The Cationic Antimicrobial Peptide LL-37 Modulates Dendritic Cell Differentiation and Dendritic Cell-Induced T Cell Polarization

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Dendritic cells (DC) are instrumental in orchestrating an appropriately polarized Th cell response to pathogens. DC exhibit considerable phenotypic and functional plasticity, influenced by lineage, Ag engagement, and the environment in which they develop and mature. In this study, we identify the human cationic peptide LL-37, found in abundance at sites of inflammation, as a potent modifier of DC differentiation, bridging innate and adaptive immune responses. LL-37-derived DC displayed significantly up-regulated endocytic capacity, modified phagocytic receptor expression and function, up-regulated costimulatory molecule expression, enhanced secretion of Th-1 inducing cytokines, and promoted Th1 responses in vitro. LL-37 may be an attractive therapeutic candidate for manipulating T cell polarization by DC. 

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Abbreviations used in this paper: DC, dendritic cell; iDC, immature DC; mDC, mature DC; h, human; MIP-3α, macrophage-inflammatory protein-3α; MFI, mean fluorescence intensity; F-actin, filamentous actin; CR, complement receptor; FSC, forward scatter; SSC, side scatter; FPRL, formyl peptide receptor-like; Tr cell, regulatory T cell.

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Thus, pre-DC recruited to sites of inflammation are likely to be exposed to high levels of LL-37 that has been produced by recruited neutrophils and resident epithelial cells. We propose that exposure to this gradient of LL-37 alters the gene expression profile and differentiation of these cells. The consequent phenotypic modifications of potential second-line DC derived in this inflammatory milieu would then alter the nature of the T cell response. To test this hypothesis, we studied the impact of LL-37 exposure on the development of monocyte-derived DC morphology, Ag uptake, maturation, Ag presentation, and T cell stimulatory capacity.

Materials and Methods

Cell purification and culture

Monocyte-derived DC were prepared based upon standard techniques (29). Briefly, 100 ml of fresh human venous blood was collected in sodium heparin Vacutainer collection tubes (BD Biosciences) from volunteers according to University of British Columbia Clinical Research Ethics Board protocol C02-0091. The blood was mixed, at a 1:1 ratio, with RPMI 1640 supplemented with 10% v/v heat-inactivated FCS, 2 mM glutamine, 1 mM sodium pyruvate (all from Invitrogen, Burlington, Ontario, Canada). Synthetic LL-37 (sequence LLGDEFKRKSFKEIGKEFKRIVORIKDDFLRNLVPRTES) and Bc2a (sequence RLARIVIVRVAR-NH2, derived from bovine calcichelin) bactericidal peptide were synthesized by F-Moc (9-Fluorenylmethoxycarbonyl) chemistry at the Nucleic Acid/Protein Synthesis Unit at University of British Columbia. Human recombinant GM-CSF and IL-4 were purchased from Research Diagnostics (Flanders, NJ). Human recombinant macrophage inflammatory protein-3α (MIP-3α) was purchased from R&D Systems (Minneapolis, MN). Salmonella typhimurium LPS was obtained from Sigma-Aldrich (Oakville, Ontario, Canada). FITC-conjugated mAbs specific for CD40, CD14, and HLA-DR, and PE-conjugated mAb specific for CD206 were purchased from BD Biosciences (Mississauga, Ontario, Canada). FITC-conjugated mAb specific for CD86, CD16, CD83, CD54, and CD11b, and PE-conjugated mAb specific for CD18 and CD32 were supplied by Caltag Laboratories (Burlingame, CA). FITC-conjugated mAb specific for CD11c, CD1a, and IgG1 isocontrol were obtained from Serotec (Raleigh, NC). FITC-conjugated mouse IgG2a isotype control, PE-conjugated mouse IgM specific for CD209, CD80, mouse IgG1, and IgG2a isotype controls, and rat IgG2a isotype control Abs were purchased from eBioscience (San Diego, CA). FITC-conjugated mAb specific for CCR7 was obtained from R&D Systems (Minneapolis, MN) and used in conjunction with FITC-conjugated F(ab′)2 rabbit anti-mouse IgG from Serotec. Pertussis toxin was supplied by List Biological Laboratories (Campbell, CA). Peptide WKYMVM was a kind gift from C. Dahlgren (Phagotec). Pertussis toxin was supplied by List Biological Laboratories (Campbell, CA). The blood was mixed, at a 1:1 ratio, with RPMI 1640 medium supplemented with 10% v/v FCS, 2 mM glutamine, and 1 mM sodium pyruvate in an E-toxa-clean (Sigma-Aldrich)-washed, endotoxin-free bottle. PBMC were separated using Ficoll-Paque Plus (Amersham Pharmacia Biotech, Baie D’Urfé, Quebec, Canada) at room temperature and washed with PBS. Monocytes were enriched with the removal of T cells by rosetting with fresh SRBC (PMI Microbiologicals, Wilsonville, OR) pretreated with Vibrio cholerae neuraminidase (Calbiochem Biosciences, La Jolla, CA) as described (30) and repeat separation by Ficoll-Paque Plus. The enriched monocytes were washed with PBS, and then cultured (1 x 106 per well) for 1 h at 37°C followed by the removal of nonadherent cells; monocytes thus purified were >95% pure as determined by flow cytometry (data not shown). Cells were cultured in Falcon tissue culture 24-well plates (BD Biosciences) or, for immunohistochemistry, on 0.4-µm pore, 24-mm Transwell-Clear culture chamber inserts (Corning Costar, Cambridge, MA). The adherent monocytes were cultured in 1 ml of medium supplemented with LL-37 (or other peptides) dissolved in endotoxin-free water (Sigma-Aldrich), or the same volume of endotoxin-free water as a control, and incubated for 1 h at 37°C, before the addition of 100 ng/ml IL-4 and 100 ng/ml GM-CSF to establish differentiation to DC phenotype. Unless otherwise stated, LL-37 was used at 50 µg/ml, previously described as optimal for monocyte chemotaxis (25). For studies using pertussis toxin, adherent monocytes were cultured for 1 h at 37°C with 100 ng/ml toxin, and then washed twice with medium before being treated as described above. For studies using WKYMVM, this synthetic peptide agonist of FPRL1 and FPRL2 was used at a concentration equimolar to 50 µg/ml LL-37 (10 µM), or at 1 µM, a dose previously shown to induce maximal neutrophil NADPH oxidase activity (31). No difference was observed between these doses. Cells were cultured at 37°C in a humidified incubator for 7 days before analysis or stimulation. Adherent cells were harvested with gentle cell scraping. LL-37-pulsed studies were performed as above; however, the medium was removed, and the cells were washed 24 h after addition of LL-37, followed by culture in fresh IL-4- and GM-CSF-supplemented medium (preincubated for 24 h in the absence of cells) for a further 6 days.

