Four-and-a-Half LIM Domain Protein 2 Is a Novel Regulator of Sphingosine 1-Phosphate Receptor 1 in CCL19-Induced Dendritic Cell Migration

Katharina König, Linda Diehl, Ursula Rommerscheidt-Fuss, Carsten Golletz, Thomas Quast, Philip Kahl, Waldemar Kolanus, Percy Knolle, Reinhard Buettner and Lukas C. Heukamp

*J Immunol* 2010; 185:1466-1475; Prepublished online 30 June 2010;
doi: 10.4049/jimmunol.0903449
http://www.jimmunol.org/content/185/3/1466

---

Supplementary Material: [http://www.jimmunol.org/content/suppl/2010/06/30/jimmunol.0903449.DC1](http://www.jimmunol.org/content/suppl/2010/06/30/jimmunol.0903449.DC1)

References: This article cites 57 articles, 28 of which you can access for free at: [http://www.jimmunol.org/content/185/3/1466.full#ref-list-1](http://www.jimmunol.org/content/185/3/1466.full#ref-list-1)

Subscription: Information about subscribing to *The Journal of Immunology* is online at: [http://jimmunol.org/subscription](http://jimmunol.org/subscription)

Permissions: Submit copyright permission requests at: [http://www.aai.org/About/Publications/JI/copyright.html](http://www.aai.org/About/Publications/JI/copyright.html)

Email Alerts: Receive free email-alerts when new articles cite this article. Sign up at: [http://jimmunol.org/alerts](http://jimmunol.org/alerts)
Four-and-a-half LIM Domain Protein 2 Is a Novel Regulator of Sphingosine 1-Phosphate Receptor 1 in CCL19-Induced Dendritic Cell Migration


We identified the four-and-a-half LIM domain protein 2 (FHL2) as a novel regulator of CCL19-induced dendritic cell (DC) migration. Initiation of migration is a hallmark of DC function and plays a central role in the induction and regulation of immune responses. In vivo, DCs continuously acquire Ag in the periphery and migrate to draining lymph nodes, under the influence of local environmental chemotactic factors like CCL19/21 or sphingosine 1-phosphate (S1P). We investigated the role of S1P- and RhoA-regulated FHL2 in this process. We found reduced nuclear localization of FHL2 in mature bone marrow-derived DCs (BMDCs), compared with immature BMDCs, following stimulation with CCL19. Furthermore, in vitro-generated murine FHL2−/−BMDCs displayed a significantly increased migratory speed, directionality, and migratory persistence toward the chemokine CCL19 compared with wild-type BMDCs. Moreover, in vivo, FHL2−/−BMDCs showed increased migration toward lymphoid organs. FHL2−/−BMDCs increased the expression of S1PR1, which was associated with greater Rac activation. An S1PR1 antagonist and knock-down of S1PR1 abrogated the increased migratory speed of FHL2−/−BMDCs. Our results identify FHL2 as an important novel regulator of DC migration via regulation of their sensitivity toward environmental migratory cues like S1P and CCL19. The Journal of Immunology, 2010, 185: 1466–1475.

Dendritic cells (DCs) play a central role in the initiation and regulation of immune responses. Depending on their maturation status, DCs are thought to be involved in the induction of immune tolerance and immunity. DCs continuously acquire Ag in the periphery and migrate to draining lymphoid organs, where they present these Ags to CD4 and CD8 T cells (1). Under noninflammatory conditions, the presentation of Ag by immature DCs is associated with the induction of immune tolerance. Upon sensing danger signals via pattern-recognition receptors (e.g., TLRs) (2, 3), DCs undergo a maturation process, leading to the upregulation of MHC and costimulatory molecules, increased inflammatory cytokine production, and, eventually, T cell priming (4–6). While migrating toward the T cell zones of the draining lymph node (LN), DCs remodel their chemokine receptor profile, by upregulating CCR7, guiding them toward its ligands CCL19 and CCL21, which are secreted by stromal cells in the T cell zone (7). This differentiation process is accompanied by a dramatic reorganization of the actin cytoskeleton (8), which is mediated via the family of Rho GTPases, including Rho, Rac, and Cdc42, and enables the rapid migration of DCs to draining lymphoid organs.

Sphingosine 1-phosphate (S1P) is a potent lysophospholipid with a wide range of biological activities (9), including regulation of cytoskeletal rearrangement and cell migration. It acts via five receptors (S1PR1–5) that couple to heterotrimeric G proteins. S1PR1–4 are expressed by a number of immune cells, whereas expression of S1PR5 is mainly confined to cells of the CNS (10). S1P possesses a chemotactic property for a variety of immune cells (11, 12), and it has an impact on the egress of B and T cells from the LN and thymus (13). Furthermore, S1P is responsible for correct positioning of B cells and DCs within the spleen (14, 15). In DCs, the expression pattern of S1PRs depends on their maturation status (15–17), which can stimulate or inhibit migration (18).

