LPA_3 receptor mediates chemotaxis of immature murine dendritic cells to unsaturated lysophosphatidic acid (LPA)

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Abstract: Increasing evidence supports roles for lipids in the biology of immune cells. In particular, bioactive lipids such as sphingosine-1-phosphate (S1P) bind to cognate G protein-coupled receptors (GPCRs) and modulate leukocyte trafficking and homeostasis. Lysophosphatidic acid (LPA) represents a family of bioactive lipids, which differ in the length and degree of saturation of the fatty acyl chain. LPA is structurally related to S1P and exerts cellular effects by binding to five known GPCRs ($LPA_{1–5}$). Its function in the immune system is less clear, although it was shown to induce chemotaxis of human dendritic cells (DCs) and activated T cells. In this study, we show that LPA can induce chemotaxis of immature but not mature mouse DCs and that only unsaturated and not saturated LPA species are efficient chemoattractants. However, both LPA species do not alter DC maturation or chemotaxis to other chemokines. The loss of DC migration capability correlated with the down-regulation of expression of the receptors LPA_3 and LPA_5, and expression of LPA_1, LPA_2, and LPA_4 did not change. A LPA_3 antagonist reduced immature DC migration to LPA by 70%, suggesting that LPA_3 mediates immature DC chemotaxis to unsaturated species of LPA. Furthermore, isolated, immature DCs from mice lacking LPA_3 exhibited a 50% reduction in migration to LPA. In summary, our results indicate that immature mouse DCs migrate preferentially in response to unsaturated LPA and that LPA_3 is important in this response. *J. Leukoc. Biol.* 82: 1193–1200; 2007.

Key Words: *sphingosine-1-phosphate · GPCR · chemoattractant · bioactive lipid · inflammation*

INTRODUCTION

Dendritic cells (DCs) are professional APCs, which reside as immature cells in peripheral tissues and act as sentinels patrolling for foreign particles. Upon antigen recognition, DCs engulf particles, begin to mature, and migrate to the draining lymph nodes to activate naïve T cells. Thus, the migratory ability of DCs is fundamental to their roles as APCs and to initiating the adaptive immune response. DC trafficking is regulated by several chemotactic signals, which include chemokines (or chemotactic cytokines) and nonchemokine factors [1]. Although the chemokines and their seven-transmembrane G-protein coupled receptors (GPCRs) are the most characterized, increasing evidence implicates bioactive lipids in DC trafficking in vivo. These lipids include the eicosanoids PGE_2 [2] and leukotriene-C_4 [3], as well as the lysophospholipid sphingosine-1-phosphate (S1P) [4]. Lysophosphatidic acid (LPA) is another lysophospholipid structurally related to S1P, that could play a role in DC migration [5, 6].

LPA comprises a family of small phospholipid molecules containing a phosphoglycerol backbone and a single fatty acyl chain, which varies in its *sn*-1 or *sn*-2 position and in its length and degree of saturation. The most prevalent LPA species includes saturated (stearoyl, 18:0; palmitoyl, 16:0) and unsaturated (oleoyl, 18:1; arachidonyl, 20:4) fatty acyl moieties. Most cell types can produce LPA during the synthesis of glycerolipids [7], but LPA can also be secreted, mainly through hydrolysis of choline from lysophosphatidylcholine by the ectoenzyme autotaxin [8, 9]. Few cell types are known to secrete LPA: adipocytes [9, 10], ovary cancer cells [11], and platelets [12].

Secreted LPA binds specific receptors present on the secreting cells in an autocrine manner or on neighboring cells in the immediate environment of LPA-producing cells. Five seven-transmembrane GPCRs designated $LPA_{1–5}$ have been identified, and all mediate cellular responses to exogenous LPA [13, 14]. For example, LPA increases cell proliferation and survival, inhibits apoptosis, induces morphological changes, inhibits gap-junctional communication between adjacent cells,

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and stimulates cell migration [13]. Genetic analyses in mouse models lacking the receptors LPA_1, LPA_2, or LPA_3 have begun to uncover roles for LPA in the brain, the reproductive system, and the heart [15, 16]. So far, these models have not been used to investigate the role of LPA in the immune system. However, some experimental evidence suggests that LPA could regulate immune cell trafficking. First, LPA receptors are expressed by lymphocytes [5, 17], DCs [6], and in lymphoid organs [13] (S. Cases, unpublished observations). In addition, LPA 18:1 can cause in vitro chemotaxis of human T cells [5] and human immature DCs [6]. It is not known whether LPA species, besides LPA 18:1, can mediate chemotaxis of DCs or which LPA receptors might be important for this effect.

In this study, we asked if LPA mediates chemotaxis of bone marrow-derived mouse immature and mature DCs and if these cells respond differently to distinct LPA species. In addition, we used RNA analyses as well as chemical and genetic inhibitions to investigate which LPA receptors are responsible for the chemotaxis of immature DCs to LPA.

MATERIALS AND METHODS

Mice

DCs were derived form the bone marrow of 8- to 12-week-old mice. Wild-type C57BL/6 mice were purchased from the Jackson Laboratory (Bar Harbor, ME, USA), and LPA_3-deficient ($LPA_3^{-/-}$) mice on a mixed genetic background (C54Bl/6×129/Sv) were obtained from Dr. Jerold Chun (The Scripps Research Institute, La Jolla, CA, USA) [18]. The Committee on Animal Research of the University of California, San Francisco (San Francisco, CA, USA), approved experiments using mice.