Monocyte-derived macrophages were generated from fresh monocyte-enriched PBMCs isolated as described and cultured in medium supplemented with 10% v/v autologous serum. Adherent cells were cultured for 7 days in Transwell-Clear culture chamber inserts. Monocyte-derived macrophages were not exposed to antimicrobial peptides in this study.

For the isolation of T lymphocytes, PBMC were isolated as described above and resuspended at 5 x 106 cells/ml. T cells were then isolated using StemSep with Human T Cell Enrichment mixture (StemCell Technologies, Vancouver, British Columbia, Canada). Purified T cells were resuspended at 2 x 106 cells/ml. T cells were >95% pure as determined by flow cytometry (data not shown).

Analyses of cytotoxicity and cell viability

Peptide cytotoxicity was assessed by collecting culture supernatants after 24 h and 7 days of culture in which the concentration of lactate dehydrogenase-I was quantified using a Cytotoxicity Detection kit (Roche Diagnostics, Laval, Quebec, Canada) according to the manufacturer’s instructions. Following the removal of nonadherent cells the number of viable adherent cells was quantified using the WST-1 assay (Roche Diagnostics) according to the manufacturer’s instructions.

Cytology and immunohistochemistry

The immunohistochemical analyses of adherent monocyte-derived macrophages and DC were performed using cells cultured on semipermeable Transwell-Clear culture chamber inserts (Corning Costar) as described. After 7 days of culture, adherent cells were washed twice in PBS at 4°C, submerged in blocking buffer (PBS, 0.1% v/v sodium azide, 0.1% v/v mixed human serum, 10% v/v FCS), stained with FITC-labeled mAb, according to the manufacturer’s instructions, washed, and fixed with 2% formaldehyde solution at 4°C. For labeling of filamentous actin (F-actin), nonadherent DC were harvested, washed, cytoplasm onto glass slides, fixed with 1:1 mix of acetone and methanol at −20°C, and labeled with Oregon Green 488 phallolidin according to manufacturer’s instructions. Specimens were all mounted in Vectashield (Vector Laboratories, Burlington, Ontario, Canada) with 1 µg/ml 4,6-diamidino-2-phenylindole. Imaging was performed using an Axioplan 2 fluorescent microscope (Carl Zeiss, Thornwood, NY), DFC-390P digital camera (Sony, Tokyo, Japan), and Northern Eclipse, version 6.0, software. To assess cell size, nonadherent DC were harvested, washed, cytoplasm onto glass slides, fixed with 1:1 mix of acetone and methanol at −20°C, and labeled with Oregon Green 488 phallolidin according to manufacturer’s instructions. Specimens were all mounted in Vectashield (Vector Laboratories, Burlington, Ontario, Canada) with 1 µg/ml 4,6-diamidino-2-phenylindole. Imaging was performed using an Axioplan 2 fluorescent microscope (Carl Zeiss, Thornwood, NY), DFC-390P digital camera (Sony, Tokyo, Japan), and Northern Eclipse, version 6.0, software.

Scanning electron microscopy

DCs cultured in 24-well plates or on 0.4-µm pore, 24-mm Transwell-Clear culture chamber inserts were washed with PBS, fixed in 2.5% glutaraldehyde in 0.1 M phosphate buffer (pH 7.4) for 1 h, and washed three times in phosphate buffer. Nonadherent cells were syringe filtered onto 0.4-µm nuclepore filters (Whatman, Clifton, NJ), which were transferred to petri dishes, while adherent samples on culture chamber inserts were processed directly. Samples were fixed using 1% osmium tetroxide in 0.1 M phosphate buffer for 1 h, washed three times in distilled water, and dehydrated through a graded ethanol series. Following critical-point drying, the specimens were mounted on aluminum stubs, sputter coated with gold/palladium, and examined with a Hitachi (Tokyo, Japan) S4700 scanning electron microscope.

FACS

Cells were harvested, counted by hemocytometer, washed twice in PBS at 4°C, and resuspended in FACS buffer (PBS, 0.1% v/v sodium azide, 0.1% v/v pooled human serum, and 10% v/v FCS). Aliquots of 1 x 106 cells were labeled with fluorescently labeled mAb or the appropriate isotype controls, according to the manufacturer’s instructions, in the dark at 4°C for 1 h, washed twice in PBS and resuspended in 2% formaldehyde in PBS. Analysis was performed based on a minimum of 10,000 cells for each
condition using a FACScalibur system and CellQuest, version 3.1, software (BD Biosciences). Data were analyzed using WinMDI 2.8 software. The mean fluorescence intensity (MFI) was established and corrected by subtraction of the MFI for the appropriate isotype control.

