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

De Yang, Qian Chen, Yingying Le, Ji Ming Wang and Joost J. Oppenheim

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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 chemoattractants (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 receptors 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 vivo 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 fMLP, C5a, and PAF. Furthermore, human monocyte-derived myeloid iDC respond chemotactically to and express the receptor for PAF (11). Therefore, the interaction of FPRL1 with agonists that are involved in inflammatory and host defense responses. Here we show that FPRL1 is down-regulated as monocytes differentiate into DC. This down-regulation occurs at both mRNA and functional levels. Therefore, the interaction of FPRL1 with its agonists is more likely to regulate the in vivo trafficking of DC precursors than DC. In contrast, FPRL1 expression is maintained at both mRNA and functional levels as monocytes differentiate into macrophages. Thus, our results demonstrate further distinctions between myeloid DC and macrophages, albeit they share a common precursor. The fact that macrophages rather than myeloid DC express functional FPRL1 suggests that this chemotactic receptor may be more involved in inflammatory reactions and innate host defense than in adaptive immune responses. The Journal of Immunology, 2001, 166: 4092–4098.

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Monocytes are the common precursors for myeloid dendritic cells (DC) and macrophages. Identification of chemotactic receptors expressed by myeloid DC, macrophages, and their precursors in the course of differentiation and maturation is important not only for elucidation of their in vivo trafficking, but also for understanding of the functional distinction between DC and macrophages. We chose to study formyl peptide receptor like-1 (FPRL1), a chemotactic receptor known to interact with several endogenous agonists that are involved in inflammatory and host defense responses. Here we show that FPRL1 is down-regulated as monocytes differentiate into DC. This down-regulation occurs at both mRNA and functional levels. Therefore, the interaction of FPRL1 with its agonists is more likely to regulate the in vivo trafficking of DC precursors than DC. In contrast, FPRL1 expression is maintained at both mRNA and functional levels as monocytes differentiate into macrophages. Thus, our results demonstrate further distinctions between myeloid DC and macrophages, albeit they share a common precursor. The fact that macrophages rather than myeloid DC express functional FPRL1 suggests that this chemotactic receptor may be more involved in inflammatory reactions and innate host defense than in adaptive immune responses. The Journal of Immunology, 2001, 166: 4092–4098.
responsive, monocyte-derived iDC and mDC were unable to re-

spond to an FPRL1-specific ligand with either chemotaxis or Ca2+ 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-regu-

lated 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 be-

tween 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 BioWhittaker (Walkersville, MD). Recombinant human (rh) TNF-α (sp. act., 2 × 10^7 U/mg), rhGM-CSF (sp. act., 10^7 U/mg), and rhIL-4 (sp. act., 2 × 10^8 U/mg) were pur-

chased from PeproTech (Rocky Hill, NJ). FITC-conjugated goat anti-

mouse IgG Ab, synthetic FMLP, and C5a were from Sigma (St. Louis, MO). FBS was purchased from HyClone (Logan, UT). Anti-CD83 was purchased from Coulter-Immunotech (Marseille, France). The other Abs used for flow cytometry were purchased from PharMingen (San Di-

ego, CA), and anti-Human CD45 (specific radioligand, 2 Cimmol) 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 spec-

trometry. The endotoxin levels in dissolved Su peptide were undetectable. Cells stably transfected with pcDNA3 encoding human FPR were isolated from Leukoteks (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 monocyte isolation 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 pu-

rity of monocytes and T cells was checked by FACSscan analysis. Cell populations with purity <95% were discarded. Rat basophilic leukemia cells stably transfected with epitope-tagged FPR (ET-FR cells) were pro-

vided by Drs. H. Ali and R. Snyderman (Duke University, Durham, NC) and maintained in the presence of 0.8 mg/ml of geneticin in DMEM sup-

plemented with 10% FBS. Monocytes, human embryonic kidney cells 293 stably trans-

fected with FPRL1 (designated FPRL1 cells) were propagated in the presence of 2 mg/ml of geneticin in DMEM supplemented with 10% FBS.