Cytokines and reagents

Recombinant murine (rm)GM-CSF and stromal cell-derived factor 1 (SDF-1; CXCL12) were purchased from R&D Systems Inc. (Minneapolis, MN, USA), and LPS from *Salmonella typhimurium* was from Sigma-Aldrich (St. Louis, MO, USA). The different LPA species used in chemotaxis assays (LPA 18:1, LPA 18:0, LPA 16:0, and LPA 20:4) and the LPA receptor antagonist VPC32179—ammonium salt of phosphoric acid mono-{2-octadec-9-enoylamino-3-[4-(pyridin-2-ylmethoxy)-phenyl]-propyl}ester—were purchased from Avanti Polar Lipids (Alabaster, AL, USA). FITC used for painting mice skin was from Sigma-Aldrich.

DC culture medium

RPMI 1640 (Invitrogen, Carlsbad, CA, USA) was supplemented with 10% FBS (Hyclone, Logan, UT, USA), 1 mM Hepes, 50 μM 2-ME, 2 mM L-glutamine, 100 U/ml penicillin, and 100 μg/ml streptomycin.

Generation of mouse DCs

Bone marrow was isolated from the mouse tibia and femur bones essentially as described [19]. On the first day of the procedure, bones were flushed twice with 3 ml medium, and the collected cells were treated with RBC lysis buffer, washed, resuspended in medium supplemented with 200 U/ml rmGM-CSF, and plated onto 10-cm bacterial petri dishes at a density of 2×10^6 cells per plate. Cells were fed on Days 3, 6, and 8. On Day 10, the suspended and loosely adherent cells were collected, resuspended in 10 ml of medium supplemented with 100 U/ml rmGM-CSF, and replated onto tissue-culture dishes. To obtain mature DCs, LPS was added at 1 μg/ml on Day 10. On Day 11, the suspended and loosely adherent cells were collected, rinsed with PBS, and resuspended in serum-free medium supplemented with fatty acid-free BSA for migration assays. Before migration assays, a fraction of the immature and LPS-matured DC populations was characterized by flow cytometry analyses of cell surface molecules. We routinely obtained over 85% of cells expressing CD11c and MHCII. LPS-induced maturation resulted in a shift of the $MHCII^{dim}/CD11c^{bright}$ cells toward $MHCII^{bright}/CD11c^{bright}$ cells and an increased expression of the costimulatory molecules CD86 and CD40.

Analysis of DC maturation by flow cytometry

Immature or LPS-matured DCs (0.5×10^6 cells) were plated in 96-well V-bottom culture plates and incubated with rat anti-mouse CD16/CD32 (1/500 in staining buffer, BD PharMingen, San Diego, CA, USA) for 30 min at 4°C to block FcRs. Cells were washed once with staining buffer and then incubated for 30 min at 4°C with fluorescent, cell type-specific antibodies: F4/80 PE (1/1000, Caltag Laboratories, Burlingame, CA, USA), CD11c PE (1/200, BD PharMingen), MHCII FITC (1/1000, BD PharMingen), CD40 PE (1/200, BD PharMingen), and CD86 PE (1/200, BD PharMingen). Cells were then washed twice with staining buffer and fixed in 1% paraformaldehyde (PFA) in PBS at 4°C until analysis with a FACSCalibur flow cytometer (BD Biosciences, San Jose, CA, USA). Data were acquired with CellQuest software (BD Biosciences) and analyzed with FlowJo software (TreeStar, Mountain View, CA, USA).

Chemotaxis assays

Costar Transwells, with 5.0-μm pore size (Fisherbrand, Hampton, NH, USA), were used for the chemotaxis assays. The cell suspensions and chemotactic agents were prepared in the assay buffer (RPMI 1640 with 1% fatty acid-free BSA). Serial dilutions of the chemotactic factors were prepared in the assay buffer, 600 μl of each dilution was placed in the lower wells, and 100 μl of the cell suspension (5×10^6 cells/ml) was placed in the upper transwell chambers. After 3.5 h of incubation at 37°C, cells, which had transmigrated into the lower wells, were collected, resuspended in 100 μl 1% PFA, and counted by flow cytometry for 1 min. The chemotactic index (CI) was calculated by dividing the number of cells, which had transmigrated in response to a chemotactic agent, by number of cells, which had transmigrated in response to medium only.

Chemokinesis assay

The assay was performed in a similar manner to the chemotaxis assay. Serial dilutions of the chemotactic agents were prepared and used for the cell suspension and to fill the lower wells.

Real-time quantitative RT-PCR

RNA was extracted from the cells with RNA Stat-60 reagent (Iso-Tex Diagnostics, Inc., Friendswood, TX, USA), and cDNA was synthesized with the SuperScript III enzyme (Invitrogen). Primers for mouse LPA_1, LPA_2, LPA_3, and cyclophilin were from Invitrogen, and sequences were as follows: LPA_1–forward CTGTGGTCATTGTGCTTGGTG, LPA_1–reverse CATTAGGGTTCTCGTTGCGC; LPA_2–forward GGCTGCACTGGGTCTGGG, LPA_2–reverse GCTGACGTGCTCCGCCAT; LPA_3–forward GCGCACAGGAATGGGAGAG, LPA_3–reverse GAGCTGGAGGATGTTGGGAG; CCR7–forward GCTGCGTCAACCCTTTCTTG, CCR7–reverse ACCGACGCGTTCCGTACAT; CCR7–forward CGTGCGTCAACCCTTTCTTG, CCR7–reverse ACCGACGCGTTCCGTACAT; cyclophilin–forward TGGAAGAGCACCAAGACAGAC, cyclophilin–reverse TGCCGGAGTCGACAATGAT. Primers for mouse LPA_4 and LPA_5 were from SuperArrays Bioscience Corp. (Frederick, MD, USA). PCR reactions were run on an ABI Prism 7900HT machine (Applied Biosystems, Foster City, CA, USA). Data were normalized to cyclophilin expression.

