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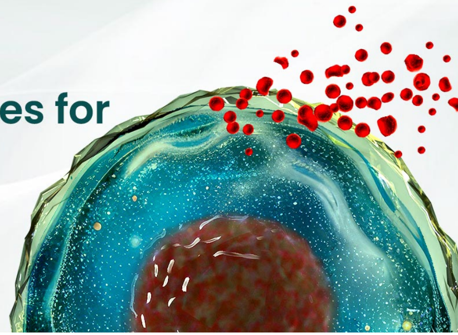

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The Chemokine Receptor CCR7 Activates in Dendritic Cells Two Signaling Modules That Independently Regulate Chemotaxis and Migratory Speed[1]

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CCR7 is necessary to direct dendritic cells (DCs) to secondary lymphoid nodes and to elicit an adaptative immune response. Despite its importance, little is known about the molecular mechanisms used by CCR7 to direct DCs to lymph nodes. In addition to chemotaxis, CCR7 regulates the migratory speed of DCs. We investigated the intracellular pathways that regulate CCR7-dependent chemotaxis and migratory speed. We found that CCR7 induced a G_i-dependent activation of MAPK members ERK1/2, JNK, and p38, with ERK1/2 and p38 controlling JNK. MAPK members regulated chemotaxis, but not the migratory speed, of DCs. CCR7 induced activation of PI3K/Akt; however, these enzymes did not regulate either chemotaxis or the speed of DCs. CCR7 also induced activation of the GTPase Rho, the tyrosine kinase Pyk2, and inactivation of cofilin. Pyk2 activation was independent of G_i and Src and was dependent on Rho. Interference with Rho or Pyk2 inhibited cofilin inactivation and the migratory speed of DCs, but did not affect chemotaxis. Interference with Rho/Pyk2/cofilin inhibited DC migratory speed even in the absence of chemokines, suggesting that this module controls the speed of DCs and that CCR7, by activating its components, induces an increase in migratory speed. Therefore, CCR7 activates two independent signaling modules, one involving G_i and a hierarchy of MAPK family members and another involving Rho/Pyk2/cofilin, which control, respectively, chemotaxis and the migratory speed of DCs. The use of independent signaling modules to control chemotaxis and speed can contribute to regulate the chemotactic effects of CCR7. *The Journal of Immunology,* **2005, 174: 4070–4080.**

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Dendritic cells (DCs)[4] migrate from peripheral tissues to lymph nodes to prime T lymphocytes in these areas and elicit an immune response (1, 2). The migration of DCs toward T cell areas requires up-regulation of CCR7, an event that takes place during the process of maturation of DCs (3–5). The two ligands of CCR7, CCL19 and CCL21, are expressed by stromal cells in the T cell zone of the lymph nodes (6–10). Moreover, CCL19 is also expressed by mature DCs (6, 11, 12), and CCL21 is expressed by endothelial cells of afferent lymphatic vessels and high endothelial veins (8–10). Mice deficient in CCL19, CCL21, or CCR7 show defective DC traffic and altered immune responses (13–15). Recently, it has also been suggested that CCR7 expressed in different metastatic cell lines may direct these cells to their niches (5, 16, 17).

Chemokine receptors confer upon cells the ability to detect and move directionally toward a chemotactic stimulus. Often in addition to chemotaxis, chemokine receptors regulate the migratory speed of the cells (also called random motility or nondirectional speed) (18, 19). In this regard, it is not clear whether the signaling pathways that regulate chemotaxis and migratory speed overlap or are independent of each other.

Although there are common rules, it is emerging that there is a high level of cell context and cell type variability in the signaling that is regulated by chemokine receptors. Generally, chemokine receptors relay intracellular signals that regulate chemotaxis through the G_i subfamily of G proteins (20). These receptors regulate a variety of signaling molecules, including MAPK family members (21–23). Mammalian cells contain three major classes of MAPKs, ERK1/2, JNK, and p38. These molecules are important regulators of chemotaxis and/or random motility in a variety of cell types (23–25). Chemokine receptors may also activate PI3K and the downstream effector Akt, which play a central role in regulation of the chemotactic response in leukocytes and other cells (21, 23, 26–30). Other mediators of chemokine receptors include kinases, such as proline-rich tyrosine kinase 2 (Pyk2) (21, 22, 31). Pyk2 binds a variety of signaling molecules, including tyrosine kinases such as Src, that impinge upon this protein in several signaling pathways (32–35). It has been shown that Pyk2 regulates leukocyte motility, although the mechanism involved is unknown (36–38).

The actin cytoskeleton is crucially involved in the regulation of chemotaxis and other motile-related functions. In this regard, reorganization of the actin cytoskeleton is an early response to CCR7 and other chemokine receptor stimulation (17, 23, 39). Actin organization is regulated by Rho GTPases and downstream effectors, including the serine/threonine kinase Rho-associated kinase (ROCK)

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[4] Abbreviations used in this paper: DC, dendritic cell; PLL, poly-L-lysine; PRNK, Pyk2-related nonkinase; PTX, pertussis toxin; Pyk2, proline-rich tyrosine kinase 2; RBD, Rho binding domain; ROCK, Rho-associated kinase.

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(40–42). ROCK phosphorylates and activates LIM kinase-1, a serine/threonine kinase that phosphorylates Ser-3 and inhibits cofilin, an actin-binding protein that, through its ability to depolymerize and sever actin filaments, is essential for regulating cell motility (43–47).

Despite the cardinal importance of CCR7 in the immune response, little is known about the signals induced from this receptor and how they are integrated to regulate the motile functions of DCs. Because stimulation of CCR7 induces both chemotaxis and an increase in the migratory speed of DCs, we investigated the mechanisms by which these two functions could be regulated in DCs. We used the following strategy. First, we identified signaling molecules that are stimulated by CCR7. Second, using selective inhibitors and/or dominant negative constructs, we investigated the roles that these molecules played in the CCR7-dependent regulation of the chemotaxis and migratory speed of DCs. By using this approach we found that CCR7 activates in human DCs two independent signaling modules, one involving G_i and a hierarchy of MAPK family members that regulate chemotaxis and another involving Rho/Pyk2/cofilin that regulates the migratory speed of DCs. Because interference with Rho/Pyk2/cofilin also inhibited the basal migratory speed even in the absence of chemokines, this indicates that this is an intrinsic module that regulates the basal speed of DCs and that CCR7 stimulates its components to cause enhancement of the migratory speed. The independent regulation of chemotaxis and migratory speed could be a mechanism that contributes to modulating the chemotactic effects of CCR7.

Materials and Methods

Reagents and materials

GM-CSF (Leucomax) was obtained from Schering-Plough. IL-4 and CCL21 were purchased from R&D Systems. CCL19 was obtained from PeproTech. TNF-α was obtained from Alexis Biochemicals. The anti-vinculin and the anti-β-actin mAbs, BSA, fibronectin, wortmannin, LY294002, and pertussis toxin (PTX) were purchased from Sigma-Aldrich. [γ-^{32}P]ATP (5000 Ci/mmol) was obtained from Hartmann Analytic. Texas Red phalloidin was purchased from Molecular Probes. Protein G-agarose was obtained from Roche. UO126, PP2, SB203580, and SP60012 were obtained from Calbiochem. The anti-RhoA (26C4), anti-Pyk2 (C-19), anti-Src, anti-ERK2, and anti-phospho-cofilin Abs were purchased from Santa Cruz Biotechnology. The anti-phospho-ERK, anti-phospho-p38, and anti-phospho-JNK Abs were obtained from Cell Signaling. The agarose-conjugated Rhotekin-Rho binding domain (RBD) was obtained from Upstate Biotechnology. C3-exoenzyme was prepared as previously described (48). Blocking anti-human CCR7 mAb (49), anti-phospho-Akt1, and anti-Akt1 were purchased from BD Pharmingen.

Cells, culture conditions, and treatment with inhibitors

Human PBMC were isolated from buffy coats over a Lymphoprep (Nycomed), and monocytes obtained from this preparation were used to derive DCs (50–52). Briefly, monocytes were resuspended at 0.5–1 $\times$ 10^6 cells/ml and cultured in complete medium containing GM-CSF (1000 U/ml) and IL-4 (1000 U/ml). Cells were cultured for 6–7 days, with cytokine addition every second day, then GM-CSF, IL-4, and 50 ng/ml TNF-α were added for an additional 72-h period. Analysis by flow cytometry showed that the treatment yielded a homogeneous population of mature DCs (CD1a$^+$-, CD14$^{low/-}$-, HLA-DR^{high}-, CCR7$^+$-, and CD83$^+$-expressing cells). When inhibitors were used, cells were pretreated with these agents for 1–2 h, as indicated in the figure legends. Inhibitors, at the indicated final concentrations, were also present during the adhesion, chemotaxis, or videomicroscopy assays. Inhibitor treatment at the dose used did not affect the viability or expression of CCR7 by the cells.

Measurement of chemotaxis and migratory speed of DCs

Chemotaxis in response to chemokines was determined by measuring the number of cells migrating through a polycarbonate filter (5-μm pore size) in 24-well Transwell chambers (Costar Europe). The upper chamber included 1 $\times$ 10^5 DCs diluted in 100 μl of RPMI 1640 medium and 0.1% BSA, and the lower chamber contained 600 μl of the same medium with or without chemokines (at 200 ng/ml, unless otherwise indicated). DCs that migrated to the bottom chamber (after 2 h at 37°C) were counted by flow cytometry using CellQuest software (BD Biosciences). In the experiments performed with GFP-expressing cells, the percentage of GFP-migrated cells was referred to as the total number of GFP-expressing cells in the input. To selectively measure the chemotactic response, independently of migratory speed, we used the chemotactic index, defined as the fold increase over basal (in the absence of chemokines). Migratory speed was measured by videomicroscopy. DCs suspended in 0.1% BSA in RPMI 1640 were allowed to attach to fibronectin (0.25 μg/ml)-coated plastic dishes for 30 min, then movement was recorded using a video camera attached to an Axiovert microscope (Zeiss) for 2 h. Tracks of individual cells (30 or more cells) were measured, and the data are presented as microns per hour. In some experiments we also assessed the random motility of DCs using a checkerboard setting where we included chemokines in the upper and lower chambers of the Transwells and then counted the number of DCs that migrated to the bottom chambers. When the inhibitors were used, they were included in the upper and bottom chambers of the Transwells during the chemotaxis assays or checkerboard settings or in the medium of the cells when the migratory speed was analyzed by videomicroscopy.

