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

Does, A.M. van der

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

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Anne M. van der Does[1]*

Sylvia J.P. Bogaards[2]*

Luigi Jonk[2]

Marty Wulferink[2]

Markwin P. Velders[2]

Peter H. Nibbering[1]

1. Dept. of Infectious Diseases, Leiden University Medical Center, The Netherlands
2. AM-Pharma, Bunnik, The Netherlands

*These authors contributed equally to this work

# 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).

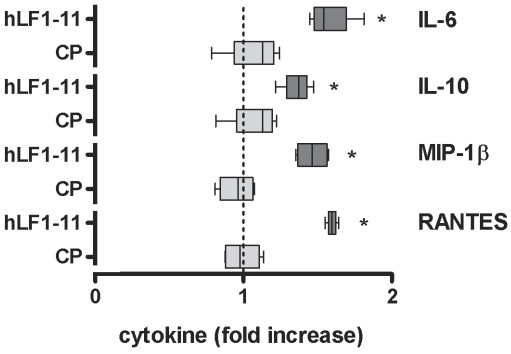



**Fig. 1 Effect of hLF1-11 on cytokine production by murine monocytes in response to LPS** Monocytes were exposed to 100 μg/ml hLF1-11 (dark gray boxes) or 100 μg/ml control peptide (CP; light gray boxes), or no peptide for 1 h and then stimulated with 100 ng/ml LPS for 18 h. Thereafter, levels of IL-1α, 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.

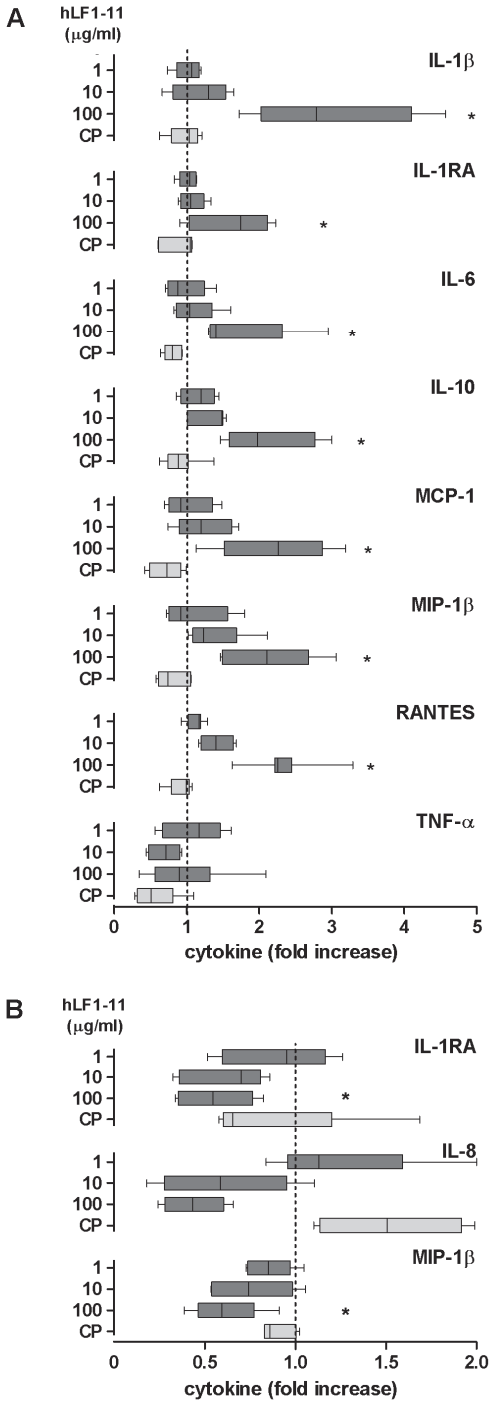



**Fig. 2 Effect of hLF1-11 on cytokine production by human monocytes in response to LPS** Monocytes were exposed to various concentrations of hLF1-11 (dark gray boxes) or 100 μg/ml control peptide (CP; light gray boxes) or vehicle for 1 h and thereafter stimulated with 100 ng/ml LPS (a) for 18 h. Levels 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).

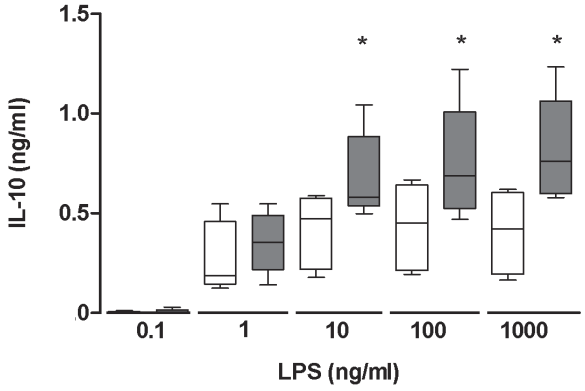



**Fig. 3 Effects of hLF1-11 on cytokine production by human monocytes stimulated with various LPS concentrations** Monocytes were exposed to hLF1-11 (dark gray boxes) or no peptide (open boxes) for 1 h and then stimulated with various concentrations LPS for 18 h. Thereafter supernatants were collected 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.