Tracking migration of cutaneous DCs in vivo in $LPA_3^{-/-}$ mice

FITC was dissolved into 50% acetone/50% dibutylphthalate (from Sigma-Aldrich) at a concentration of 5 mg/ml. Mice were painted on the shaved abdomen or flank with 25–50 ml of this solution and killed at 24 and 48 h after painting to record the number of fluorescently labeled DCs (FITC+/CD11c+), which had arrived in the draining lymph nodes.

Statistical analysis of data

The data are presented as means ± SD. Each data point was calculated from triplicate or duplicate samples as indicated. Statistical analyses were performed by one-way ANOVA followed by a Tukey's multiple comparison

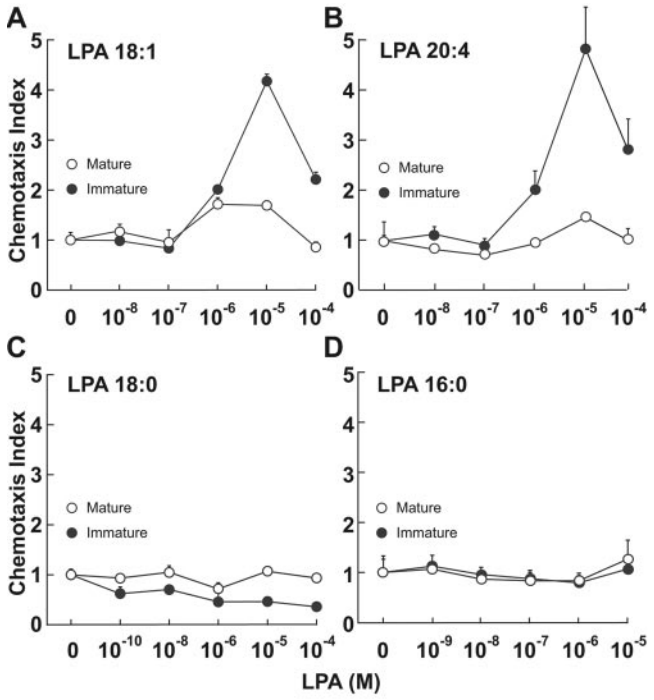

Fig. 1. Immature murine DCs migrate in response to unsaturated LPA species. DCs were differentiated in vitro from bone-marrow precursors, and a subset was treated with LPS to induce maturation. Immature (●) or LPS-matured (○) DCs were placed in the transwell insert, and migration was recorded as a function of increased concentrations in the bottom well of various LPA species: LPA 18:1 (A), LPA 20:4 (B), LPA 18:0 (C), and LPA 16:0 (D). Data points result from triplicate measurements, and one representative experiment for each LPA species tested is shown. When data from three experiments performed with independent preparations of bone marrow-derived DCs were compiled, the increased migration of immature DCs to a concentration of 10^{-5} M LPA 18:1 or to 10^{-5} M LPA 20:4 was statistically significant ($P<0.01$ for LPA 18:1, and $P<0.05$ for LPA 20:4).

post-test for chemotaxis assay curves and by one-sample *t*-test for gene expression studies; **, $P < 0.01$; *, $P < 0.05$.

RESULTS

LPA 18:1 and LPA 20:4 but not LPA 16:0 or LPA 18:0 induced chemotaxis of immature DCs

Previous chemotaxis studies used only LPA 18:1, which contains the monounsaturated fatty acyl chain from oleic acid. They showed that LPA 18:1 is chemotactic for immature human DCs generated in vitro from peripheral blood leukocytes isolated from buffy coats [6]. To determine if LPA 18:1 can also stimulate chemotaxis of mouse DCs, we generated DCs from bone marrow of C57Bl/6 mice and measured the migration of immature or LPS-matured DCs through the membrane of transwell inserts toward increasing concentrations of LPA 18:1 in the bottom wells. We found that immature DCs exhibited a robust, chemotactic response, which peaked at 10 μM LPA and decreased at 100 μM LPA 18:1 (**Fig. 1A**). No chemotactic response was observed with LPS-matured DCs (Fig. 1A), although background motility in the absence of the chemoattractant tended to be higher. Mature DCs did not lack an inherent ability to migrate: They showed efficient chemotaxis to the CCR7 ligand CCL21 (CI=13.11±3.7, $n=3$).

To confirm further that LPA 18:1 triggered DC chemotaxis and did not simply increase random mobility by chemokinesis, we performed a "checkerboard" analysis (**Table 1**). LPA 18:1 was added to both compartments of the transwell chamber in increasing concentrations. Migration of immature DCs only occurred when a true concentration gradient of LPA 18:1 was present. When concentrations were equal, DCs did not migrate to the bottom well.