The four-and-a-half LIM domain protein 2 (FHL2) is a member of the LIM-only subclass of the LIM protein superfamily, which is defined by the presence of one or more LIM domains that mediate protein–protein interactions (19). FHL2 is strongly expressed in heart and skeletal muscle, as well as at lower levels in most epithelial tissues. We previously showed that S1P triggers a signal-transduction cascade mediating nuclear translocation of the LIM-only protein FHL2 in response to activation of the RhoA GTPase (20, 21). Following nuclear translocation, FHL2 can function as a transcriptional coactivator for, among others, the androgen receptor (20, CREB (22), AP-1, RunX1 (23), and serum response factor (24), linking extracellular signals to gene expression (25). We previously showed that FHL2-deficient mice have a severely delayed wound healing response due a defect in fibroblast migration and a reduced collagen contraction (26, 27). Furthermore,
Rho GTPase-mediated nuclear translocation of FHL2 correlates with enhanced metastatic behavior in prostate carcinomas (28).

Together, these data point to a pivotal signaling role for FHL2 in cellular migration.

Because DC migration toward lymphatic organs is dependent on extracellular chemotactic signals that influence migratory behavior via the differential activation of Rho GTPases, we investigated the role of FHL2 in DC migration. In this study, we showed that, in the absence of FHL2, CCL19-induced DC migratory speed, persistence, and directionality were markedly increased in vitro and in vivo. These were associated with increased expression levels of S1PR1, which resulted in increased Rac activation. This identified FHL2 as an important novel regulator of chemotactic DC migration.

**Materials and Methods**

**Transgenic mice**

FHL2-deficient mice were provided by R. Bassel-Duby (University of Texas Southwestern Medical Center, Dallas, TX). All animals were housed in accordance with institutional guidelines and approved by the regional board. FHL2−/− mice were bred onto a C57BL/6 background for >11 generations.

**FHL2 RT-PCR and quantitative real-time PCR**

RNA was isolated from day-7 BMDCs, heart, lung, colon, and skeletal muscle using an RNaseasy kit (Qiagen, Hilden, Germany), according to the manufacturer’s instructions. Five hundred nanograms total RNA was converted to cDNA with SuperScript III H−. Reverse Transcriptase using oligonucleotide (dT)12-18 Primer (Invitrogen, Karlsruhe, Germany). For the amplification of exon1 of FHL2, the forward primer 5′-TTGCTGAAAGACAGGGTGTACG-3′ and the reverse primer 5′-TTGCAGTCCACGGCAGGTGGG-3′ were used. Actin was served as a positive control and was amplified with the primer 5′-CTAGTGCCTGCTGACTGACGG-3′ and 5′-GATGTCGACCCAGCGAACC-3′. One microliter of cDNA was de-natured for 5 min at 95˚C, followed by an annealing step of 60˚C and ending with elongation at 72˚C.

The expression of S1PR1, S1PR2, S1PR3, and S1PR4 was analyzed using the Quantitect SYBR Green PCR Kit (Qiagen) with the 7900HT Fast Real-Time PCR System (Applied Biosystems, Darmstadt, Germany). Ex-Pressing normalized to β-actin was used as an internal control.

**Immunoprecipitation and Western blot**

Twenty micrograms of total protein was separated on NuPAGE 4–12% Bis-Tris gels (1.5 mm × 15 well; Invitrogen) and blotted onto nitrocellulose membrane (Bio-Rad, Munich, Germany). The membrane was stained with anti-FHL2 (Cell Biosciences, Palo Alto, CA) or anti-β-actin mAb (Sigma-Aldrich, Munich, Germany) for 1 h at room temperature and visualized with ECL Western blotting detection reagents (GE Healthcare, Freiburg, Germany).

For immunoprecipitation, 1 mg protein of total wild-type (wt) and FHL2−/− BMDC lysates was incubated with 4 μg polyclonal Ab directed against FHL2 (a kind gift R. Schuele, Freiburg, Germany). The complexes were precipitated using protein G PLUS-Agarose (Santa Cruz Biotechnology, Heidelberg, Germany) at 4˚C overnight. After washing in radioimmuno-precipitation assay buffer, the beads were boiled in Laemmli buffer and analyzed by Western blot.

**BMDC culture**

Bone marrow was flushed from the hind legs of mice and cultured for 7 d in IMDM (PAA, Pasching, Austria) supplemented with penicillin/streptomycin (PAA), 1-glutamine (2 mM PAA), 8% heat-inactivated FCS (PAA), 20 μM 2-ME (Merck, Darmstadt, Germany), and GM-CSF–containing culture supernatant, unless otherwise stated. To induce maturation of the BMDCs, the cultures were supplemented with 100 ng/ml LPS for an additional 24 h or 30 ng/ml murine TNF-α (PeproTech, Hamburg, Germany) for an additional 48 h. SEW2871 was purchased from Cayman (Tallinn, Estonia).

**Flow cytometry**

Cells were washed in PBS containing 1% FCS and incubated for 30 min at 4˚C with anti-FcyRII/III mAb to block unspecific binding of the following Ab reagents. All mAbs used were purchased from BD Pharmingen (Hamburg, Germany) or eBioscience (San Diego, CA). Biotin- and PE-labeled mAbs (used at 5–20 μg/ml) included Abs against MHC class II (clone Z90, MHC class I (clone AF6-88.5), CD11c (clone N418), CD80/B7-1 (clone 1G10), CD86/B7-2 (clone GL1), and CD40 (clone 3/23), streptavidin-PerCp, and streptavidin-Cy5.5. Isotype controls included purified rat IgG2a and hamster IgG. After incubation with mAbs for 30 min at 4˚C, cells were washed with PBS containing 1% FCS. Data were acquired on a FACSArray (BD Biosciences) and analyzed using FlowJo software (Tree Star, Ashland, OR).

