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*J Immunol* 2012; 188:5012-5019; Prepublished online 20 April 2012;
doi: 10.4049/jimmunol.1102777
http://www.jimmunol.org/content/188/10/5012

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The Human Lactoferrin-Derived Peptide hLF1-11 Exerts Immunomodulatory Effects by Specific Inhibition of Myeloperoxidase Activity

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Because of their ability to eliminate pathogens and to modulate various host immune responses, antimicrobial peptides are considered as candidate agents to fight infections by (antibiotic-resistant) pathogens. We recently reported that hLF1-11 (GRRRRSVQWCA), an antimicrobial peptide derived from the N terminus of human lactoferrin, displays diverse modulatory activities on monocytes, thereby enhancing their actions in innate immune responses. The aim of this study was to identify the cellular target of hLF1-11 that mediates these effects. Results revealed that hLF1-11 binds and subsequently penetrates human monocytes, after which it inhibits the enzymatic activities of myeloperoxidase (MPO). Moreover, a chemical inhibitor of MPO (aminobenzoic acid hydrazide) mimicked the effects of hLF1-11 on the inflammatory response by monocytes and on monocyte–macrophage differentiation. Computer-assisted molecular modeling predicted that hLF1-11 can bind to the edge of and within the crevice of the active site of MPO. Experiments with a set of hLF1-11 peptides with amino acid substitutions identified the stretch of arginines and the cysteine at position 10 as pivotal in these immunomodulatory properties of hLF1-11. We conclude that hLF1-11 may exert its modulatory effects on human monocytes by specific inhibition of MPO activity. *The Journal of Immunology, 2012, 188: 5012–5019.

Over the past 60 years, the excessive use of antibiotics in humans and in animals has resulted in the emergence of multidrug resistance of a variety of microorganisms (1–3). Today’s acknowledgment of antibiotic resistance as a global threat underscores the need for better antibiotic stewardship and novel antibiotics with a mode of action different from that of current anti-infectives. In the past decades, the development of antimicrobial agents has mainly focused on ways to eliminate the pathogen, either by a direct microbicidal activity or by affecting bacterial multiplication, allowing the host’s 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 host’s immune system, thereby enhancing its ability to cope with pathogens.

Promising candidates in this respect may be found in the class of antimicrobial peptides (4–6); that is, cationic, relatively short peptides that are active against a variety of microorganisms, including multidrug-resistant pathogens. In addition to their direct antimicrobial activities, many antimicrobial peptides also display immunomodulatory properties. For example, they have been reported to mediate chemotaxis of neutrophils and monocytes (7) and induce the production of cytokines/chemokines by immune cells (8, 9). They can also stimulate angiogenesis and wound healing (10–12) and even modulate monocyte–macrophage differentiation (13) and monocyte–dendritic cell differentiation (14). Obviously, identification of the intracellular target(s) of antimicrobial peptides in immune cells could provide the basis for further development of agents that modulate the ( innate) immune response.

In addition to its antimicrobial effects (15–17), the lactoferrin-derived peptide hLF1-11—a synthetic peptide comprising the first 11 N-terminal residues of human lactoferrin—enhances cytokine and chemokine production by murine and human monocytes in response to microbial stimuli (18). Moreover, this peptide 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 (13). The aim of this study was to identify the cellular target(s) of hLF1-11 that mediates its immunomodulatory effects.

Materials and Methods

Peptides

The synthetic peptide comprising the first 11 aa of human lactoferrin (further referred to as hLF1-11; GRRRRSVQWCA) was purchased from Pepsysyntha (Torrance, CA). The control peptide GAARRAVQWAA, N-terminal biotinylated hLF1-11 (biotin–hLF1-11), N-terminal biotinylated control peptide (biotin–control peptide), and a set of alanine-substituted peptides were from Iosgen (De Meern, The Netherlands). The purity of the peptides was determined by reverse-phase HPLC and exceeded 97%.
additional set of peptides (i.e., GAAAASVQWCA, GAARRSVOCA, GARRASVQWA, GKKKSVQWCA, GRRRSSVQWA, GRRRSSQWUA, GRRRSSCVQWA, GRRRSSVQWA, and GAARRAVQWA) was synthesized and purified as described before (19); the purity of these peptides exceeded 88%. U represents cysteic acid. The peptides were tested for endotoxin contamination by incubation with human monocytes for 24 h. No IL-6, IL-10, IL-12p40, or TNF-α were detected in the supernatants of these cultures, indicating that these peptides were endotoxin free.