Next, we hypothesized that different LPA species may exhibit differences in their ability to induce DC chemotaxis. To test this hypothesis, we analyzed the migration of immature and mature DCs to additional LPA species: another unsaturated LPA containing the fatty acyl chain from arachidonic acid, LPA 20:4, and two naturally occurring saturated species, LPA 16:0 and LPA 18:0. LPA 20:4 triggered migration of immature DCs to the same extent as LPA 18:1 (Fig. 1B). However, neither immature nor LPS-matured DCs could migrate through the membrane of transwell inserts toward increasing concentrations of the saturated species LPA 18:0 (Fig. 1C) or LPA 16:0 (Fig. 1D). LPS-matured DCs were unresponsive to various concentrations of all the LPA species tested (Fig. 1).

LPA does not affect immature DC maturation or chemotaxis to SDF-1/CXCL12

Saturated LPA species were shown to be able to activate LPA receptors overexpressed in insect cells or mammalian cells [20, 21]. However, they did not trigger chemotaxis of immature DCs. We explored the possibility that they affect the behavior of immature cells differently. First, we hypothesized that high concentrations of saturated LPA, such as LPA 18:0, trigger internalization of LPA receptors involved in chemotaxis to unsaturated LPA, such as LPA 18:1, thus decreasing the migratory response to this ligand. To test this hypothesis, we preincubated immature DCs with LPA 18:0 for 24 h before testing their migratory response to LPA 18:1 but observed a normal, chemotactic response (**Fig. 2A**). Another possible role for LPA 18:0 is to modulate chemotaxis to other chemokines, such as the suppression by S1P of CD4 T cell chemotaxis to

TABLE 1. LPA 18:1 Triggers Chemotaxis, Not Chemokinesis

LPA in bottom compartment (M)	LPA in top compartment (M)			
	0	10^{-7}	10^{-6}	10^{-5}
0	1 (0.1)	1.2 (0.16)	1.4 (0.35)	1.2 (0.4)
10^{-7}	1.2 (0.03)	1.25 (0.12)	1.5 (0.14)	1.4 (0.17)
10^{-6}	1.8 (0.2)	1.9 (0.2)	1.65 (0.31)	1.2 (0.03)
10^{-5}	3.15 (1.19)*	2.7 (0.24)*	2.9 (0.42)*	1.7 (0.15)

DCs were differentiated in vitro from bone-marrow precursors, and their migration was assessed in conditions where an LPA gradient was created or not between both compartments of a transwell separated by a porous membrane (the DC-containing top compartment and the bottom well compartment). The chemotaxis index (CI) (the number of cells transmigrated in response to LPA-containing medium divided by the number of cells transmigrated in response to medium only) was determined for three wells per condition. The table shows the mean CI with the standard deviation in parentheses. The increased migration was significant when indicated (*, $P<0.05$).

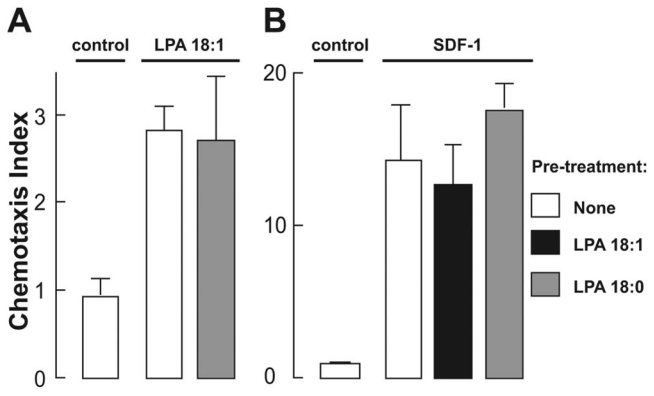

Fig. 2. Preincubation with LPA has no effect on chemotaxis of immature DCs to SDF-1 or LPA 18:1. DCs were differentiated in vitro from bone-marrow precursors and pretreated with 10 μM LPA 18:0 or LPA 18:1 for 24 h at 37°C before recording their migration to (A) 10 μM LPA 18:1 or (B) 250 ng/ml SDF-1 or using the transwell system. Each bar represents the mean ± SD of the CI calculated for three independent experiments performed in triplicate. Mean control CI is for DC migration to culture medium only.

CCL21 [22]. However, we found that treating DCs with LPA 18:0 did not affect their migration response to 250 ng/ml SDF-1 (Fig. 2B). Pretreatment of DCs with LPA 18:1 also had no effect on migration to LPA or SDF-1 (Fig. 2). Finally, saturated LPA could induce maturation of immature DCs, which are unresponsive to chemotactic stimuli by unsaturated LPA. To test this hypothesis, we incubated immature DCs with LPA 18:0 and monitored DC maturation by analyzing cell-surface expression of costimulatory molecules by flow cytometry. We found that the low percentage of immature DCs expressing CD40 and CD86 is unaffected by treatment with LPA 18:0 (**Fig. 3**), and LPS-induced maturation led to the expected increase in DCs expressing high levels of CD40 and CD86 cells (Fig. 3). We also found that LPA 18:0 did not enhance the expression of CCR7 or MHCII (not shown) and that LPA 18:1 had no effect on any cell-surface molecule analyzed (Fig. 3).