**Intracellular staining of IL-12 and TNF-α**

Immature and LPS-matured BMDCs were incubated in the presence of Golgi Stop and Golgi Plug, according to the manufacturer’s recommendations (BD Biosciences) for 5 h. After surface staining with CD11c PE, BMDCs were fixed with 4% paraformaldehyde, permeabilized with 0.5% saponin, and stained with Abs against IL-12 and TNF-α conjugated to Alexa Fluor 647.

**Transwell migration assay**

A total of 2.5 × 10⁵ BMDCs were added to the top chamber of 5-μm-pore, polycarbonate 24-well tissue-culture inserts (Costar, Cambridge, MA) in 100 μl IMDM supplemented with 0.5% BSA. The lower wells were filled with 600 μl IMDM/0.5% BSA containing 10 ng/ml CCL19 (R&D Systems, Wiesbaden-Nordenstadt, Germany) or with medium alone. Cells were allowed to migrate to 37°C for 3 h and then were harvested from the bottom chamber using 600 μl IMDT in PBS and stained for CD11c. A total of 5000 BD Calibrate beads (BD Biosciences, San Jose, CA) were added to each sample, and samples were acquired on a FACSArray.

**Time-lapse video microscopy**

A total of 1 × 10⁵ BMDCs were allowed to adhere to collagen-coated IBIDI µSlides (IBIDI, Munich, Germany) for 45 min at 37°C. Time-lapse images were taken every 15 s for 30 min on an IX81 microscope (Olympus, Hamburg, Germany), to determine the migratory speed without cytokine stimulation, using the CellR Imaging System (Olympus). After 45 min, a chemokine gradient was set up with 40 μl 20 μg/ml CCL19 (R&D Systems) in the µSlide, according to the manufacturer’s instructions, after which the cells were imaged for an additional 60 min at 37°C. Migratory speed, directionality, and distance were determined using Track-it software (Olympus).

**In vivo BMDC migration**

BMDCs were labeled with 1 μM CFSE or 10 μM of the fluorescent dye 5- and 6-chloromethyl SNARF-1 acetate (SNARF) (Invitrogen), mixed in equal proportions, and 2 × 10⁶ cells in 20 μl PBS were injected into the hind footpads of wt mice. Twenty-six hours later, the popliteal LNs were isolated. Forty-eight hours post injection, the mixture was stained with CD11c and analyzed by flow cytometry as well, to confirm that wt and FHL2−/− BMDCs were injected at the same proportion.

**Rac1 activation assay**

Wt and FHL2−/− day-7 BMDCs were incubated for 2 d in IMDM containing 0.5% FCS and were subsequently incubated for 5 h in serum-free IMDM to starve the cells. Protein lysates were collected, and the activity of Rac1 was measured by the G-LISA activation assay (Cytoskeleton, Hamburg, Germany), to determine the migratory speed without cytokine stimulation, using the CellR Imaging System (Olympus). After 45 min, a chemokine gradient was set up with 40 μl 20 μg/ml CCL19 (R&D Systems) in the µSlide, according to the manufacturer’s instructions, after which the cells were imaged for an additional 60 min at 37°C. Migratory speed, directionality, and distance were determined using Track-it software (Olympus).

**Immunofluorescence**

BMDCs were allowed to adhere on a collagen-coated surface for 45 min at 37°C. BMDCs were fixed with 4% paraformaldehyde for 15 min on ice and were subsequently permeabilized on ice for 1 h in 0.2% Triton/PBS. Actin filaments were stained with 0.4 U/ml phalloidin Alexa Fluor 546 (Invitrogen) in 5% milk powder (Nestlé Research Laboratories, Vevey, Switzerland). BMDCs were incubated with medium containing 0.5 mg/ml dextran coupled to FITC.
protein prior to Western blotting of wt and FHL2−/− BMDCs. Counterstaining with CD4 (clone GK1.5) and CD8 (clone 53-6.7) Abs. BMDCs. Three days later, proliferation was assessed by flow cytometry.

**Ag-presentation assay**

On day 7, 2 × 10⁵ wt and FHL2−/− BMDCs, purified by AutoMacs using CD11c beads (Miltenyi Biotec, Bergisch Gladbach, Germany) according to the manufacturer’s instructions, were seeded per well and incubated with 0, 0.01, 0.1, or 1 mg/ml OVA. Two days before isolation of T cells from OTI mice, NK T cells were depleted by injection of 300 μg anti-NK1.1 Ab (clone PK136). A total of 1 × 10⁵ purified naïve CD4⁺ and CD8⁺ T cells from OTII and OTI mice, respectively, were cocultured with BMDCs. Twenty-four hours later, supernatants were collected and subjected to a commercially available quantitative IL-2 (clones JES6-1A12 and JES6-5H4) ELISA kit (BD Biosciences).

**T cell proliferation assay**

On day 7, 4 × 10⁵ wt and FHL2−/− BMDCs, purified by AutoMacs using CD11c beads (Miltenyi Biotec) according to the manufacturer’s instructions, were seeded per well and incubated with or without 1 mg/ml OVA for 2 h. A total of 2 × 10⁵ purified CD4⁺ and CD8⁺ T cells from OTII and OTI mice, respectively, labeled with 0.1 μM CFSE, were added to BMDCs. Three days later, proliferation was assessed by flow cytometry counterstaining with CD4 (clone GK1.5) and CD8 (clone 53-6.7) Abs.

