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

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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).

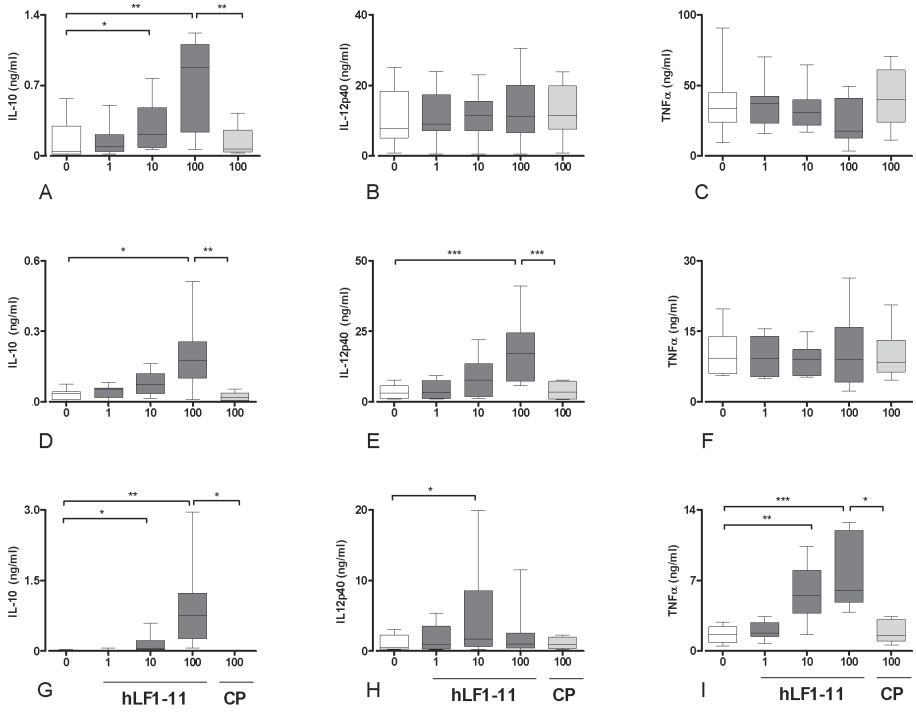

Fig. 1 Cytokine profiles of hLF1-11-exposed GM-CSF-driven macrophages in response to LPS, LTA, and heat-killed *C. albicans* Monocytes were cultured with rhGM-CSF in the presence of hLF1-11 (1, 10, and 100 µg/ml) (light-gray bars), control peptide (CP; 100 µg/ml) (dark-gray bars), or saline (open bars) for 6 days. Thereafter, the cells were stimulated with 100 ng/ml LPS (A to C), 1 µg/ml LTA (D to F), or 1x10^7 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-

