Differential Regulation of Formyl Peptide Receptor-Like 1 Expression During the Differentiation of Monocytes to Dendritic Cells and Macrophages

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Dendritic cells (DC) are the most potent APC and play a crucial role in the initiation of primary Ag-specific immune responses (1, 2). DC precursors circulate in blood and differentiate, while undergoing extravasation, into immature DC (iDC). Immature DC populate most nonlymphoid tissues and have the unique properties of migrating to sites of Ag entry and actively internalizing and processing Ags. After Ag uptake and processing, DC become functionally mature (mDC), and acquire the capacity to traffic to the T cell areas of secondary lymphoid organs and the ability to activate Ag-specific naive T cells. Thus, DC precursors, iDC and mDC, have different trafficking patterns.

Regulation of DC trafficking in vivo, like trafficking of other leukocytes, is presumably influenced by many mediators (3). However, the capacity of iDC and mDC to migrate to different anatomical sites is primarily determined by their differential expression of receptors specific for chemokines and classical chemotaxotactic (2, 4). Of the 17 chemokine receptors identified to date, in vitro studies have shown that iDC express CXC chemokine receptor 1, -2, and -4 and CCR1, -2, -3, -4, -5, and -6 and are able to migrate in response to their respective ligands (4–6). In contrast, mDC express CXC chemokine receptor 4 and CCR7 and thus are able to migrate, respectively, in response to stromal cell-derived chemokine-1/CXCL12 and CCR7 ligands, including 6Ckine/secondary lymphoid organ chemokine/exodus-2/CCL21 and macrophage inflammatory protein-3/EBI1 ligand chemokine/exodus-3/CCL19 (2, 4, 7). The in vivo contribution of CCR6 to iDC migration and that of CCR7 to mDC trafficking have been confirmed recently (8–10), indicating that in vitro investigation of the expression of chemotactic receptors by DC provides a useful step for defining DC trafficking.

The receptors for classical chemoattractants, including bacterial formyl peptides such as fMLP, C5a, and platelet-activating factor (PAF) may also contribute to regulating trafficking of DC precursors and DC. Monocytes, the precursors of myeloid DC, are known to express the receptors for and be able to respond to iMLP, C5a, and PAF. Furthermore, human monocyte-derived myeloid iDC respond chemotactically to and express the receptor for PAF (11). DC isolated from both rat respiratory tract tissue and mouse skin or generated in vitro from human peripheral blood monocytes can be chemotactated by iMLP and C5a (12–14). We have previously found that while iDC express both C5aR and formyl peptide receptor (FPR), mDC only express C5aR, but not FPR (15). In humans two functional receptors for iMLP exist: the high affinity receptor FPR and a second receptor, termed FPR-like 1 receptor (FPRL1), which was cloned in 1992 by several independent groups (16–18). FPRL1 has a low affinity for iMLP (17, 19, 20). Subsequently, FPRL1 was also reported to be a functional high affinity receptor for lipoxin A4 and given another name, LXA4R (21). FPRL1 has ~90% homology with FPR at the nucleotide level (16–18), and the genes for FPRL1 and FPR are located in the same region (q13.3) of chromosome 19 (16, 20, 22). Thus, it has been proposed that FPRL1 and FPR expression may be coordinately regulated (20).

Because functional FPR is present in iDC and down-regulated in mDC (15), we investigated whether FPRL1 is also expressed by iDC and, if so, whether mDC also down-regulate their FPRL1 expression. To our surprise, although human monocytes were fully
responsive, monocyte-derived iDC and mDC were unable to respond to an FPRL1-specific ligand with either chemotaxis or Ca\textsuperscript{2+} flux, indicating that FPRL1 expression might be turned off by the differentiation of monocytes to iDC. This was supported by the fact that monocytes, unlike monocyte-derived iDC, did show FPRL1 expression at the mRNA level. Furthermore, because monocytes are the common precursors for both DC and macrophages, we investigated whether FPRL1 would also be down-regulated as monocytes differentiate into macrophages. However, monocyte-derived macrophages preserved FPRL1 expression at both mRNA and functional levels. Thus, FPRL1 expression in the course of monocyte differentiation into DC and macrophages is differentially regulated. These results reveal another distinction between DC and macrophages and suggest that FPRL1 may serve as a marker to distinguish iDC and macrophages.

