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

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The human lactoferrin-derived antimicrobial peptide hLF1-11 drives monocyte-dendritic cell differentiation toward dendritic cells that promote antifungal responses and induce Th17 polarization

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Chapter 5

Abstract The hLF1-11 peptide comprising the first 11 N-terminal residues of human lactoferrin, exerts antimicrobial activity *in vivo*, enhances the inflammatory response of monocytes, and directs monocyte-macrophage differentiation toward cells with enhanced antimicrobial properties. Since its effects on mononuclear phagocytes are already seen after short incubation of monocytes with hLF1-11, we hypothesized that the differentiation of these cells toward dendritic cells (DCs) may also be affected by hLF1-11. We therefore determined the effects of hLF1-11 and a control peptide on human monocyte-dendritic cell differentiation and subsequent T-cell activation. The DCs thus obtained were assessed for their antimicrobial activity against the yeast *C. albicans*. Also, cytokine and reactive oxygen species production in response to this yeast was quantified. Results revealed that -compared to control DCs- hLF1-11-differentiated DCs displayed enhanced antimicrobial activity against *C. albicans* and produced enhanced amounts of IL-6 and IL-10 upon stimulation with *C. albicans*, whereas IL-12p40 production was reduced. Moreover, 6 day-cultured hLF1-11-differentiated DCs and control (peptide-differentiated) DCs were stimulated with a memory mix for 24 h and co-cultured with autologous CD4+ T cells for 72 h; hereafter cytokine production in supernatants as well as in T cells was assessed. The hLF1-11-differentiated DCs induced an enhanced IL-17 but reduced IFN-γ production by T cells as compared to control DCs. Collectively, the hLF1-11 peptide drives monocyte-dendritic cell differentiation toward DCs that promote antifungal responses and induce Th17 polarization. The ability of hLF1-11 to modulate the hosts' immune response may be key to its potential for treatment of infections with (multi-drug resistant) pathogens.

Introduction

In tissues dendritic cells (DCs) have the capacity to recognize and process pathogens and following their migration to the lymph nodes, present pathogen-derived antigens to T cells thereby activating an adaptive immune response. Dendritic cells can differentiate from a precursor cell depending on factors present in the local microenvironment. For example, when monocytes upon entering tissue encounter inflammatory mediators like cytokines, chemokines, complement components and antimicrobial peptides (AMPs), they differentiate into immature dendritic cells (iDC). These cells are known for abundant expression of pathogen recognition receptors, efficient antigen uptake and processing capacities. Upon tissue injury, microbial infection or other danger signals, iDCs mature into antigen presenting cells that produce inflammatory mediators, migrate toward lymphoid organs and instruct T lymphocytes to proliferate and differentiate into specific T cell subsets. This polarization of T cells depends greatly on the DCs and the inflammatory mediators present in their environment. Important molecules that influence differentiation of monocytes and functional properties of immune cells are antimicrobial peptides (AMPs). AMPs are cationic, relatively short and are active against a variety of microorganisms including multi-drug resistant pathogens (1). The principal mechanism of action of antimicrobial peptides was long thought to be perturbation of the microbial cell membrane. However, an increasing number of studies shows the diversity in the mechanisms of action of these peptides that have therefore been renamed as host defense peptides (HDPs) (2, 3). These mechanisms of action include direct killing of pathogens (4, 5), modulation of pathogen development (6, 7) and modulation of immune cells (8-10), the latter is now increasingly recognized as being an important contribution to clearance of infection. For possible therapeutic application of HDPs, it will be important to understand the interactions of these peptides with the hosts' immune cells. For example, cathelicidin-based peptides IDR-1 (11) and IDR1002 (12) have been developed on the basis of their ability to affect the host immune system by enhancement of chemokine production by innate immune cells. The cathelicidin LL-37 is able to direct both monocyte-macrophage differentiation (13) as well as differentiation toward dendritic cells that promote a Th1 response *in vitro* (14) through interaction with an intracellular target (15). LL-37 is also able to modulate the adaptive immune response by directly affecting the maturation of DCs (16). We recently reported that the antimicrobial peptide comprising the first 11 N-terminal residues of human lactoferrin, further referred to as hLF1-11, enhances the inflammatory response of monocytes and modulates monocyte-macrophage differentiation; an additional mechanism of action to its already established

antimicrobial effects (5,7,17-20). hLF1-11 enhances inflammatory mediator production by murine and human monocytes (21) and promotes differentiation of GM-CSF-stimulated monocytes toward a macrophage subset that shows enhanced responsiveness to microbial stimuli and demonstrates increased clearance of pathogens (8). These effects were already obtained after incubation of monocytes with hLF1-11 for 60 min, indicating that hLF1-11 can modulate monocytes at an early stage, resulting in long-term alterations. Since monocytes can also differentiate toward (immature) dendritic cells, we have here investigated the effects of hLF1-11 on monocyte-dendritic cell differentiation. We found that when hLF1-11 was present during GM-CSF and IL-4-driven differentiation of monocytes toward dendritic cells, the resulting immature DCs displayed enhanced antimicrobial properties against *C. albicans* and -upon maturation- induced IL-17 production by T cells while reducing IFN-γ production.