**Isolation and culture of human monocytes**

Human monocytes were isolated fromuffycoats from healthy donors by Ficoll amidotrizoate density centrifugation. Monocytes were further purified by CD14-positive selection using anti-CD14–conjugated magnetic microbeads (Miltenyi Biotec, Bergisch Gladbach, Germany) according to the manufacturer’s protocol. The resulting suspension comprised ~96% monocytes with a viability exceeding 98% as determined by annexin V and propidium iodide staining 2 h after isolation. Monocytes were resuspended in RPMI-1640 (Invitrogen, Breda, The Netherlands) supplemented with 2 mM L-glutamine (Invitrogen), 100 U/ml penicillin (PAA, Pasching, Germany), 100 mM streptomycin (PAA), and 10% inactivated FBS (Invitrogen). Monocytes were cultured in RPMI-1640 (Invitrogen, Breda, The Netherlands) supplemented with 2 mM L-glutamine (Invitrogen), 100 U/ml penicillin (PAA, Pasching, Germany), 100 mM streptomycin (PAA), and 10% inactivated FBS (Invitrogen), further referred to as standard medium. Monocytes were cultured at a concentration of 1 × 10⁶ cells/ml of standard medium at 37˚C and 5% CO². Monocytes were exposed to the different peptides or the myeloperoxidase (MPO) inhibitor aminobenzoic acid hydrazide (ABAH; Sigma-Aldrich Chemie BV, Zwijndrecht, The Netherlands) 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 using cytostet ELISA kits (Invitrogen). To investigate the involvement of MPO in the effects of hLF1-11 on GM-CSF–driven monocyte–macrophage differentiation, monocytes were incubated right after isolation in standard medium supplemented with GM-CSF (5 ng/ml) in the presence of either hLF1-11, control peptide, the MPO inhibitor ABAH, or solvent control. MPO oxidizes ABAH to a radical that reduces MPO to its ferrous intermediate. Ferrous MPO reacts either with oxygen to allow enzyme turnover or with hydrogen peroxide to give irreversible inactivation (20). After 6 d of culture, the cells were stimulated with LPS (100 ng/ml) for 20 h; thereafter, supernatants were collected for assessment of the production of IL-10 using cytostet ELISA kits.