Immunofluorescence and cytometry

Immunofluorescence was determined as previously described (32, 42, 52). For cytometric analysis, DCs were preincubated with human poly-IgGs (50 μg/ml) and then incubated at 4°C with the appropriate mAb. The cells were subsequently analyzed on a FACScan cytometer using CellQuest software. The mAbs used were purchased from BD Biosciences.

Expression constructs and nucleoporation

The pEGFP-C1 (GFP) expression vector was obtained from BD Clontech. The pEGFP-N19Rho, pcDNA3, and PRNK plasmids have been described previously (53, 54). DCs were transfected with the Amaxa nucleoporator system following the manufacturer's instructions. After nucleoporation, DCs were maintained in complete medium, and experiments were performed 18 h after transfection.

Cell adhesion assays

Adhesion assays were performed as previously described (32). Briefly, 96-well, flat-bottom plates were precoated with 0.25 μg/ml fibronectin in PBS and blocked with 1% BSA in PBS. DCs (50 $\times$ 10^3 cells/well) were added to wells containing, or not, CCL19 or CCL21 (both at 200 ng/ml) or PMA (at 50 ng/ml). Incubation of plates continued for 60 min at 37°C. Cells were stained with crystal violet and quantified as described previously (32).

Cell lysis and immunoprecipitation

To examine total or phosphorylated ERK1/2, p38, JNK, β-actin, cofilin, Pyk2, and PRNK, DCs (300 $\times$ 10^3 cells) were extracted by boiling in SDS-PAGE sample buffer (100 mM Tris-HCl (pH 6.8), 0.05 mM sodium orthovanadate, 0.5 mM EDTA, 3% SDS, 1 mM EDTA, 2% 2-ME, and 5% glycerol). To analyze total and phosphorylated Akt, DCs were solubilized in Akt lysis buffer (20 mM Tris-HCl (pH 7.5), 120 mM NaCl, 1% Nonidet P-40, 10% glycerol, 1 mM sodium pyrophosphate, 20 mM sodium fluoride, and 1 mM sodium orthovanadate plus a protease inhibitor mixture (Sigma-Aldrich)) and subsequently extracted in SDS-PAGE sample buffer. After extraction, all samples were separated in SDS-PAGE and analyzed by Western blot. When Src kinase and Pyk2 activities were examined, DCs were lysed in Src-Pyk2 lysis buffer (10 mM Tris-HCl (pH 7.65), 5 mM EDTA, 50 mM NaCl, 30 mM sodium pyrophosphate, 50 mM sodium fluoride, 2 mM sodium orthovanadate, and 1% Triton X-100 plus a protease inhibitor mixture). Pyk2 or Src was then immunoprecipitated with protein G-agarose-conjugated anti-Pyk2 or anti-Src Abs. Immunoprecipitates were washed with lysis buffer and either used for in vitro kinase reactions (see below) or extracted in SDS-PAGE sample buffer.

In vitro kinase assays of Src and Pyk2

Pyk2 and Src kinase assays were performed as previously described (32, 55). Briefly, Pyk2 and Src immunoprecipitates were washed in lysis buffer, then dissolved in kinase buffer (20 mM HEPES and 3 mM $MnCl_2$, pH 7.35). The reactions, which were started by adding 10 μCi of [γ-^{32}P]ATP, were conducted at 30°C for 15 min and stopped on ice by adding 10 mM EDTA. Finally, pellets were extracted in SDS-PAGE sample buffer and analyzed by SDS-PAGE. After fixing and drying the gels, autoradiography was conducted at -80°C.

Rho activity

DCs (1 $\times$ 10^6 cells) were dissolved in Rho lysis buffer (25 mM HEPES (pH 7.5), 150 mM NaCl, 1% Igepal CA-630, 10 mM $MgCl_2$, 1 mM EDTA,

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2% glycerol, 5 mM sodium fluoride, and 0.2 mM sodium orthovanadate). Active Rho (Rho-GTP) was isolated directly on glutathione-agarose beads using GST-tagged Rhotekin-RBD. The bead pellets were washed in Rho lysis buffer, suspended in SDS-PAGE sample buffer, and boiled. Samples were separated by SDS-PAGE and subjected to Western blotting with an anti-RhoA Ab.

Western blot analysis

Samples fractionated by SDS-PAGE were electrotransferred to membranes. After blocking with 5% nonfat milk protein in PBS, filters were incubated with Abs dissolved in 1× PBS plus 0.05% Tween 20 solution containing 5% BSA and 50 mM sodium fluoride. Membranes were incubated with suitable peroxidase-conjugated secondary Abs, and immunoreactive bands were visualized using ECL reagents.

Statistical analysis

Statistical analysis was performed using a two-tailed Student's *t* test (Microsoft Excel software). A value of $p < 0.05$ was considered significant.

Results

CCL19 and CCL21 induce chemotaxis and increase the migratory speed of DCs

We assessed the concentrations of CCL19 and CCL21 that gave the maximum chemotactic response of the DCs in Transwell migration assays. The highest migration was observed at 200 ng/ml for both chemokines (Fig. 1*A*); therefore, we used this concentration in all subsequent experiments. At this concentration the chemotactic response of the DCs was completely dependent on CCR7, because it was abrogated by pretreatment of the cells with a blocking anti-CCR7 mAb (not shown) (49). To determine whether CCL19 and CCL21, in addition to chemotaxis, regulate the migratory speed of the DCs, we analyzed the motility of the cells by videomicroscopy. Both chemokines induced almost a 2-fold increase in the migratory speed of DCs compared with unstimulated controls (Fig. 1*B*). Because the speed at which cells move on the substrate is inversely correlated with the number and size of focal contacts (56), we used an anti-vinculin Ab to examine these structures in control and chemokine-stimulated DCs. In keeping with the high motile phenotype of the chemokine-stimulated DCs, the number and size of focal contacts in CCL19- and CCL21-stimulated cells were reduced compared with controls (Fig. 1*C*). Because the speed of motile cells can be regulated by the degree of adhesion (57), we analyzed whether stimulation with CCL19 or CCL21 modifies the adhesion of DCs to the substrate. We plated DCs onto fibronectin-coated dishes and measured the adhesion of DCs stimulated, or not, with CCL19 or CCL21. As shown in Fig. 1*D*, we did not find differences in the adhesion to fibronectin between chemokine-treated and untreated DCs (Fig. 1*D*). Taken together, the results indicate that CCL19 and CCL21 induced strong chemotaxis and a significant increase in the speed of the DCs, which were not caused by changes in the adhesion of the DCs.

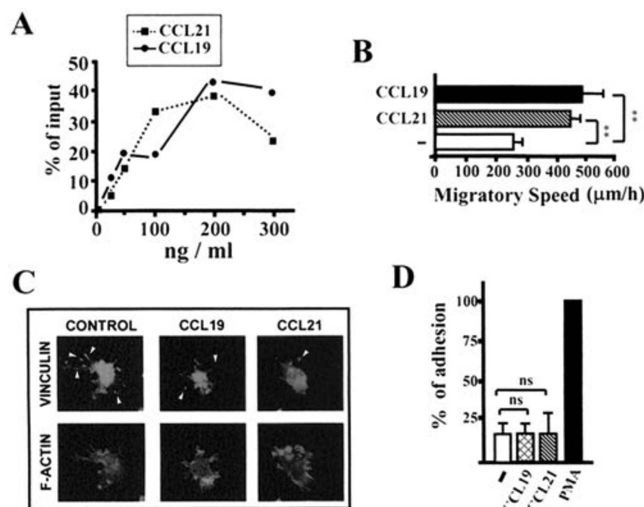

FIGURE 1. CCR7 regulates chemotaxis and migratory speed in DCs. *A*, Migration of DCs toward increasing concentrations of CCL19 and CCL21. DCs were allowed to migrate in chemotactic Transwell assays as described in *Materials and Methods*. A representative experiment of three performed is shown. *B*, DCs were plated onto dishes coated with fibronectin (0.25 μg/ml), and then either left unstimulated (−) or stimulated with CCL19 or CCL21. Motility was determined by videomicroscopy for 2 h. The trajectories of the cells were measured, and migratory speed was expressed as microns per hour. The results represent the mean ± SEM of three independent experiments. **, $p < 0.01$. *C*, DCs were plated onto fibronectin-coated coverslips (0.25 μg/ml), then were either left unstimulated (CONTROL) or stimulated with CCL19 or CCL21 for 60 min. The DCs were double-stained with Texas Red-phalloidin to label F-actin and with an anti-vinculin mAb (using anti-mouse IgG FITC-conjugated as a secondary Ab) to label vinculin. Arrowheads mark the positions of representative focal contacts. Note that smaller and fewer focal contacts were observed in the chemokine-treated cells. *D*, DCs were either left unstimulated (−) or stimulated for 60 min with 200 ng/ml CCL19 or CCL21 or 50 ng/ml PMA, which was used as a positive control. Cells were fixed and stained with 0.5% crystal violet in 20% methanol, and adhesion was quantified. The results represent the mean ± SEM of three independent experiments. ns, no significant difference.

In subsequent experiments we dissected the signaling pathways induced from CCR7 that regulate chemotaxis and migratory speed of DCs. For this purpose, we used the following strategy. First, we identified signaling molecules that were activated upon stimulation of CCR7 with its ligands. Second, we used pharmacological inhibitors and/or dominant negative constructs to interfere with these proteins and assess whether they regulated the chemotaxis and/or the migratory speed of the DCs. We quantified chemotaxis by measuring the chemotactic index, and in parallel, we measured the migratory speed of the cells by videomicroscopy (see *Materials and Methods*). Using these two parameters, we could dissect the effects that the various agents exerted on chemotaxis and/or migratory speed of the DCs.

CCR7 stimulates G_i-dependent MAPK family member activation

MAPK family members (ERK1/2, p38, and JNK) have been implicated in regulating chemotaxis in some systems and random motility in others (23–25). First, we analyzed whether CCR7 induced activation of these kinases in DCs. DCs were stimulated with CCL19 or CCL21 for various time periods, the cells were lysed, and the lysates were analyzed by Western blotting using Abs specific for the dual phosphorylated/active forms of the three MAPKs mentioned. Stimulation with CCL19 or CCL21 resulted in a transient and potent phosphorylation of ERK1/2 and JNK and a slight, but consistent, stimulation of p38 (Fig. 2*A*). Phosphorylation of ERK1/2, JNK, and p38 started after 0.5 min of stimulation, reached a maximum after 5–10 min, and returned to levels close to baseline by 60 min (Fig. 2*A*). To determine whether G_i regulates the activation of MAPKs, DCs were pretreated with PTX, an inhibitor that uncouples G_i proteins from serpentine transmembrane receptors. Control and PTX-treated DCs were stimulated with CCL19 and CCL21, and activation of MAPKs was analyzed. Treatment with this inhibitor completely abrogated the CCR7-dependent activation of the three MAPKs analyzed (Fig. 2*B*), indicating that G_i was mediating their activation.