Endocytosis and phagocytosis assays

For quantitative analysis of the endocytic activity of both LL-37-derived and control monocyte-derived DC, 1 × 10^5 cells were resuspended in HBSS and incubated with 1 mg/ml FITC-labeled dextran (molecular mass, 40,000 Da) for 1 h at either 37 or 4°C. The reaction was stopped by washing with ice-cold PBS, and mean FITC fluorescence intensity was determined by flow cytometry. CD11b-mediated and FcγR-mediated adhesion and phagocytosis were assessed using complement-coated SRBC (IgMC SRBC) and IgG-coated sheep erythrocytes (IgG SRBC), respectively, prepared as previously described (32). DC were suspended in HBSS as above, with 0.1% w/v gelatin. IgMC SRBC or IgG SRBC were added at a ratio of 20:1 and incubated, gently rotating, at 37°C for 1 h. PBS at 4°C was added to stop the reaction, and the cells were washed and resuspended in 200 µl of PBS. Three drops of distilled water were added rapidly to half of each sample, to lyse the exposed erythrocytes, followed immediately by 5 ml of PBS to prevent DC lysis. Samples were washed, fixed in 2% formaldehyde solution, cytospun onto glass slides, and stained with Dif-Quik (Dade Behring). The number of particles associated with each DC were counted by light microscopy for 60 cells per sample and performed in triplicate for each condition.

Induction of DC maturation

Monocyte-derived DC were stimulated at day 7 of culture. Cells were harvested, washed twice with PBS, resuspended in fresh medium (without IL-4, GM-CSF, or peptides), and counted. A total of 1 × 10^5 cells per well were incubated for 24 h in Teflon vials (Savillex, Minnetonka, MN) in medium containing 200 ng/ml S. typhimurium LPS (repurified as previously described (28)) or the same volume of endotoxin-free water as a control. Alternatively, 5 × 10^4 cells per well were incubated in 24-well tissue culture plates for 48 h for ELISA analysis of supernatants.

Chemotaxis assays

DC chemotaxis to recombinant human chemokine MIP-3β was performed using a Transwell chemotaxis assay with cells preincubated for 24 h with S. typhimurium LPS or endotoxin-free water as described above. A total of 5 × 10^4 cells was added in 100 µl of RPMI 1640 medium, supplemented with 0.5% w/v filtered BSA, to the apical compartment of a 5-µm pore, 24-mm Transwell polycarbonate culture chamber insert (Corning Costar). A volume of 600 µl of the same medium, additionally containing 100 ng/ml MIP-3β, or the same volume of 0.1% w/v BSA in PBS (as a carrier control) was added to the basal compartment. After 2-h incubation at 37°C, the inserts were removed, their basal surfaces were washed, and the number of cells in the lower chamber was assessed by light microscopy, counting five defined fields of view per well. Studies were performed in triplicate for each condition.

DC-derived cytokine analysis

Following 7 days of culture, 5 × 10^5 monocyte-derived DC per well were exposed to S. typhimurium LPS or endotoxin-free water in triplicate as described above. Supernatants were collected after 48 h and stored at −70°C for analysis by ELISA. Supernatants were analyzed using commercial ELISA kits for IL-12 p70, IL-4, IL-6, TNF-α, and IL-10 (BD Biosciences) performed according to the manufacturer’s instructions and read using a Model 3550 Microplate reader (Bio-Rad Laboratories, Mississauga, Ontario, Canada).

T cell proliferation assays

Assays were set up in triplicate using round-bottom 96-well plates containing 200 µl of complete medium (RPMI 1640, Biofluids, Rockville, MD) supplemented with 10% FBS (Invitrogen), 20 mM HEPES, and 2 mM l-glutamine. A total of 1 × 10^6 DC in each well was coincubated with T cell-derived cytokine analysis

T cells (1 × 10^6) and DC (1 × 10^6) were coincubated in each well as described above. A volume of 100 µl of supernatant was removed from each well of the proliferation assay plates immediately before the addition of [3H]thymidine and stored at −70°C for analysis by cytometric bead array (BD Biosciences) following the manufacturer’s recommended protocol.

Statistical analysis

All data were expressed as mean ± SEM. Statistical significance of differences between groups was established using paired Student’s t tests comparing matched control and treated DC populations generated simultaneously from the same donors. A value of p < 0.05 was taken to denote statistical significance.

Results

LL-37 modifies iDC morphology

Freshly purified human monocytes were cultured for 7 days with IL-4 and GM-CSF (29) to derive iDC, in the presence or absence of 50 µg/ml LL-37 or Bac2a (a related cationic peptide derived from the bovine cathelicidin bactericin (33)). No cytotoxicity resulted from peptide exposure under these conditions, as measured using a lactate dehydrogenase-1 detection assay (data not shown). As expected, control cells differentiated into nonadherent iDC (Fig. 1a). In contrast, a proportion of LL-37-derived iDC were strongly adherent, with confluency of ~30% at day 7, after the removal of nonadherent cells (Fig. 1b). This phenotype was observed with cells from all 11 donors evaluated. Bac2a-derived iDC did not develop the adherent phenotype. A WST-1 tetrazolium salt cleavage assay confirmed the viability of adherent LL-37-derived iDC and the absence of viable adherent cells on washed wells of control iDC, and Bac2a-derived iDC (data not shown).