LPA_3 is involved in chemotaxis of immature DCs to LPA 18:1

Work published by Bandoh et al. [20] showed that LPA_3 was preferentially activated by unsaturated LPA species, and the saturated LPA species 18:0 and 16:0 were poor agonists. Therefore, the selective chemotactic activity we found for unsaturated LPA species suggests that LPA_3 is a candidate receptor to transduce this effect. In addition, as only immature DCs migrated to LPA, and the chemotactic effect was lost upon maturation, we reasoned that receptors, which are down-regulated when DCs mature, are likely to play a role in this process. We measured LPA receptor mRNA expression by real-time PCR in immature or LPS-matured DCs and found that upon LPS-induced maturation, LPA_1 was up-regulated twofold, and levels of LPA_2 and LPA_4 remained low, but LPA_3 was considerably down-regulated, and LPA_5 tended to decrease as well (**Fig. 4A**). We also measured CCR7 expression as a control, and as expected, mRNA levels of CCR7 were increased significantly by LPS maturation. In addition, a comparison of the relative levels of the different LPA receptors expressed as a percentage of cyclophilin expression (Fig. 4B) revealed that LPA_3 and LPA_5 are the most highly expressed receptors in immature DCs, and LPA_1 is highest in mature DCs. Altogether, these data suggest that LPA_3 and/or LPA_5 were the key receptors involved in immature DC chemotaxis to LPA.

Little is known about LPA_5, and no antagonists or $LPA_3^{-/-}$ mice are available. However, these tools are available for LPA_3

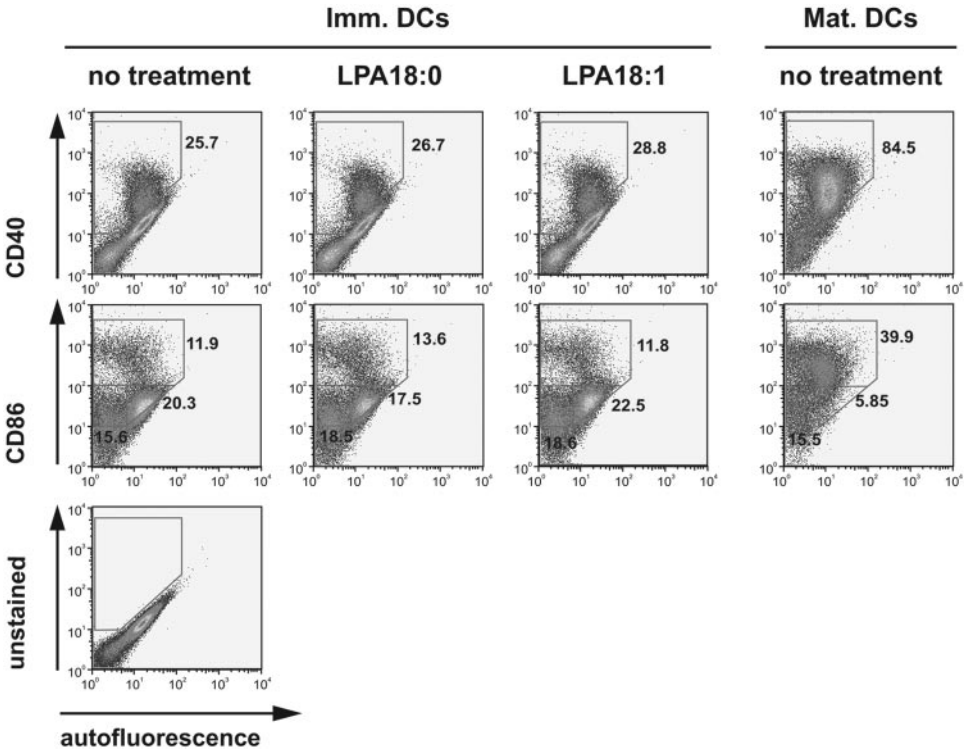

Fig. 3. Expression of costimulatory receptors in LPA-treated immature DCs (Imm. DCs), which were differentiated in vitro from bone-marrow precursors and were incubated with or without 10 μM LPA 18:0 or LPA 18:1 for 24 h at 37°C before analyzing cell-surface expression of maturation markers and costimulatory molecules by flow cytometry. LPS-treated DCs are shown as controls for mature DCs (Mat. DCs). Representative FACS plots for CD40 and CD86 are shown, and the gates were set using unstained, control, immature DCs. For CD86 staining, three gates are displayed, as three subpopulations of CD86+ cells are clearly visible (CD86 low and dim autofluorescence, CD86 low and bright autofluorescence, and CD86 high).

