. Vitamin D insufficiency is common still in both industrialized and developing nations (36). Recent studies have shown that vitamin D insufficiency is associated with a somewhat higher risk of active tuberculosis (37-39). Of note, LL-37 shows activity against *M. tuberculosis* and therefore induction of LL-37 transcription by

vitamin D could possibly be helpful by treatment of mycobacterial infections (40-42). Obviously, the safety and consequences of administration of butyrate and vitamin D should be further investigated before application in humans (34). Moreover, additional compounds that induce endogenous production of antimicrobial/host defense peptides need to be identified, but these preliminary results already seem to indicate the potency of this application. Third, as hLF1-11 is a potent inhibitor of myeloperoxidase one can think of applications of hLF1-11 as an agent for diseases in which myeloperoxidase plays an unfavourable role. Myeloperoxidase has emerged as a potential participant in the promotion and/or propagation of atherosclerosis (43). Liu *et al* showed that pre-treatment of rabbits that were subjected to myocardial ischaemia and reperfusion with ABAH significantly reduced cardiac caspase-3 activity, providing direct evidence that myeloperoxidase is a significant contributor to post-ischaemic cardiomyocyte apoptosis (44). These results can have implications as they suggest therapeutic interventions, for example hLF1-11 may (in part) exert cardioprotective effects by inhibition of myeloperoxidase. Lastly, antimicrobial/host defense peptides have been mentioned as potent components of vaccine formulations (5,45). The presence of these peptides could (re)direct the development of antigen presentating cells toward a type driving a favorable immune response to the presented antigen.

**Conclusion**

In conclusion, the future of antimicrobial peptides with immune modulating activity appears bright as these peptides might be developed further into a novel class of anti-infectives to which microbial drug-resistance is unlikely to develop quickly. Obviously, research on antimicrobial/host defense peptides (including hLF1-11) has to be extended before these peptides can be safely introduced in medical practice. It is encouraging to see that the safety of hLF1-11 has not been a concern in clinical trial phase I and IIa. Together, our findings strongly support the notion that the therapeutic potential of hLF1-11 and other small host defense peptides should be investigated further.

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# Nederlandse samenvatting

**Inleiding**

De ontdekking van penicilline eind jaren twintig door Alexander Fleming luidde het tijdperk van de antibiotica in. Vanaf de jaren ‘40 werden infecties bij mens en dier behandelbaar door gebruik van penicilline en later ontdekte antibiotica. Inmiddels heeft het gebruik én misbruik van deze middelen erin geresulteerd dat veel bacteriën en andere ziekteverwekkers resistent zijn geworden tegen veel van de antibiotica. Overmatig of onjuist gebruik van antibiotica heeft zelfs geleid tot micro-organismen die tegen alle bekende antibiotica totaal resistent zijn, gedefinieerd als pan-drug resistentie. Vaak wordt vergeten dat antibiotica slechts hulpmiddelen zijn, bedoeld om het afweersysteem van de patiënt in de gelegenheid te stellen de infectie te overkomen. Geen wonder dus dat problemen rond antibiotica resistentie voornamelijk spelen in een ziekenhuis setting, waar antibiotica toegepast worden om infecties te bestrijden bij patiënten met een verminderde afweer zoals transplantatiepatiënten of hiv-geïnfecteerden.