Monocytes from the same donors were cultured to generate either LL-37-derived adherent iDC or untreated monocyte-derived macrophages (cultured in autologous serum). Adherent LL-37-derived iDC were strongly positive for cell surface CD1a, but negative for CD14, whereas those cultured in autologous serum were strongly positive for cell surface CD14, but negative for CD1a (Fig. 1, c–f). This suggested that the former were indeed DC, and that LL-37 was not inducing the development of macrophages. Scanning electron microscopy revealed LL-37-derived iDC to be larger cells with more numerous surface filopodia (Fig. 1, j and k), in contrast to the control iDC on which small lamellae were more prominent (i). F-actin labeling also demonstrated the increased size of LL-37-derived iDC, with punctate staining that could represent the filopodia observed by scanning electron microscopy (Fig. 1, g and h). The mean cell size by light microscopy of stained cytospins of pooled harvested adherent and nonadherent LL-37-derived iDC was significantly greater than control iDC (p = 0.008) with a mean difference of 33 ± 6% (16.9 ± 1.8 and 12.9 ± 1.7 µm, respectively; n = 4 donors). FACS analysis of these cells also clearly demonstrated a significant increase in both forward scatter (FSC) (p = 7 × 10^{-5}; Fig. 2, a and b) and side scatter (SSC) (p = 2 × 10^{-3}; a and b) in comparison with controls, with mean increases of 20 ± 9 and 75 ± 8%, respectively (n = 13 from 6 donors). These effects were dose dependent, with cell size (as indicated by FSC) significantly increased by exposure to as little as 5 µg/ml LL-37 and maximally increased by 50 µg/ml, whereas SSC (perhaps representing the increased surface structure complexity) increased significantly in a dose-dependent manner over the range of 25–100 µg/ml LL-37 (Fig. 2c).
Cell surface receptor expression is altered on LL-37-derived iDC

To further characterize LL-37-derived iDC and confirm their expression of surface markers characteristic of iDC, FACS analysis was performed using a panel of specific mAbs (Table I, Fig. 3b). As previously reported, control iDC expressed CD1a, but little, if any, CD14, CD83, or CCR7 (3). The level of expression of these surface markers on LL-37-derived iDC was not significantly different from controls, suggesting that these were indeed iDC. In contrast, LL-37-derived iDC expressed significantly enhanced surface levels of CD86, CD11b, CD11c, and CD18, and significantly decreased surface expression of CD209, CD16, and CD32 (Table I). No significant differences were observed in the expression of CD80, CD40, CD206, HLA-DR, or CD54. Changes in surface marker expression were dose dependent over the range of 1–100 μg/ml LL-37, with significant effects observed for some markers even at 5 μg/ml (Fig. 3a). Surface expression of the costimulatory molecule CD86 in LL-37-derived iDC increased dramatically with increasing LL-37 concentration. The increases in expression of CD11b and CD18 (cocomponents of complement receptor (CR)3) were observed to be proportional when comparing the percentage change in individual donors. The percentage decreases in CD16 closely replicated those in the more highly expressed CD32, with the maximal effect upon these FcγRs observed at 25 μg/ml.

Ag uptake is altered in LL-37-derived iDC

To address the functional significance of the altered receptor expression on LL-37-derived iDC, the Ag uptake capabilities of these cells was studied. The majority of both control and LL-37-derived iDC associated with at least one complement-coated SRBC (IgMC SRBC); however, the proportion of LL-37-derived iDC associated with ≥5 or ≥10 particles was significantly greater than for control iDC (p = 0.01 and 0.03, respectively; Fig. 4a). No significant internalization of IgMC SRBC was observed for either control or
LL-37 iDC, confirming that LL-37 did not activate these iDC. In contrast to this increased IgMC SRBC binding, the proportion of LL-37-derived iDC associated with at least 1, or ≥5, IgG-coated SRBC (IgG SRBC) was significantly reduced in comparison to control iDC ($p = 0.02$ and $0.01$, respectively; Fig. 4b). Although the percentage of LL-37-derived iDC that had internalized IgG SRBC was lower than that of control iDC (23 ± 11 and 38 ± 13%, respectively), this did not reach statistical significance. In addition, the endocytic capacity of these cells was studied by examining the binding and uptake of FITC-labeled dextran. A significantly greater uptake was observed in LL-37-derived iDC ($p = 0.005$), with a 105 ± 15% increase in mean FITC-labeled dextran internalization in comparison with control iDC (Fig. 4, c and d). A trend toward greater binding of FITC-labeled dextran was also observed (at 4°C), but did not reach statistical significance. Thus, these data indicate that LL-37-derived iDC have a functionally modified profile of Ag uptake as predicted by the alterations observed in their surface receptor expression.

Cell surface receptor expression is altered on LL-37-derived mDC

To examine the maturation of LL-37-derived DC, iDC were stimulated with LPS. Both the control and LL-37-derived mDC thus generated had a normal maturation profile by FACS, with increased expression of CD86, CD80, CD83, HLA-DR, CD54, and CCR7 in comparison with iDC (Table I, Fig. 3c). However, LL-37-derived mDC displayed significantly greater expression of CD11b, CD86, and CD83 in control with controls. No significant differences were observed in the expression of CD80, HLA-DR, CD54, or CCR7.