Materials and Methods

Reagents and cells

DMEM and RPMI 1640 were purchased from BioWhitaker (Walkersville, MD). Recombinant human (rh) TNF-α (sp. act., 2 × 10\textsuperscript{7} U/mg), rhGM-CSF (sp. act., 10\textsuperscript{7} U/mg), and rhIL-4 (sp. act., 2 × 10\textsuperscript{7} U/mg) were purchased from PeproTech (Rocky Hill, NJ). FITC-conjugated goat anti-mouse IgG Ab, synthetic fMLP, and C5a were purchased from Sigma (St. Louis, MO). FBS was purchased from HyClone (Logan, UT). Anti-CD83 mouse IgG Ab, and rhM-CSF (sp. act., 10\textsuperscript{7} U/mg), and rhIL-4 (sp. act., 2 × 10\textsuperscript{7} U/mg) were purchased from Coulter-Immunotech (Marseilles, France). The other Abs used for flow cytometry were purchased from PharMingen (San Diego, CA). Anti-CXCR4 (specific radioactivity, 2 Ci/mmol) was purchased from New England Nuclear (Boston, MA). Su peptide, a shorter version (without the N-terminal 5 aa) of T21/DP107 corresponding to aa 563–595 of HIV envelope protein gp41, was synthesized and purified by the Department of Biochemistry, Colorado State University (Fort Collins, CO). The purity was >90%, and the amino acid composition was verified by mass spectrometry. The endotoxin levels in dissolved Su peptide were undetectable. The human T21/DP107 or PB PB were isolated from Leukobacs (courtesy of the Transfusion Medicine Department, National Institutes of Health Clinic Center, Bethesda, MD) by routine Ficoll-Hypaque density gradient centrifugation. Monocytes were purified from human PBMC with the use of MACS CD14 monoclonal antibody kit (Miltenyi Biotech, Auburn, CA) according to the manufacturer’s recommendation. Human T cells were purified from PBMC by the use of human CD3 T cell enrichment columns (R&D Systems, Minneapolis, MN) following the manufacturer’s recommendation. The purity of monocytes and T cells was checked by FACScan analysis. Cell populations with purity <95% were discarded. Rat basophilic leukemia cells stably transfected with epitope-tagged FPR (ET-FR cells) were provided by Drs. H. Ali and R. Snyderman (Duke University, Durham, NC) and maintained in the presence of 0.8 mg/ml of genitin in DMEM supplemented with 10% FBS. Human embryonic kidney cells 293 stably transfected with FPRL1 (designated FPRL1 cells thereafter) were maintained in the presence of 2 mg/ml of genitin in DMEM supplemented with 10% FBS.

DC and macrophage culture

DC were generated as described previously (15). Briefly, monocytes were differentiated to iDC by incubating them at 1 × 10\textsuperscript{6} in RPMI 1640 medium (RPMI 1640 plus 10% FBS, 2 mM glutamine, 25 mM HEPES, 100 U/ml penicillin, and 100 µg/ml streptomycin) in the presence of rhGM-CSF (50 ng/ml) and rhIL-4 (50 ng/ml) at 37°C in a humidified CO\textsubscript{2} (5%) incubator for 7 days. To induce DC maturation, iDC were cultured in the same cytokine cocktails with added rhTNF-α (50 ng/ml) for 48 h at 37°C in a humidified CO\textsubscript{2} (5%) incubator. Macrophages were generated by incubation of purified monocytes at 1 × 10\textsuperscript{6} in RPMI 1640 medium in the presence of rhM-CSF (50 ng/ml) at 37°C in a humidified CO\textsubscript{2} (5%) incubator for 7 days with the addition of fresh rhM-CSF-containing medium every 2–3 days (23).

Chemotaxis assay

Migration of monocytes, DC, ET-FR, and FPRL1 cells in response to chemotactic factors was assessed using a 48-well microchemotaxis chamber technique as previously described (24). Briefly, different concentrations of chemotactic factors were placed in the wells of the lower compartment of the chamber (Neuro Probe, Cabin John, MA), and cells (10\textsuperscript{6} cells/ml) were added to wells of the upper compartment. The lower and upper compartments were separated by either a 5-µm pore size, uncoated (for monocyte and DC) or a 10-µm pore size, collagen-coated (for ET-FR and FPRL1 cells) polycarbonate filter (Osmonics, Livermore, CA). After incubation at 37°C in humidified air with 5% CO\textsubscript{2} (1.5 h for monocyte and DC, 5 h for ET-FR and FPRL1 cells), the filters were removed and stained, and the cells migrating across the filter were counted with the use of a BiQuant semiautomatic counting system. The results are presented as the number of cells per high power field.