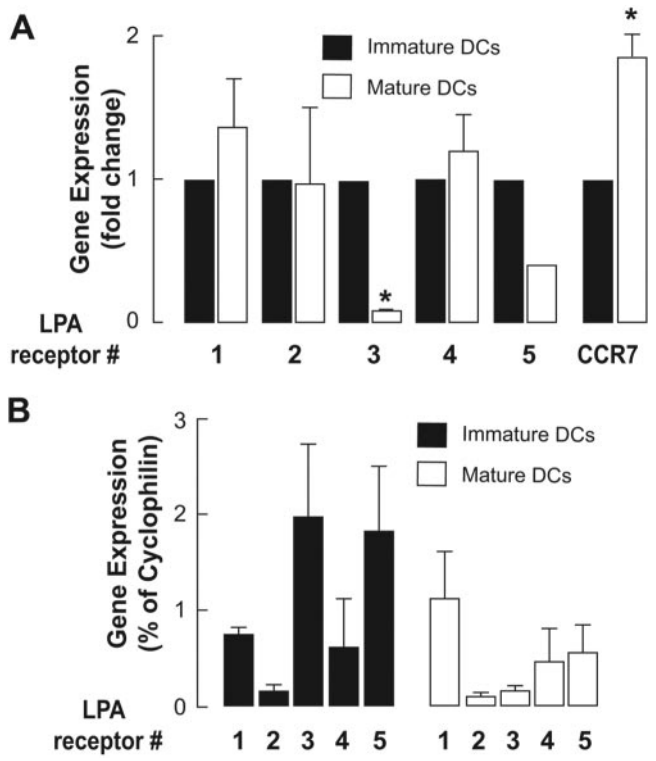

Fig. 4. Immature and LPS-matured DCs express different levels of the five known LPA receptors. Real-time PCR was performed on mRNA extracted from bone marrow-derived, immature and LPS-matured DCs, and results were normalized to the expression of cyclophilin. (A) Average fold changes in expression between immature and LPS-matured DCs. CCR7 expression is shown as a positive control for LPS-induced maturation (*, $P<0.05$). (B) Comparison of expression levels of the different LPA receptors as a percentage of cyclophilin expression. Means and SDs are for three to four independent DC preparations.

and allowed us to ask whether this receptor is involved in chemotaxis of immature DCs to LPA 18:1. We first examined the effect of the LPA_3 antagonist VPC32179. In pilot experiments, we found that adding 10 μM VPC32179 to immature DCs just before the chemotaxis assay had no effect (data not shown). However, preincubation of immature DCs with 10 μM VPC32179 for 18 h decreased their migration to LPA 18:1 and to LPA 20:4 by 70% (**Fig. 5A**). A the same time, overnight exposure of immature DCs to VPC32179 did not affect chemotaxis in a nonspecific manner, as the CI for migration to SDF-1 was not statistically different in untreated and treated DCs (CI=9.41±0.18 vs. 5.75±0.99). One possible mechanism for VPC32179 inhibition of chemotaxis could be down-regulation of the expression of LPA receptors. So far, we found no difference in LPA receptor mRNA levels determined by real-time PCR in DCs pretreated with VPC32179 for 3 h or 18 h (data not shown). If LPA_3 is essential for DC chemotaxis to LPA, we also reasoned that migration of $LPA_3^{-/-}$ DCs to LPA should be compromised. We generated DCs from the bone marrow of $LPA_3^{-/-}$ mice and from $LPA_3^{+/-}$ littermate controls, which are undistinguishable from wild-type mice to the best of our knowledge, and assayed the migration of immature and LPS-matured DCs to increasing concentrations of LPA 18:1. Migration of control $LPA_3^{+/-}$ DCs was similar to that of wild-type DCs. However, the migration of $LPA_3^{-/-}$ immature DCs was reduced by 50% (Fig. 5B). The lack of LPA_3 in DCs did not inhibit chemotaxis in a nonspecific manner, as the CI for migration to SDF-1 is similar in $LPA_3^{-/-}$ and $LPA_3^{+/-}$ DCs (CI= 60.61±8.6 vs. 51.54±2.1). The fact that $LPA_3^{-/-}$ DCs retained half of their migration capacity to LPA suggests that another LPA receptor may be involved in mediating this response. Using real-time PCR, we analyzed the expression of LPA_1, LPA_2, LPA_4, and LPA_5 in immature and LPS-matured DCs prepared from a pool of three $LPA_3^{-/-}$ and three $LPA_3^{+/-}$ mice and found no significant up-regulation of any of these receptors in $LPA_3^{-/-}$ cells compared with $LPA_3^{+/-}$ cells (data not shown). In the absence of LPA_3, LPA_5 and LPA_2 are expressed in immature DCs and are not up-regulated in nonmigrating, LPS-treated DCs, suggesting that these receptors may be involved in mediating the observed, residual chemotaxis of $LPA_3^{-/-}$ immature DCs to LPA 18:1.

Next, we took advantage of $LPA_3^{-/-}$ mice to investigate the role of LPA_3 in DC migration in vivo. As immature DCs reside