Door toenemende resistentie voor de gebruikelijke antibiotica is het noodzakelijk dat er snel alternatieven voor deze antibiotica ontwikkeld worden. Het aantal nieuwe middelen dat de komende jaren op de markt komt is echter zeer beperkt, en wat er beschikbaar komt zijn vaak variaties op al bekende antibiotica. Naast onderzoek gericht op nieuwe typen van antibiotica verrichtten universiteiten en bedrijven ook onderzoek naar componenten van de afweer van mens en dier, of varianten daarvan, welke als basis zouden kunnen dienen voor een nieuw model van infectie-bestrijdende medicijnen. Een groep moleculen binnen dit onderzoek zijn de antimicrobiële peptiden (AMPs), kleine eiwitten die in staat zijn om ziekteverwekkers te doden. Deze peptiden kunnen van nature voorkomen in het lichaam van mens of dier, of ze vormen fragmenten van (grotere) eiwitten. Deze peptiden kunnen bovendien *in silico* ontworpen worden met behulp van computer software. Ondanks een grote diversiteit in herkomst hebben AMPs een aantal eigenschappen gemeen. Zo zijn ze vaak positief geladen (kationisch), actief tegen bacteriën (en door hun geheel ander werkingsmechanisme vaak ook actief tegen bacteriën die resistent zijn tegen antibiotica) en kunnen ook anti-schimmel, anti-virale of anti-parasitaire activiteiten vertonen. Daarnaast hebben sommige AMPs anti-carcinogene of afweerregulerende eigenschappen. AMPs werden in de jaren tachtig van de twintigste eeuw eerst geïsoleerd uit poppen van de zijdemot door de Zweedse onderzoeker Hans Boman en zijn medewerkers. In de afgelopen 30 jaar zijn er veel meer AMPs geïsoleerd uit een verscheidenheid aan planten, bacteriën, dieren en de uit mens. Een interessante eigenschap van AMPs betreft hun regulerende effect op het afweersysteem van de gastheer. Aangezien het afweersysteem geen onderscheid maakt tussen antibiotica

resistente en gevoelige micro-organismen, zijn AMPs evenzogoed in staat de gastheer te helpen beide groepen micro-organismen te elimineren. Echter, om AMPs in te kunnen zetten als therapeutisch middel moet nog veel kennis vergaard worden, te beginnen met inzicht in de werkingsmechanismen van deze peptiden.

In dit proefschrift is onderzoek verricht naar twee AMPs van menselijke oorsprong. Ten eerste een AMP bestaande uit de eerste elf aminozuren van het humane antimicrobiële eiwit lactoferrine, genaamd hLF1-11. Lactoferrine is een lichaamseigen eiwit dat aanwezig is in moedermelk, speeksel, traanvocht, en in granules, blaasjes, die aanwezig zijn in een bepaald type afweercel genaamd de neutrofiele granulocyt. Het eiwit bestaat uit 693 aminozuren en heeft een breed scala aan eigenschappen waaronder antimicrobiële, ontstekingsremmende en afweerregulerende activiteiten. Lactoferrine is bij het publiek bekend geworden vanwege stier Herman. Bij deze genetisch gemodificeerde stier was een stukje DNA ingebouwd zodat zijn vrouwelijke nakomelingen humaan lactoferrine zouden produceerden in hun melk. Een van de belangrijkste werkingsmechanismen van lactoferrine is het binden van ijzer: hiermee wordt een molecuul dat van essentieel belang is voor de groei van bacteriën weggevangen en wordt verdere uitbreiding van de infectie tegengegaan. Binnen de eerste 47 aminozuren van de N-terminus van het lactoferrine eiwit bevinden zich twee kationische domeinen. Uit onderzoek is gebleken dat het eerste kationische domein, aminozuur 1 tot 11, het meest actieve domein is wat betreft de activiteit van lactoferrine tegen bacteriën en schimmels. Het peptide dat zo ontstaat, hLF1-11 genaamd, is in staat gebleken om de uitgroei van methicilline-resistente *Staphylococcus aureus* (MRSA) en fluconazole-resistente *Candida albicans* in een muizenmodel in te dammen. Ondanks dat hLF1-11 *in vivo* in staat blijkt micro-organismen in hun uitgroei te remmen, blijkt bij *in vitro* experimenten dat het doden van de pathogeen alleen optreedt in media met een lage zout concentratie en niet bij een fysiologische zoutconcentratie (d.w.z., gelijk aan de zoutconcentratie in het lichaam). Op grond van onder andere deze bevinding werd verondersteld dat hLF1-11 meerdere werkingsmechanismen heeft om infecties te bestrijden, mogelijk los van een direct antimicrobieel effect. In dit proefschrift is onderzocht of hLF1-11 een additioneel werkingsmechanisme heeft, waarbij door versterken van het afweersysteem van de gastheer, een infectie beter kan bestreden worden. Daarnaast is onderzocht, hoe hLF1-11 deze effecten in afweercellen bewerkstelligt. De achtergrond gedachte is om hierbij nieuwe therapeutische interventiemogelijkheden bij infecties aan het licht te brengen. Ten tweede is onderzoek gedaan naar een welbekend humaan AMP, genaamd LL-37. Dit peptide, dat 37 aminozuren lang is, wordt geproduceerd door een verscheidenheid aan