**Transfection of BMDCs with S1PR1 siRNA**

A total of 4 × 10⁶ day-7 BMDCs were electroporated with a square wave protocol (300 V, 6 ms; Bio-Rad) with 5 μg S1PR1 siRNA, as well as with 5 μg AllStars Negative Control siRNA (Qiagen). Forty-eight hours later, BMDCs were allowed to migrate in a Transwell, as described previously, and the expression of S1PR1 was assessed with quantitative RT-PCR (qRT-PCR).

**Statistics**

Statistics were calculated with Excel (Microsoft, Redmond, WA) and GraphPad Prism (GraphPad, San Diego, CA). Error bars indicate SEM. The Student t test was used to analyze data for significant differences; p values <0.05 were considered significant.

**Results**

**Nuclear localization of FHL2 is lost in mature but not immature BMDCs following stimulation with CCL19**

To determine FHL2 expression and localization, we stained immature or LPS-matured wt BMDCs for FHL2. Mature and immature BMDCs express FHL2, which localizes to the nucleus and the cytoplasm (Fig. 1A). To further analyze whether FHL2 has a functional role in DC migration, we also stained mature and immature BMDCs that were stimulated with the CCR7 chemokine receptor ligand CCL19. In immature BMDCs, CCL19 treatment did not alter FHL2 localization, which remained mainly in the nucleus and cytoplasm. Interestingly, CCR7 ligation in mature DCs led to the loss of nuclear localization of FHL2 redistributing toward the cell membrane. Together, these data indicate that the migratory chemokine CCL19 influences FHL2 function in DCs.

To further functionally address the role of FHL2 in CCL19-induced processes in DC migration, we generated BMDCs from an FHL2-deficient mouse strain. To confirm the loss of FHL2 expression in FHL2−/− mice, we performed RT-PCR on various tissues and in vitro-generated BMDCs of C57BL/6 and FHL2−/− mice (Fig. 1B). FHL2 mRNA is highly expressed in the heart, skeletal muscle, gastrointestinal tract, and lung at lower levels in BMDCs of wt mice, whereas expression is lost in FHL2-deficient mice. Moreover, FHL2 protein is not detectable by Western blot in heart tissue that has the highest levels of FHL2 expression in wt mice (Fig. 1C). Because the expression of FHL2 is relatively low in BMDCs (Fig. 1B), we performed immunoprecipitation of FHL2 protein prior to Western blotting of wt and FHL2−/− BMDC lysates. As expected, we confirmed that FHL2 expression is not detectable at the protein level in FHL2−/− BMDCs (Fig. 1D).

![FIGURE 1.](http://www.jimmunol.org/)

**Enhanced in vitro and in vivo migration of FHL2-deficient BMDCs**

To examine the effect of FHL2 deficiency on CCL19-induced DC migration, immature wt or FHL2−/− BMDCs were placed in the upper chamber of a Transwell and left to migrate toward CCL19 or medium alone (Fig. 2A). Wt and FHL2−/− BMDCs migrated toward CCL19 when this was added to the lower chamber of the Transwell. Furthermore, significantly more FHL2−/− BMDCs than wt BMDCs migrated into the lower chamber in the given time period, indicating that FHL2 deficiency significantly increases the CCL19-induced migration rate of BMDCs. When CCL19 was added only to the upper chamber or simultaneously to the upper and lower Transwell chambers, no increased migration (above basal levels) was observed (data not shown).

Directional migratory speed in DCs is known to be regulated by CCR7, among others (7). Because FHL2 can act as a transcriptional coactivator or corepressor (20–23, 29) and, thus, might affect CCR7, among others (7). Because FHL2 can act as a transcriptional coactivator or corepressor (20–23, 29) and, thus, might affect CCR7 expression, we investigated whether CCR7 expression was increased in FHL2−/− BMDCs. However, staining of immature (Fig. 2B) or mature (data not shown) wt and FHL2−/− BMDCs with CCL19-IgG fusion protein did not reveal differences in CCR7 expression levels between wt and FHL2−/− BMDCs, indicating that the enhanced CCL19-induced migration of FHL2−/− BMDCs was not due to altered CCR7 surface expression.

In vitro DC migration assays only partially resemble DC migration in vivo (30). Therefore, we determined whether FHL2−/− BMDCs also had altered migratory properties in vivo. To this end,
we injected equal amounts of immature or TNF-α-matured wt and FHL2−/− BMDCs, which were fluorescently labeled with SNARF (FHL2−/−) and CFSE (wt), into the footpad of C57BL/6 recipient mice. After 26 h, single-cell suspensions of the draining popliteal LN were prepared, stained for CD11c, and analyzed by flow cytometry. The total number of CD11c+ BMDCs recovered from the popliteal LN was not due to a survival advantage over wt BMDCs. Collectively, this indicates that FHL2 deficiency in BMDCs leads to enhanced migratory activity in vitro and in vivo.