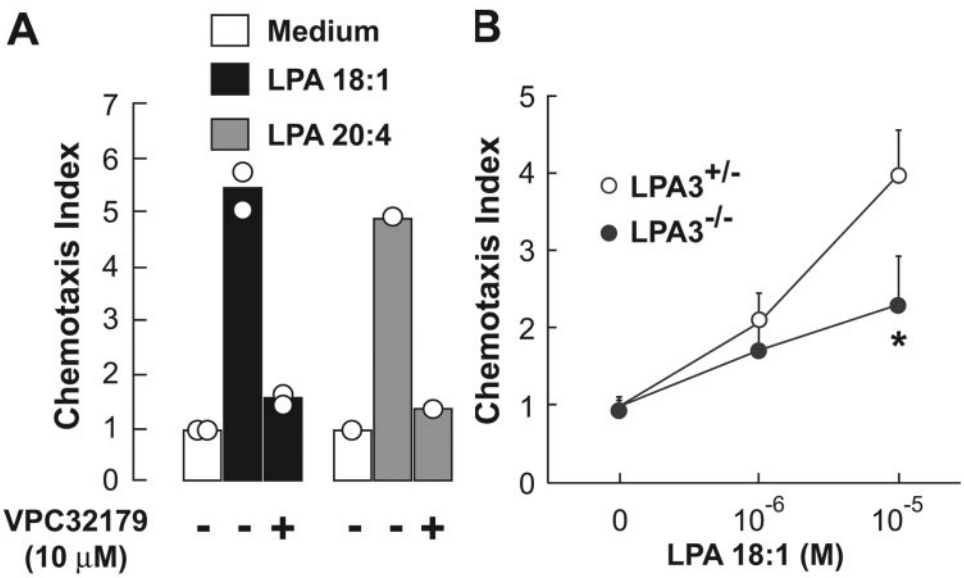

Fig. 5. Inhibition of LPA_3 impairs DC chemotaxis to LPA 18:1. (A) Immature DCs were preincubated with 10 μM LPA_3 receptor antagonist (VPC32179) for 24 h at 37°C before being placed in transwell inserts for determining their migration to 10 μM LPA 18:1 or LPA 20:4. Individual points show the mean of triplicate measurements from independent DC preparations. Inhibition of migration was observed in response to LPA 18:1 in two separate experiments and in response to LPA 20:4 in a third experiment. (B) DCs were differentiated in vitro from bone-marrow precursors from three pooled $LPA_3^{+/-}$ and $LPA_3^{-/-}$ mice, and a subset was treated with LPS to induce maturation. DC migration was recorded as a function of increased concentrations of LPA 18:1 in the bottom wells. Triplicate measurements were performed to determine the CI for DCs of each genotype (*, $P<0.05$).

primarily in peripheral tissues, we first hypothesized that the absence of LPA_3 could impair the recruitment of DCs in a peripheral tissue such as the skin. To test this hypothesis, we compared the number of DCs in the skin of $LPA_3^{-/-}$ and $LPA_3^{+/-}$ control mice in the steady-state and 48 h after sensitization by FITC painting. We collected skin samples of equal sizes from the flank of mice and stained the tissues with an antibody against the DC marker CD11c but found no difference in DC numbers between genotypes (Supplemental Fig. 1). Another possibility is that altered, immature DC behavior in the absence of LPA_3 perturbed DC migration to draining lymph nodes. We analyzed by flow cytometry the composition of lymph nodes in the steady-state in $LPA_3^{-/-}$ mice and $LPA_3^{+/-}$ littermate controls but found no difference in the percentage of CD11c+/MHCII+ DCs in $LPA_3^{-/-}$ mice and $LPA_3^{+/-}$ inguinal lymph nodes (2.87%±0.71 and 3.19%±0.69, respectively, $n=6$ mice per genotype) or in cervical lymph nodes (2.06%±0.31 and 2.39%±0.69, respectively). We also investigated the migratory ability of resident, cutaneous $LPA_3^{-/-}$ DCs after stimulation by painting the skin with FITC. We observed no difference in the number of FITC-positive DCs recovered from $LPA_3^{-/-}$ and $LPA_3^{+/-}$ inguinal draining lymph nodes 48 h after FITC painting ($1.57\times10^5\pm7\times10^4$ and $1.99\times10^5\pm2.7\times10^4$ cells, respectively, $n=5$–7 mice per genotype). Altogether, results from these experiments suggest that LPA_3 deficiency was not sufficient to impair the migration of stimulated epidermal and dermal DCs or that compensation by activation of other LPA receptors occurred in vivo.

DISCUSSION

In this study, we report that physiological concentrations of LPA induce chemotaxis of immature mouse DCs and that this effect is specific for unsaturated LPA species only. We also provide evidence that LPA_3 is an important mediator of DC chemotaxis.

Chemotaxis of immature mouse DCs occurred at physiologically relevant concentrations (1–10 μM). Although plasma LPA levels in the steady-state are normally low (less than 1 μM) [23], they can rise to 10 μM in serum, partly as a result of platelet activation [23, 24], to 80 μM in ovary cancer ascites [25, 26] and possibly in wound repair, such as reported in an injured cornea [27]. The average CI for migration of immature mouse DCs was 4, which is somewhat higher than those reported for immature, human DCs in response to LPA (CI=1.7 [6]) and for immature mouse DCs in response to another chemotactic phospholipid, S1P (CI=2 [28]).

We also found that LPA species, which differ in length and saturation of their fatty acyl chains, were not equally able to trigger DC chemotaxis: out of four LPA species tested, only those containing unsaturated fatty acyl chains (oleoyl-LPA or LPA 18:1 and arachidonyl LPA or LPA 20:4) were chemotactic, and those containing saturated chains (palmitoyl LPA or LPA 16:0 and stearoyl LPA or LPA 18:0) were not. There is currently limited information about physiological or pathological situations associated with changes in the relative abundance of different LPA species. However, it is clear that various LPA species exist in the body, and the enzymes involved in LPA synthesis and degradation are able to tightly regulate their relative concentration. For example, LPA produced by platelets through phospholipase D was shown to be enriched in the unsaturated LPA species 18:2 and 20:4 [24, 29], and LPA acyl chains generally differ between human plasma and serum [23]. Therefore, the fact that only some LPA species are chemotactic to DCs could invoke selectivity in the migratory response and allow recruitment of immature DCs to specific areas, where the appropriate LPA is produced.