Results

Determination of monocytes to DC results in functional down-regulation of FPRL1

Due to the simultaneous expression of FPR and FPRL1 by monocytes, an FPRL1-specific agonistic ligand was required to probe the functional expression of FPRL1. Su peptide, a peptide modified (lacking the N-terminal 5 aa residues) from T21/DP107 (25), activates FPRL1 specifically (J. M. Wang, unpublished observation) and was therefore chosen for this study. Fig. 1 shows that Su peptide only induced the migration of FPRL1-transfected, not FPR-transfected, cells, confirming the FPRL1 specificity of Su peptide. To investigate the expression of FPRL1 in the course of
DC differentiation and maturation, we generated iDC by culturing purified monocytes (DC precursors) for 7 days in the presence of GM-CSF and IL-4. Mature DC were generated by culturing iDC for 2 days in the presence of GM-CSF, IL-4, and TNF-α. To ensure that iDC and mDC generated in this manner show characteristics of iDC and mDC, their surface marker expression and capacity to stimulate allogeneic MLR were evaluated. As shown in Fig. 2A, iDC were CD1a<sup>1</sup>, CD14<sup>2</sup>, CD40 low , CD83<sup>2</sup>, CD86 low , and HLA-DR medium , whereas mDC were CD1a<sup>1</sup>, CD14<sup>2</sup>, CD40 high , CD83<sup>1</sup>, CD86 high , and HLA-DR high . In addition, iDC were unable to stimulate allogeneic MLR, whereas mDC stimulated marked proliferation of allogeneic T cells at a DC:T cell ratio ranging from 1:100 to 1:10, as detected by [3 H]TdR incorporation (Fig. 2B). These data confirmed that monocyte-derived iDC and mDC used in this study had the characteristics of iDC and mDC, respectively.

A comparison of the responsiveness of monocytes, iDC, and mDC to Su peptide revealed that both iDC and mDC failed to migrate in response to Su peptide, although monocytes did (Fig. 3A). The possibility that iDC and mDC were unable to migrate was ruled out, because they migrated as well as monocytes to SDF-1α (Fig. 3A). Furthermore, Su peptide induced dose-dependent Ca<sup>2+</sup> flux in monocytes, but could not induce significant Ca<sup>2+</sup> flux in iDC derived from monocytes isolated from the same donor (Fig. 3B). Mature DC also did not mobilize Ca<sup>2+</sup> in response to Su peptide (data not shown). These results suggest that differentiation of monocytes to DC is associated with down-regulation of functional FPRL1 expression.

**Differentiation of monocytes to DC coincides with down-regulation of FPRL1 expression at mRNA level**

Due to the unavailability of either FPRL1-specific Ab or radiolabeled FPRL1-specific ligand, we were unable to investigate FPRL1 expression at the protein level during the differentiation of monocytes to DC. However, when FPRL1 expression was monitored by RT-PCR, iDC and mDC did not, while monocytes did, express FPRL1 at the mRNA level (Fig. 4). Amplification of GAPDH by the use of 100 ng of total RNAs isolated from monocytes, monocyte-derived iDC, and mDC under similar RT-PCR conditions yielded bands of nearly identical intensities, confirming that equal amounts of RNAs were used in the RT-PCR for FPRL1 amplification (Fig. 4). Thus, FPRL1 was down-regulated at the mRNA level by the differentiation of monocytes to DC.