DC and macrophage culture

DC were generated as described previously (15). Briefly, monocytes were differ-

entiated to iDC by incubating them at 1 × 10^7/ml 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 CO2 (5%) incubator for 7 days. To induce DC maturation, iDC were cultured in the presence of rhM-CSF (50 ng/ml) at 37°C in a humidified CO2 (5%) incubator. Macrophages were generated by incubation of purified monocytes at 1 × 10^7/ml in RPMI 1640 medium in the presence of rhM-CSF (50 ng/ml) at 37°C in a humidified CO2 (5%) incubator for 7 days with the addition of fresh rhM-CSF-containing me-

dium every 2–3 days (23).

Chemotaxis assay

Migration of monocytes, DC, ET-FR, and FPRL1 cells in response to che-

motactic 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 (10^6 cells/ml) were added to wells of the upper compartment. The lower and upper compart-

ments were separated by either a 5-μm pore size, uncoated (for monocyte and DC) or a 10-μm pore size, coated-en (for ET-FR and FPRL1 cells) polycarbonate filter (Osmonics, Livermore, CA). After incubation at 37°C in humidified air with 5% CO2 (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 BioQuant semiautomatic counting system. The results are presented as the number of cells per high power field.

Calcium flux

Monocytes, macrophages, or DC (10^7 cells/ml in RPMI 1640 containing 10% FBS) were loaded by incubating with 5 μM fura-2 (Molecular Probes, Eugene, OR) at 24°C for 30 min in the dark. Subsequently, the cells were washed with and resuspended (10^6 cells/ml) in saline buffer (138 mM NaCl, 1 mM CaCl2, 10 mM HEPES, 5 mM glucose, and 1% BSA, pH 7.4). Each 2 ml of the cell suspension was then transferred into a quartz cuvette, which was placed in a luminescence spectrometer LS50 B (Perkin-Elmer, Beaconsfield, U.K). Ca2+ mobilization of the cells was measured by recording the ratio of fluorescence emitted at 510 nm after sequential excitation at 340 and 380 nm in response to chemotactic factors.

Fluorescence-activated cell sorting

Monocytes, DC, and macrophages (10^6/sample) were first washed three times with FACS buffer (PBS, 1% FBS, and 0.02% NaN3, pH 7.4), and then stained with mouse mAbs against CD1a, CD14, CD40, CD83, CD86, and HLA-DR or with isotype-matched control Ab (final concentration, 5 μg/ml) at room temperature for 30 min. After washing three times with FACS buffer, the cells were suspended in FACS buffer containing FITC-

conjugated goat anti-mouse IgG for 30 min at room temperature. Finally, the stained cells were washed twice with FACS buffer and twice with PBS, fixed with 1% paraformaldehyde in PBS at 4°C overnight, and analyzed the next day with a flow cytometer (EPICS, Coulter, Miami, FL).

Mixed leukocyte reaction

Allogeneic MLR was performed as previously described (5). Briefly, pu-

rified allogeneic T cells (10^7/well) were cultured with different numbers of DC in a 96-well flat-bottom plate for 7 days at 37°C in humidified air with 5% CO2. The proliferative response of T cells was examined by pulsing the DC in a 96-well flat-bottom plate for 7 days at 37°C in humidified CO2 (5%) incubator. Macrophages were generated by incubation of purified monocytes at 1 × 10^7/ml in RPMI 1640 medium in the presence of rhM-CSF (50 ng/ml) at 37°C in a humidified CO2 (5%) incubator for 7 days with the addition of fresh rhM-CSF-containing me-

dium every 2–3 days (23).

RNA isolation and RT-PCR

Total RNA from monocytes, DC, and macrophages was isolated by the use of Trizol reagent (Life Technologies, Grand Island, NY). The RNAs were cleaned by treatment with RNase-free DNAse I (Stratagene, La Jolla, CA). RT-PCR was performed by the use of ProSTAR HP Single-Tube RT-PCR System (Stratagene, La Jolla, CA). Briefly, 100 ng of total RNA was used in the RT-PCR. After RT at 37°C for 15 min and inactivation of Moloney murine leukemia virus reverse transcriptase at 95°C for 1 min, FPRL1 and GAPDH cDNA fragments were amplified by 40 cycles of PCR (denaturing at 95°C for 30 s, annealing at 60°C for 30 s, and extension at 68°C for 2 min); reaction with the last extension was performed at 68°C for 10 min. The primers for FPRL1 were 5’-CTGCTGCTGCTGCTGCCAAG-3’ and 5’-AATATCCCTGACCCCATCCTCA-3’, which enabled the amplification of an 1.1-kb cDNA fragment of FPRL1 as verified by sequencing. The primers for human GAPDH were 5’-AATGACATCAAGAAGGTGGT-3’ and 5’-GTCTTACTCCTTGGAGGCCATGT-3’, which resulted in the amplification of a fragment of 246 bp as previously described (5). PCR products were identified on 1–2% agarose gel after ethidium bromide staining and photodocumented.