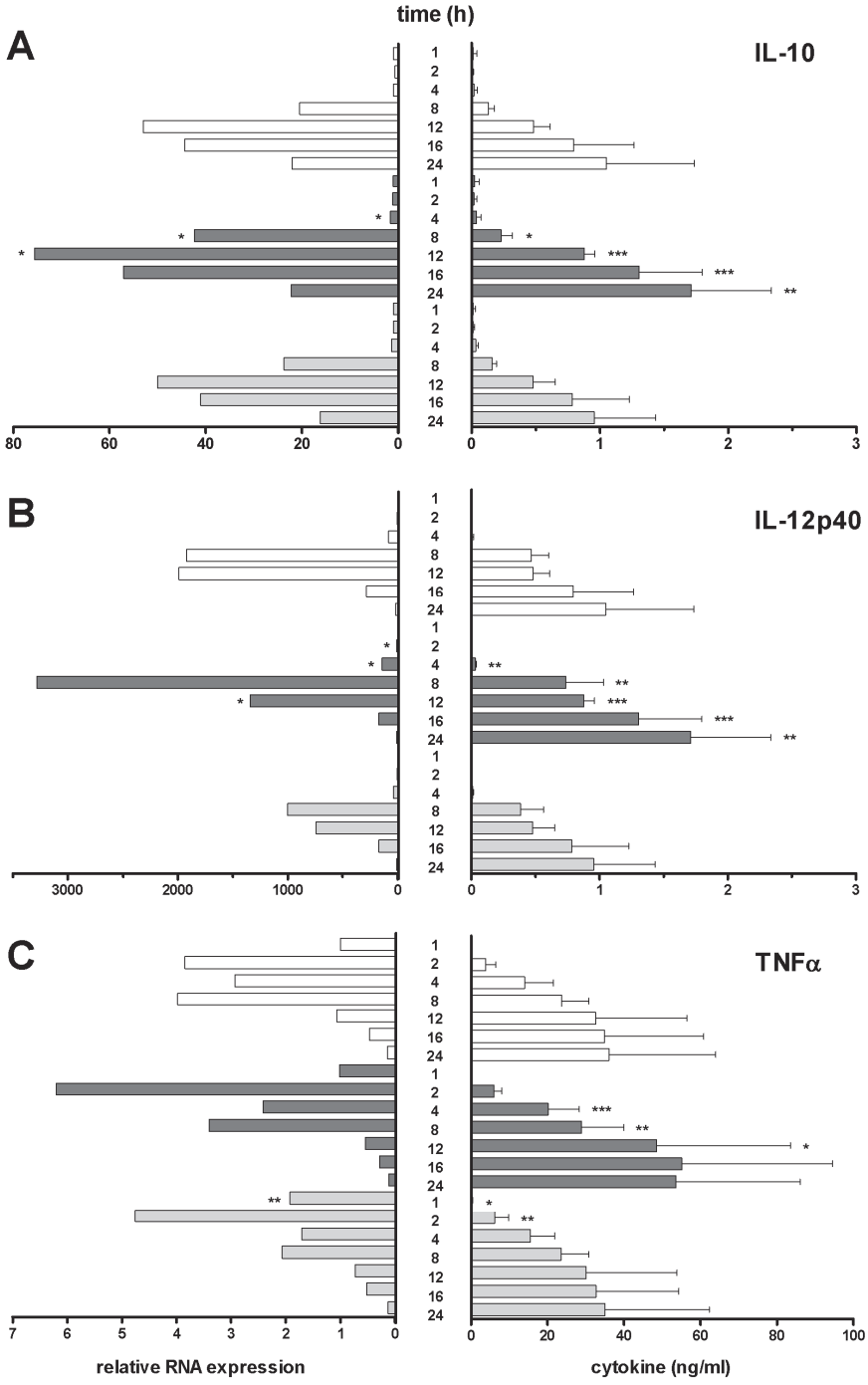
**Interaction of hLF1-11 with LPS in human monocyte cultures** To exclude the possibility that hLF1-11 exerts its effects on cytokine production by interacting with LPS, two experiments were performed. First the order of stimulation was reversed, freshly isolated monocytes were stimulated with LPS and 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)

