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

Does, A.M. van der

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

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Anne M. van der Does[1*]

Henry Beekhuizen[1*]

Bep Ravensbergen[1]

Tim Vos[1]

Tom H. Ottenhoff[1]

Jaap T. van Dissel[1]

Jan W. Drijfhout[2]

Pieter S. Hiemstra[3]

Peter H. Nibbering[1]

1. Dept. of Infectious Diseases, Leiden University Medical Center (LUMC), The Netherlands
2. Dept. of Immunohaematology and Blood Transfusion, LUMC, The Netherlands
3. Dept. of Pulmonology, LUMC, The Netherlands

*These authors contributed equally to this work

Chapter 4

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

Introduction

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

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

Materials and Methods

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

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

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

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

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

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

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

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

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

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

Results

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

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

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

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

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

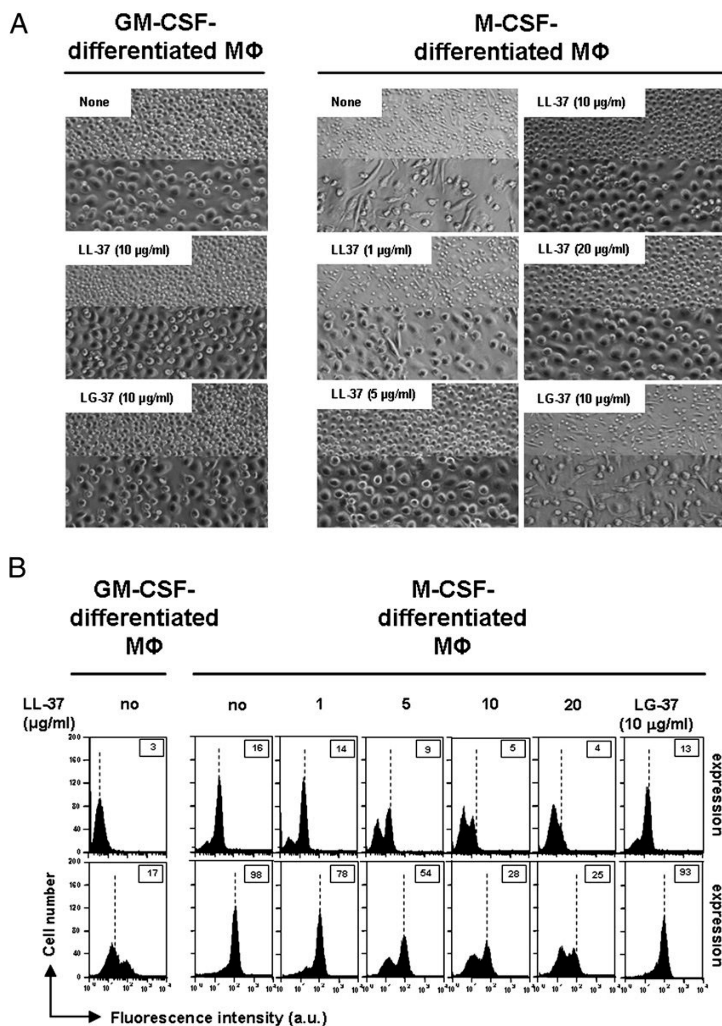
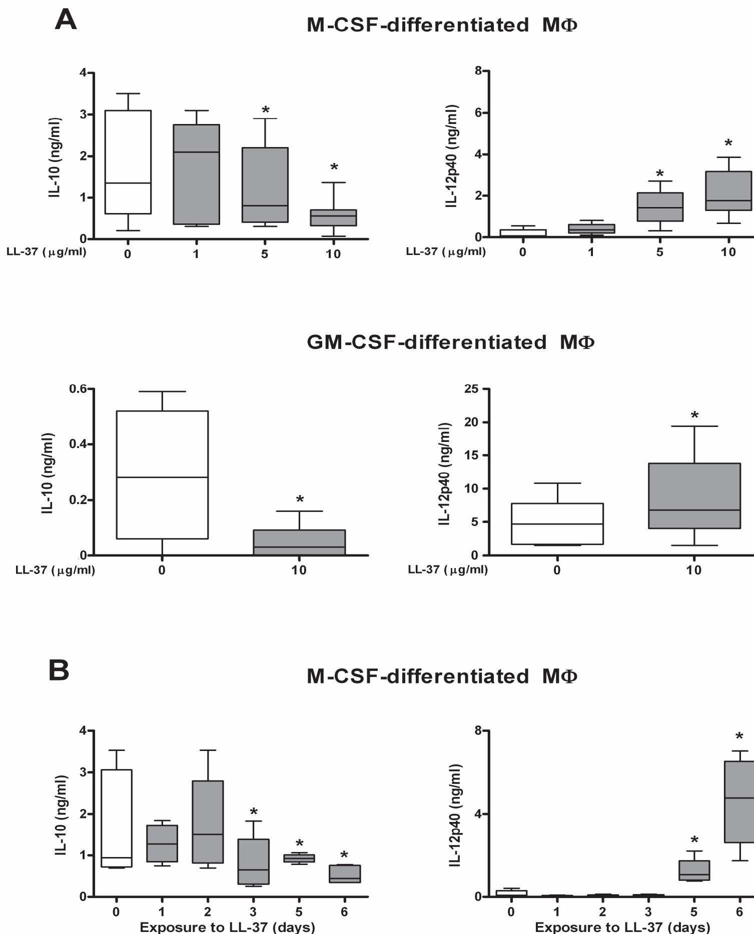