Results

Differential expression of monocytes to DC results in functional down-

regulation of FPRL1

Due to the simultaneous expression of FPR and FPRL1 by mono-

cytes, 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 observa-

tion) 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 showed 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>+</sup>, CD14<sup>+</sup>, CD40 low, CD83<sup>−</sup>, CD86 low, and HLA-DR medium, whereas mDC were CD1a<sup>+</sup>, CD14<sup>+</sup>, CD40 high, CD83<sup>+</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 chemotactant human C5a...
ious concentrations of Su peptide. Su peptide was added to monocytes and iDC (derived from the same monocyte population) was stained with 1 µg of isotype-matched control mouse IgG or mouse mononclonal IgG Abs specific for the human leukocyte surface molecules as indicated. After removal of the unbound IgG, the cells were further stained with FITC-conjugated goat anti-mouse IgG Ab, washed, and analyzed with a flow cytometer. Shown is the mean fluorescence intensity (MFI) of every marker for monocytes (○) and macrophages (□) from one representative experiment.

(Fig. 5A, triangles). In addition, iDC (D7) generated from monocytes in a similar manner maintained their responsiveness to fMLP and the expression of FPR, a chemotactic receptor that shows considerable homology with FPRL1 (data not shown) (15). Therefore, the down-regulation of FPRL1 expression and function during DC differentiation begins after 3-day culture and is completed by the late stage of differentiation of monocytes to iDC.

Macrophages differentiated from monocytes maintain functional expression of FPRL1

To determine whether differentiation of monocytes to macrophages also results in a down-regulation of FPRL1 expression, purified human peripheral blood monocytes were cultured in the presence of M-CSF for 7 days in vitro, a condition known to induce macrophage, but not DC, differentiation (23, 26). The resulting cells had several characteristics typical for macrophages: 1) they were adherent; 2) they expressed similar levels of CD11b, CD14, and CD86 as monocytes (Fig. 6); and 3) they up-regulated their expression of CD16, CD40, and HLA-DR (Fig. 6). Macrophages and iDC differentiated from the same batch of purified monocytes were then tested for their capacity to migrate in response to Su peptide, the FPRL1-specific agonist. In contrast to iDC that did not respond, macrophages migrated chemotactically to Su peptide (Fig. 7A). Moreover, macrophages mobilized Ca2+ in a dose-dependent manner in response to Su peptide (Fig. 7B). When total RNAs were isolated from monocytes, iDC, and macrophages and tested for FPRL1 expression by RT-PCR, comparable levels of FPRL1 mRNA were expressed by monocytes and macrophages, whereas FPRL1 mRNA was absent in iDC (Fig. 7C). Therefore, differentiation of monocytes to macrophages is associated with persistent FPRL1 expression and function.

Discussion

A comparison of the responsiveness to an FPRL1-specific ligand of, 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 chemotactic 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 (PAF-R). 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 PAF-R (12, 27, 28).

Table I. Expression of various chemoattractant receptors during the differentiation and maturation of monocyte-derived DC

<table>
<thead>
<tr>
<th>Chemoattractant Receptor</th>
<th>DC Precursor (monocyte)</th>
<th>iDC</th>
<th>mDC</th>
<th>Reference</th>
</tr>
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<tbody>
<tr>
<td>FPRL1</td>
<td>+</td>
<td>–</td>
<td>–</td>
<td>Present study</td>
</tr>
<tr>
<td>FPR</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>15</td>
</tr>
<tr>
<td>C5aR</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>15</td>
</tr>
<tr>
<td>PAF-R</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>11</td>
</tr>
</tbody>
</table>
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 monocyte 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 Fcε 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 arachidonic 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 reflect 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.

Acknowledgments

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References