Materials and methods

Peptides The human lactoferrin-derived peptide hLF1-11 (GRRRRSVQWCA; 1.374 kDa) was purchased from Peptisyntha (Torrance, CA) and the control peptide (GAARRAVQWAA; 1.115 kDa) from Isogen (De Meern, The Netherlands). The control peptide shows no activity against pathogens *in vitro* and *in vivo* (7). The purity of both peptides was >97% as determined by reverse-phase high-performance liquid chromatography (HPLC). Stocks of the peptides were made in phosphate-buffered saline (PBS, Dept. of Pharmacy LUMC, Leiden, The Netherlands) and stored at -20˚C. Endotoxin concentrations were below detection level.

Cell culture Peripheral blood mononuclear cells (PBMCs) were isolated from buffy coats of healthy donors using Ficoll-Amidotrizoate density gradient centrifugation. Monocytes were further purified by CD14-positive selection using antiCD14-conjugated magnetic microbeads (Miltenyi Biotec, Bergisch Gladbach, Germany) according to manufacturers' protocol. Next, monocytes (>95% pure, viability >96% as determined by propidium iodide staining) were incubated for 7 days in culture medium (RPMI 1640, GIBCO Invitrogen, Breda, The Netherlands) containing 10% heat-inactivated fetal bovine serum (FCSi, Greiner Bio-One B.V., Alphen a/d Rijn, The Netherlands), 2 mM penicillin, 2 mM streptomycin (both PAA GmbH, Pasching, Germany) and 2 mM L-glutamine (GIBCO Invitrogen) supplemented with 10 ng/ml recombinant human granulocyte macrophage-colony stimulating factor (rhGM-CSF) and 10 ng/ml recombinant human IL-4 (both Biosource,

Camarillo, CA). To study the effect of hLF1-11 on differentiation of monocytes into iDCs, at the start of the culture monocytes were exposed to various concentrations of either, hLF1-11 (up to 100 μg/ml) or control peptide (100 μg/ml) or saline as a control. On day 6, the immature DCs were matured with heat-killed (30 min at 100 °C) *Candida albicans* ($1x10^7$ CFU/ml strain Y01-19, Pfizer Inc., Groton, CT) for 20 h. Thereafter supernatants were harvested for assessment of cytokine (and chemokine) levels, or immature and mature DCs were harvested at day 7 and used for analysis of their capacity to take up dextran, phagocytose *S. aureus* and *C. albicans*, produce reactive oxygen species (ROS), and polarize CD4+ T cell differentiation in co-culture experiments.

Flow cytometric analysis of cell-surface molecule expression by dendritic cells For measurements of the expression of a variety of cell-surface molecules, the following monoclonal antibodies were used: PE-conjugated antibodies directed against CD11b, CD40, CD54, CD80, CD83 and CD86 and FITC-labeled antibodies against HLA-DR, CD14, CD206 and CD209 were all obtained from BD Biosciences (Heidelberg, Germany). PE-conjugated antibody against Dectin-1 was purchased from R&D Systems and Alexa Fluor 647-conjugated antibody against CD197 from BD Biosciences. DCs were harvested and resuspended in ice cold 0.2% PBS/BSA, washed twice and then incubated with the selected antibodies for 30 min on ice in the dark. Cell-surface molecule expression was assessed on a FACSCalibur and analysed by BD CellQuest software (BD Biosciences). Results are expressed as MFI corrected for background measurements.

Flow cytometric analysis of dextran-FITC uptake by dendritic cells The endocytic property of the various iDCs was examined by incubation of the cells with 1 mg/ml FITC-labeled dextran (Invitrogen) for 2 h at 37°C in culture medium. Background measurements were performed at 4°C. After washing, dextran-FITC fluorescence was assessed using a FACSCalibur and analysed by BD CellQuest software (BD Biosciences). Results are expressed as median fluorescence intensity (MFI) corrected for background measurements.