**Interaction of hLF1-11 with human monocytes**

Monocytes were incubated with 10 μg hLF1-11–biotin/ml at 37˚C and 4˚C for several intervals up to 70 min, then washed with ice-cold PBS and incubated with PE-labeled streptavidin (Invitrogen) for 15 min on ice. Afterward, the monocytes were washed, and the mean fluorescence intensity (MFI) of the cells was assessed by flow cytometry on a FACSCalibur and analyzed using BD CellQuest software (BD Biosciences, Heidelberg, Germany). To find out whether the hLF1-11 peptide enters cells, we incubated monocytes with 100 μg hLF1-11–biotin/ml 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 24-well slides (Nunc, Rochester, NY) by a 15-min incubation. Next, the cells were carefully washed with PBS supplemented with 0.05% Tween 20 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 (Invitrogen) for 20 min to stain the membranes. Thereafter, cells were washed and mounted with hard Vectashield containing DAPI (Vector Laboratories, Amsterdam, The Netherlands). High-quality, three-dimensional stacks of optical sections were obtained at room temperature with a Marianas digital imaging microscopy workstation (Zeiss 200M; Zeiss, Göttingen, Germany) using a 1.63 oil-immersion objective (EC-plan neofluar, NA 1.25), a CCD camera (Sensicam; PCO, Kelheim, Germany) and a Z-step size of 0.25 μm. Finally, three-dimensional 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 × 10⁵/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 × 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 manufacturer’s protocol. In short, hLF1-11–biotin or control peptide–biotin was immobilized on a streptavidin column. Next, these columns were washed with 0.05% Tween 20 and permeabilized with 90% methanol, and washed with PBS. Thereafter, the interaction of this peptide with the cells was visualized with Alexa 647-labeled streptavidin (left). The cells were stained with rhodamine-conjugated wheat germ agglutinin (a membrane marker; second image from left) and DAPI (to stain the nucleus; second image from right). Thereafter, cells were analyzed on a Marianas digital imaging microscope using a 63× oil-immersion objective and a Z-step size of 0.25 μm. An overlay of the three images is displayed in the right-most panel.
were incubated for 2 h with monocyte lysate supernatant, and after several washings, proteins were eluted with the manufacturer’s 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 5× concentrated nonreducing loading buffer (100 mM Tris-HCl, 4% w/v SDS, 50% glycerol, and 0.05% bromophenol blue) and then subjected to SDS-PAGE on a 10% 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% Na2S2O3 and washed three times for 5 min in water, followed by staining of the proteins for 20 min in 0.2% (w/v) silver nitrate and 0.076% formaldehyde. After washing, the gel was developed using 6% (w/v) Na2CO3, 0.05% formaldehyde, and 0.0004% (w/v) Na2S2O3 for a maximum of 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 NH4HCO3 followed by dehydration with 100% acetonitrile (ACN) for 10 min. For reduction and alkylation, dried gel particles were first incubated with 10 mM DTT for 30 min at 56°C. After 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 NH4HCO3 and dehydration with 100% ACN, the gel particles were completely dried in a centrifugal vacuum concentrator (Eppendorf). The dried gel particles were reswollen 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 NH4HCO3). Subsequently, 20 μl of 25 mM NH4HCO3 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 of 0.1% trifluoroacetic acid was used to extract peptides from the gel plugs, and this extract was pooled with extract 1.

MALDI-TOF/TOF analyses were performed on an Ultraflex II mass spectrometer (Bruker Daltonics, Bremen, Germany) using dihydroxybenzoic acid (5 mg/ml of 50% ACN0.1% trifluoroacetic acid) as a matrix. The mass spectrometer was used in the positive ion reflectron 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 August 23, 2010, 89,486 sequences) using the Mascot search algorithm (Mascot 2.2; Matrix Science, London, U.K.). 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 mass tolerance was set to 50 ppm.

**Assays for MPO enzymatic activity**

The effect of hLF1-11 and the other peptides on MPO enzymatic activities was assessed using an MPO inhibitor screening assay kit of Cayman Europe (Tallinn, Estonia). The assay for OCl− (hypochlorite) and other strong oxidants uses the nonfluorescent 2-[6-(4-aminophenoxy)-3-oxo-3H-xanthen-9-yl]-benzoic acid (APF) to yield the highly fluorescent compound fluorescein. The peroxidation assay uses the peroxidase component of MPO. The reaction between hydrogen peroxide and 10-acetyl-3,7-dihydroxyphenoxazine (ADHP) produces the highly fluorescent compound resorufin. Both enzymatic activities of MPO were assessed following the manufacturer’s instructions. In short, formation of hypochlorite (and other strong oxidants) was assayed by mixing several concentrations of hLF1-11 or control peptide (range 0.5–250 μg/ml) with MPO (final concentration of 114 ng/ml) in the presence of a reaction mixture containing H2O2 (40 μM) and APF (final concentration of 40 μM). For the peroxidation assay, similar concentrations of hLF1-11 or control peptide and MPO were mixed with H2O2 (final concentration of 20 μM) and ADHP (final concentration of 100 μM). MPO alone was used as a positive control.

![FIGURE 2.](http://www.jimmunol.org/) Identification of human MPO as the principal binding partner of hLF1-11 in monocytes. To investigate the intracellular binding partner of hLF1-11, biotinylated hLF1-11–coupled and control peptide (CP)-coupled streptavidin columns were used in pull-down assays with monocytic cell lysates as input. After washing, bound proteins were eluted and subjected to SDS-PAGE under nonreducing conditions. hLF1-11 specifically bound to a protein with an apparent molecular mass of 110 kDa (inset). Tryptic digestion followed by MALDI-TOF MS analysis and database searches using the Mascot search algorithm showed that this protein corresponds to human MPO (IPI00236554, Mascot score 241). Fragments indicated with an asterisk correspond to matched tryptic peptides of MPO (total sequence coverage 35%).