Chemotaxis of LL-37-derived mDC is normal

In response to maturation, DC alter their expression profile of chemokine receptors, down-regulating expression of CCR5 and CCR6, but up-regulating expression of CCR7 (4). No surface expression of CCR7 was observed on control iDC or LL-37-derived iDC by FACS analysis (Table I, Fig. 3b). A significant increase in expression was observed following maturation ($p < 0.05$; $n = 5$ donors), with no significant difference between control mDC and LL-37-derived mDC (Table I, Fig. 3c). CCR7 up-regulation was also demonstrated functionally in both LL-37-derived and control mDC as chemotaxis across a gradient of the chemokine MIP-3β. Chemotaxis was induced by MIP-3β in mDC, but not in iDC. No significant difference was observed between LL-37-derived and control mDC or iDC (Fig. 5a).

LL-37-derived mDC produce a characteristic Th-1-inducing cytokine profile

The release of cytokines following maturation with LPS was quantified by ELISA (Fig. 5, b–f). LL-37-derived mDC secreted significantly more IL-12 and IL-6 ($p < 0.05$; $n = 10$ donors) and significantly less IFN-γ ($p < 0.05$; $n = 10$ donors), than paired control mDC from the same donors. In addition, assessed on an individual donor basis, LL-37-derived mDC secreted significantly more TNF-α ($p < 0.05$) in 9 of the 10 donors evaluated; however, considerable variation in absolute levels of cytokine expression was observed between different donors, as previously reported (7). No consistent relationship between IL-10 secretion and LL-37 derivation was observed. In contrast to these LPS-matured DC, LL-37-derived and control immature cells did not demonstrate substantial expression of any of the cytokines studied.

LL-37-derived mDC stimulate enhanced proliferation of IFN-γ-secreting T cells

The capacity of LL-37-derived mDC to activate and induce the proliferation of T lymphocytes, and the functional significance of their altered cytokine and CD86 expression were studied using allogenic T cells. Both LL-37-derived and control DC induced proliferation, but no significant difference was observed over a range of DC/T cell ratios (Fig. 6a). However, T cells stimulated with LPS-matured LL-37-derived mDC produced significantly more IFN-γ than controls ($p = 0.03$; Fig. 6b). This difference was observed for all five donors tested. No significant T cell IL-4 production was detected. No significant IFN-γ was detected from mDC alone.

LL-37-induced DC modulation occurs early in differentiation, via a G protein-coupled receptor

To establish the temporal contribution of LL-37 to the modulation of DC development, monocytes were exposed to a pulse of LL-37 for only the first 24 h of culture. These LL-37-pulse-derived iDC displayed the same adherent phenotype and significant cell size increase ($p = 1 \times 10^{-6}$) comparable with LL-37-derived iDC, with intermediate SSC, significantly greater than controls ($p = 2 \times 10^{-6}$), but less than LL-37-derived iDC ($p = 0.01$). Significantly enhanced expression of CD86 (Fig. 7) and CD11b (data not shown) were also replicated using this pulse exposure with almost
identical magnitude. These data suggest that many of the modifications observed in LL-37-derived DC result from peptide interaction with the pre-DC in the first day of differentiation. To begin dissecting the mechanism underlying these observations, monocytes were pretreated with pertussis toxin to inhibit G\textsubscript{i} protein-coupled receptor activity before a 24-h LL-37 pulse. Pertussis toxin inhibited LL-37-dependent up-regulation of both CD86 (p = 0.04) and CD11b (p = 0.02) significantly, but incompletely, with a significant degree of up-regulation still observed in comparison to pertussis toxin-treated control cells (p = 0.04 and 0.005, respectively; Fig. 7 and data not shown). LL-37-induced changes in FSC and SSC were also partially, but significantly inhibited (data not shown). There were no significant changes in any of these markers in cells treated only with pertussis toxin in comparison with control cells. A previous study implicated formyl peptide receptor-like (FPRL)1 as a G\textsubscript{i} protein-coupled receptor for LL-37 (25). However, a synthetic peptide activator of FPRL1 and FPRL2 (WKYMVM; Ref. 31) substituted for LL-37 had no significant effect on iDC expression of CD86 (Fig. 7) or CD11b, nor on FSC or SSC (data not shown). Finally, characterization of Bac2a-derived iDC suggests peptide specificity, with no significant increase in CD86 (Fig. 7) or CD11b expression nor SSC (data not shown), but cells were larger, with significantly increased FSC (p = 0.03). These data suggest that the development of iDC can be specifically modulated by the interaction of pre-DC with LL-37, and that at least some of these modifications result from the activation of an as-yet-undefined G\textsubscript{i} protein-coupled receptor.

### Discussion

The ability of DC to perform their physiological role is dependent upon appropriate development from pre-DC, Ag capture, maturation, chemotaxis, and Ag presentation to T cells. We have demonstrated that LL-37-derived DC had significantly up-regulated endocytic capacity, modified expression of phagocytic receptors, enhanced costimulatory molecule expression and secretion of Th-1 inducing cytokines, and generated an enhanced Th1 response in vitro. These modifications were superimposed upon retention of basic DC phenotype and appropriate maturational modifications, including changes in chemokine receptor expression that facilitate DC migration to the T cell areas. Thus, we have demonstrated that the cationic peptide LL-37 is a multipotent, tissue microenvironmental modifier of DC differentiation, capable of affecting all temporal stages of the DC life cycle.

Endocytic and phagocytic Ag capture are crucial sentinel functions of iDC. We found that LL-37 significantly and selectively altered the processes of endocytosis and phagocytosis and the expression of phagocytic receptors by iDC. The endocytic capacity of LL-37-derived iDC was significantly enhanced. This is thought to increase the density of Ag presentation, which will enhance T cell stimulation (7). Thus, iDC differentiation in the presence of high concentrations of LL-37 at sites of inflammation may directly impact on the Ag loading and presentation capabilities of DC.