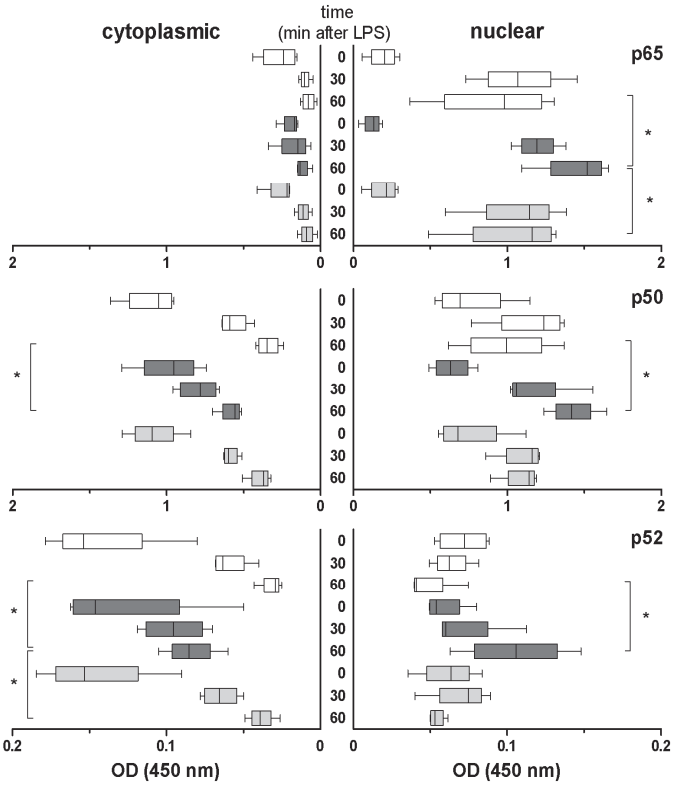



**Fig. 5 Effect of hLF1-11 on NF-κB translocation in human monocytes** Freshly isolated monocytes were exposed to hLF1-11 (dark gray boxes), control peptide (CP; light gray boxes), control peptide (open boxes) for 1 h and then stimulated with LPS for 30 or 60 min. Then cytoplasmic (left) and nuclear (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).

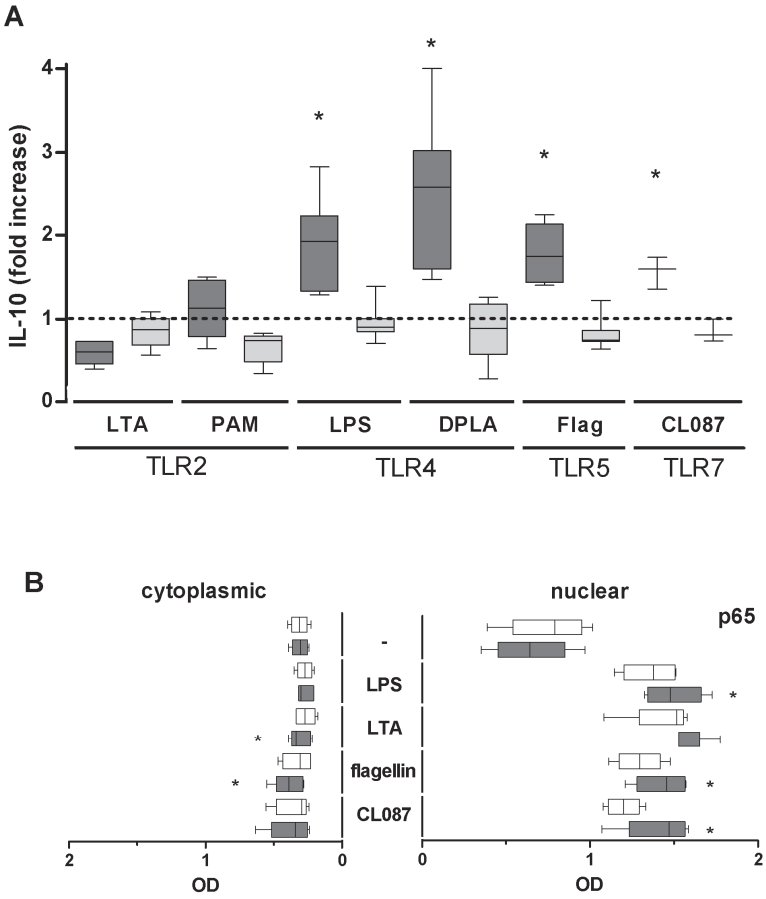



**Fig. 6 Effect of hLF1-11 on cytokine production and NF-κB translocation by human monocytes in response to different TLR-stimuli** Freshly isolated monocytes were exposed to hLF1-11 (dark gray boxes), control peptide (CP; light gray boxes), or no peptide (open boxes) for 1 h and then stimulated with 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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