**FHL2−/− BMDCs display enhanced migratory directionality, speed, and persistence in vitro**

We used time-lapse video microscopy to measure the influence of FHL2 expression on chemotactic directionality, migratory speed, and directional persistence of BMDCs more closely (Fig. 3). Briefly, immature BMDCs were transferred into collagen-coated IBIDI µSlide chambers, and images were taken every 15 s for 1 h after establishing a CCL19 gradient. Quantitative analysis of single BMDC tracks revealed that wt and FHL2−/− BMDCs showed little directional migration when no chemokine gradient was applied (Fig. 3A, 3B control). Once the chemokine gradient was applied, wt and FHL2−/− BMDCs showed directed migration toward CCL19 (from the right). FHL2−/− BMDCs covered a significantly greater distance (mean distance, 100 μm; n = 90), measured as a direct line from their point of origin, than did wt BMDCs (mean distance, 53 μm; n = 75) (Fig. 3B). This was due, in part, to a less tortuous migratory path (Fig. 3A), as well as to a significantly increased mean migratory speed: 40 μm/h for wt BMDCs and 140 μm/h for FHL2−/− BMDCs (Fig. 3C). Furthermore, FHL2−/− BMDCs showed a greater degree of directional persistence (Fig. 3A). Similar differences between FHL2−/− and wt BMDCs were observed for immature and mature BMDCs, although the overall speed of mature BMDCs was greater (data not shown). Thus, time-lapse video microscopy confirmed our observations in Transwell and in vivo experiments (i.e., that FHL2 loss leads to increased migratory levels, as well as increased persistence and directionality).

**FHL2−/− BMDCs have fewer filopodia and increased Rac activation**

Because reorganization of the actin cytoskeleton is the basis for cellular migration (31), the cytoskeletal actin conformation of immature FHL2−/− and wt BMDCs was examined by immunofluorescent staining with phalloidin (Fig. 4A). The morphology of FHL2−/− and wt BMDCs differed significantly: although 90% of immature wt BMDCs on collagen showed long protruding filopodia and only <10% of cells were small and veiled (Fig. 4A, left panels), up to 50% of the cells in an FHL2−/− BMDC culture showed a small and veiled phenotype (Fig. 4A, right panels, quantification in Fig. 4B). The loss of filopodia and formation of vesicles or lamellipodia are associated with DC maturation and are thought to allow their rapid migration into secondary lymphoid organs (32–34).

The formation of protrusions at the leading edge of DCs during migration is controlled by Rac and Cdc42 (35). Cdc42 activity induces filopodia in immature DCs, whereas Rac activity induces the loss of filopodia and the formation of lamellipodia upon maturation (35, 36). Rac and Cdc42 belong to the family of small Rho GTPases, which cycle between active GTP-bound and inactive GDP-bound states (37, 38). To analyze whether the observed increased migration of FHL2−/− BMDCs was due to increased Rac1 activation, we determined the amount of Rac-GTP in immature wt and FHL2−/− BMDCs. We found a significantly greater amount of active Rac in FHL2−/− BMDCs compared with wt BMDCs (Fig. 4C), indicating that deregulated control of the Rac GTPase is responsible for the observed migratory changes in FHL2−/− BMDCs.

**FHL2 deficiency does not lead to spontaneous BMDC maturation**

Increased migration and the formation of lamellipodia are associated with DC maturation. To determine whether BMDCs spontaneously...
mature in the absence of FHL2, the expression of maturation markers, as well as the production of proinflammatory cytokines by immature wt and FHL2−/− BMDCs, was examined (Fig. 5). However, the expression levels of CCR7 (Fig. 2B), CD40, CD80, CD86, and MHC class I and II (Fig. 5A) did not differ between immature wt and FHL2−/− BMDCs. Furthermore, these markers were upregulated to the same extent on wt and FHL2−/− BMDCs by incubation with LPS or TNF-α (data not shown). Also, the ability to produce IL-12 and TNF-α upon maturation with LPS did not differ between wt and FHL2−/− BMDCs (Fig. 5B), indicating that FHL2−/− BMDCs are not constitutively mature. Together, these data show that the enhanced migratory capacity of FHL2−/− BMDCs toward CCL19 is associated with the formation of lamellipodia, but it does not correlate with their maturation status.

Ag uptake and presentation in BMDCs are not influenced by FHL2

The actin cytoskeleton, regulated by Rho GTPases, is also involved in Ag uptake and presentation by DCs (39, 40). Therefore, we compared Ag uptake by immature wt and FHL2−/− BMDCs. To this end, BMDCs were incubated with fluorescently labeled dextran or OVA for 30 min at 37°C or 4°C. However, we did not observe any significant differences between wt and FHL2−/− BMDCs, indicating that FHL2 does not influence Ag uptake in BMDCs (Fig. 6A). To test whether the loss of FHL2 affects Ag presentation by DCs, we incubated immature FHL2−/− and wt BMDCs with various concentrations of the protein OVA for 1 h, cocultured them with OVA-specific CD8+ (OTI) or CD4+ (OTII) T cells, and analyzed T cell proliferation (Fig. 6B) and IL-2 production after 24 h (Fig. 6C, 6D). There was no difference in the proliferation profile of CD8+ and CD4 T cells stimulated by FHL2−/− or wt BMDCs (Fig. 6B). At lower OVA concentrations, FHL2−/− BMDCs induced slightly greater IL-2 production by OTI and OTII T cells; however, these differences were not statistically significant. Together, based on these data, we concluded that although FHL2 influences the migratory behavior of DCs, it does not influence Ag uptake or presentation or T cell activation.