Assays for the phagocytosis of *S. aureus* and *C. albicans* by immature dendritic cells Phagocytosis of pHrodo-labeled *S. aureus* was performed as described for macrophages (8). Briefly, a stock suspension of pHrodo-labeled *S. aureus* (Invitrogen) was prepared according to manufacturer's protocol. pHrodo is a dye that is non-fluorescent at neutral pH and bright red in acidic environments (e.g. phagolysosome). Equal volumes of iDCs

($1x10^6$/ml) 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, at 4˚C. Thereafter, pHrodo fluorescence of the iDCs was assessed on a FACSCalibur. Results are expressed as the percentage of pHrodo-positive iDCs.

Phagocytosis of *C. albicans* by iDCs was assessed by FACS analysis as described for macrophages (8). In short, overnight cultured *C. albicans* were washed twice in PBS and then labeled with 0.5 µM carboxy fluorescein succinimidyl ester (CFSE, Invitrogen) for 30 min at 37°C in the dark, centrifuged and resuspended in RPMI 1640 supplemented with 20% human serum (HuS). iDCs were washed twice in PBS/0.2% bovine serum albumin (BSA) and then labeled with PE-conjugated antibody against CD54 for 30 min (on ice in the dark). Next, these labeled iDCs were mixed with CFSE-labeled *C. albicans* in a 1:1 ratio and incubated for various intervals at 37°C under slow rotation in the dark. The percentage of iDCs associated with *C. albicans* was assessed by determining the percentage of double positive iDCs (CD54+/CFSE+) using a FACSCalibur and analysed by BD CellQuest software. Control experiments were performed at 4°C to correct for binding of *C. albicans* to iDCs. Results are expressed as percentage iDCs that were positive for both CD54 and CFSE.

Flow cytometric analysis of ROS production by dendritic cells Intracellular ROS production by iDCs was quantified using the fluorescent probe 2',7'-dichlorofluorescein-diacetate (DCFH-DA) (Invitrogen). In short, iDCs were loaded with 10 µM DCFH-DA for 20 min at 37˚C in the dark. Thereafter, iDCs were incubated with $1x10^7$ heat-killed *C. albicans* for various intervals. ROS production was measured on a FACSCalibur. Results are expressed as median fluorescence intensity (MFI) with interquartile range.

Determination of cytokine levels by ELISA Enzyme-linked immunosorbent assay (ELISA) cytosets were used to determine the concentrations of interleukin (IL)-6, IL-10, IL-12p40 and TNF-α (Invitrogen) according to manufacturers' instructions.

T cell isolation and DC-T cell co-cultures CD4+ T cells (>95% purity) were obtained from the CD14-negative fraction after monocyte isolation (stored at -80˚C) by CD4-positive selection using anti-CD4-conjugated magnetic microbeads (Miltenyi Biotec) according to manufacturers' protocol. They were resuspended in IMDM (Lonza, Verviers, Belgium) containing 10% heat-inactivated fetal bovine serum (FBSi, Gibco), antibiotics and 2 mM L-glutamine and left overnight to recover. Immature DCs were reseeded in a 96-wells plate ($1x10^5$/well). Three hours thereafter cells were stimulated with a mix of LPS (100 ng/ml;

Sigma-Aldrich, Zwijndrecht, The Netherlands), purified protein derivative of *Mycobacterium tuberculosis* (PPD, Statens Serum Institute, Copenhagen, Denmark; 5 µg/ml), tetanus toxoid (TT; 1% of the stock, 80 IE/ml) and heat-killed *C. albicans* (1 x 10^6/ml) for 24 h. Thereafter, DC's were co-cultured with 1 x 10^6 autologous T cells for 72 h in the presence of TT (1% of the stock, 80 IE/ml) and supernatants were collected and assessed for IL-17, IL-10, IFN-γ, IL-2 and IL-4 levels using a custom-made multiplex bead-array (Bio-Rad, Hercules, CA), according to manufacturers' protocol. For intracellular cytokine staining, cells in the co-culture were incubated with Brefeldin A (BFA, 3 µg/ml, Sigma) during the last 18 hours of co-culture, replicates (n = 10) pooled and T-cells were labeled extracellular using anti-CD3-AMCyan, anti-CD4-PECy7 and anti-CD25-FITC (all BD Biosciences) antibodies. Next, cells were fixed and intracellular labeled using Intrastain reagents (DakoCytomation, Heverlee, Belgium) with antibodies directed against IL-17-PE, (Ebioscience, San Diego, CA), IFN-γ-Alexa700 (BD Biosciences) and IL-10-APC (Miltenyi Biotec) before acquisition on a LSRII flowcytometer (BD Biosciences) and analysed using FlowJo software version 8.7.3 (Tree Star Inc, Ashland, Oregon). The core population of live $CD3^+$ T cells was analyzed for IL-17 production, while activated and $CD3^{dim}$ T cells were also included in IFN-γ production analysis.