Fig. 1 Effects of LL-37 on the M-CSF–driven and GM-CSF–driven monocyte–macrophage differentiation regarding morphology and cell-surface molecule expression Monocytes were cultured for 6 d in medium containing GM-CSF (to generate Mϕ-1) or M-CSF (to generate Mϕ-2) in the presence of LL-37, the scrambled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

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



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

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

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

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

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

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

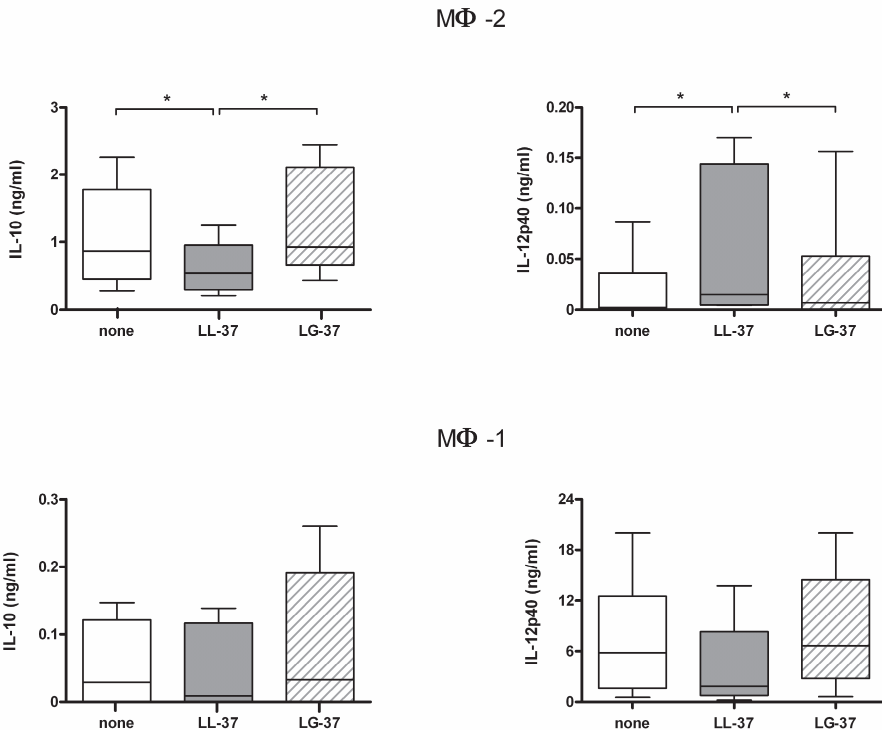


Fig. 3 Effects of LL-37 on fully differentiated macrophages Fully differentiated Mϕ-2 and Mϕ-1 were cultured for an additional 6 d in medium supplemented with LL-37 (10 µg/ml; gray boxes), LG-37 (10 µg/ml; hatched boxes), or no peptide (white boxes) and then stimulated with LPS (100 ng/ml) for 24 h.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