**Down-regulation of FPRL1 expression and function is completed at the late stage of DC differentiation**

Differentiation of monocytes to iDC in vitro took 7 days when cultured in the presence of GM-CSF and IL-4. To investigate the kinetics of FPRL1 down-regulation, cells were removed from the culture at various time points for chemotaxis and extraction of total RNA. Cells cultured for 1 and 3 days (D1 and D3) responded chemotactically to Su peptide equally well as freshly isolated...
monocytes (Fig. 5A). However, the migratory response of the cultured cells to Su peptide decreased significantly after 5-day incubation (D5) and was absent after 7-day incubation (D7) when DC differentiation was complete (Fig. 5A). Similarly, the expression of FPRL1 mRNA was unchanged in comparison with monocytes on D1 and D3, but was greatly decreased on D5 and absent in D7 cells (Fig. 5B). Bands of GAPDH with similar intensity were obtained for each 100 ng of total RNA isolated from monocytes and D1, D3, D5, and D7 cells, assuring that identical amounts of RNAs were used for the amplification of FPRL1 (Fig. 5B). The down-regulation of FPRL1-mediated chemotactic responsiveness was not due to a decrease in cell motility, because the random migrations (without Su peptide) of monocytes and D1, D3, D5, and D7 cells were similar (Fig. 5A), and all cell populations migrated equally well as monocytes in response to another chemoattractant human C5a.

FIGURE 4. Expression of FPRL1 mRNA by monocytes and monocyte-derived DC. Total RNA was isolated from monocytes, monocyte-derived iDC, and mDC. The RT-PCR products of FPRL1 (upper panel) and GAPDH (lower panel) were displayed by 1 and 1.5% agarose gels, respectively. The anticipated sizes for FPRL1 and GAPDH are 1100 and 246 bp, respectively. The marker is a 1-kb DNA ladder.

FIGURE 5. The kinetics of FPRL1 expression in the process of DC differentiation. Purified monocytes were incubated in the presence of rhGM-CSF and rhIL-4 for 7 days in humidified air containing 5% CO2 to allow differentiation to iDC. A fraction of monocytes (Mo) or cells cultured for 1, 3, 5, and 7 days (D1, D3, D5, and D7) was collected for chemotaxis assay and for total RNA extraction. A. Chemotaxis of monocytes (upper panel), iDC (middle panel), and mDC (lower panel) in response to Su peptide. SDF-1 at a final concentration of 100 ng/ml was used as a positive control. The results are shown as the average (mean ± SD) of triplicate wells. B. Ca2+ mobilization by monocytes (upper panel) and iDC (lower panel) in response to various concentrations of Su peptide. Su peptide was added 80–90 s after the beginning of the recording. One experiment representative of three is shown.
Differential FPRL1 Expression by DC and Macrophages

Expression of various chemoattractant receptors during the differentiation and maturation of monocyte-derived receptors

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Discussion

A comparison of the responsiveness to an FPRL1-specific agonist, and FPRL1 expression at the mRNA level by DC, macrophages, and their common precursors (monocytes) revealed that 1) FPRL1 is down-regulated at both functional and mRNA levels after the differentiation of monocytes to iDC; 2) FPRL1 down-regulation occurs at a later stage of DC differentiation; and 3) FPRL1 expression is maintained at both functional and mRNA levels after the differentiation of monocytes to macrophages. Although we could not investigate FPRL1 expression at the protein level due to the unavailability of appropriate reagents, it is likely that DC also lack FPRL1 protein expression because they did not respond to an FPRL1-specific agonist, nor did they express FPRL1 mRNA. Based on our previous studies and those of others, the functional expression of chemoattractant receptors by monocyte-derived DC and monocytes is summarized in Table I. Because chemoattractant receptors mediate cell migration through interacting with their ligands (3, 4, 20), the migration of DC precursors, iDC, and mDC, must be differentially regulated by agonistic ligands specific for FPRL1, FPR, C5aR, and PAF receptor (PAFR). Indeed, the migration of monocytes to sites of inflammation is regulated by classical chemoattractants (20). The migration of iDC to sites of Ag deposition or inflammation is also regulated by chemoattractants specific for FPR, C5aR, and PAFR (12, 27, 28).
Because mDC maintain the functional expression of C5aR and PAFR (11, 15). C5a and PAF presumably also contribute to the control of mDC trafficking from peripheral tissues to secondary lymphoid organs and presumably contribute to the induction of adaptive immune responses. The results showing functional expression of FPRL1 by monocyctic precursors of DC, but not by DC, suggest that endogenous ligands acting on FPRL1 may play a role in regulating the extravasation of DC precursors.

It has long been known that monocytes are the precursors for macrophages (29, 30). Monocytes have recently been shown to be able to differentiate into DC both in vitro, either by treatment with appropriate cytokines (23, 26) or by incubation with endothelial cells grown on a collagen matrix (31), and in vivo (32). Therefore, DC and macrophages share the same precursors. However, DC and macrophages display unique characteristics and exhibit distinct physiological functions. First, DC express more specialized receptors (e.g., mannose, Fcγ, and Fce receptors) to facilitate Ag capture and specific delivery to the Ag processing compartment (33–35), whereas macrophages express more scavenger receptors (36, 37).