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FIGURE 3. MPO activity in the presence of hLF1-11 and control peptide. The peroxidation activity (A) and the formation of hypochlorite (and other strong oxidants) (B) by human MPO were assessed in the presence of several concentrations of hLF1-11 (dark gray) or control peptide (light gray). The results of a representative experiment (out of three independent experiments) are displayed.

Results

Binding and internalization of hLF1-11 by human monocytes

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

Identification of the intracellular target of hLF1-11

Next, we sought to isolate the intracellular binding partner(s) of hLF1-11 by using a pull-down assay with monocyte lysates and an hLF1-11–coated column followed by SDS-PAGE of bound proteins. Results revealed that the hLF1-11 column bound a single protein with an apparent molecular mass of ~110 kDa, whereas no protein was retained by the control peptide-coated column (Fig. 2, inset). After elution from the column, the hLF1-11 binding protein was subjected to in-gel tryptic digestion and identified as human MPO using MALDI-TOF mass spectrometry followed by database searching (Fig. 2). MPO was identified as...
the single binding partner of hLF1-11 in three out of three independent experiments.

Effects of hLF1-11 on the enzymatic activities of human MPO
To investigate whether hLF1-11 affects the enzymatic activities of MPO, we determined both the formation of hypochlorite (and other strong oxidants) and the peroxidation activity of MPO in the presence of hLF1-11 or a control peptide. hLF1-11 inhibited both activities of MPO in a dose-dependent fashion (Fig. 3A, 3B). Surprisingly, the control peptide did not inhibit the peroxidation activity of MPO, but inhibited the formation of hypochlorite (and other strong oxidants), although the control peptide was ~25 times less efficient than hLF1-11.

Effect of hLF1-11 on the production of ROS by monocytes in response to LPS
As MPO amplifies the potency of ROS after initiation of the oxidative burst, we considered the possibility that binding of hLF1-11 to MPO results in reduced ROS production by human monocytes. Results showed that hLF1-11–incubated monocytes produced significantly less ROS after LPS stimulation than that of control (peptide-treated) monocytes (Fig. 4A). In addition, the MPO inhibitor ABAH also reduced the LPS-stimulated ROS production by human monocytes (Fig. 4B), indicating that inhibition of MPO by this chemical mimics the effect of hLF1-11 on the LPS-induced ROS production.

Comparison of the effects of hLF1-11 and the MPO inhibitor ABAH on LPS-induced responses by monocytes and on monocyte–macrophage differentiation
To investigate whether inhibition of MPO by hLF1-11 can explain the immunomodulatory actions of hLF1-11, we compared the effects of hLF1-11 and ABAH on IL-10 production by LPS-stimulated human monocytes (18) and on the GM-CSF-driven monocyte–macrophage differentiation (13). For this purpose, we incubated human monocytes with 100 μM ABAH, 72 μM hLF1-11 peptide, or the combination of these two MPO inhibitors, as well as with the proper controls (diluents of these compounds) for 1 h and then stimulated the cells for 18 h with LPS. Thereafter, the IL-10 levels in the supernatants of the various monocyte cultures were quantified by ELISA. Results showed that hLF1-11–incubated and ABAH-incubated monocytes produced significantly higher levels of IL-10 upon stimulation with LPS than those of control monocytes (Fig. 5A). In addition, monocytes incubated with the combination of ABAH (100 μM) and the hLF1-11 peptide (72 μM) did not produce significantly more IL-10 in response to LPS than that of MPO monocytes exposed to either of these compounds alone (Fig. 5A). Notably, the LPS-stimulated IL-10 production by monocytes did not differ if the cells were exposed first to ABAH, washed, and then exposed to the peptide or to these compounds in the reversed order before addition of LPS (data not shown). To investigate the possibility that inhibition of MPO by ABAH reduces the responsiveness of the monocytes to hLF1-11, we preincubated monocytes with 500 μM ABAH or its diluent (0.5% DMSO) for 1 h, washed and reincubated the cells for an additional hour with hLF1-11 or no peptide, and then LPS was added. Eighteen hours thereafter, the culture supernatants were harvested and the levels of IL-10 quantitated. Results revealed that hLF1-11 enhanced the LPS-stimulated IL-10 production by DMSO-preincubated monocytes (p = 0.007), but not by ABAH-preincubated monocytes (p = 0.24); (the LPS-induced IL-10 production by DMSO-preincubated cells exposed to hLF1-11 amounted to 1718 [487–3180; n = 8] pg/ml and by control cells to 483 [101–2817; n = 8] pg/ml) and by ABAH-pretreated monocytes; the respective values were 1543 (802–3707; n = 8) and 1020 (557–2143; n = 8) pg of IL-10/ml. These results suggest that MPO is a major mediator of the modulatory effects of the hLF1-11 peptide on monocytes.