**SIPR1 is upregulated in FHL2−/− BMDCs**

In addition to the CCR7 ligands CCL19 and CCL21, the lysophospholipid S1P plays an important role in DC migration and positioning. The SIPR1−4 are differentially expressed depending on the maturation status of DCs (17), and this influences their migratory behavior. Therefore, we analyzed the expression pattern of SIPR1−4 in immature and mature wt and FHL2−/− BMDCs by qRT-PCR. Consistent with previous reports (15, 17), we found that after maturation, SIPR1 and SIPR3 mRNA levels were upregulated in wt BMDCs, whereas SIPR4 mRNA levels were diminished, and SIPR2 mRNA levels were unchanged (Fig. 7A). Interestingly, in immature and mature FHL2−/− BMDCs, we found significantly greater levels of SIPR1 mRNA, whereas SIPR2−4 mRNA levels did not differ significantly from the levels in the wt BMDCs. Because ligation of the SIPR1 by S1P in DCs is associated with migration initiation or enhancement (15), these data suggest that FHL2 regulates DC migration by inhibiting the expression of SIPR1.

**Downregulation of SIPR1 using siRNA abrogates the increased migratory speed of FHL2−/− BMDCs**

To investigate whether upregulation of SIPR1 in FHL2−/− BMDCs functions as an accelerating receptor (18), leading to the observed

---

**FIGURE 3.** Greater migratory speed and directionality toward a CCL19 gradient in FHL2−/− BMDCs compared with wt BMDCs. Wt and FHL2−/− BMDCs were generated as described. At day 7, immature BMDCs were harvested, and 105 BMDCs were left to adhere to collagen-coated IBIDI μSlides for 45 min at 37°C and were filmed simultaneously. After 45 min, a CCL19 gradient was established, and BMDCs were filmed for an additional 60 min. Time-lapse images were taken every 15 s. A, Single tracks of migrating BMDCs were analyzed using Track-it software. Mean distance (D) and speed (C) of migrating BMDCs were calculated using Track-it software. Values are depicted as mean ± SEM. The tracks of ≥70 BMDCs were analyzed in each group. Statistical significance was calculated using the Student t test: *p ≤ 0.05; **p ≤ 0.01; ***p ≤ 0.001. Representative of three independent experiments.
increased migration of FHL2−/− BMDCs, we suppressed expression of S1PR1 by transfecting immature wt and FHL2−/− BMDCs with S1PR1-specific or unspecific control siRNA (scrambled). As expected, in FHL2−/− BMDCs transfected with control siRNA, S1PR1 mRNA levels were significantly higher compared with wt BMDCs transfected with control siRNA (Fig. 7B). Furthermore, in wt and FHL2−/− BMDCs, we could successfully knock-down S1PR1 mRNA levels by transfection with S1PR1-specific siRNA. This led to 72% knock-down in wt BMDCs and to 75% knock-down in FHL2−/− BMDCs (Fig. 7B). Knock-down of S1PR1 in FHL2−/− BMDCs significantly reduced their migratory rate to about half that of the FHL2−/− BMDCs transfected with control siRNA, which equaled the migratory rate of wt BMDCs (Fig. 7C). Interestingly, although some S1PR1 knock-down was achieved in wt BMDCs, this did not influence their migratory rate. In a second approach, we used the selective S1PR1 antagonist SEW2871 (41), which leads to receptor internalization, to inhibit S1PR1 function in FHL2−/− BMDCs (Fig. 7D). Again, the increased migratory rate in FHL2−/− BMDCs was significantly reduced by S1PR1 inhibition, but this effect was only visible in FHL2−/− BMDC and not wt BMDCs.

Ligation of S1PR1 by additional S1P enhanced CCL19-induced BMDC migration to a minor extent in a Transwell assay (Supplemental Fig. 1B). This small increase was probably due to the fact that, even without the external addition of S1P to the culture medium, there is S1P in the medium. This indicates that the overexpression of S1PR1 in FHL2−/− BMDCs enhances their migration. Ag uptake, which is known to be independent of S1PR1, was not altered in FHL2−/− BMDCs (Fig. 6A); furthermore, it was not influenced by the addition of S1P (Supplemental Fig. 1A). Also, positioning of DCs in LNs and spleen was not altered in FHL2-deficient animals (data not shown). In summary, these data indicate that in FHL2−/− BMDCs, repression of S1PR1 is lost, leading to its overexpression, and that signaling via S1PR1 is responsible for the increased migratory phenotype of FHL2−/− BMDCs.