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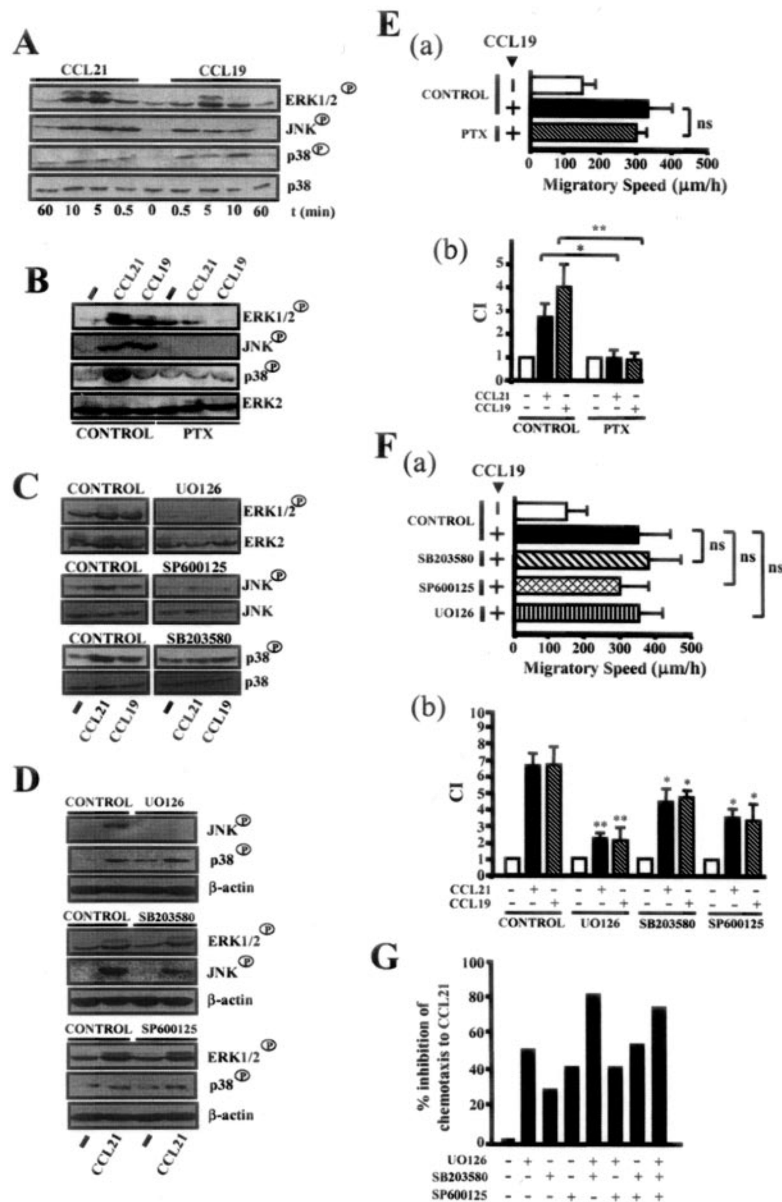

FIGURE 2. CCR7 induces PTX-sensitive activation of ERK1/2, JNK, and p38 and hierarchy of MAPK family members. *A*, DCs (300,000 cells) were stimulated for the indicated times with CCL21 or CCL19 (both at 200 ng/ml), then lysed and analyzed by SDS-PAGE, followed by Western blotting with Abs against phospho-ERK1/2, phospho-JNK, or phospho-p38. To show equal loading, the membrane was stripped and probed with an Ab against p38. *B*, Control or PTX-treated (100 ng/ml; 120 min) DCs were either left unstimulated (−) or stimulated with CCL19 or CCL21 (both at 200 ng/ml) and subsequently lysed and subjected to Western blot, as described in *A*, with Abs against phospho-ERK1/2, phospho-JNK, or phospho-p38. To show equal loading, the membrane was stripped and probed with an Ab against ERK2. *C*, DCs were left untreated (control) or were pretreated for 60 min with UO126 (5 μM), SP60012 (30 μM), or SB203580 (20 μM) to inhibit phosphorylation/activation of ERK1/2, JNK, or p38, respectively. Then DCs were either left unstimulated (−) or stimulated with 200 ng/ml CCL21 or CCL19 for 5 min. The cells were lysed and analyzed by Western blotting with Abs against phospho-ERK1/2, phospho-JNK, or phospho-p38. To show equal loading, the membrane was stripped, and respective blots were reprobed with Abs against ERK2, JNK, and p38. *D*, DCs were left untreated (control) or were pretreated with UO126, SB203580, or SP600125, as described in *C*, to inhibit phosphorylation of ERK1/2, p38, or JNK, respectively. Cells were lysed and subjected to Western blot with Abs against phospho-JNK, phospho-ERK1/2, or phospho-p38. To show equal loading, the membranes were stripped, and the respective blots were reprobed with Abs against β-actin. *A–D*, A representative experiment of three performed is shown. *E*, Cells were washed in RPMI 1640 medium, then either treated with vehicle (Control) or pretreated with PTX (100 ng/ml) for 120 min. *a*, Migratory speed in the absence (−) or the presence (+) of CCL19 was measured by videomicroscopy. The results represent the mean ± SEM of three independent experiments. ns, no significant difference. *b*, DC migration against no stimulus (−) or against 200 ng/ml CCL21 or CCL19 was measured in Transwell assays. The chemotactic response of the cells was expressed as a chemotactic index (CI). The results represent the mean ± SEM of three independent experiments. *, $p < 0.05$; **, $p < 0.01$. *F*, *a*, DCs, untreated (Control) or pretreated with UO126, SB203580, or SP600125, as described in *C*, were plated onto dishes coated with fibronectin (0.25 μg/ml), and the motility of unstimulated control (−) or CCL19-stimulated (+) DCs was followed by video microscopy. The results represent the mean ± SEM of three independent experiments. ns, no significant difference. *b*, DCs were left untreated (Control) or were pretreated with UO126, SB203500, or SP600125, as described in *C*. Then DC migration against no stimulus (−) or against CCL21 or CCL19 was measured in Transwell assays. The chemotactic response of the cells was expressed as a chemotactic index (CI). The results represent the mean ± SEM of three independent experiments. The CI values corresponding to the CCL21- or CCL19-stimulated DCs in the presence of UO126, SB203500, and SP600125 were compared with their corresponding CCL19- or CCL21-stimulated untreated control values. *, $p < 0.05$; **, $p < 0.01$. *G*, DCs were left untreated (control) or were pretreated with UO126, SB203500, SP600125, or the indicated combination of these inhibitors, as described in *C*. Chemotaxis toward CCL21 was analyzed in Transwell assays. Data are presented as the percent inhibition of migration compared with migration measured in the absence of any inhibitor. The results are representative of two independent experiments.

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Hierarchy among MAPK family members in CCR7-mediated stimulation of DCs

To analyze the possible relationship among MAPK family members after stimulation of CCR7, we used pharmacological agents to inhibit these kinases. DCs were pretreated with UO126, SB203580, and SP600125 to inhibit ERK1/2, p38, and JNK, respectively. As expected, treatment with the inhibitors blunted the increase in the phosphorylation of all MAPKs induced by stimulation with CCL19 or CCL21 (Fig. 2*C*). We analyzed the possible relationships among ERK1/2, JNK, and p38 using the inhibitors of these enzymes. Inhibition of ERK1/2 (with UO126) or p38 (with SB203580) completely or partially blunted JNK activity, respectively (Fig. 2*D*; see also Fig. 7). In contrast, inhibition of JNK (with SP600125) did not affect either ERK1/2 or p38 activation, indicating that JNK was downstream of ERK1/2 and p38 (Fig. 2*D*; see also Fig. 7). Finally, because inhibition of p38 phosphorylation (by treating DCs with SB203580; Fig. 2*C*) did not affect activation of ERK1/2 (Fig. 2*D*, *middle panel*), and inhibition of ERK1/2 (by treating DCs with U0126) did not blunt p38 activation (Fig. 2*D*, *upper panel*), this implies that p38 and ERK1/2 are activated independently of each other. Taken together, the data indicate that CCR7-stimulated activation of MAPKs takes place with a specific hierarchy, where p38 and ERK1/2 are activated independently, and both regulate the activity of JNK (see Fig. 7).

MAPKs regulate CCR7-dependent chemotaxis, but not the migratory speed, of DCs

We analyzed the role of MAPKs in regulation of the motile functions of DCs. Because MAPKs were inhibited by PTX, we analyzed the involvement of G_i proteins in regulation of CCR7-dependent chemotaxis and migratory speed. PTX treatment did not affect the migratory speed of DCs in either the absence (not shown) or the presence of chemokines (Fig. 2*Ea*). PTX treatment did not affect the adhesion of cells to the substrate (not shown). However, inhibition of G_i completely blunted the ability of DCs to chemotax toward CCL19 or CCL21 (Fig. 2*Eb*). Next, we examined the effect of inhibition of ERK1/2, p38, or JNK on migratory speed and chemotaxis. Inhibition of any of these MAPKs did not affect the migratory speed of DCs in the absence (not shown) or the presence of CCL19 or CCL21 (Fig. 2*Fa*). The inhibitors did not affect the adhesion of DCs to the substrate (not shown). In contrast, inhibition of MAPKs blunted the ability of DCs to chemotax toward CCL19 or CCL21 (Fig. 2*Fb*). The strongest inhibition of chemotaxis was observed when the activity of ERK1/2 was inhibited, and less potent inhibitory effects were observed when JNK or p38 was blocked (Fig. 2*Fb*). To determine whether MAPK members exert a synergistic effect on chemotaxis, we treated DCs with combinations of two or three inhibitors and analyzed the chemotactic responses of the cells (Fig. 2*G*). The results of these experiments, which are presented as percent inhibition with respect to the maximum migration obtained in the presence of CCL21, showed that the effects exerted by MAPKs were not synergistic (Fig. 2*G*). Interestingly, although simultaneous inhibition of the three MAPKs led to almost 75% inhibition of the chemotactic response of the cells, this response was not completely abrogated (Fig. 2*G*), suggesting that, apart from MAPKs, additional molecules can regulate CCR7-mediated chemotaxis (see Figs. 2*G* and 7). Taken collectively, the results indicate that ERK1/2, JNK, and p38 regulate, in a G_i-dependent and nonsynergistic manner, CCR7-dependent chemotaxis, but not the migratory speed, of DCs.