Statistical analysis 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 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

Morphology and cell-surface molecule expression by hLF1-11-differentiated and control (peptide-differentiated) iDCs and mature DCs First, we inspected the morphology of the dendritic cells that had been differentiated in the presence of hLF1-11 or the control peptide. The morphology, density and attachment of the cells to the wells did not differ between hLF1-11 and control (peptide-differentiated) DCs. Next, we compared cell-surface molecule expression between hLF1-11-differentiated and control (peptide-differentiated) iDCs. Results revealed that hLF1-11-differentiated iDCs expressed significantly higher levels of CD14, CD80, HLA-DR and Dectin-1, a receptor involved in *C. albicans* recognition, on their cell-surface as compared to control (peptide-differentiated) iDCs (Table I). Upon maturation by *C. albicans*, the expression of the maturation marker CD83 as well as the co-stimulatory molecule CD86 by hLF1-11-differentiated DCs was significantly decreased as compared to control (peptide-differentiated) DCs (Table I).

Table 1 Cell-surface molecule expression by hLF1-11 and control (peptide-differentiated) DCs

		Immature DCs			*C. albicans*-matured DCs		
		Control	hLF1-11	Control peptide	Control	hLF1-11	Control peptide
CD14	LPS co-receptor	**2** (1-3)	**4*** (2-7)	**2** (1-3)	**3** (2-7)	**2** (1-6)	**2** (0-6)
CD83	Maturation marker	**1** (0-4)	**2** (0-3)	**1** (0-3)	**32** (21-54)	**22*** (12-42)	**36** (26-74)
CD197	CCR7	**8** (5-17)	**12** (7-19)	**10** (7-16)	**29** (26-34)	**27** (21-42)	**32** (24-38)
CD54	ICAM-1	**199** (123-241)	**138** (92-245)	**183** (109-256)	**881** (512-1190)	**1025** (500-1434)	**850** (356-1155)
CD40	Co-stimulatory	**52** (30-81)	**57** (31-89)	**56** (20-68)	**86** (52-127)	**77** (42-127)	**70** (26-104)
CD80	B7.1	**5** (0-9)	**7*** (0-19)	**4** (0-7)	**32** (24-83)	**29** (18-90)	**30** (29-99)
CD86	B7.2	**14** (0-26)	**7** (2-48)	**11** (4-25)	**407** (233-459)	**247*** (217-375)	**382** (317-466)
HLA-DR	MHC class II	**46** (24-100)	**63*** (36-252)	**45** (21-73)	**170** (97-399)	**138** (86-517)	**147** (68-365)
CD11b	CRIII	**294** (131-382)	**261** (104-299)	**288** (201-379)	**278** (183-320)	**224** (189-304)	**269** (226-396)
CD206	Mannose receptor	**45** (32-71)	**33** (22-152)	**54** (27-75)	**30** (17-45)	**31** (16-44)	**33** (19-53)
CD209	DC-SIGN	**44** (29-66)	**44** (19-74)	**42** (23-54)	**41** (23-49)	**41** (26-51)	**33** (23-50)
Dectin-1	β-glucan receptor	**13** (9-23)	**19*** (13-39)	**12** (7-19)	**20** (10-25)	**19** (11-28)	**21** (14-34)

Results are expressed as median fluorescence intensity (MFI) and corrected for background fluorescence. Data are expressed as median and range within experiments with at least six different donors. *, $p < 0.05$

Endocytic and phagocytic properties of hLF1-11-differentiated and control (peptide-differentiated) DCs To investigate whether hLF1-11-differentiated DCs also displayed differences in their functional activities, we compared endocytic and phagocytic capacities of iDCs differentiated in the presence of hLF1-11 with those of control (peptide-differentiated) iDCs. No significant difference in dextran uptake between hLF1-11-

differentiated iDCs and control (peptide-differentiated) iDCs was observed (Fig. 1A). In addition, phagocytosis of *S. aureus* by hLF1-11-differentiated iDCs did not differ from that by control (peptide-differentiated) iDCs (Fig. 1B). However, the percentage of *C. albicans*-phagocytosing hLF1-11-differentiated iDCs was significantly higher than that of control (peptide-differentiated) iDCs (Fig. 1C). Control experiments at 4˚C revealed that approximately 10% of the iDCs bound *C. albicans* at all intervals, independent of the presence of hLF1-11 or control peptide during differentiation (data not shown).