FIGURE 5. Comparison of the LPS-induced IL-10 production by ABAH- and hLF1-11–incubated monocytes and by macrophages resulting from monocytes differentiated by GM-CSF in the presence or absence of these agents. Monocytes were cultured in the presence of ABAH (100 μM), hLF1-11 (72 μM), both, or diluent 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 recombinant human GM-CSF in the presence of ABAH (100 μM) or hLF1-11 (72 μM) for 7 d. Thereafter, IL-10 production by the resulting macrophages after LPS was assessed by ELISA (B). Lastly, 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 (C). All data are expressed as boxes and whiskers; boxes represent medians and second and third interquartiles, and whiskers represent range within experiments with 6–24 different donors. *p < 0.05 (compared with control monocytes incubated with hLF1-11), **p < 0.01, ***p < 0.001 (compared with control monocytes).
Furthermore, monocytes differentiated by GM-CSF to macrophages in the presence of ABAH displayed significantly enhanced levels of IL-10 in response to LPS similar to macrophages that differentiated from monocytes in the presence of GM-CSF and hLF1-11 (Fig. 5B). To determine which amino acid(s) in hLF1-11 is (are) most essential for this immunomodulatory activity, we compared the effect of a set of peptides with a single amino acid substitution to alanine on IL-10 production by LPS-stimulated monocytes. Notably, results revealed that the cysteine at position 10 was pivotal for the LPS-induced IL-10 production by monocytes (Fig. 5C).

**Structural model of MPO with hLF1-11 and identification of amino acids within hLF1-11 primarily responsible for its MPO-inhibiting activity**

As both the crystal structures of human MPO and hLF1-11 (as part of human lactoferricin) have been solved (21, 22), we could generate a binding model of the two molecules by submitting the respective structures as receptor and ligand to the ClusPro protein–protein docking server (see Materials and Methods). This predicted that hLF1-11 docks in a crevice containing the heme pocket (Fig. 6A). This crevice has previously been identified as the catalytic site of MPO (24). The docking model furthermore predicts a binding mode in which several electrostatic interactions between hLF1-11 peptide and MPO are involved (Fig. 6B). In summary, the predicted docking site of hLF1-11 on MPO may occlude the active site and block the catalytic activity of MPO. These findings thus provide a molecular model to explain the observation that hLF1-11 is able to inhibit the enzymatic activities of MPO.

Next, we wanted to study in more detail which amino acids are important for the MPO-inhibitory effect of hLF1-11 using an assay for the formation of hypochlorite (and other strong oxidants) by MPO. In agreement with the results observed with the cellular assay (Fig. 5C), the cysteine was also essential for the inhibitory activity of hLF1-11 on this enzymatic activity of MPO (data not shown). As a cysteine can be oxidized and can thereby act as a radical scavenger, we wanted to discriminate whether hLF1-11 acted mainly by scavenging or that the peptide inhibited the enzymatic activity of MPO differently. Substitution of the cysteine with cysteic acid (the oxidized form of cysteine) rendered the hLF1-11 peptide virtually ineffective in affecting the production of hypochlorite/hydroxyl radical by MPO (data not shown), indicating that the cysteine is indeed able to scavenge radicals produced by MPO. Moreover, experiments with cysteine-containing peptides without any homology to hLF1-11 showed considerable inhibition of the MPO activity, as has been observed previously (25). Together, these data indicate that the presence of cysteines within the assay mixture was sufficient to inhibit the hypochlorite (and other strong oxidants) formation by MPO.