cellen in het menselijk lichaam, zoals neutrofielen, monocyten en epitheelcellen. Het komt tot expressie tijdens een infectie. LL-37 is niet alleen actief tegen een brede diversiteit aan ziekteverwekkers, maar is ook in staat de afweer reactie van de mens te reguleren, bijvoorbeeld door afweercellen naar een ontstekingshaard te rekruteren, en door het aangeboren en adaptieve afweersysteem te beïnvloeden. In dit proefschrift is onderzocht of LL-37 vergelijkbare effecten heeft als gevonden voor hLF1-11 op een specifiek onderdeel van het afweersysteem, en of deze effecten AMP specifiek zijn.

**Dit proefschrift**

In **hoofdstuk 1** van dit proefschrift wordt de ontdekking van antimicrobiële eiwitten en peptiden kort beschreven en wordt het onderzoek samengevat dat voorafgegaan is aan hetgeen in dit proefschrift beschreven wordt. Ook wordt informatie gegeven over het lichaamseigen antimicrobiële peptide LL-37 en over het afweersysteem van de mens. Dit afweersysteem bestaat uit een aangeboren en een adaptief, verworven systeem. Het aangeboren afweersysteem reageert op alles dat als gevaarlijk beschouwd wordt; specificiteit ligt besloten in het type reactie dat een lichaamsvreemd molecule oproept. De belangrijkste afweercellen die hierbij betrokken zijn, betreffen de neutrofielen en mononucleaire fagocyten. Deze laatste groep bestaat uit monocyten, macrofagen en dendritische cellen. Deze cellen zijn in staat ziekteverwekkers te fagocyteren (‘opeten’), wat de infectie zal helpen beteugelen of zelfs geheel op zal ruimen. Tevens kunnen ze componenten van deze ziekteverwekkers op hun oppervlakte presenteren aan T-lymfocyten welke deel uit maken van het adaptieve afweersysteem. Enkele cellen uit het brede repertoire aan T-cellen herkennen de structuur en zullen hierop reageren met een specifieke respons tegen de ziekteverwekker.

Het dierexperimenteel model waar hLF1-11 in beproefd is bestrijkt 48 uur. Dit ligt binnen de reactietijd van de aangeboren afweer. Daarnaast bleek hLF1-11 actief in neutropene dieren (d.w.z., in complete afwezigheid van neutrofiele granulocyten) en daarom is onderzocht of hLF1-11 mogelijk effecten uitoefent op mononucleaire fagocyten, afweercellen die in neutropenie immers nog wel aanwezig zijn. Daarbij, hLF1-11 wordt *in vivo* intraveneus ingespoten waardoor monocyten waarschijnlijk deel uit maken van de eerste groep afweercellen die met hLF1-11 in contact komen.

In **hoofdstuk 2** staan de resultaten beschreven over de stimulerende effecten van hLF1-11 op zowel monocyten van muizen als monocyten geïsoleerd uit het bloed van mensen. hLF1-11 bleek de reactie van monocyten op structuren van pathogene micro-organismen, te versterken. Een opmerkelijke bevinding was dat de reactie van monocyten op alle

pathogene structuren die we getest hebben door incubatie van de cellen met hLF1-11 verhoogd werd, met uitzondering van de reactie op componenten van Gram-positieve bacteriën. Zodra afweercellen een infectie in hun nabijheid waarnemen produceren ze moleculen die bewerkstelligen dat monocyten vanuit de bloedbaan het weefsel in migreren (chemotactische factoren) en daar differentiëren tot macrofaag of dendritische cel. Omdat hLF1-11 een modificerend effect had op de reactie van monocyten op pathogene structuren, was een logische vervolgvraag of monocyten die met hLF1-11 geïncubeerd worden ook beïnvloed zouden worden in hun differentiatie tot macrofaag of dendritische cel. De resultaten van onderzoek naar deze vraag staan beschreven in **hoofdstuk 3** en **hoofdstuk 4**.