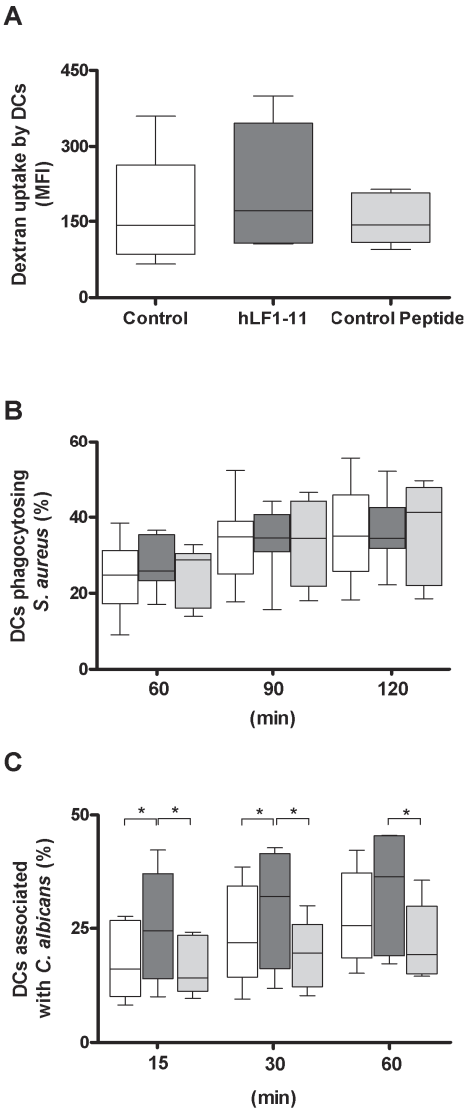

Fig. 1 Endocytosis of dextran-FITC and uptake of *C. albicans* and *S. aureus* by hLF1-11- or control (peptide-differentiated) immature dendritic cells
Monocytes were cultured with rhGM-CSF and IL-4 in the presence of hLF1-11 (100 µg/ml, dark gray bars), control peptide (100 µg/ml, light gray bars) or no peptide (open bars). At day 7, iDCs were harvested, washed and cultured for 2 h in the presence of dextran-FITC, thereafter the amount of dextran uptake by these cells was assessed using flow cytometry (A). Furthermore, after 60, 90 or 120 min co-incubation of the iDCs with pHrodo-labeled *S. aureus*, the percentage of pHrodo-positive iDCs was determined using flow cytometry (B). Lastly, iDCs were co-incubated with CFSE-labeled *C. albicans* and after 15, 30 and 60 min co-incubation, the percentage of iDCs associated with *C. albicans* was determined using flow cytometry (C). Boxes represent medians and second and third interquartiles, whiskers represent range within experiments with 6-10 different donors. *, $p<0.05$

ROS production by hLF1-11-differentiated and control (peptide-differentiated) iDCs in response to *C. albicans* Since hLF1-11-differentiated iDCs displayed enhanced phagocytosis of *C. albicans*, we considered the possibility that more antimicrobial functions of these cells were enhanced in response to *C. albicans*. We therefore determined the production of reactive oxygen species by the various iDCs in response to this yeast. Results revealed that hLF1-11-differentiated iDCs produced significantly more ROS 30 and 60 min after stimulation with *C. albicans* than control (peptide-differentiated) iDCs (Fig. 2). ROS levels decreased 90 min after stimulation with *C. albicans*, however hLF1-11-differentiated DCs still produced at that point significantly more ROS than control peptide-differentiated DCs.

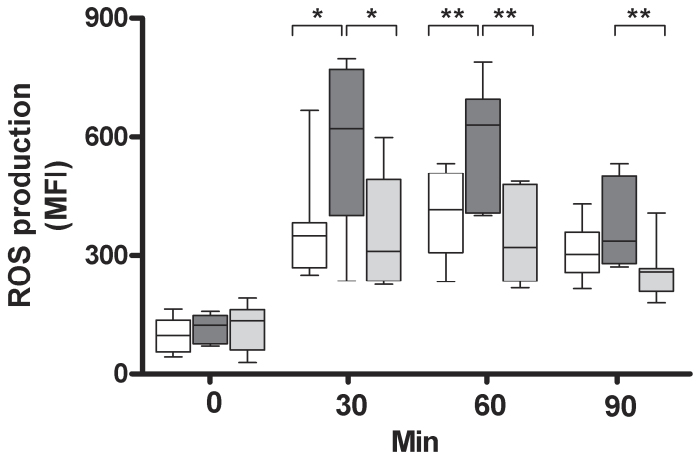

Fig. 2 Intracellular ROS production by hLF1-11 and control (peptide-differentiated) immature dendritic cells Monocytes were cultured with rhGM-CSF and IL-4 in the presence of hLF1-11 (100 µg/ml, dark gray bars), control peptide (100 µg/ml, light gray bars) or no peptide (open bars) and labeled at day 7 with DCFH-DA. Next, the labeled iDCs were stimulated with heat-killed *C. albicans* and the MFI was assessed directly and after 30, 60 and 90 min as a measure of ROS production. Boxes represent medians and second and third interquartiles, whiskers represent range within experiments with 8 different donors. *, $p<0.05$; ** $p<0.01$