PI3K/Akt does not regulate CCR7-dependent chemotaxis nor migratory speed of DCs

Searching for additional molecules that could regulate chemotaxis, and because PI3K/Akt kinase has been involved in regulating chemotaxis in several cell types (23), we examined whether stimulation of CCR7 induces activation of the PI3K effector Akt. For this purpose, we performed Western blots with Abs against phosphorylated/active forms of Akt. As shown in Fig. 3*A*, chemokines in-

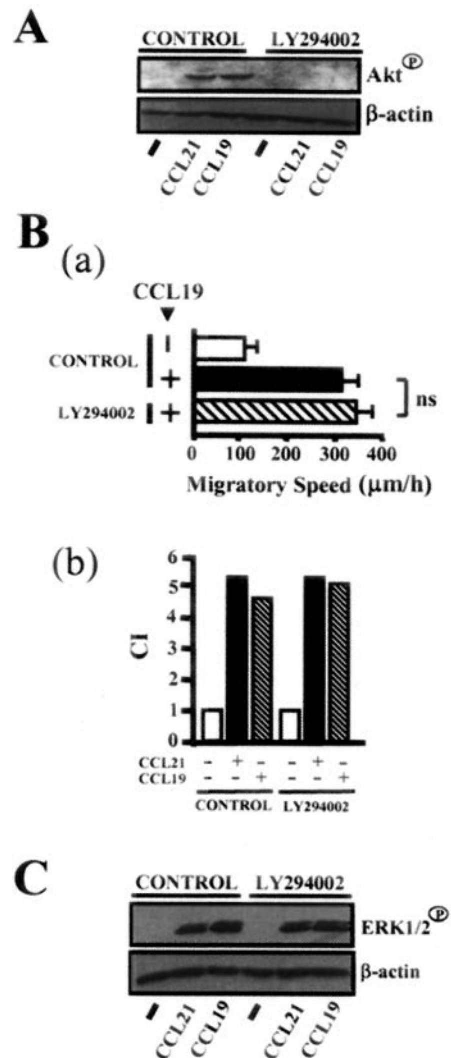

FIGURE 3. PI3K/Akt does not regulate CCR7-mediated chemotaxis or migratory speed in DCs. *A*, DCs, untreated (control) or pretreated with LY294002 (100 μM; 60 min), were either left unstimulated (−) or stimulated with CCL21 or CCL19 for an additional 60 min. Then DCs were lysed and subjected to Western blot with Abs against active/phosphorylated Akt. To show equal loading, the membrane was stripped and reprobed with an anti-β-actin Ab. *B*, *a*, Control DCs or DCs pretreated with LY294002, as described in *A*, were plated onto dishes coated with fibronectin (0.25 μg/ml), and migratory speed was analyzed by videomicroscopy. The results represent the mean ± SEM of three independent experiments. ns, no significant difference. *b*, Chemotaxis against CCL19 or CCL21 in control or LY294002 treated DCs was measured in Transwell assays. The chemotactic response of the cells was expressed as a chemotactic index (CI). The results are representative of three independent experiments. *C*, DC, untreated (control) or pretreated with LY204002, as described in *A*, were either left unstimulated (−) or stimulated with CCL19 or CCL21 for 5 min. Cells were lysed and subjected to SDS-PAGE and Western blot with Abs against phospho-ERK1/2. To show equal loading, the membrane was stripped and reprobed with an anti-β-actin Ab.

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duced a potent increase in the phosphorylation of Akt.

Because persistent inhibition of PI3K for long periods (e.g., after transfection with dominant negative PI3K) leads to prohibitive levels of cell death, we used pharmacological inhibitors to analyze the involvement of PI3K/Akt in the regulation of the motility of DCs. To inhibit PI3K/Akt, we pretreated the cells with two structurally different PI3K inhibitors, LY294002 (Fig. 3*A*) and wortmannin (not shown). The inhibitors completely abrogated the increase in phosphorylation of Akt elicited by CCL19 or CCL21 (Fig. 3*A* and not shown). However, surprisingly, neither migratory speed in the absence (not shown) or the presence of chemokines (Fig. 3*Ba*) nor chemotaxis (Fig. 3*Bb*), was altered in inhibitor-treated DCs compared with untreated control DCs. Because Akt has been shown to be upstream of ERK1/2 (28), which, as demonstrated above (Fig. 2*Fb*), plays an important role in the regulation of CCR7-dependent chemotaxis, we analyzed whether PI3K/Akt was upstream of ERK1/2. As shown in Fig. 3*C*, inhibition of PI3K/Akt with LY294002 did not blunt ERK1/2 activation. In summary, our results show that PI3K/Akt does not regulate CCR7-dependent chemotaxis nor migratory speed of DCs.

Rho regulates migratory speed, but not CCR7-dependent chemotaxis

Because CCR7 regulates actin organization (17), and the latter can be controlled by the small GTPase Rho (40), we studied whether CCR7 induces activation of Rho. We stimulated cells with CCL21, DCs were lysed, and active Rho was pulled down using GST-tagged RBD bound to glutathione-agarose beads. As observed in Fig. 4*A*, stimulation of CCR7 induced activation of Rho. To determine whether Rho was regulating CCR7-dependent chemotaxis and/or migratory speed, DCs were pretreated for 12 h with the C3 exoenzyme, a selective Rho inhibitor. We have shown previously that this treatment leads to efficient inhibition of Rho in leukocytes (35, 48). Videomicroscopic analysis showed that the basal migratory speed was potently inhibited in DCs treated with C3 exoenzyme (Fig. 4*Ba*). The migratory speed was also inhibited in C3-treated cells when they were stimulated with chemokines (Fig. 4*Ba*). When we transfected DCs with GFP vector or with a dominant negative Rho (pEGFP-N19Rho) (35), the migratory speed was also drastically inhibited in cells that overexpressed pEGFP-N19Rho (not shown). Adhesion experiments suggested that the observed differences in motility were not due to the effects of the Rho inhibitors (C3 exoenzyme or N19Rho-GFP) on the adhesion of DCs to substrate (not shown). Taken together, the results point out the crucial role of Rho in regulation of the migratory speed of DCs in both unstimulated and cells stimulated by chemokines.

A parallel analysis of the chemotactic response to CCL19 or CCL21 chemokines of DCs treated with C3-exoenzyme, showed that inhibition of Rho had only a slight effect on the chemotactic response of the cells (Fig. 4*Bb*). Similarly, analysis of the chemotaxis response of DCs that express the dominant negative Rho (N19Rho-GFP) demonstrated that interfering with Rho had no effect on the chemotaxis of DCs toward CCL19 or CCL21 (not shown).

Finally, consistent with the lack of a regulatory role for Rho on the regulation of CCR7-dependent chemotaxis, pretreatment of DCs with the C3 exoenzyme to inhibit Rho did not block activation of the chemotactic regulator ERK1/2 (Fig. 2*Fb*) upon stimulation of DCs with CCL19 or CCL21 (Fig. 4*C*). This result emphasizes that the signals relayed from CCR7 that regulated migratory speed are independent of those that regulate chemotaxis. In summary, the results indicate that Rho regulates CCR7-dependent migratory speed, but not chemotaxis, in DCs.

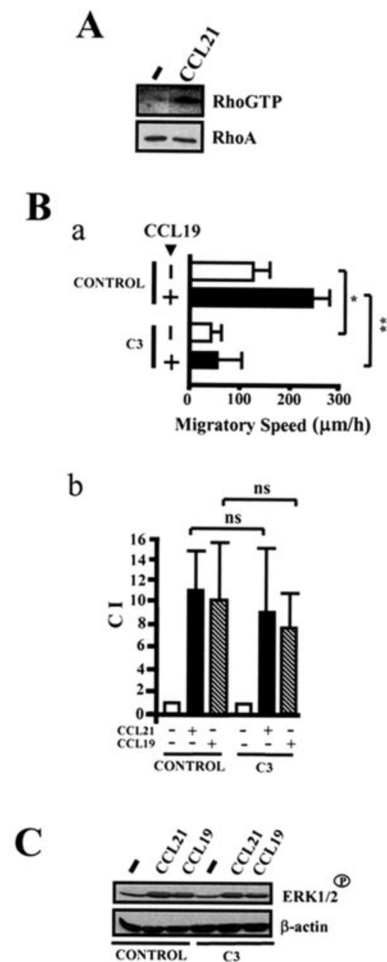

FIGURE 4. Rho regulates the migratory speed, but not the chemotaxis, of DCs. *A*, DCs were either left unstimulated (−) or stimulated with CCL21 (200 ng/ml) for 0.5 min. Then DCs were lysed, and active Rho (Rho-GTP) was pulled down using GST-tagged RBD bound to glutathione-agarose beads (see *Materials and Methods*). The level of RhoA isolated on the beads was assessed by Western blotting with an anti-RhoA Ab. To show equal loading, an aliquot of the lysed cells before the pulldown was used to perform a Western blot with the anti-RhoA Ab. The results are representative of three independent experiments. *B*, DCs were either left untreated (control) or pretreated with C3 exoenzyme (50 ng/ml; 12 h), then were either left unstimulated (−) or stimulated (+) with CCL19 (200 ng/ml). *a*, DCs were plated onto fibronectin-coated dishes (0.25 μg/ml), and the motility of the DCs was followed by videomicroscopy. The results represent the mean ± SEM of three independent experiments. *, $p < 0.05$; **, $p < 0.01$. *b*, The chemotactic response of the cells was expressed as a chemotactic index (CI). The results represent the mean ± SEM of three independent experiments. ns, no significant difference. *C*, DCs untreated (control) or pretreated with C3 exoenzyme, as described in *B*, were either left unstimulated (−) or stimulated with CCL19 or CCL21 for 5 min. DCs were then lysed and subjected to SDS-PAGE and Western blot with Abs against phospho-ERK1/2. To show equal loading, the membrane was stripped, and the blot was reprobed with anti-β-actin Ab.