As the docking model (Fig. 6A, 6B) suggested that also the arginines were of importance for the interaction of hLF1-11 with MPO, we considered the possibility that the arginines interact with human MPO but are not involved in the inhibition of its enzymatic activities. To investigate this possibility, we immobilized human MPO (purified from extracts of human neutrophils, a kind gift of Mrs. N. Klar-Mohamad, Department of Nephrology, Leiden University Medical Center) on the surface of a 96-well plate, preincubated this with hLF1-11 or other peptides, washed away the unbound peptides (thereby removing the unspecific scavenging activity), and then determined the hypochlorite (and other strong oxidants) formation. Results revealed that peptides in which two or more arginines had been replaced by alanines were much less effective than the hLF1-11 peptide in inhibiting this enzymatic activity. Substitution of all four arginines with lysine did not affect the inhibitory activity of the peptide. Furthermore, positioning of the cysteine immediately after the arginine stretch did not affect the inhibitory activity of the hLF1-11 peptide. Substitution of cysteine with cysteic acid rendered the hLF1-11 peptide virtually ineffective in inhibiting the hypochlorite (and other strong oxidants) formation by MPO (Fig. 6C). Together, these results suggest that the arginines in the hLF1-11 peptide are necessary to bind to the crevice of the active site and that the cysteine subsequently interferes with the ROS within the active site.

**Discussion**

We previously reported that the hLF1-11 peptide modulates the LPS-induced inflammatory responses of monocytes and drives the GM-CSF–driven monocyte differentiation toward macrophages displaying enhanced antimicrobial activities (13, 18). In addition, a 60-min exposure of monocytes to hLF1-11 before addition of GM-CSF was sufficient to direct the differentiation toward macrophages with enhanced effector functions compared with control monocytes.

**FIGURE 6.** Model of human MPO with the hLF1-11 peptide docked in the active site and effects of peptides on the hypochlorite (and other strong oxidants) formation by immobilized human MPO. This model shows the molecular surfaces for human MPO. Atoms of hLF1-11 residues are shown in a red-stick representation. Residues surrounding hLF11, making up the peroxidase substrate pocket, are colored blue for better visualization (A). Hydrogen bonds and polar contacts between side chains of hLF1-11 (red) and human MPO (blue) are shown (dotted line). Numbering of residues according to PDB 3FPF. The interaction model was generated by the fully automatic ClusPro protein–protein docking server and visualized using Pymol. (B) To investigate the predicted role of the arginines and cysteine, wells were coated overnight with 100 μL of 19 μg/mL human MPO at 4°C. The next day, the nonadherent MPO was removed by washes, the peptide was added, and 1 h thereafter, unbound hLF1-11 was removed by washes, and the amount of hypochlorite (and other strong oxidants) formed was assessed using the reaction mixture containing hydrogen peroxide and substrate. Results are expressed as IC50%, i.e., the concentration of peptide inhibiting 50% of the chlorination activity (C). Data are expressed as means and SEM of three to four independent experiments. U, Cysteic acid.
GM-CSF macrophages (18). Together, a short exposure of monocytes to hLF1-11 is sufficient to exert its immunomodulatory effects. Because this peptide also inhibits the enzymatic activities of MPO, we suggest that these effects may be linked. This suggestion is supported by the following observations. First, hLF1-11 binds to and subsequently penetrates monocytes. Second, hLF1-11, but not a control peptide, bound to a single protein in lysates of monocytes. This protein was subsequently identified as MPO. Third, hLF1-11 inhibited the peroxidation and chlorination activity of purified human MPO. Lastly, the MPO-inhibitor ABAH mimicked the modulatory effects of hLF1-11 on LPS-induced inflammatory responses of monocytes and the GM-CSF–driven monocyte–macrophage differentiation. In addition, monocytes preincubated with a high concentration of ABAH (500 μM) were less responsive to LPS than control monocytes using IL-10 production as readout.