Differential cytokine production patterns in hLF1-11-differentiated DCs and control (peptide-differentiated) DCs Another functional property of DCs is the production of inflammatory mediators. We therefore assessed pro- and anti-inflammatory cytokine production by the various DC groups in response to *C. albicans*. Results showed that heat-killed *C. albicans*-matured hLF1-11-differentiated DCs produced significantly more IL-6 and IL-10 and less IL-12p40 than control (peptide-differentiated) DCs (Fig. 3). The production of TNF-α did not differ between the various groups of DCs (Fig. 3).

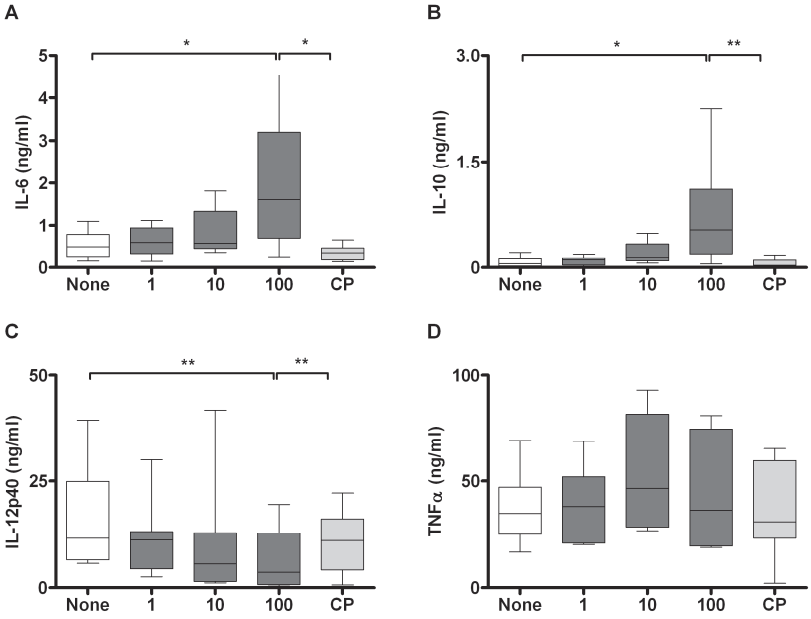

Fig. 3 Cytokine profiles of hLF1-11- and control (peptide-differentiated) dendritic cells in response to heat-killed *C. albicans* Monocytes were cultured with rhGM-CSF and IL-4 in the presence of hLF1-11 (1, 10, 100 µg/ml; dark gray bars) or control peptide (CP, 100 µg/ml; light gray bars) for 6 days. Thereafter, cells were stimulated with $1x10^7$ heat-killed *C. albicans* for 20 h and then supernatants were collected and assessed for IL-6 (A), IL-10 (B), IL-12p40 (C) and TNF-α (D) levels. Values are expressed as fold increase of cytokine production compared to control DCs (none, no peptide; open bars). Data are expressed as boxes and whiskers, boxes represent medians and second and third interquartiles, whiskers represent range within experiments with 7-8 different donors. *, $p<0.05$; **, $p<0.01$ as compared to control and control peptide-differentiated DCs

Polarization of CD4+ T cells by hLF1-11-differentiated or control (peptide-differentiated) DCs Since the innate activities of hLF1-11-differentiated DCs differed from that of control (peptide-differentiated) DCs, we compared their T cell activating capacities. First, the supernatants of the co-cultures of hLF1-11-differentiated DCs -matured by a memory mix- with autologous CD4+ T cells, were found to contain significantly more IL-17 ($p<0.05$) and more IL-10 ($p=0.06$), but less IFN-γ (Fig 4A) than co-cultures with control (peptide-differentiated) DCs. In these cultures, IL-2 was out of range and no IL-4 was detected (data not shown). Next we determined the percentage of T-cells expressing the various cytokines (IL-17, IL-10 and IFN-γ) by performing intracellular cytokine staining. Results revealed that enhanced percentages of IL-17-producing T cells and reduced percentages of IFN-γ producing T cells were found in the co-cultures with hLF1-11-differentiated DCs as compared to control (peptide-differentiated) DCs (Fig. 4B). IL-10 producing T cells were present at very low frequencies and no difference in percentages of IL-10-producing T cells

were found between the different groups (Fig. 4B), suggesting that IL-10 in the supernatants of the co-culture (Fig 4A) is mainly derived from the DCs.