LL-37 profoundly affected the expression of function and of several phagocytic receptors. The decreased expression of DC-specific ICAM-3-grabbing nonintegrin (CD209) on LL-37-derived iDC may have important consequences for pathogen clearance.

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### Table I. DC surface marker expression

| Marker | Control iDC/mDC | LL-37-derived iDC/mDC | Number of Repeats (Donors) |
|--------|----------------|----------------------|--------------------------|
| CD86 (B7.2) | 8.1 (±1.2) | 29 (±6)** | 26 (10) |
| CD11b (α\textsubscript{M} integrin) | 100 (±16) | 211 (±36)** | 24 (11) |
| CD11c (α\textsubscript{X} integrin) | 72 (±11) | 117 (±25)** | 13 (7) |
| CD18 (β2 integrin) | 210 (±18) | 378 (±70)* | 8 (6) |
| CD209 (DC-SIGN) | 79 (±24) | 45 (±9)** | 8 (5) |
| CD16 (F\textsubscript{c},RII) | 5.3 (±0.9) | 1.7 (±0.4)** | 13 (6) |
| CD32 (F\textsubscript{c},RI) | 57 (±13) | 17 (±3.3)** | 15 (10) |
| CD80 (B7.1) | 6.5 (±0.8) | 7.8 (±2.0) | 20 (9) |
| CD40 | 22 (±5) | 25 (±0.4) | 6 (5) |
| CD206 (MMR) | 87 (±35) | 65 (±11) | 6 (4) |
| CD1a | 137 (±48) | 196 (±75) | 6 (6) |
| CD14 | 1.4 (±0.2) | 0.6 (±0.1) | 5 (5) |
| CD83 | 0.02 (±0.1) | 0.6 (±0.2) | 21 (14) |
| HLA-DR | 101 (±16) | 97.8 (±16) | 8 (6) |
| CD54 (ICAM-1) | 122 (±22) | 140 (±22) | 7 (4) |
| CCR7 | 0.6 (±0.3) | 0.6 (±0.3) | 5 (5) |

| LPS-matured DC (mDC) | |
|-----------------------|-----------------------|--------------------------|
| CD11b (α\textsubscript{M} integrin) | 70 (±15) | 164 (±34)** | 10 (5) |
| CD86 (B7.2) | 55 (±7) | 99 (±17)** | 10 (5) |
| CD83 | 10 (±2) | 15 (±2.0)* | 11 (7) |
| CD80 (B7.1) | 10 (±2) | 10 (±4.0) | 8 (6) |
| HLA-DR | 249 (±69) | 208 (±43) | 4 (3) |
| CD54 (ICAM-1) | 249 (±69) | 222 (±26) | 5 (3) |
| CCR7 | 2.7 (±0.5) | 2.9 (±0.9) | 6 (5) |

* DC were derived from monocytes over 7 days in the presence or absence of 50 μg/ml LL-37, fluorescently labeled with specific mAb and analyzed by flow cytometry. The MFI was determined for LL-37-derived and control DC from the same donor as described. Surface marker expression is demonstrated in the table by mean MFI.

\( *, p < 0.05; **, p < 0.01; ***, p < 0.005, \) from the \( n \) value tabulated (derived from the specified number of different donors).

Statistical comparisons of the MFI was by paired t test.
This DC-specific lectin has been implicated as a receptor used by various microorganisms associated with chronic infection, including HIV and *Mycobacterium tuberculosis* (34). Thus, LL-37-induced down-regulation at sites of inflammation might be advantageous to the host, by denying pathogens a protected niche within mononuclear cells. We also demonstrated marked LL-37-induced alterations in the expression and function of CR3 and CR4, and FcγRII and -III. Both CR3 and CR4 are important cell adhesion molecules and also function as competent opsonic and nonopsonic phagocytic receptors (35). The dramatic enhancement of integrin expression by LL-37 could substantially impact upon DC migration (36). It may also enhance their capacity to phagocytose complement-opsonized and unopsonized pathogens with consequences for maturation and activation of such cells in vivo. In contrast to CR3 and CR4, FcγR expression and activity was significantly reduced. However, the consequences of Ag recognition by FcγRII, the predominant FcγR on iDC, depends on the relative contributions of the activating and inhibitory cytoplasmic regions (37). Thus, further studies are required to assess the functional implications of this decrease.

The effective Ag-presenting function of mDC requires the establishment of an immunological synapse with the T cell, and three primary signals as follows: 1) cognate presentation of Ag by MHC class II molecules, 2) expression and engagement of costimulatory molecules, namely CD80 (B7.1) and CD86 (B7.2), amplifying the signaling processes by up to 100-fold (5), and 3) the production of specific polarizing cytokines predisposing to a Th1, Th2, Th3, or regulatory T (Tr) cell response (1, 38). LL-37-derived iDC displayed normal maturation in response to LPS, with an increase in HLA-DR expression on LL-37-derived mDC comparable with control cells. This suggests that the capacity of these cells to present Ag, and hence provide signal 1, was normal. In contrast, the expression of CD86 (signal 2) was significantly altered. Whereas control iDC normally only express high levels of CD86 upon maturation (4), LL-37-derived iDC showed a dramatic, dose-dependent enhancement of CD86 expression in all donors, without changes to other markers associated with maturation. CD86 expression by these cells was further up-regulated by exposure to LPS, confirming that these DC were immature before activation. This also resulted in the enhanced CD86 phenotype observed in LL-37-derived iDC being carried across to maturation. These observations are in marked contrast to the recently described activity of another cationic peptide, murine -defensin-2, reported to directly mature DC in a Toll-like receptor-4-dependent manner (39), although it should be noted that this peptide was studied in the form of fusion proteins constructed from murine -defensin-2 and tumor Ags and does not represent an endogenous ligand. Thus, LL-37 constitutes a modulator of DC differentiation but does not alter maturation, nor directly activate and mature iDC.