CCL19 and CCL21 induce Rho-dependent and G_i and Src-independent stimulation of tyrosine kinase activity of Pyk2

Because tyrosine kinase Pyk2 has been involved in the signaling from chemokine receptors (21, 34, 37), we examined whether stimulation of DCs with CCL19 and CCL21 modulates the activity of this kinase. DCs were stimulated with chemokines for different time periods and lysed. Then Pyk2 was immunoprecipitated with specific anti-Pyk2 Abs, and immunoprecipitates were used to perform in vitro kinase reactions. As shown in Fig. 5*A*, CCL19 and

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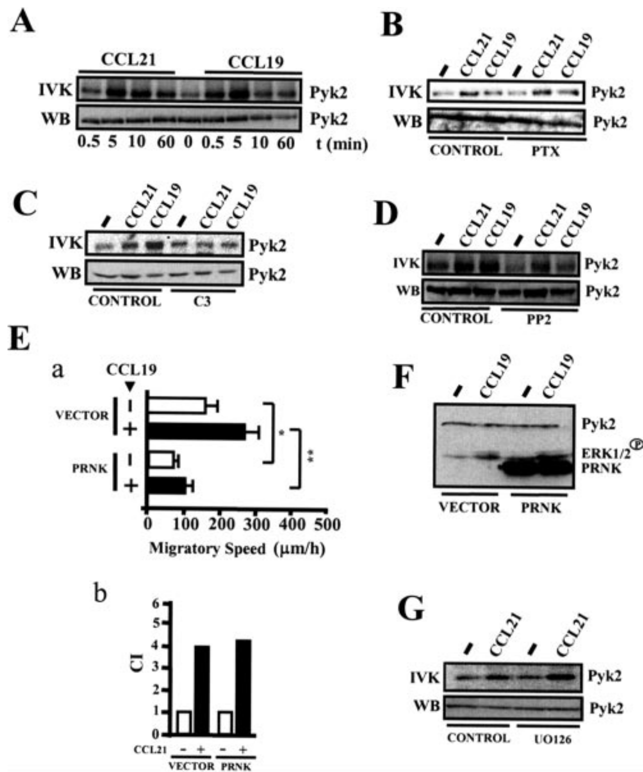

FIGURE 5. CCR7 stimulates Rho-dependent and G_i-independent activation of the tyrosine kinase Pyk2. *A*, Time course of CCL21- and CCL19-stimulated Pyk2 activity in DCs. DCs were stimulated for the indicated times with 200 ng/ml CCL19 or CCL21 and lysed. Lysates were incubated with anti-Pyk2 Abs to precipitate Pyk2. Kinase activities in the resulting immunoprecipitates were measured by in vitro kinase (ivk) reaction (see *Materials and Methods*). Pyk2 immunoprecipitates were also analyzed by SDS-PAGE, followed by transfer to membranes and Western blotting with anti-Pyk2 Abs (WB). *B–D*, DCs were either left untreated (control) or pretreated PTX (100 ng/ml; *B*), C3 exoenzyme (50 ng/ml; 16 h; *C*), or PP2 (20 μM; *D*). Then DCs were suspended in RPMI 1640 and stimulated for 5 min with either CCL21 or CCL19. Pyk2 was immunoprecipitated, and in vitro kinase activity was determined. *E*, DCs were transfected with empty vector or with a dominant negative Pyk2 (PRNK). *a*, The migratory speed of DCs was followed by videomicroscopy. The results represent the mean ± SEM of three independent experiments. *, $p < 0.05$; **, $p < 0.01$. *b*, The chemotactic index (CI) in response to CCL21 was determined. The results are representative of three independent experiments. *F*, DCs transfected with vector or PRNK were either left unstimulated (−) or stimulated for 5 min with CCL19 or CCL21. Cells were lysed and subjected to SDS-PAGE and Western blotting with Abs against phospho-ERK1/2. The membrane was stripped, and blots were reprobed with anti-Pyk2 Abs that recognize endogenous Pyk2 and PRNK. A representative blot of three experiments performed is shown. *G*, DCs were either left untreated (control) or pretreated with UO126 (5 μM). Then DCs were suspended in RPMI 1640 and stimulated for 5 min with CCL21. Pyk2 was immunoprecipitated, and Pyk2 in vitro kinase activity was determined (ivk). Pyk2 immunoprecipitates were also analyzed by SDS-PAGE, followed by transfer to membranes and Western blotting with anti-Pyk2 Abs (WB).

CCl21 induced a transient increase in the activity of Pyk2 with similar kinetics. Autophosphorylation of Pyk2 increased as early as 0.5 min after the addition of chemokines to the cells, reached a maximum after 5–10 min, and returned to basal levels after 60 min (Fig. 5*A*). To determine whether G_i proteins mediate the CCR7-dependent stimulation of Pyk2, we pretreated DCs with PTX. As shown in Fig. 5*B*, pretreatment with PTX did not inhibit the activation of Pyk2 induced by CCL19 or CCL21, indicating that such activation is not mediated by G_i proteins. Because we observed that stimulation of DCs with chemokines also induced activation of Rho (Fig. 4*A*), we determined whether Rho was upstream of Pyk2. Treatment with C3 exoenzyme abrogated the activation of Pyk2 induced by CCL19 or CCL21 (Fig. 5*C*), indicating that Rho was mediating this activation.

Because Src family members can regulate the activity of Pyk2 (34),we also analyzed whether Src was mediating Pyk2 activation. Pretreatment of DCs with PP2, a selective inhibitor of the Src family, did not prevent the CCR7-dependent stimulation of Pyk2, indicating that Src was not mediating the chemokine-induced stimulation of Pyk2 (Fig. 5*D*). The lower basal activity of Pyk2 observed in PP2-pretreated cells (Fig. 5*D*) is in agreement with previous findings that Pyk2 constitutively associates with Src (34). Consistent with the lack of a role for Src in the activation of Pyk2, stimulation of DCs with CCL19 or CCL21 failed to induce activation of this tyrosine kinase (not shown). Taken together, the results demonstrate that CCR7 induces stimulation of Pyk2 activity downstream of Rho, which is not mediated by G_i or Src.

Pyk2 regulates the migratory speed, but not the chemotaxis, of DCs

To examine the role of Pyk2 in regulation of CCR7-mediated chemotaxis or migratory speed, we transfected DCs with a dominant negative Pyk2 construct (PRNK) (53). Overexpression of PRNK (Fig. 5*F*) lead to a potent inhibition of migratory speed in the absence or presence of chemokines (Fig. 5*Ea*), but failed to affect the chemotactic response of DCs to CCL21 (Fig. 5*Eb*). The changes in motility were not related to differences in adhesion to the substrate, because such adhesion was not altered by overexpression of PRNK (not shown). Finally, consistent with the independence of the pathways regulating chemotactic and migratory speed, overexpression of PRNK did not inhibit stimulation of the chemotactic regulator ERK1/2 (Fig. 5*F*) nor did inhibition of ERK1/2 block activation of Pyk2 (Fig. 5*G*). In summary, the results indicate that the tyrosine kinase Pyk2 regulates CCR7-dependent migratory speed, but not chemotaxis, in DCs.

CCR7 induces phosphorylation/inactivation of cofilin that is mediated by Pyk2

Because cell motility involves regulation of the actin cytoskeleton, and the actin-severing protein cofilin regulates actin organization, we analyzed whether CCR7 induces changes in the activity of this protein. We used an Ab that recognizes phosphorylated Ser-3, corresponding to an inactive form of cofilin. Stimulation of DCs with CCL21 induced a transient phosphorylation/inactivation of cofilin. The level of phosphorylation increased after 0.5 min of stimulation with chemokines, reached a maximum after 5–10 min, and returned to basal levels after 60 min (Fig. 6*A*). Inhibition of Rho by treating cells with C3 exoenzyme blunted the CCR7-dependent phosphorylation/inactivation of cofilin, indicating that the protein was downstream of Rho (Fig. 6*B*). Because the time course of Pyk2 activity paralleled that of cofilin, we analyzed whether cofilin was downstream of Pyk2. DCs were transfected with the dominant negative Pyk2 (PRNK), then phosphorylation of cofilin was compared in vector- and PRNK-transfected cells. Most interestingly, overexpression of PRNK blunted the increase in phosphorylation of cofilin (Fig. 6*C*), implying that cofilin is downstream of Pyk2. In summary, the results indicate that CCR7 induces phosphorylation/inactivation of cofilin that is mediated by Rho and Pyk2.

Discussion

Despite the importance of the chemokine receptor CCR7 in regulation of the migration of DCs to the lymph node and, consequently, for the immune response (15), little is known about the signaling pathways triggered and the mechanism(s) by which this

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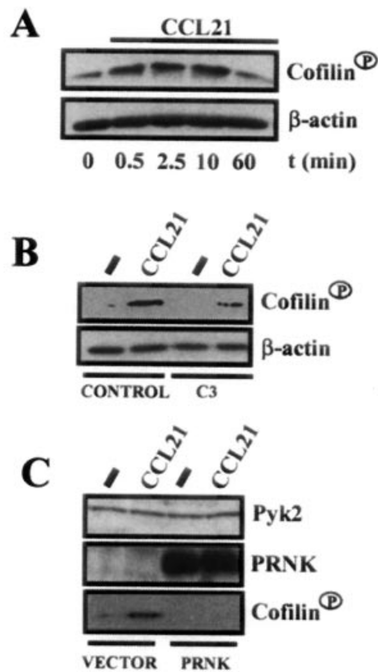

FIGURE 6. CCR7 induced transient phosphorylation of cofilin that is mediated by Rho and Pyk2. *A*, Time course of CCL21-stimulated phosphorylation of cofilin. DCs were stimulated for the indicated times with 200 ng/ml CCL21 and lysed. Lysates were used to perform Western blotting with the anti-Ser-3-phosphorylated cofilin. Blots were striped and reblotted with anti-β-actin to show equal loading. *B*, Untreated DCs (control) or DCs pretreated with C3 (50 ng/ml; 12 h) were either left unstimulated (−) or stimulated with CCL21 for 0.5 min. DCs were then lysed and subjected to SDS-PAGE and Western blotting with Abs against phospho-cofilin. To show equal loading, the membrane was stripped, and respective blots were reprobed with Abs against β-actin. *C*, DCs were transfected with empty vector (VECTOR) or with a dominant negative form of Pyk2 (PRNK). Vector- or PRNK-transfected DCs were either left unstimulated (−) or stimulated for 0.5 min with CCL21. Cells were lysed and subjected to SDS-PAGE and Western blotting with Abs against phospho-cofilin. To show equal loading and expression of PRNK, the membrane was stripped, and blots were reprobed with an anti-Pyk2 Ab (C19) that recognizes the C terminus of both Pyk2 and PRNK.

receptor regulates the motile functions of DCs. Stimulation of CCR7 with CCL19 and CCL21 induces a chemotactic response as well as an increase in the migratory speed of DCs, as shown by videomicroscopy (Fig. 1) and checkerboard analysis (19) (not shown). This simultaneous stimulation of chemotaxis and migratory speed has been observed for several chemokine receptors (18); however, to date it has not been determined whether similar or different signaling pathways regulate both processes.