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

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

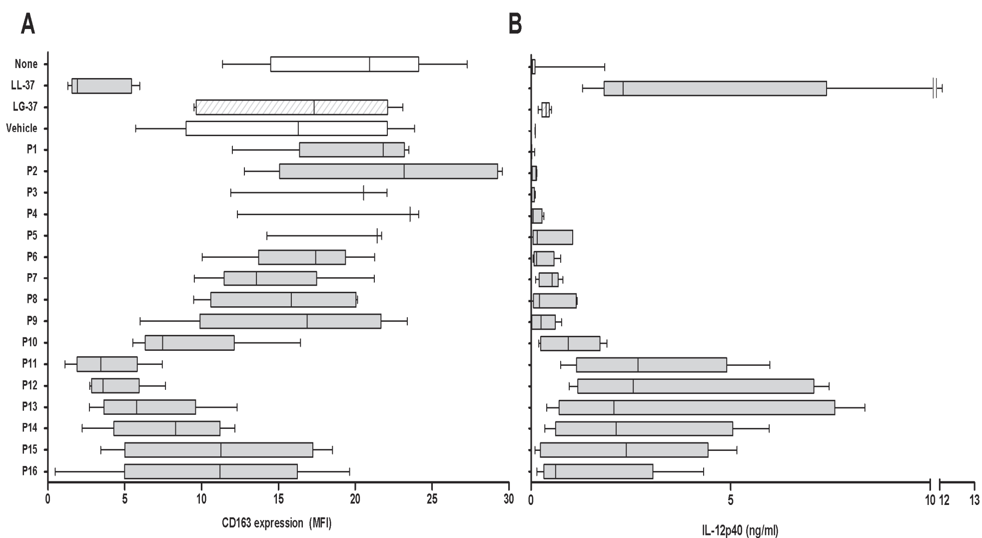


Fig. 4 Effects of LL-37-derived peptides on the M-CSF–driven macrophage differentiation. Monocytes were cultured for 6 d in medium containing M-CSF in the presence of LL-37–derived peptides (peptides 1–16; 6.25 µg/ml; gray boxes) or as controls LL-37 (10 µg/ml; gray boxes), LG-37 (10 µg/ml; hatched boxes),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

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

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

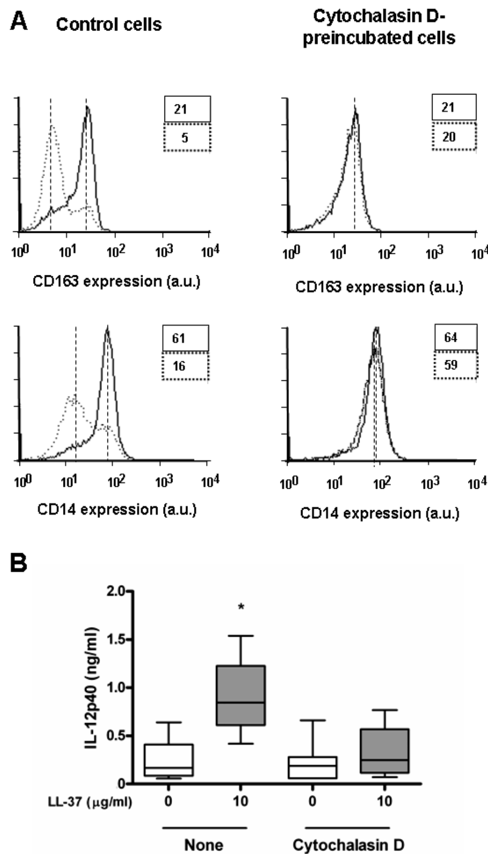


Fig. 5 Effect of preincubation of the monocytes with cytochalasin-D on the modulation of the M-CSF–driven macrophage differentiation by LL-37 Monocytes were preincubated with cytochalasin- D for 30 min or left untreated, then washed and incubated in medium containing M-CSF and LL-37 (or as control no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

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

Discussion

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

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

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

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