In **hoofdstuk 3** hebben we monocyten tot macrofagen gedifferentieerd in de aan- of afwezigheid van hLF1-11, en daarna de karakteristieken van de cellen onderzocht. Hierbij hebben wij ons gericht op de differentiatie naar een macrofaag met specifiek ontstekingsbevorderende eigenschappen (de zogenaamde type-1 macrofaag), dat is een macrofaag zoals je die zou verwachten bij een infectie. Macrofagen die gedifferentieerd waren in de aanwezigheid van hLF1-11, bleken naast ontstekingsbevorderende ook ontstekingsremmende moleculen te produceren. Daarnaast reageerden deze macrofagen eerder op pathogene bacteriële structuren dan controle cellen, en waren ze in staat om pathogenen sneller te fagocyteren en vervolgens te doden. Opvallend was dat een korte blootstelling van slechts een uur aan het begin van de incubatie al voldoende was om de differentiatie van monocyten vergelijkbaar te beïnvloeden als wanneer hLF1-11 continu in de kweek aanwezig bleef, d.w.z. voor 7 dagen. Dit suggereert dat het effect van hLF1-11 al vroeg en snel optreedt, nog voor differentiatie van monocyt naar macrofaag. Aangezien een monocyt ook kan differentiëren tot dendritische cel, zijn we in **hoofdstuk 4** het effect van hLF1-11 op deze differentiatie nagegaan. Als monocyten differentiëren tot een dendritische cel in de aanwezigheid van hLF1-11, blijkt deze cel vooral effectiever tegen *C. albicans* te zijn, in vergelijking met controle cellen. Het was opmerkelijk dat, waar de fagocytose van *C. albicans* in aan hLF1-11 blootgestelde dendritische cellen hoger is in vergelijking met controle cellen, de opname van MRSA niet aangedaan en onveranderd is. Wel produceren dendritische cellen die gedifferentieerd zijn in de aanwezigheid van hLF1-11 meer zuurstofradicalen en de moleculen interleukine-6 en 10 na stimulatie met *C. albicans*. Zodra een dendritische cel een pathogeen gefagocyteerd heeft, presenteert de cel fragmenten hiervan aan T lymfocyten; de dendritische cel wordt dan rijpe dendritische cel genoemd. De uitrijping van de dendritische cel in aanwezigheid van een pathogene structuur blijkt bij hLF1-11-gedifferentieerde dendritische cellen achter te blijven.

Interessant is dat deze cellen in staat blijken om een zogenaamde Th17 reactie te induceren, na opkweken van de cellen met T-lymfocyten. Een Th17 reactie wordt in de literatuur geassocieerd met een anti-schimmel reactie, en is dus consistent met onze eerdere resultaten betreffende een versnelde opname van *C. albicans*.

In **hoofdstuk 5** worden de resultaten beschreven van onderzoek naar de moleculaire mechanismen achter de effecten van hLF1-11 op mononucleaire cellen. Met behulp van fluorescentie microscopie is onderzocht of het peptide aan monocyten bindt en/of in de cel migreert. Dit zou immers verduidelijken of hLF1-11 via een receptor op de cel aangrijpt, of het peptide een target, bindingspartner, binnenin de cel heeft. Met dit onderzoek hebben we kunnen aantonen dat hLF1-11 inderdaad in de cel migreert en bindt aan een targetmolecuul in de cel. Via een zogenaamd 'fishing' experiment met het peptide als aas in een 'vijver' van gelyseerde, uiteengevallen monocyten, hebben we het molecuul dat het sterkste aan hLF1-11 bindt geïdentificeerd als zijnde myeloperoxidase (MPO), een enzym betrokken bij de vorming van zuurstofradicalen. Om na te gaan hoe relevant deze binding is voor de eerder waargenomen effecten van hLF1-11 op monocyten werd onderzocht of hLF1-11 na binding aan MPO het molecuul remt in zijn enzymatische activiteit. Dit bleek het geval. Vervolgens konden we met een commerciëel verkrijgbare remmer van MPO vergelijkbare effecten in monocyten en macrofagen induceren als degene die met hLF1-11 werden verkregen. Door het MPO te binden en de enzymwerking te onderdrukken blijkt hLF1-11 in staat om haar regulerend effect op afweercellen en daarmee het afweersysteem uit te oefenen.