**Discussion**

Regulated migration of DCs is central to the induction of physiological immune responses and, possibly, to the maintenance of tolerance (42, 43). DCs have evolved specialized responses to specific external signals to regulate maturation, chemotaxis, migratory speed, Ag uptake, and Ag processing. Rho family GTPases are involved in the regulation of all of these processes. Several extracellular ligands control the migratory behavior of DCs. First, signaling through the chemokine receptor CCR7, by its ligands CCL19 and CCL21, controls chemotaxis via G protein-coupled receptors and MAPK family members and migratory speed via the activation of Rac or Cdc42 activity (44). Second, S1P signaling via S1PRs acts via heterotrimeric G proteins on members of the small GTPase family (Cdc42/Rac/Rho), triggering cytoskeletal rearrangements that influence cellular morphology and movement in DCs (15). We previously identified FHL2, a four-and-a-half LIM domain adapter molecule and transcriptional modulator, whose nuclear translocation is also mediated by S1P. Furthermore, we showed nuclear FHL2 to be involved in cell migration, wound healing, and cancer invasion (26–28). The role of FHL2 in these processes and its regulation by S1P-mediated signaling suggested that FHL2 might play a novel regulatory role in DC migration and triggered this study.

Indeed, in our initial experiments (Fig. 1), we found that stimulation of DCs with CCL19 led to a loss of nuclear localization of FHL2 only in mature DCs. Because maturation of DCs is tightly linked to a marked increase in their migratory propensity, dictated by the need for mature DCs only to cover long distances to convey Ag to draining LNs, a loss of FHL2 nuclear localization only in mature DCs is intriguing.

One mechanistic explanation may be a shift in the balance of Rho and Rac signaling in mature DCs caused by the strong upregulation of CCR7 expression: stimulation of mature DCs with CCL19 leads to increased Rac and decreased RhoA signaling downstream of CCR7 (45, 46). Because Rho signaling is required for nuclear localization of FHL2, strong CCR7 signaling can explain the redistribution of FHL2 to the cytoplasm.

To determine whether FHL2 plays a functional role in DC migration, we used BMDCs derived from FHL2-deficient mice, which showed a highly significantly increased migratory capacity in vitro and in vivo.

Enhanced migration of DCs is induced after their maturation in response to, for instance, bacterial or viral stimuli. Therefore, we assumed that the loss of FHL2 might lead to a constitutive maturation of FHL2-deficient BMDCs. However, the enhanced migratory property of FHL2−/− BMDCs did not correlate with their maturation status, because wt and FHL2−/− BMDCs expressed the same levels of maturation markers and inflammatory cytokines. Additionally, normal maturation could be induced in wt or FHL2−/− BMDCs, as measured by the upregulation of typical maturation markers, such as CD86, CD40, and MHC class I and II. This indicates that FHL2 regulates DC migratory function independently of DC maturation.
The molecular functions of FHL2 are 2-fold. On the one hand, it can bind to the actin cytoskeleton, ADAM-17, and α and β integrins (20–22, 25, 26, 29, 47–49), thereby functioning as a structural protein. On the other hand, FHL2 can translocate to the nucleus and act as a transcriptional coactivator or corepressor in a tissuespecific manner. We showed in fibroblasts that translocation of protein. On the other hand, FHL2 can translocate to the nucleus and act as a transcriptional coactivator or corepressor in a tissue-specific manner. We showed in fibroblasts that translocation of
FHL2 from the cytosol into the nucleus could be achieved by S1P-induced RhoA activation, but it does not involve the GTPases Cdc42 or Rac1 (21).

The CCR7 receptor was the most obvious target to be transcriptionally regulated by FHL2 that could explain the phenotype of FHL2<sup>−/−</sup> DCs; when upregulated, it can be responsible for the enhanced migratory properties of DCs (50, 51). However, CCR7 expression levels were not different between wt BMDCs and FHL2<sup>−/−</sup> BMDCs, indicating that FHL2 does not influence DC migration by regulating CCR7 expression levels.

The S1PR family is the other G protein-coupled system known to lead to the activation of small Rho GTPases (15, 52) and with a described function in DC migration. In DCs, S1P can bind to four receptors, which are differentially regulated during DC maturation (15, 17) and are thought to have opposing functions in cell migration. S1P binding to S1PR1 and S1PR3 usually promotes cell migration, via Gi pathways, and Rac activation, associated with lamellipodia formation (53). In contrast, ligation of S1PR2 inhibits cell migration via stimulating G<sub>12/13</sub> and Rho activation and antagonizing Rac, causing assembly of actin stress fibers (18, 52, 54). Czeloth et al. (16) showed that immature DCs lack expression of S1PR1, and they express only low levels of S1PR2 and S1PR4. Upon DC maturation, S1PR1 and S1PR3 are highly upregulated, promoting migration, whereas expression of S1PR2 and S1PR4 declines, which decreased the inhibition of migration (15).

**FIGURE 7.** Enhanced migration of FHL2<sup>−/−</sup> BMDCs is due to greater levels of S1PR1. A, mRNA was isolated from day-7 immature or day-9 TNF-α-matured wt and FHL2<sup>−/−</sup> BMDCs. The expression of S1PR1–4 was determined by qRT-PCR and normalized to the expression of 18s RNA. Gray bars, wt BMDCs; black bars, FHL2<sup>−/−</sup> BMDCs. B and C, A total of 4 × 10<sup>6</sup> day-7 wt and FHL2<sup>−/−</sup> BMDCs were electroporated with siRNA targeted against S1PR1 or nontargeting siRNA. B, Two days later, RNA was isolated from electroporated BMDCs and converted to cDNA. The knock-down of S1PR1 was quantified by RT-PCR. C, In parallel, the electroporated BMDCs were analyzed for their migratory behavior in a Transwell assay. D, Day-7 wt and FHL2<sup>−/−</sup> BMDCs were incubated for 16 h with 10 µM SEW2871, harvested, and analyzed for their migratory behavior in a Transwell assay. Statistical significance was calculated using the Student t test: *p < 0.05; **p ≤ 0.01; ***p ≤ 0.001. Representative of three independent experiments.