To analyze the signaling components that regulate CCR7-dependent chemotaxis and migratory speed of DCs, we used the following strategy. First, we identified molecules that were activated after stimulation of CCR7. Second, we used dominant negative constructs and pharmacological inhibitors to analyze the hierarchy among the signaling molecules identified. Third, we assessed the effect(s) that inhibition of molecules activated by CCR7 exerted on either the chemotactic response or the migratory speed of DCs. To dissect the effects on chemotaxis from those on migratory speed, we determined, in parallel, the chemotactic index in Transwell assays and the migratory speed using videomicroscopy. The migratory speed data obtained by videomicroscopy were corroborated using a checkerboard setting analysis with similar results (not shown). Because the migratory speed study was performed in DCs that were plated on fibronectin-coated dishes, the results apply to mature DCs that are motile on this substrate. Additional studies need to be performed to confirm that these findings can be extended to other substrates.

Using the experimental strategy described, we found that in DCs, CCR7 regulates two signaling modules, one formed by G_i and a specific hierarchy of MAPK family members that regulates chemotaxis (see below) and another formed by Rho/Pyk2/cofilin that regulates the migratory speed of DCs (Fig. 7). For the module that regulates the migratory speed (Rho/Pyk2/cofilin) we made an interesting observation. When we analyzed the effect that interference with these molecules exerted on DC motility, we observed inhibition of migratory speed in both the absence (not shown) and the presence of chemokines (Fig. 4*B*, *a* and *b*, and Fig. 5*E*, *a* and *b*). This result suggests that Rho/Pyk2/cofilin regulate the basal motility of DCs independently of the presence of chemokines. Therefore, the stimulation of CCR7 by its ligands leads to an increase in the activity of these signaling molecules and consequently to an increase in the migratory speed of the cells (see Figs. 4*A*, 5*A*, and 6*A*). The stimulation by CCR7 ligands of the intrinsic migratory speed axis behaves as an accelerator system that increases the speed at which DCs move toward the maximum concentrations of CCL19 and CCL21. In the in vivo context, this regulatory process would have the obvious advantage of more rapidly directing DCs to lymph node regions.

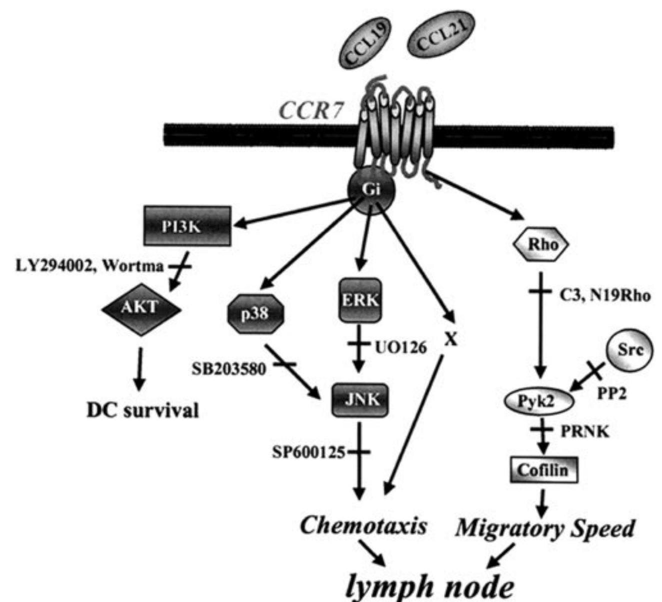

FIGURE 7. Model explaining CCR7-mediated independent regulation of chemotaxis and migratory speed in DCs. CCR7 induces G_i-mediated activation of p38, ERK1/2, and JNK, with ERK1/2 and p38 upstream of JNK. Together, these molecules constitute a signaling module that regulates CCR7-dependent chemotaxis. An additional unknown molecule(s), denoted X, participates in the regulation of chemotaxis, because simultaneous inhibition of the three MAPKs did not completely abrogate chemotaxis (see Fig. 2*G*). CCR7 also regulates Rho and Pyk2 activation and cofilin phosphorylation/inactivation. Rho is upstream of Pyk2, and the latter is upstream of cofilin. Src can phosphorylate Pyk2, but does not mediate the effects of CCR7. Rho/Pyk2/cofilin constitute a module that regulates the intrinsic or basal migratory speed of DCs. Stimulation of CCR7 activates the signaling components of this module, resulting in enhanced motility. CCR7 also stimulates PI3K/Akt; however, these molecules do not regulate chemotaxis or migratory speed, but are involved in regulating DC survival (70). Also shown are inhibitors of the molecules tested (see text for additional details).

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Importantly, we found that the signaling molecules that regulate chemotaxis and migratory speed were regulated independently of each other, as shown by the fact that selective interference with components of the chemotactic module did not affect migratory speed and vice versa (Fig. 7 and see below). The independence of both modules was corroborated by the experimental demonstration that selective inhibition of a key signaling regulator of chemotaxis (i.e., ERK1/2) failed to affect a key signaling component of the migratory speed (i.e., Pyk2; see Fig. 5*G*). In contrast, overexpression of a dominant negative Pyk2 construct (PRNK), which inhibited migratory speed (Fig. 5*E*), did not blunt stimulation of the chemotactic regulator ERK1/2 (Fig. 5*F*). Finally, we also observed that interference with the signaling molecules identified did not affect adhesion of cells to substrate (Fig. 1*D* and not shown), suggesting that the proteins identified do not exert their effects on the adhesive function of the DCs.

Regarding the specific molecules that regulate chemotaxis, we found that MAPK family members played a crucial role. In this regard, CCR7 induced a G_i-dependent activation of ERK1/2, p38, and JNK (Fig. 2, *A* and *B*). We found that upon stimulation of CCR7, ERK1/2 and p38 were activated independently of each other. However, the use of selective inhibitors showed that both kinases regulated JNK activation (Fig. 2*D*). ERK1/2, p38, and JNK played an important role in the regulation of CCR7-dependent chemotaxis, because simultaneous inhibition of these three kinases blunted almost 75% of the chemotactic response of the cells (Fig. 2, *F* and *G*). These experiments also showed that the effects of MAPKs were not synergistic (Fig. 2*G*). Interestingly, because inhibition of these enzymes did not completely blunt CCR7-dependent chemotaxis, this implies that additional unidentified molecules could be regulating this process. In contrast to results obtained in other cell types (24, 25), the three MAPKs played no role in regulation of the migratory speed of DCs (Fig. 2*Fa*), indicating the importance of cell context in the regulation of chemotaxis.

Interestingly, we found that PI3K/Akt regulated neither CCR7-dependent chemotaxis nor migratory speed in DCs (Fig. 3). Previously it has been shown that PI3K/Akt plays a role in the regulation of both functions in several cell types (23, 26–28, 30, 58, 59). However, it is emerging that in certain cell settings, including DCs, chemotaxis can take place even when PI3K/Akt is completely inhibited (60–64). In this regard, a similar lack of a regulatory role for PI3K/Akt on migration has been reported for T cells (60, 64–66). Furthermore, Dumstrei et al. (67) suggested recently that PI3K does not regulate chemotaxis in primordial germ cells, and Lacalle et al. (68) showed that PTEN (Phosphatase and tensin homolog deleted on chromosome 10), an enzyme that degrades the products of PI3K, does not regulate directed motility in HL60 cells. A similar lack of control of PI3K/Akt on cell migration has been observed for other chemokine receptors different from CCR7, including CXCR3 (60) and CCR4 (64). Of note, although the lack of a regulatory role for PI3K/Akt in chemotaxis is not exclusive of DCs or CCR7 receptor, however, because it has been reported that, apart from DCs, CCR7 does not control chemotaxis in T cells, it is possible that this receptor may be coupled to the machinery that regulate chemotaxis independently of PI3K/Akt at least in the two cell types indicated. In this regard, we observed that inhibition of PI3K/Akt by treating DCs with LY294002 specifically blunted CXCR4-dependent, but not CCR7-dependent, chemotaxis DCs (not shown). Additional studies will be required to clarify whether CCR7 uses preferentially PI3K/Akt-independent mechanisms to regulate cell migration. Taken together, the results emphasize the context dependence of the regulation of motile functions and indicate that there are alternative pathways that regulate chemotaxis which do not require PI3K/Akt activation (60–64, 69). Because we have shown previously that CCR7 induces activation of PI3K/Akt, and these molecules are involved in inhibiting apoptosis of DCs, we suggest that regulation of apoptosis, instead of motility, could be the main role of CCR7-stimulated activation of PI3K/Akt in DCs (70).

We identified Rho/Pyk2/cofilin as the components of a module that regulates CCR7-dependent migratory speed based on the following data. 1) We observed that stimulation of CCR7 induced activation of Rho (Fig. 4*A*). We also observed that inhibition of Rho with C3 exoenzyme (Fig. 4*B*) or a dominant negative form of Rho (N19Rho; not shown) inhibited the motility of DCs, but did not affect chemotaxis. In agreement with our results, Rho has been shown to regulate basal motility in HL60 cells (71). Furthermore, a low basal motility is also observed in lymphocytes deficient in p115Rho, a guanine nucleotide exchange factor for Rho, which maintains this GTPase in an active form (72). 2) We observed that Pyk2 is activated after stimulation of CCR7 in a G_i-independent and RhoA-dependent manner (Fig. 5). We suggest that Pyk2 is involved in regulation of the migratory speed of DCs, because overexpression a dominant negative Pyk2 (PRNK) reduced the motility of the cells, but did not affect chemotaxis (Fig. 5). 3) Finally, CCR7-dependent activation of the chemotactic regulator ERK1/2 was not affected by inhibition of Rho or Pyk2, emphasizing that these molecules regulate only the migratory speed of DCs (Figs. 4 and 5).

Pyk2 is a kinase activated by integrin and chemokine receptors that regulate cell motile functions (22, 27, 31, 35). The potential relevance of the activation of Pyk2 by these receptors is underscored by previous reports that Pyk2 regulates the migratory speed of leukocytes (36–38). However, the mechanism(s) by which Pyk2 regulates cell speed is unknown. In this regard, we made the important observation that stimulation of CCR7 induced phosphorylation/inactivation of cofilin (Fig. 6). Overexpression of the dominant negative Pyk2 (PRNK) completely abolished the phosphorylation/inactivation of cofilin, suggesting that Pyk2 is upstream of this molecule (Fig. 6*C*). Because cofilin can regulate cell motility through its ability to depolymerize and sever actin filaments at the leading edge (43–47), our results imply that cofilin may mediate the effects of Pyk2 on cell motility. This is an important finding, because, to the best of our knowledge, this is the first report suggesting that cofilin could mediate the effects of Pyk2 on cell motility.