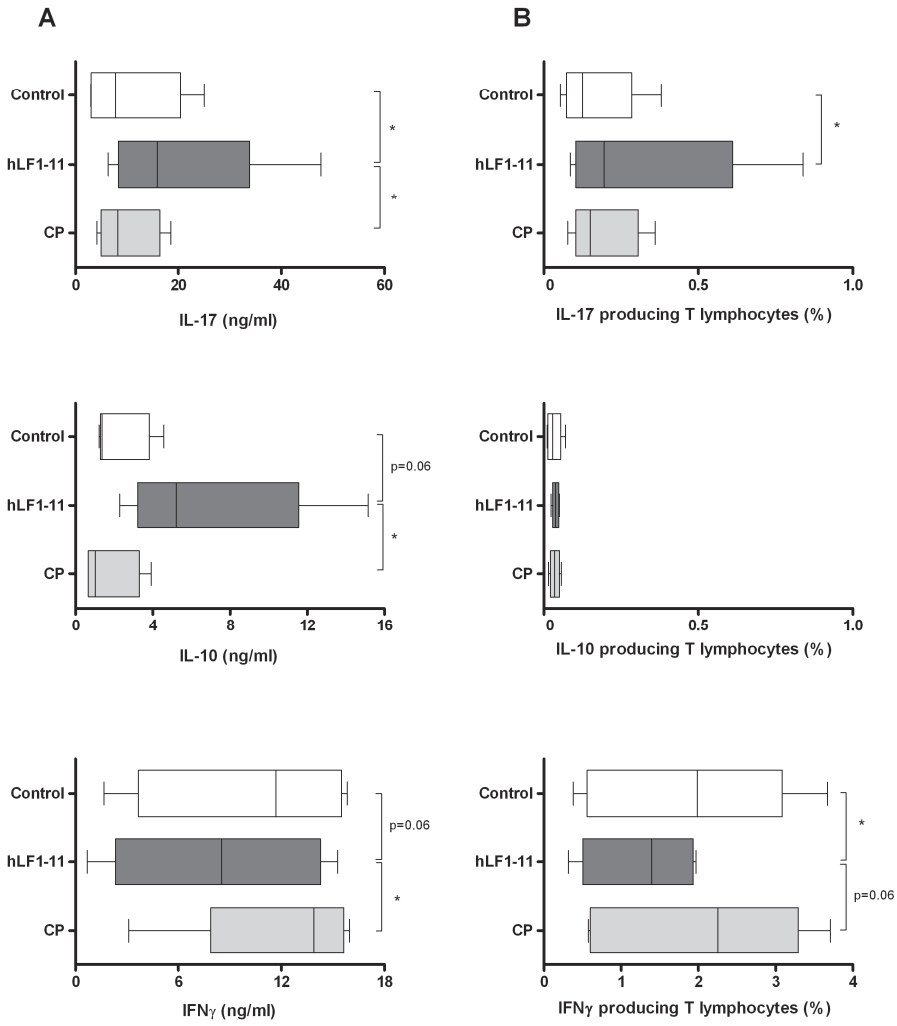

Fig. 4 Cytokine profile by CD4+ T cells after co-culture with hLF1-11 and control (peptide-differentiated) dendritic cells Monocytes were cultured with rhGM-CSF and IL-4 in the presence of hLF1-11 (100 µg/ml; dark gray bars), control peptide (CP, 100 µg/ml; light gray bars), or saline (open bars) for 6 days. Thereafter, cells were stimulated with heat-killed *C. albicans* ($5x10^5$/ml), purified protein derivative of *M. tuberculosis* (PPD; 5 µg/ml), tetanus toxoid (TT; 150 lf/ml) and LPS (100 ng/ml) for 24 h. Next, cells were washed and CD4+ T cells from the same donor and TT were added to the culture. 72 h later, supernatants were harvested and assessed for IL-17, IL-10 and IFN-γ (A) and IL-4, IL-2 levels (data not shown). Next, intracellular cytokine production by the T►

►lymphocytes in the co-culture was assessed by addition of brefeldin A for the last 16 hours of the co-culture (3 µg/ml). T cells were stained for intracellular IL-17, IL-10 and IFN-γ (B) Data are expressed as boxes and whiskers, boxes represent medians and second and third interquartiles, whiskers represent range within experiments with 6-8 different donors. *, $p<0.05$