FHL2 from the cytosol into the nucleus could be achieved by S1P-induced RhoA activation, but it does not involve the GTPases Cdc42 or Rac1 (21).

The CCR7 receptor was the most obvious target to be transcriptionally regulated by FHL2 that could explain the phenotype of FHL2<sup>−/−</sup> DCs; when upregulated, it can be responsible for the enhanced migratory properties of DCs (50, 51). However, CCR7 expression levels were not different between wt BMDCs and FHL2<sup>−/−</sup> BMDCs, indicating that FHL2 does not influence DC migration by regulating CCR7 expression levels.

The S1PR family is the other G protein-coupled system known to lead to the activation of small Rho GTPases (15, 52) and with a described function in DC migration. In DCs, S1P can bind to four receptors, which are differentially regulated during DC maturation (15, 17) and are thought to have opposing functions in cell migration. S1P binding to S1PR1 and S1PR3 usually promotes cell migration, via Gi pathways, and Rac activation, associated with lamellipodia formation (53). In contrast, ligation of S1PR2 inhibits cell migration via stimulating G<sub>12/13</sub> and Rho activation and antagonizing Rac, causing assembly of actin stress fibers (18, 52, 54). Czeloth et al. (16) showed that immature DCs lack expression of S1PR1, and they express only low levels of S1PR3. Upon DC maturation, S1PR1 and S1PR3 are highly upregulated, promoting migration, whereas expression of S1PR2 and S1PR4 declines, which decreased the inhibition of migration (15).

**FIGURE 8.** Model of FHL2-mediated repression of S1PR1 expression and regulation of DC migration. A, In immature wt DCS, signaling via S1PR2 leads to Rho activation, causing translocation of FHL2 into the nucleus, where FHL2 represses transcription of S1PR1 by binding to the transcription factor of the promoter region. Additionally, low expression levels of CCR7R blocks Rac activity. B, Maturation of DCS leads to downregulation of S1PR2. Subsequent diminished Rho activation inhibits nuclear translocation of FHL2 into the nucleus, which has the same effect as FHL2 deficiency (i.e., reduced repression of S1PR1 transcription). S1P signaling via greater S1PR1 levels increases Rac activation, which leads to an increased migratory rate of mature or FHL2<sup>−/−</sup> DCs.
FHL2-deficient DCs showed no change in the expression profile of S1PR2–4 compared with wt DCs. This is consistent with our observation that S1PR3-dependent Ag uptake and presentation (17, 35, 55) were not altered in FHL2−/− DCs. However, we found that the expression of S1PR1 was highly upregulated in immature and mature FHL2−/− DCs. We conclude that this accounts for the increased migratory properties of immature and mature FHL2−/− DCs, which is supported by our finding that siRNA-mediated knock-down of S1PR1 or antagonist-mediated internalization of S1PR1 in FHL2−/− BMDCs decreased their migratory rate to that of wt BMDCs.

Forced overexpression of S1PR1 was reported to cause increased levels of active GTP-bound Rac (56, 57), which we also observed in FHL2−/− BMDCs. Thus, the increased migratory rate and formation of lamellipodia found in FHL2−/− DCs are most likely a direct result of the increased levels of active GTP-bound Rac.

The question remains how FHL2 regulates DC migration and S1PR1 expression under physiological circumstances. In fibroblasts, we showed that S1P signaling causes RhoA- and ROCK-dependent translocation of FHL2 into the nucleus (21), where it can function as coactivator or corepressor for several transcription factors (22–25). This is most likely due to S1PR2-mediated activation of ROCK and A kinase (18) (Fig. 8).

In summary, we propose the following chain of events (Fig. 8). In immature wt DCs, S1PR2 is highly expressed, and CCR7 expression is relatively low. This leads to active RhoA signaling and nuclear translocation of FHL2 (Fig. 8A). In the nucleus, FHL2 inhibits the transcription of S1PR1. During DC maturation, S1PR2 expression is downregulated, and CCR7 is upregulated (15). This reduces nuclear translocation of FHL2 into the nucleus, which has the same effect as FHL2 deficiency (Fig. 8B) (i.e., a reduced repression of S1PR1 transcription by FHL2, which, in turn, leads to an upregulation of S1PR1). Higher S1PR1 levels lead to increased S1P signaling and an additional increase in Rac activation. This, in turn, leads to an increased migratory rate of mature or FHL2−/− DCs.

Interestingly, other DC functions, such as Ag uptake and presentation, were not altered in FHL2−/− DCs compared with wt DCs. Although these processes are also regulated by Rho GTPases, it was shown that Cdc42 (35, 55), rather than Rac, is involved in that, furthermore, this process is regulated via S1PR3 (17), which is not differentially expressed in wt and FHL2−/− DCs. Our data identify FHL2 as a key regulator of DC migration by switching between S1PR2 expression in immature DCs and S1PR1 expression in mature DCs.

Acknowledgments

We thank Roland Schüle for kindly providing the anti-FHL2 polyclonal Ab.

Disclosures

The authors have no financial conflicts of interest.

References