The finding that CCR7 transmit signals that modulate chemotaxis and migratory speed is relevant for regulation of the functions of DCs in vivo (73). As mentioned above, when CCR7-expressing DCs move from tissues toward lymph nodes, an increase in the speed of the cells can allow them to reach these regions more rapidly. Moreover, once in the nodes, where the cells are surrounded by chemokines, the increased motility of the DCs can enhance the likelihood of encountering Ag-specific T cells at this important meeting point for DCs and T cells (73). It is possible that on the way to lymph nodes or in the nodes, the speed of DCs could be enhanced by environmental factors that stimulate receptors that control signaling components of the migratory speed module.

Our data are consistent with recent models suggesting that the signaling module that controls chemotaxis is separated from other biochemical modules that regulate motility (74). In this regard, it would be interesting to analyze whether the modular mechanisms that we observed for CCR7 could be extended to other chemokine receptors and leukocytes. The knowledge that independent signaling modules regulate CCR7-dependent chemotaxis and migratory speed may open the possibility of a more selective intervention to modulate CCR7-dependent immune response in both pathological and normal states.

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Disclosures

The authors have no financial conflict of interest.

References

1. Banchereau, J., and R. M. Steinman. 1998. Dendritic cells and the control of immunity. *Nature 392:245*.
2. Banchereau, J., F. Briere, C. Caux, J. Davoust, S. Lebecque, Y.-J. Liu, B. Pulendran, and K. Palucka. 2000. Immunobiology of dendritic cells. *Annu. Rev. Immunol. 18:767*.
3. Dieu, M. C., B. Vanbervliet, A. Vicari, J. M. Bridon, E. Oldhaam, S. Ait-Yahia, F. Briere, A. Zlotnik, S. Lebecque, and C. Caux. 1998. Selective recruitment of immature and mature dendritic cells by distinct chemokines expressed in different anatomical sites. *J. Exp. Med. 188:373*.
4. Sallusto, F., P. Schaerli, P. Loetscher, C. Schaniel, D. Lenig, C. R. Mackay, S. Qin, and A. Lanzavecchia. 1998. Rapid and coordinated switch in chemokine receptor expression during dendritic cell maturation. *Eur. J. Immunol. 28:2760*.
5. Allavena, P., A. Sica, A. Vecchi, M. Locati, S. Sozzani, and A. Mantovani. 2000. The chemokine receptor switch paradigm and dendritic cell migration: its significance in tumor tissues. *Immunol. Rev. 177:141*.
6. Luther, S. A., H. L. Tang, P. L. Hyman, A. G. Farr, and J. G. Cyster. 2000. Coexpression of chemokines ELC and SLC by T zone stromal cells and deletion of the ELC gene in the plt/plt mouse. *Proc. Natl. Acad. Sci. USA 97:12694*.
7. Ngo, V. N., H. L. Tang, and J. G. Cyster. 1998. Epstein-Barr virus-induced molecule 1 ligand chemokine is expressed by dendritic cells in lymphoid tissues and strongly attracts naive T cells and activated B cells. *Eur. J. Immunol. 188:181*.
8. Gunn, M. D., K. Tangemann, C. Tam, J. G. Cyster, S. D. Rosen, and L. T. Williams. 1998. A chemokine expressed in lymphoid high endothelial venules promotes the adhesion and chemotaxis of naive T lymphocytes. *Proc. Natl. Acad. Sci. USA 95:258*.
9. Stein, J. V., A. Rot, Y. Luo, M. Narasimhaswamy, H. Nakano, M. D. Gunn, A. Matsuzawa, E. J. Quackenbush, M. E. Dorf, and U. H. von Andrian. 2000. The CC chemokine thymus-derived chemotactic agent 4 (TCA-4, secondary lymphoid tissue chemokine, 6Ckine, exodus-2) triggers lymphocyte function-associated antigen 1-mediated arrest of rolling T lymphocytes in peripheral lymph node high endothelial venules. *J. Exp. Med. 191:61*.
10. Warnock, R. A., J. J. Campbell, M. E. Dorf, A. Matsuzawa, L. M. McEvoy, and E. C. Butcher. 2000. The role of chemokines in the microenvironmental control of T versus B cell arrest in Peyer's path high endothelial venules. *J. Exp. Med. 191:77*.
11. Ansel, K. M., V. N. Ngo, P. L. Hyman, S. A. Luther, R. Forster, J. D. Sedgwick, J. L. Browning, M. Lipp, and J. G. Cyster. 2000. A chemokine driven positive feedback loop organizes lymphoid follicles. *Nature 406:309*.
12. Baekkevold, E. S., T. Yamanaka, R. T. Palframan, H. S. Carlsen, F. P. Reinholdt, U. H. von Andrian, P. Brandtzaeg, and G. Haraldsen. 2001. The CCR7 ligand ELC (CCL19) is transcytosed in high endothelial venules and mediates T-cell recruitment. *J. Exp. Med. 193:1105*.
13. Gunn, M. D., S. Kyuwa, C. Tam, T. Kakiuchi, A. Matsuwa, L. T. Williams, and H. Nakano. 1999. Mice lacking expression of secondary lymphoid organ chemokine have defects in lymphocyte homing and dendritic cells localisation. *J. Exp. Med. 189:451*.
14. Nakano, H., and M. D. Gunn. 2001. Gene duplication at the chemokine locus on mouse chromosome 4: multiple strain specific haplotypes and the deletion of secondary lymphoid organ chemokine and EBI-1 ligand chemokine genes in the plt mutation. *J. Immunol. 1. 66:361*.
15. Forster, R., A. Schubel, D. Breitfeld, E. Kremmer, I. Renner-Muller, E. Wolf, and M. Lipp. 1999. CCR7 coordinates the primary immune response by establishing functional microenvironments in secondary lymphoid organs. *Cell 99:23*.
16. Moore, M. A. 2001. The role of chemoattraction in cancer metastases. *BioEssays 23:674*.
17. Muller, A., B. Homey, H. Soto, N. Ge, D. Catron, M. E. Buchanan, T. McClanahan, E. Murphy, W. Yuan, S. N. Wagner, et al. 2001. Involvement of chemokine receptors in breast cancer metastasis. *Nature 410:50*.
18. Campbell, J. J., S. Qin, K. B. Bacon, C. R. Mackay, and E. C. Butcher. 1996. Biology of chemokine and classical chemoattractant receptors: differential requirement for adhesion-triggering versus chemotactic responses in lymphoid cells. *J. Cell Biol. 134:255*.
19. Kellermann, S. A., S. Hudak, E. R. Oldham, Y.-J. Liu, and L. M. McEvoy. 1999. The CC chemokine receptor-7 ligands 6Ckine and macrophage inflammatory protein-3β are potent chemoattractants for in vitro and in vivo-derived dendritic cells. *J. Immunol. 162:3859*.
20. Thelen, M. 2001. Dancing to the tune of chemokines. *Nat. Immunol. 2:129*.
21. Ganju, R. K., S. A. Brubaker, J. Meyer, P. Dutt, Y. Yang, S. Qin, W. Newman, and J. Groopman. 1998. The α-chemokine, stromal cell-derived factor-1α, binds to the transmembrane G-protein-coupled CXCR-4 receptor and activates multiple signal transduction pathways. *J. Biol. Chem. 273:23169*.
22. Ganju, R. K., P. Dutt, L. Wu, W. Newman, H. Avraham, S. Avraham, and J. E. Groopman. 1998. β-Chemokine receptor CCR5 signals via the novel tyrosine kinase RAFTK. *Blood 91:791*.
23. Wong, M. M., and E. N. Fish. 2003. Chemokines: attractive mediators of the immune response. *Semin. Immunol. 15:5*.
24. Klenke, R. L., S. Cai, A. L. Giannini, P. J. Gallagher, P. de Lorerolle, and D. A. Cheresh. 1997. Regulation of cell motility by mitogen-activated protein kinase. *J. Cell Biol. 137:481*.
25. Huang, C., Z. Rajfur, C. Borchers, M. D. Schaller, and K. Jacobson. 2003. JNK phosphorylates paxillin and regulates cell migration. *Nature 424:219*.
26. Sotsios, Y., and S. G. Ward. 2000. Phosphoinositide 3-kinase: a key biochemical signal for cell migration in response to chemokines. *Immunol. Rev. 177:217*.
27. Wang, J. F., I. W. Park, and J. E. Groopman. 2000. Stromal cell-derived factor-1α stimulates tyrosine phosphorylation of multiple focal adhesion proteins and induces migration of hematopoietic progenitor cells: roles of phosphoinositide-3 kinase and protein kinase C. *Blood 95:2505*.
28. Curnock, A. P., M. K. Logan, and S. G. Ward. 2002. Chemokine signalling: pivoting around multiple phosphoinositide 3-kinases. *Immunology 105:125*.
29. Devreotes, P., and C. Janetopoulos. 2003. Eukaryotic chemotaxis: Distinction between directional sensing and polarization. *J. Biol. Chem. 278:20445*.
30. Wang, J., P. Herzmark, O. D. Weiner, S. Srinivasan, G. Servant, and H. R. Bourne. 2002. Lipid products of PI_3Ks maintain persistent cell polarity and directed polarity in neutrophils. *Nat. Cell. Biol. 4:513*.
31. Ganju, R. K., S. A. Brubaker, R. D. Chernock, S. Avraham, and J. E. Groopman. 2000. β-Chemokine receptor CCR5 signals through SHP1, SHP2, and Syk. *J. Biol. Chem. 275:17263*.
32. Rodríguez-Fernández, J. L., M. Gómez, A. Luque, N. Hogg, F. Sánchez-Madrid, and C. Cabañas. 1999. The interaction of activated integrin lymphocyte function-associated antigen 1 with ligand intercellular adhesion molecule 1 induces activation and redistribution of focal adhesion kinase and proline-rich tyrosine kinase 2 in T lymphocytes. *Mol. Biol. Cell 10:1891*.
33. Schlaepfer, D. D., C. R. Hauck, and D. J. Sieg. 1999. *Prog. Biophys. Mol. Biol. 71:435*.
34. Avraham, H., S.-Y. Park, K. Schinkmann, and S. Avraham. 2000. RAFTK/Pyk-2 mediated cellular signalling. *Cell. Signal. 12:123*.
35. Rodriguez-Fernandez, J. L., L. Sanchez-Martin, M. Rey, M. Vicente-Manzanares, S. Narumiya, J. Teixido, F. Sanchez-Madrid, and C. Cabañas. 2001. Rho and ROCK modulate the tyrosine kinase PYK2 in T-cells through regulation of the activity of the integrin LFA-1. *J. Biol. Chem. 276:40518*.
36. Guinamard, R., M. Okigaki, J. Schlessinger, and J. V. Ravetch. 2000. Absence of marginal zone B cells in Pyk-2-deficient mice defines their role in the humoral response. *Nat. Immunol. 1:31*.
37. Gismondi, A., J. Jacobelli, R. Strippoli, F. Mainiero, A. Soriani, L. Cifaldi, M. Piccoli, L. Frati, and A. Santoni. 2003. Proline-rich tyrosine kinase 2 and Rac activation by chemokine and integrin receptors controls NK cell transendothelial migration. *J. Immunol. 170:3065*.
38. Okigaki, M., C. Davis, M. Falasca, S. Harroch, D. P. Felsenfeld, M. P. Sheetz, J. Schlessinger, and S. P. Palecek. 2003. Pyk2 regulates multiple signaling events crucial for macrophage morphology and migration. *Proc. Natl. Acad. Sci. USA 100:10740*.
39. Vicente-Manzanares, M., and F. Sanchez-Madrid. 2004. Role of the cytoskeleton during leukocyte responses. *Nat. Rev. Immunol. 4:1*.
40. Bishop, A. L., and A. Hall. 2000. Rho GTPases and their effector proteins. *Biochem. J. 348:241*.
41. Higgs, H. N., and T. D. Pollard. 2001. Regulation of actin filament network formation through Arp2/3 complex: activation by a diverse array of proteins. *Annu. Rev. Biochem. 70:649*.
42. Riol-Blanco, L., T. Iglesias, N. Sánchez-Sánchez, G. De la Rosa, L. Sánchez-Ruiloba, N. Cabrera-Poch, A. Torres, I. Longo, J. García-Bordas, N. Longo, et al. 2004. The neuronal protein Kidins220 localizes in a raft compartment at the leading edge of motile immature dendritic cells. *Eur. J. Immunol. 34:108*.
43. Chen, J., D. Godt, K. Gunsalus, I. Kiss, M. Goldberg, and F. A. Laski. 2001. Cofilin/ADF is required for cell motility during *Drosophila* ovary development and oogenesis. *Nat. Cell. Biol. 3:204*.
44. Ono, S. 2003. Regulation of actin filament dynamics by actin depolymerizing factor/cofilin and actin-interacting protein 1: new blades for twisted filaments. *Biochemistry 42:13363*.
45. Pollard, T. D., and G. G. Borisy. 2003. Cellular motility driven by assembly and disassembly of actin filaments. *Cell 112:453*.
46. Bailly, M., and G. E. Jones. 2003. Polarised migration: cofilin holds the front. *Curr. Biol. 13:R128*
47. Rogers, S. L., U. Wiedemann, N. Stuurman, and R. D. Vale. 2003. Molecular requirements for actin-based lamella formation in *Drosophila* S2 cells. *J. Cell Biol. 162:1079*.
48. Morii, N., and Narumiya, S. 1995. Preparation of native and recombinant *Clostridium botulinum* C3 ADP-ribosyltransferase and identification of Rho proteins by ADP-ribosylation. *Methods Enzymol. 256:196*.
49. Sallusto, F., D. Lenig, R. Forster, M. Lipp, and A. Lanzavecchia. 1999. Two subsets of memory T lymphocytes with distinct homing potentials and effector functions. *Nature 401:708*.
50. Romani, N., S. Gruner, E. Brang, B. Kampgen, G. Lenz, P. Trockenbacher, O. Konwalinka, R. M. Fritsch, G. Steinman, and Schuler. 1994. Proliferating dendritic cells progenitors in human blood. *J. Exp. Med. 180:83*.
51. Sallusto, F., and A. Lanzavecchia. 1994. Efficient presentation of soluble antigen by cultured human dendritic cells is maintained by granulocyte/macrophage colony-stimulating factor plus interleukin-4 and down regulated by tumor necrosis factor α. *J. Exp. Med. 179:1109*.
52. Relloso, M., A. Puig-Kroger, O. Muñiz Pello, J. L. Rodríguez-Fernández, G. de la Rosa, N. Longo, J. Navarro, M. A. Muñoz-Fernández, P. Sánchez-Mateos, and Corbi, A. L. 2002. DC-SIGN (CD209) Expression is IL-4 dependent

