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 exerts immunomodulatory effects by specific inhibition of myeloperoxidase activity

Anne M. van der Does[1]

Paul J. Hensbergen[2]

Sylvia J.P. Bogaards[3]

André M. Deelder[2]

Jaap T. Van Dissel[1]

Peter H. Nibbering[1]

1. Dept. of Infectious Diseases, LUMC, Leiden, The Netherlands
2. Biomolecular Mass Spectrometry Unit, Dept. of Parasitology, LUMC, Leiden, The Netherlands
3. Dept. of Physiology, VUMC, Amsterdam, The Netherlands

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™ 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 (3×10^7/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 1×10^{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* (1×10^{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 (1×10^{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.
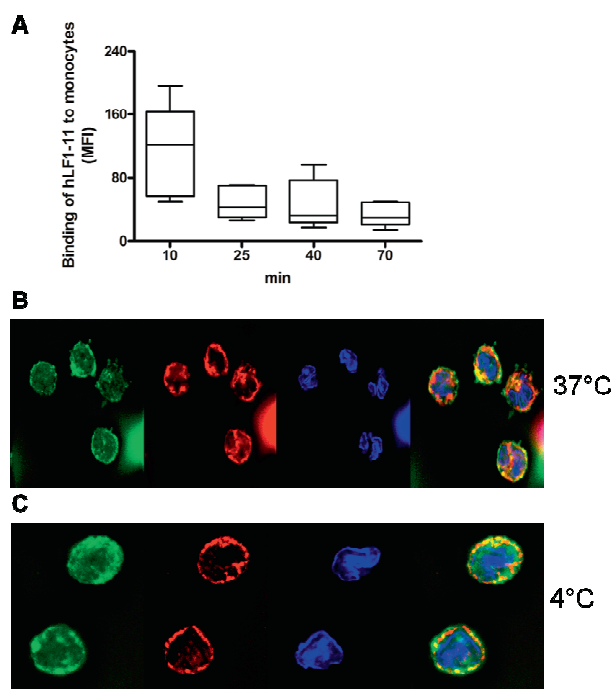
Fig. 1 Binding and uptake of hLF1-11 by human monocytes To assess whether hLF1-11 binds to monocytes, human monocytes were incubated with biotinylated hLF1-11 for several intervals. Thereafter, the cells were washed, incubated with streptavidin-PE for 15 min, washed again and then the mean fluorescence 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.
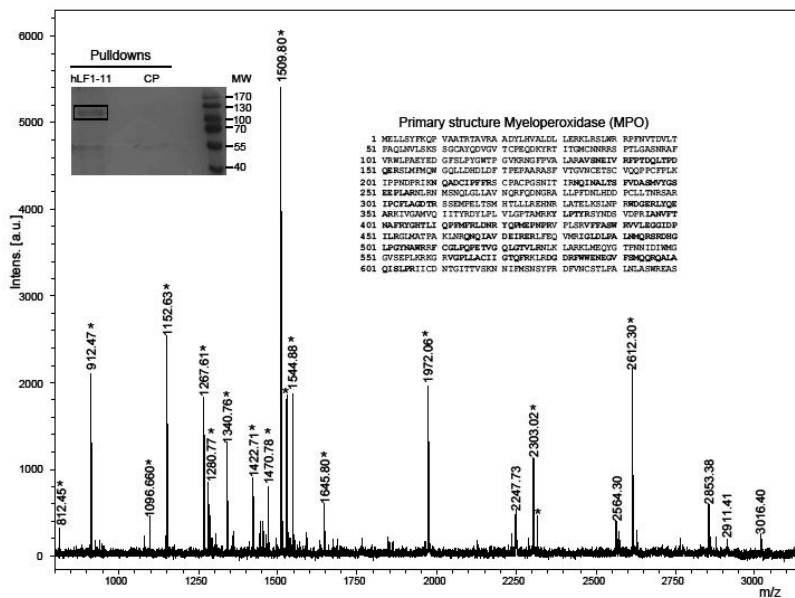

Fig. 2 Identification of myeloperoxidase as the principal binding partner of hLF1-11 in monocytes To investigate the intracellular binding partner of hLF1-11, biotinylated hLF1-11- and control peptide (CP)-coupled streptavidin columns were used in pull-down assays using monocytic cell lysates as input. 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.
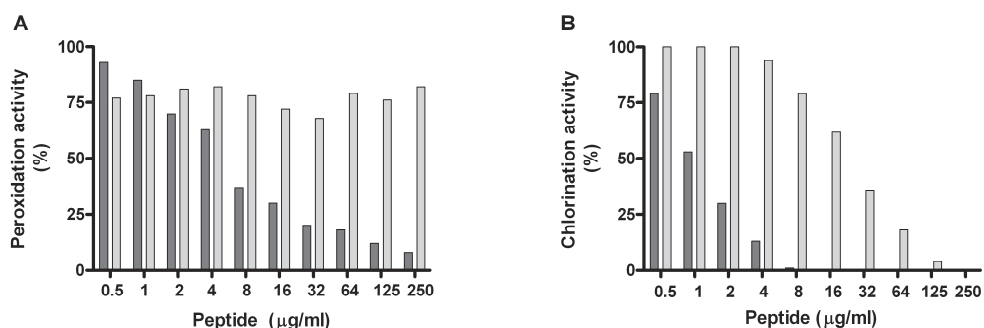