In addition, DC, relative to macrophages, synthesize higher levels of MHC class II molecules and express more accessory molecules, especially after maturation (1, 5, 15, 23, 26, 32, 35). Furthermore, DC can prime naïve T cells in vitro and in vivo (1, 2). Our results showing that macrophages, but not iDC, express FPRL1 highlight another distinction between iDC and macrophages and suggest that FPRL1 may be used as a marker to distinguish between iDC and macrophages.

FPRL1 uses a variety of specific ligands, including Su peptide (used in the present study), lipoxin A4 (21, 38), a lipid derivative of arachidonate metabolism (39), a synthetic peptide analogous to HIV type 1 gp120 (40), and serum amyloid A (41). Very recently, we established that LL-37, the antimicrobial domain of human cathelicidin/human cationic antimicrobial protein 18 (42), is also an FPRL1-specific ligand (24). Of these, lipoxin A4, serum amyloid A, and LL-37 are endogenously generated predominantly during inflammation, atherosclerosis, and bacterial infection (38, 39, 42–46). Furthermore, serum amyloid A is able to induce tissue infiltration of leukocytes into the site of injection in vivo (47).

Thus, FPRL1 may be involved in attracting macrophages and their precursors, monocytes, but not iDC, to tissues where FPRL1-specific endogenous ligands are locally generated. The selective expression of FPRL1 by macrophages may also reflect a physiologically fundamental capacity for macrophages, unlike iDC, to respond to endogenous FPRL1-specific agonistic ligands. One of the endogenous FPRL1-specific ligands, lipoxin A4, has been reported as a potent inhibitor of acute inflammation by suppressing CD11/18 expression and chemokine production of FPRL1-positive cells (38, 48–50). Serum amyloid A, another endogenous FPRL1-specific agonistic ligand, has also been reported to inhibit the oxidative burst response of neutrophils stimulated by fMLP (51).

Thus, the interaction of FPRL1 with its endogenous ligands may have a negative feedback inhibitory effect on FPRL1-positive cells, especially when endogenous FPRL1-specific ligands are produced systemically in large amounts, such as during severe infection. It would be counterproductive for DC to sense lipoxin A4, serum amyloid A, and LL-37, because DC should not be inhibited even during severe systemic inflammation to perform Ag uptake, processing, and presentation. The physiological significance of the differential down-regulation of FPRL1 in DC, but not in macrophages, needs to be clarified by more in-depth investigation.

FPRL1 was identified and cloned from differentiated HL-60 neutrophils (16–18). FPRL1 possesses 69% identity at the amino acid level to FPR (16–18, 20). The FPR and FPRL1 genes are clustered next to each other within a narrow region on human chromosome 19q13.3 (16, 20, 22). FPRL1 and FPR even share some ligands, including fMLP (17, 19, 20), a synthetic peptide termed T21/DP107 that is analogous to an ectodomain of HIV gp41 (25), and a synthetic hexapeptide (Try-Lys-Tyr-Met-Val-d-Met-NH2) termed W peptide (52, 53). Because FPR is expressed by iDC at functional, protein, and mRNA levels (12, 14, 15), it was thus expected that monocyte-derived iDC would also express FPRL1. In this sense the finding that FPRL1 is down-regulated after the differentiation of monocytes to iDC is unique. Human FPRL1 is expressed not only by monocytes, but also by macrophages (the present study), neutrophils (20), lymphocytes (41), and enterocytes (50). The expression of FPRL1/LXA4R by a human enterocyte cell line, the T84 human colonic adenocarcinoma cell line, has recently been shown to be up-regulated by IL-13 and IFN (50). However, how FPRL1 expression in leukocytes is controlled is not clear. Therefore, the process by which FPRL1 down-regulation occurs only as monocytes differentiate into DC, but not as monocytes differentiate into macrophages, needs further investigation.

Collectively, the finding that the functional FPRL1 is selectively expressed by monocyte-derived macrophages but not by monocyte-derived DC suggests that the interaction between FPRL1 and its specific endogenous ligands may play an important role in regulating the trafficking of DC precursors and the accumulation of macrophages at inflammatory sites and is more involved in inflammatory reactions than in adaptive immune responses.

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