Discussion

In this study we demonstrate that the presence of hLF1-11 during differentiation of monocytes into dendritic cells results in DCs that promote antifungal responses and induce Th17 polarization. This conclusion is based on the following findings. First, phagocytosis of *C. albicans* and ROS production in response to this yeast were elevated in hLF1-11-differentiated DCs as compared to the control (peptide-differentiated) DCs, while phagocytosis of *S. aureus* by hLF1-11-differentiated DCs was not enhanced. Second, the production of IL-6 and IL-10 by hLF1-11-differentiated mDCs in response to *C. albicans* was significantly enhanced, while IL-12p40 production was reduced as compared to control (peptide-differentiated) DCs. Incubation of hLF1-11-differentiated DCs with bacterial stimuli like LPS or LTA resulted in a significant ($p< 0.05$) reduction of TNF-α and IL-12p40 (without affecting the IL-6 and IL-10 levels) as compared to control (peptide-differentiated) DCs (data not shown). These data indicate that the presence of hLF1-11 during monocyte-DC differentiation has differential effects on the cytokine profile of the resulting mDC upon maturation by fungal or bacterial stimulation. Interestingly, we reported earlier that the presence of hLF1-11 during GM-CSF-driven differentiation of monocytes into macrophages, results in macrophages that also display enhanced production of IL-10 but not IL-12p40 or TNF-α in response to *C. albicans* (8). In addition, these macrophages displayed enhanced antimicrobial properties against *C. albicans*, but also *S. aureus* and displayed enhanced expression of pathogen recognition receptors like dectin-1. One possible explanation for these partial overlapping effects of hLF1-11 on monocyte-macrophage and monocyte-DC differentiation is the following. GM-CSF is known to be involved in regulation and enhancement of myeloperoxidase -the intracellular target of hLF1-11 (van der Does *et al*, submitted)- during monocyte-macrophage differentiation, whereas GM-CSF and IL-4 or with IL-4 alone diminished myeloperoxidase expression as compared to freshly isolated monocytes. As dendritic cells are obtained *in vitro* by incubation of monocytes with a combination of GM-CSF and IL-4, possible hLF1-11 is less able to affect monocyte-DC differentiation than monocyte-macrophage differentiation. Some properties of hLF1-11-differentiated dendritic cells and macrophages might therefore be overlapping between these cell types, while others are

not. Interestingly, as Dectin-1 expression was also significantly enhanced by hLF1-11-differentiated iDCs, it is tempting to speculate that myeloperoxidase might be involved in signaling leading to dectin-1 expression.

The most striking finding of this study is that hLF1-11 drives differentiation of monocytes toward DCs that promote Th17 polarization. The development of Th17 cells is linked to activation of several receptors including dectin-1 and CD206 (24,25,26), as recognition of *C. albicans* by these receptors is associated with Th17 responses. Also cytokines like IL-6, IL-1β and IL-23 can facilitate Th17 polarization. Th17 responses are thought to be important in host defense against fungi and *S. aureus* (27,28,29,30) especially at epithelia and mucosa. In addition, IL-17 is involved in the influx of neutrophils and can induce production of cytokines/chemokines and antimicrobial peptides by epithelial cells (31). Although Th17 cells are part of the adaptive immune response, they serve mainly to regulate innate immune responses (32). Besides promoting Th17 polarization, hLF1-11-differentiated DCs also reduced IFN-γ production by CD4+ T cells. It could be that this reduction of Th1 polarization is the consequence of the enhanced IL-10 production by the hLF1-11-differentiated DCs in the co-cultures (33). Interestingly, the effects of hLF1-11 on monocyte-DC differentiation differ from those reported for other AMPs/HDPs, such as the human cathelicidin LL-37 and α- and β-defensins. Davidson *et al*. (14) showed that the presence of LL-37 during monocyte-DC differentiation resulted in DCs promoting IFN-γ-producing T cells. Kandler *et al.* (16) have shown that this peptide inhibited the response iDCs toward microbial stimuli such as LPS, thereby indicating that LL-37 reduced maturation of these cells. Human defensins HNP-1 and hBD-1 promoted the activation and maturation of DCs and stimulated the production of TNF-α, IL-6, and IL-12p70 but not IL-10. Clearly, AMPs/HDPs differentially affect the differentiation and subsequent maturation of DCs (34). The concentration of hLF1-11 in the present study (100 μg/ml) is determined by dose response experiments with monocytes, macrophages and dendritic cells. hLF1-11 has been shown to be effective in mice up to concentrations of 4 mg/kg body weight, which corresponds to injection of 100 μg/mice. However, it should be realized that extrapolation of *in vitro* to *in vivo* levels is difficult since the local concentration of the peptide at the site of infection remains unknown. Together, these data show that hLF1-11 is able to modulate monocyte-dendritic cell differentiation, resulting in DCs displaying enhanced antimicrobial activities against *C. albicans* and promoting Th17 polarization after co-culture with CD4+ T cells. The immunomodulatory properties of hLF1-11 may aid in balancing the immune responses of the host leading to the resolution of infections with (multi-drug) resistant pathogens.

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