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and is negatively regulated by IFN, TGF-α, and anti-Inflammatory agents. *J. Immunol. 168:2634*.
53. Ivankovik-Dikic, I., E. Gronroos, A. Blaukat, B.-U. Barth, and I. Dikic. 2000. Pyk2 and FAK regulate neurite outgrowth induced by growth factors and integrins. *Nat. Cell. Biol. 2:574*.
54. del Pozo, M. A., M. Vicente-Manzanares, R. Tejedor, J. M. Serrador, and F. Sánchez-Madrid. 1999. Rho GTPases control migration and polarization of adhesion molecules and cytoskeletal ERM components in T lymphocytes. *Eur. J. Immunol. 29:3609*.
55. Rodríguez-Fernández, J. L., and E. Rozengurt. 1996. Bombesin, bradykinin, vasopressin, and phorbol esters rapidly and transiently activate Src family tyrosine kinases in Swiss 3T3 cells. *J. Biol. Chem. 271:27895*.
56. Rodríguez-Fernández, J. L., B. Geiger, D. Salomon, and A. Ben-Ze'ev. 1993. Suppression of vinculin expression by antisense transfection confers changes in cell morphology, motility, and anchorage-dependent growth of 3T3 cells. *J. Cell Biol. 122:1285.*
57. Palecek, S. P., J. C. Loftus, M. H. Ginsberg, D. A. Lauffenburger, and A. F. Horwitz. 1997. Integrin-ligand binding properties govern cell migration speed through cell-substratum adhesiveness. *Nature 385:537*.
58. Sotsios, Y., G. C. Whittaker, J. Westwick, and S. G. Ward. 1999. The CXC chemokine stromal cell-derived factor activates a G_i-coupled phosphoinositide 3-kinase in T-lymphocytes. *J. Immunol. 163:5954*.
59. Chung, C. Y., S. Funamoto, and R. A. Firtel. 2001. Signaling pathways controlling cell polarity and chemotaxis. *Trends Biochem. Sci. 26:557*.
60. Smit, M. J., P. Verdijk, E. M. H. van der Raaij-Helmer, M. Navis, P. J. Hensbergen, R. Leurs, and C. P. Tensen. 2003. CXCR3-mediated chemotaxis of human T cells is regulated by a G_i-and phospholipase C-dependent pathway and not via activation of MEK/p44/p42 MAPK nor Akt/PI-3 kinase. *Blood 102:1959*.
61. Chodniewicz, D., and D. V. Zhelev. 2003. Chemoattractant receptor-stimulated F-actin polymerization in the human neutrophils is signaled by 2 distinct pathways. *Blood 101:1181*.
62. Chodniewicz, D., and D. V. Zhelev. 2003. Novel pathways of F-actin polymerization in the human neutrophils. *Blood 102:2251*.
63. Scandella, E., Y. Men, D. F. Legler, S. Gillessen, L. Prikler, B. Ludewig, and M. Groettrup. 2004. CCL19/CCL21-triggered signal transduction and migration of dendritic cells requires prostaglandin E_2. *Blood 103:1595*.
64. Ward, S. G. 2004. Do phosphoinositide 3-kinases direct lymphocyte navigation? *Trends Immunol. 25:67*.
65. Cinamon, G., V. Shinder, and R. Alon. 2001. Shear forces promote lymphocyte migration across vascular endothelium bearing apical chemokines. *Nat. Immunol. 2:515*.
66. Constantin, G., M. Majeed, C. Giagulli, L. Piccio, J. Y. Kim, E. C. Butcher, and C. Laudanna. 2000. Chemokines trigger immediate β_2 integrin affinity and mobility changes: differential regulation and roles in lymphocyte arrest under flow. *Immunity 13:759*.
67. Dumstrei, K., R. Mennecke, and E. Raz. 2004. Signaling pathways controlling primordial germ cell migration in zebrafish. *J. Cell Sci. 117:4787*.
68. Lacalle, R. A., C. Gómez-Moutón, D. F. Barber, S. Jiménez-Baranda, E. Mira, C. Martínez-A, A. C. Carrera, and S. Mañes. 2004. PTEN regulates motility but not directionality during leukocyte chemotaxis. *J. Cell Sci. 117:6207*.
69. Cronshaw, D. G., C. Owen, Z. Brown, and S. G. Ward. 2004. Activation of phosphoinositide 3-kinases by the CCR4 ligand macrophage-derived chemokine is a dispensable signal for T lymphocyte chemotaxis. *J. Immunol. 172:7761*.
70. Sánchez-Sánchez, N., L. Riol-Blanco, G. de la Rosa, J. García-Bordas, D. Martín, N. Longo, A. Cuadrado, C. Cabañas, P. Sánchez-Mateos, and J. L. Rodríguez-Fernández. 2004. Chemokine receptor CCR7 induces intracellular signaling that inhibits apoptosis of mature dendritic cells. *Blood 104:619*.
71. Xu, J., F. Wang, A. Van Keymeulen, P. Herzmark, A. Straight, K. Kelly, Y. Takuwa, N. Sugimoto, T. Mitchison, and H. R. Bourne. 2003. Divergent signals and cytoskeletal assemblies regulate self-organizing polarity in neutrophils. *Cell 114:201*.
72. Girkontaite, I., K. Missy, V. Sakk, A. Harenberg, K. Tedford, T. Potzel, K. Pfeffer, and K. D. Fischer. 2001. Lsc is required for marginal zone B cells, regulation of lymphocyte motility and immune responses. *Nat. Immunol. 2:855*.
73. Mempel, T. R., S. E. Henrickson, and U. H. von Andrian. 2004. T-cell priming by dendritic cells in lymph nodes occurs in three distinct phases. *Nature 427:154*.
74. Iglesias, P. A., and A. Levchenko. 2002. Modelling the cell's guidance system. *Science STKE 148:re12*.

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