Ten slotte hebben we in **hoofdstuk 6** onderzocht of het lichaamseigen AMP LL-37 eveneens de differentiatie van monocyten naar macrofagen beïnvloedt. Dit bleek niet alleen het geval, maar ook waren de effecten verschillend van die van hLF1-11 op de differentiatie van monocyt naar macrofaag. LL-37 was in staat om de differentiatie van monocyt tot een ontstekingsremmende macrofaag om te buigen naar een macrofaag met ontstekingsbevorderende eigenschappen. LL-37 kon dit effect zelfs bereiken bij al uitgedifferentieerde macrofagen. Aangezien LL-37 normaliter vrijkomt tijdens ontstekingsreacties en door het afweersysteem ingezet wordt tijdens infectie lijkt in theorie op dat moment een ontstekingsbevorderende macrofaag meer van pas te komen dan een ontstekingsremmende macrofaag, bij de beteugeling van de infectie.

**Tot slot**

Deze samenvatting begon met de constatering dat er een hoge nood is voor nieuwe antibiotica, en dan vooral antimicrobiële middelen die niet slechts een variatie zijn op de

al bekende middelen. Tegen veel middelen die op een enkele target binnen de bacterie aangrijpen, blijken op den duur resistente micro-organismen geselecteerd te worden. Een manier om dit probleem te omzeilen zou zijn om niet rechtstreeks op de bacterie aan te grijpen, maar indirect, door sturing en ondersteuning van het afweersysteem. Uit het onderzoek beschreven in dit proefschrift blijkt dat hLF1-11 in staat is het aangeboren afweersysteem te versterken door de functie van de betrokken afweercellen te stimuleren. Het adaptieve, verworven afweersysteem blijkt eveneens beïnvloed te worden, al zal verder onderzoek moeten uitwijzen of dit werkelijk bijdraagt aan het beteugelen van infectie *in vivo*. Interessant is dat hLF1-11 ook ontstekingsremmende eigenschappen in de onderzochte afweercellen oproept. Zo'n mechanisme zou de mate van ontsteking afzwakken en daarmee omliggende weefselschade verminderen. De identificatie van myeloperoxidase als belangrijke speler in mediatie van de effecten van hLF1-11, suggereert een nieuwe therapeutische target voor stimulatie en versterking van het afweersysteem.

Aangezien LL-37 andere effecten op afweercellen heeft dan hLF1-11, maakt dit onderzoek duidelijk dat het mogelijk is om het afweersysteem met antimicrobiële peptiden op verschillende wijzen te beïnvloeden. In de toekomst zou het mogelijk kunnen worden de afweerreactie tijdens infectie specifiek te sturen door de productie van lichaamseigen AMPs te activeren, of AMPs van een andere bron in te zetten als geneesmiddel. Doordat AMPs de beperkingen van de huidige antibiotica omzeilen zou een dusdanige ontwikkeling kunnen bijdragen aan het snel groeiende probleem van de behandeling van antibiotica-resistente micro-organismen.

# Curriculum Vitae

De schrijfster van dit proefschrift werd geboren op 18 september 1982 te 's-Gravenhage. Na het behalen van haar V.W.O. diploma bij het Adelbert College te Wassenaar in 2001, begon zij in datzelfde jaar met de studie Biologie aan de Universiteit Leiden. Tijdens haar studie koos zij voor de richting medische biologie en liep zij haar 3e en 4e jaars stages bij de afdeling Infectieziekten in het Leids Universitair Medisch Centrum onder begeleiding van prof.dr. Jaap van Dissel, dr. Peter Nibbering en dr. Mariena van der Plas. In het laatste jaar van haar studie Biologie liep zij stage bij de afdeling Parasitologie onder begeleiding van prof.dr. André Deelder en dr. Paul Hensbergen om zich te verdiepen in proteomics technieken. In mei 2006 heeft zij haar doctoraal diploma ontvangen om in juli 2006 te starten als promovendus met het in dit proefschrift beschreven onderzoek. Per september 2010 is zij werkzaam als postdoc in het laboratorium van prof.dr Lennart Lindbom bij de afdeling Fysiologie en Farmacologie van het Karolinska Institutet in Stockholm, Zweden.

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