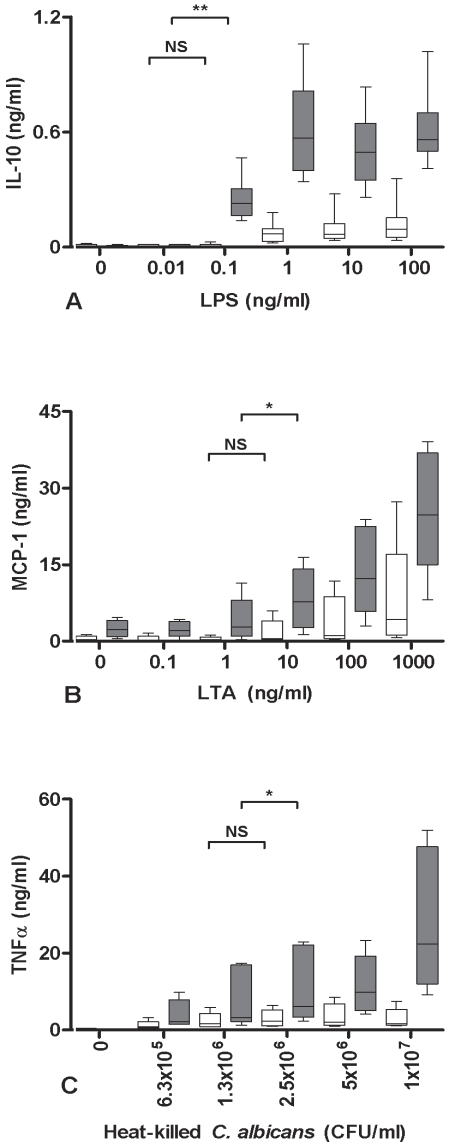

Fig. 2 Responsiveness of hLF1-11-incubated GM-CSF-driven macrophages to LPS, LTA, or heat-killed *C. albicans* Monocytes were incubated with rhGM-CSF and either with (gray bars) or without (open bars) hLF1-11 (100 µg/ml) and stimulated on day 6 with various concentrations of LPS (A), ultrapure LTA (B), 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.

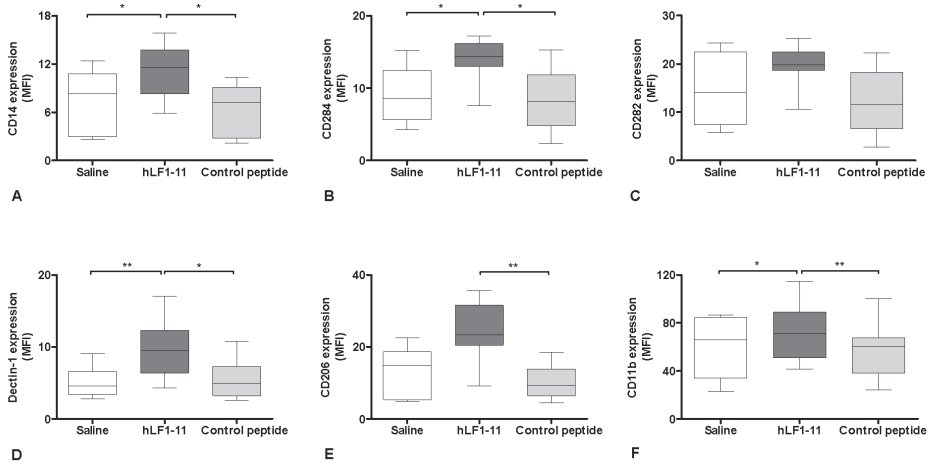

Fig. 3 Comparison of surface molecule expression by hLF1-11 or control peptide-exposed GM-CSF-driven macrophages Monocytes were cultured in the presence of rhGM-CSF and hLF1-11 (100 µg/ml; dark gray boxes), control peptide (100 µg/ml; light gray boxes), or no peptide (saline; open boxes). On day 7, macrophages 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.

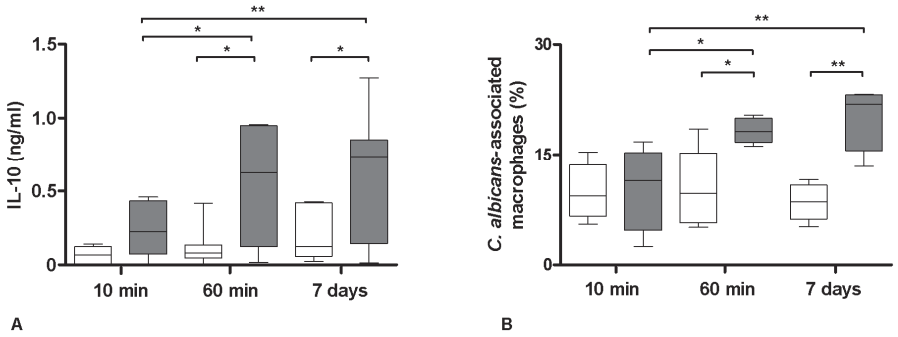

Fig. 4 Effect of the presence of hLF1-11 on the GM-CSF-driven differentiation of monocytes into macrophages
(A) Monocytes were cultured in the presence of rhGM-CSF. At the start of the culture, hLF1-11 was added (100 µg/ml, filled bars) for 10 min or 60 min or for constant exposure for 7 days. Saline 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.

Acknowledgements

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