Enhanced CD86 expression on mDC would be expected to confer amplified T cell stimulatory capacity to these cells (5) and possibly favor a Th2 response (40). However, LL-37-derived mDC

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**FIGURE 3.** Comparative FACS analysis of LL-37-derived and control iDC. Monocyte-derived iDC were generated in the presence or absence of LL-37 as described, harvested at day 7, stained with fluorescent-labeled mAb, and analyzed by FACS. a, The increases in CD86, CD18, CD11b, and CD11c, and decreases in CD16 and CD32 surface expression on LL-37-derived iDC are shown by MFI over the range of 0–100 μg/ml LL-37. Values represent mean ± SEM; n = 5 donors for CD86, CD18, CD11b, and CD11c; n = 3 donors for CD16 and CD32. Significance of changes was determined vs control DC. *, p < 0.05. **, p < 0.01. b and c, Representative FACS histograms depicting the MFI (x-axis) vs the number of events (y-axis) for 10,000 gated events are shown for control iDC and LL-37-derived iDC (50 μg/ml LL-37) (b), or control mDC and LL-37-derived mDC (c). The thin gray line represents staining with the appropriate isotype control Ab; the shaded gray area and the thick black line represent the control DC and LL-37-derived DC, respectively.
The suppressive effects of Tr cells (42). Furthermore, LL-37-de-
cytokine is known to enhance B cell proliferation and might block
derived mDC consistently produced increased levels of IL-6. This
remains to be determined. In addition to enhanced IL-12, LL-37-
expression of other polarizing cytokines, such as IL-18 and IL-23,
maturation (1, 38, 41). Although the balance of IL-12 vs IL-4
of a Th-1 inducing cytokines (signal 3), particularly IL-12, by the
DC-induced Th1 responses, but does not initiate
them. The consequences of LL-37-induced modifications to the
critical DC signals remain to be determined in the context of a
broader range of Ag-dependent responses. This will establish
whether LL-37-derived DC amplify the expression of polarizing
cytokines of a nature defined by the maturing stimulus, or are
primed to skew the magnitude and the nature of the cytokine re-
sponse, and consequent T cell polarization. In addition, the T cell-
polarizing capacity of DC is temporally controlled. LPS-ma-
tured DC produce an initial IL-12, IL-6, and TNF-α response with Th1-generating capacity, but over time, this IL-12 release
has been shown to diminish, with an increased IL-10 response, and these same exhausted mDC then promote Th2 polarization
(7). In our study, DC supernatants were collected 48 h after LPS
stimulation to assess patterns of change in total cytokine pro-
duction over that period, and the temporal control of DC cyto-
kine release remains to be established. Finally, the role of T
cell-DC interactions in stimulating LL-37-derived DC cytokine
production, and thus the Th polarization, remains to be ex-
plored, including the effects of CD40 ligation and IFN-γ. Nev-
evertheless, it is evident from our study that this innate host de-
finite peptide, LL-37, has the capacity to influence adaptive
immunity via modulation of DC differentiation. Further studies
are required to develop this model in vivo.

The recruitment of pre-DC to sites of inflammation is likely to
be a rapid event, and thus, any potential DC modulator must also
act quickly. We demonstrated that LL-37 modulation of DC re-
quired only a short exposure at an early stage of differentiation
from pre-DC to manifest a wide spectrum of phenotypic changes.
Furthermore, the concentrations of LL-37 at which we observed
these effects were consistent with those observed in vivo during
inflammation (21–23), probably produced predominantly by neu-
rophils and epithelial cells (16, 19, 22). Although LL-37 expres-
sion has also been reported in Langerhans cells, which might also
contribute (24), no LL-37 expression was evident at the protein or
RNA level in monocyte-derived DC in the immature or mature
state (data not shown). It seems likely that this can be attributed to
differences between Langerhans cells and monocyte-derived
DC, and the cellular milieu. Modulation of DC differentiation
was not a nonspecific consequence of exposure to a cationic
peptide, but rather was mediated, at least partly, by a specific
G1-coupled receptor or receptors. This suggested a role for

stimulated the proliferation of T cells secreting significantly higher
levels of IFN-γ. This most likely relates to the enhanced secretion
of Th-1 inducing cytokines (signal 3), particularly IL-12, by the
LL-37-derived DC in response to LPS-induced maturation. DC-
derived IL-12 has been demonstrated to be a critical component in
promotion of a Th1 response. This is in contrast to IL-4, a
cytokine antagonistic of Th1 responses and down-regulated in LL-
37-derived mDC, and IL-10, a cytokine involved in the generation
of a Tr cell response and capable of acting on iDC to prevent full
maturation (1, 38, 41). Although the balance of IL-12 vs IL-4
levels is likely to be critical in determining Th cell polarization, the
expression of other polarizing cytokines, such as IL-18 and IL-23,
remains to be determined. In addition to enhanced IL-12, LL-37-
derived mDC consistently produced increased levels of IL-6. This
cytokine is known to enhance B cell proliferation and might block
the suppressive effects of Tr cells (42). Furthermore, LL-37-de-