The role of saturated LPA species in DC biology remains to be determined. We have tested the hypothesis that saturated LPA species such as LPA 18:0, which did not cause immature DC chemotaxis, could induce maturation or affect migration to other chemokines. Although we found no such effect in vitro, the possibilities still exist in vivo that LPA acts synergistically or in opposition with a combination of factors involved in DC maturation, a process that is still not fully elucidated.

We then asked if immature DC chemotaxis to LPA was mediated by a particular receptor. Several results support a role for LPA_3 in this process. First, LPA_3 expression in immature and mature DCs correlates with its function in chemotaxis, as LPA_3 is down-regulated (with LPA_5) in nonmigrating, mature DCs. Our data also suggest that LPA receptors preferentially activated by unsaturated LPA species are more likely to mediate DC chemotaxis, and studies using insect cells have shown that the LPA_3 receptor was preferentially activated by unsaturated LPA species, and the saturated LPA species 18:0 and 16:0 were poor agonists [20]. In the same system, LPA_1 and LPA_2 showed no marked ligand preference. In RH7777 cells, although all LPA species could induce intracellular calcium mobilization and did not discriminate clearly among LPA receptors, saturated LPA species, such as LPA 16:0 and LPA 18:0, were less potent than unsaturated ones [21]. To our knowledge, there are currently no data about ligand preference for the two other known receptors LPA_4 or LPA_5.

It is more important that a role for LPA_3 in immature DC chemotaxis is supported by our functional studies. We showed that VPC32179, a chemical antagonist of LPA_3, was able to decrease the chemotactic response of immature DCs to LPA 18:1 by ~70%. In addition, DCs isolated from LPA_3 knockout mice lost half of their ability to chemotact to LPA 18:1. The fact that neither chemical treatment with a LPA_3 antagonist nor the genetic disruption of LPA_3 could abrogate DC chemotaxis completely may be attributable to the function of at least one other LPA receptor. Many cells coexpress different receptor subtypes, and functional redundancy has been described: for instance, mice lacking LPA_1 and LPA_2 receptors exhibit no major physiological abnormalities [30], suggesting that at least an additional receptor compensates for the lack of these two. Expression of the LPA_5 receptor, like that of LPA_3, is down-regulated in nonmigrating, LPS-treated DCs, and its expression level was similar in $LPA_3^{-/-}$ and $LPA_3^{+/-}$ DCs. Therefore, LPA_5 is likely to be important for immature DC chemotaxis to LPA. However, the potential role of other LPA receptors cannot be excluded at this point, and more functional studies are required to analyze the contribution of each of them to DC chemotaxis. Functional redundancy might also explain the fact that we did not observe an alteration in DC migration in vivo in $LPA_3^{-/-}$ mice. Alternatively, LPA might not be required for DC

migration in the steady-state in vivo but only in inflammatory situations, where it could cooperate with other danger and inflammatory signals.

LPA might influence DCs differently in the steady-state and in inflammatory conditions. In the steady-state in naïve animals, DCs migrate at constant rates from peripheral tissues to corresponding draining lymph nodes and help maintain peripheral tolerance. LPA in the tissue environment, such as secreted by adipocytes or fibroblasts, might evoke DC chemotactic responses, perhaps playing a role in maintaining the cells in their local environment by competing with other chemotactic signals. Such a model has been proposed for T cell chemotaxis to the structurally related phospholipid S1P [22]. In the presence of an infection or an inflammatory insult, antigen uptake coupled to stimulation by various danger signals led to DC maturation and their enhanced migration to draining lymph nodes, where they stimulate T cells to activate an adaptive immune response [1]. Increased LPA production upon a specific stimulus, such as platelet activation in wound repair, might provide a concomitant signal to attract innate immune cells such as immature DCs to fight potential infections. As in chemotaxis assays, high concentrations of LPA inhibit the migration response; another possibility is that rising LPA concentrations in inflamed tissues may act by desensitizing the corresponding receptors and allowing DCs to respond fully to other chemokines, thus participating in increasing DC trafficking to draining lymph nodes to promote immune responses. Finally, as aberrant LPA metabolism was recorded in cancer cells, and patients and autocrine activation loops were described, in particular, for ovary and prostate cancer cells [31], our data suggest that LPA production by cancer cells or by the cancer stroma might alter DC responses to tumor antigens. However, as the composition of LPA produced by cancer cells is poorly documented, we can only speculate about the nature of the effect on DCs. If DCs are attracted to the tumor site at a higher rate and can mature, an enhanced immune response could result, but if DCs are maintained in an immature state at the tumor front, they would not be able to migrate to the draining lymph node to prime an adaptive immune response efficiently.

In summary, we have shown that unsaturated but not saturated species of the bioactive lysophospholipid LPA trigger chemotaxis of immature mouse DCs. Our results also suggest that the LPA_3 receptor plays an important role in immature but not mature DC chemotaxis. It is interesting that LPA_3 has been shown to play an important role in the migration of ovary cancer cells [32]. More comprehensive analyses will be required to understand the in vivo function of LPA-mediated DC migration. As this report has highlighted the ability of mouse DCs to migrate to unsaturated LPA species, we believe the use of genetically modified mice lacking one or more LPA receptors will greatly advance our knowledge of the involvement of LPA in leukocyte migration.

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