Other major findings of this study pertain to the specific amino acids in hLF1-11 that are pivotal to its inhibitory effects on MPO. In our experiments with immobilized MPO in which we removed excess peptide by washes, we found that hLF1-11, but not peptides with two or fewer arginines, inhibited the production of hypochlorite (and other strong oxidants) by MPO. In addition, this peptide, but not peptides only lacking the cysteine residue and such peptides substituted with cysteic acid, inhibited this enzymatic activity of MPO. In agreement, the structural docking model of MPO–hLF1-11 indicated that the four arginine residues of this peptide might bind to the edge of the cleft of the active site of MPO and that the cysteine is close to the heme group in the activity center. As hLF1-11 contains 5 nmol (4 nmol of arginine and 1 nmol of cysteine) of a residue that can compete with APF, the possibility that this peptide competes with the fluorescent substrate for reaction with hypochlorite was considered. However, in experiments in which excess peptide remained present during the enzymatic reaction, a peptide with cysteine substituted by alanine (still having four arginines) did not inhibit the cleavage of APF by MPO, indicating that the guanidinium groups of the arginines in hLF1-11 do not effectively compete with APF for hypochlorite. On the basis of these results, we suggest that hLF1-11 comprises two regions that are important for its ability to inhibit the enzymatic activity of MPO; that is, the arginines that bind to the crevice of the active site by electrostatic interactions and the cysteine that interferes with ROS. In addition, the arginines may mediate binding to and entry into cells (26, 27). Together, our results with human monocytes and (immobilized) MPO indicate that hLF1-11 inhibits the enzymatic activities of both intracellular and extracellular MPO.

The current findings show that MPO is a negative regulator of the production of IL-10 by LPS-stimulated monocytes. In agreement with this finding, others have reported that neutrophils from MPO-deficient mice express higher levels of IL-10 in response to LPS than those of cells isolated from wild-type mice (28). Furthermore, we previously described that hLF1-11 enhanced the mRNA expression of IL-10 and the NF-κB activation and translocation in LPS-stimulated monocytes (18). Apparently, MPO negatively affects a signal transduction pathway(s) regulating the production of IL-10 and possibly more cytokines, as ABAH, an inhibitor of MPO, mimicked these actions of hLF1-11 on IL-6, IL-2p40, and TNF-α production by these monocytes (data not shown). In agreement, several studies have shown the involvement of MPO and its products in multiple intracellular signaling pathways (29, 30). Moreover, the presence of anti-neutrophil cytoplasmic autoantibodies with specificity for MPO as present in patients with Wegener’s disease enhances the phagocytic activity, IL-8 production, and glucose uptake by neutrophils (31).

Alongside its roles in the host defense, MPO also promotes oxidative damage of host tissues at sites of inflammation and has been implicated in diseases such as coronary artery disease (32), Alzheimer’s disease (33), and kidney disease (34). Accordingly, there is considerable interest in the development of therapeutically useful MPO inhibitors (35). In this context, Liu et al. (36) have shown that rabbits pretreated with ABAH and subsequently submitted to myocardial ischemia and reperfusion displayed significantly reduced cardiac caspase-3 activity, suggesting that MPO is a significant contributor to postischemic cardiomyocyte apoptosis. Consistent with this notion, MPO deficiency appeared to be associated with lower incidence of cardiovascular heart diseases (37). Finally Askari et al. (38) reported a significant enhanced preservation of left ventricle function after myocardial infarction in MPO-deficient mice compared with that after myocardial infarction in wild-type mice.

In conclusion, immunomodulatory effects of hLF1-11 are mediated by its ability to inhibit MPO activity. As repeated i.v. administrations of 5 mg hLF1-11 were safe for healthy human volunteers (39), the current findings merit further research into the possibility of hLF1-11 as a potential therapeutic agent in diseases in which MPO plays an unfavorable role.

Disclosures
The authors have no financial conflicts of interest.

References

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