FIGURE 4. Ag uptake by LL-37-derived and control iDC. In compar-
ison with control iDC, LL-37-derived iDC demonstrated significantly in-
creased association with complement-coated SRBC (IgM SRBC) (a), signi-
dificantly decreased association with Ab-coated SRBC (IgG SRBC) (b), and significantly increased uptake of FITC-labeled dextran (c and d). The
percentage of cells associated with IgM SRBC or IgG SRBC after a 1-h
incubation was assessed by counting 60 cells per sample by light micros-
copy and performed in triplicate for every condition. The
x-axis indicates the proportion of cells categorized as associated with a specified minimum number of particles. The uptake of FITC-labeled dextran was performed at
37°C to establish total cell association and internalization, and at 4°C, at
which temperature internalization will not occur. Uptake was determined by
flow cytometry (d shows a representative FACS plot; the solid gray area
represents control iDC at 37°C, the black line represents LL-37-derived
iDC at 37°C, the gray line represents control iDC at 4°C, and the broken
gray line represents LL-37-derived iDC at 4°C) and displayed as the mean
percentage increase in LL-37-derived iDC compared with control iDC in c.
Values represent mean ± SEM. *p < 0.05; **p < 0.01; n = 4 donors
for each study.
FPR1, the only LL-37 receptor identified to date (25). However, FPR1 stimulation failed to induce a similar DC phenotype, suggesting the involvement of as-yet-unidentified receptors. Future studies are required to define these receptors and the downstream signaling cascades responsible for the LL-37-dependent DC modulation.

Interestingly, overexpression of GM-CSF in mice has been shown to recruit DC, secreting high levels of TNF-α and IL-6 with increased Ag capture and enhanced T cell and NK cell stimulatory capacities (45). In our in vitro human model, repeated medium supplementation with GM-CSF failed to replicate the LL-37-derived DC phenotype, and GM-CSF receptor expression was unaltered (data not shown). Nevertheless, given the critical nature of

**FIGURE 5.** Chemotaxis and cytokine production by LL-37-derived mDC and control cells. a, Chemotaxis of LL-37-derived mDC and control mDC was not significantly different in response to 100 ng/ml MIP-3β in a Transwell assay. Minimal chemotaxis was observed using IDC or toward a BSA carrier control. Values represent mean ± SEM, n = 2 donors. b–f, Cytokine production in triplicate wells of 5 × 10^4 LL-37-derived DC or control cells was assessed by ELISA after 48-h incubation with 200 ng/ml repurified S. typhimurium LPS. Box plots represent the median, 25th percentile, 75th percentile, and range of cytokine concentrations from LL-37-derived and control DC (n = 10 donors). Paired t tests were performed comparing LL-37-derived and control DC derived from the same donor (n = 10 donors); *, p < 0.05. Donor-specific variation in absolute values required logarithmic y-axes to display IL-12 and TNF-α (b and e).

**FIGURE 6.** T cell stimulatory capacity of LL-37-derived mDC and control cells. a, Coincubation of DC with allogenic T cells performed over a range of DC/T cell ratios, for 96 h, with assessment of [3H]thymidine incorporation over an additional 18 h, revealed no significant difference in DC-induced T cell proliferation between LL-37-derived and control DC. b, Analysis of supernatants from T cell proliferation assays demonstrated significantly increased production of IFN-γ by cells stimulated with LL-37-derived mDC, in comparison with control mDC, but no significant differences in low-level IL-4 production. For both studies, bars represent mean values ± SEM for LL-37-derived and control IDC and mDC. Studies were performed in triplicate; n = 5 donors; *, p < 0.05.

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**FIGURE 7.** Modulation of CD86 expression. DC were derived from monocytes over 7 days in the presence of 50 μg/ml LL-37, 50 μg/ml Bac2a, or the FPRL1 agonist WKYMVM (10 μM), or over 7 days with a 50 μg/ml LL-37 pulse exposure for the first 24 h, with or without pertussis toxin (PTx) pretreatment. IDC were fluorescently labeled with specific mAb and analyzed by flow cytometry. Mean CD86 surface expression is shown and compared with the appropriate matched control IDC prepared in parallel from the same donors. Statistical comparison of the MFI was by paired t test. *, p < 0.05; **, p < 0.005; n = 11 from 6 donors (control, LL-37 pulse, and LL-37 study); n = 4 from 4 donors (control, LL-37 pulse, PTx, PTx LL-37 pulse study); n = 5 from 3 donors (control, Bac2a study); and n = 3 from 3 donors (control, WKYMVM study).
GM-CSF in DC differentiation and the similarity between LL-37-derived DC and this murine DC subset, it seems likely that LL-37 impacts upon the GM-CSF pathway. Indeed, recent data demonstrate that LL-37 and GM-CSF act synergistically to induce phosphorylation and activation of the mitogen-activated protein kinases extracellular signal-regulated kinase 1/2 and p38 in human peripheral blood-derived monocytes.5

In conclusion, we propose that LL-37-derived DC may represent highly stimulatory second-line DC, generated in an LL-37-rich inflammatory milieu in vivo. This modification of DC differentiation may enhance DC production of Th1 cytokines in response to maturation stimuli, establish prolonged T cell stimulation, and generate a more robust Th1 response to harmful Ags. Our data implicate LL-37 as a potent modiﬁer of DC differentiation. Thus, it appears to function as a bridge between the innate and adaptive immune systems, indirectly facilitating the generation of an enhanced Th1 response. This endogenous host modiﬁcation could be very valuable in defending against potential pathogens, particularly at sites where LL-37 has shown to be concentrated in inﬂammation. LL-37 has tremendous therapeutic potential in the development of DC-based immunotherapies for infectious diseases and cancer.

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