Fig. 3 Myeloperoxidase activity in the presence of hLF1-11 and control peptide The peroxidation (A) and the chlorination (B) activity by myeloperoxidase were assessed in the presence of several concentrations of hLF1-11 (dark gray) or control peptide (light gray). The peroxidation assay utilizes the 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.
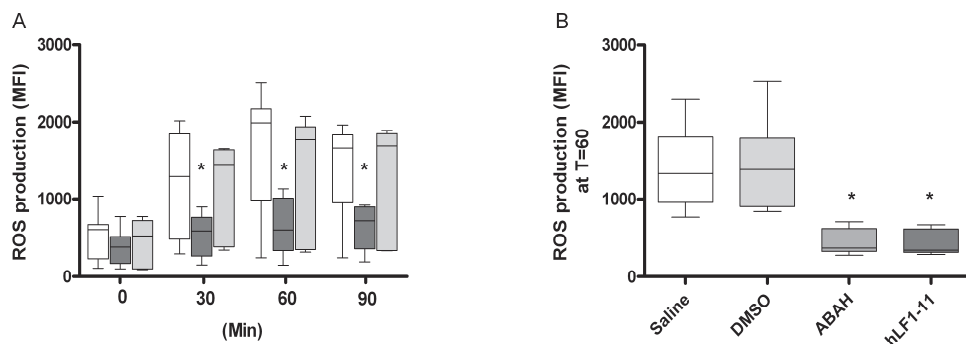
Fig. 4 Intracellular ROS production by hLF1-11- or control peptide-incubated monocytes in response to LPS
Monocytes were cultured in the presence of hLF1-11 (100 µg/ml, dark gray), control peptide (100 µg/ml, light gray) or no peptide (open) for 1 h and then labeled with DCFH-DA. Next, the labeled monocytes 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).
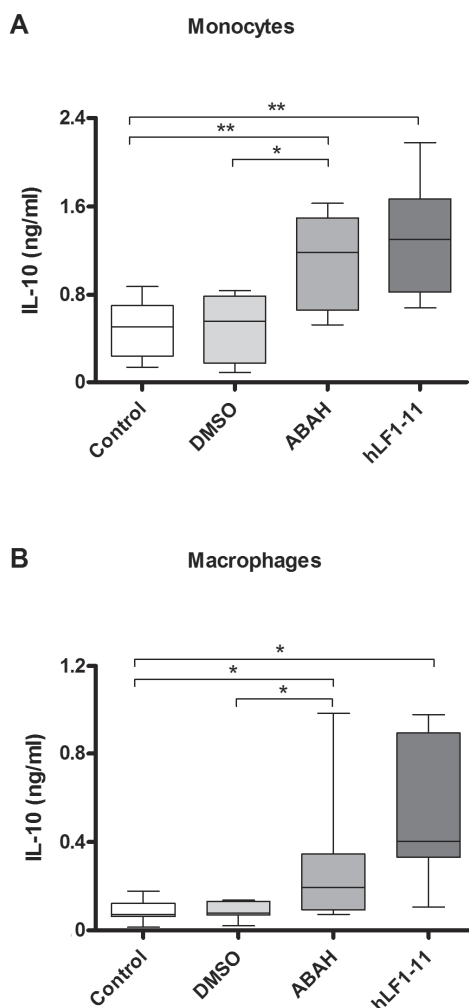
A Monocytes

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.

B 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).
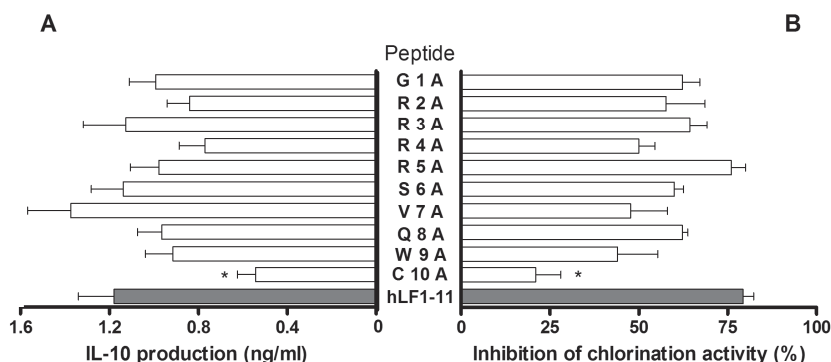
Fig. 6 Effects of hLF1-11 and single alanine substituted analogs on myeloperoxidase activity and on IL-10 production by monocytes in response to LPS Human monocytes were incubated with hLF1-11 or derived peptides in which one amino acid within the hLF1-11 sequence was replaced by an alanine (all 100 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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