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Sphingosine-1-Phosphate Mediates Migration of Mature Dendritic Cells[1]

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Sphingosine-1-phosphate (S1P) represents a potent modulator of diverse cellular activities, including lymphocyte trafficking and maintenance of lymphocyte homeostasis. The five known receptors for S1P ($S1P_{1–5}$) belong to the family of G protein-coupled receptors. Upon binding S1P, they act downstream via heterotrimeric G proteins on members of the small GTPase family (Cdc42/Rac/Rho), evoking a S1P receptor-dependent activation pattern of Cdc42, Rac, and Rho, respectively. This, in turn, triggers cytoskeletal rearrangements determining cellular morphology and movement. In this study we investigated the effects of S1P on murine dendritic cells (DC). Mature DC, but not immature in vitro differentiated DC, were found to migrate to S1P, a phenomenon that correlated to the up-regulation of $S1P_1$ and $S1P_3$ in maturing DC. The same pattern of S1P receptor regulation could be observed in vivo on skin DC after their activation and migration into the lymph node. The migration-inducing effect of S1P could be severely hampered by application of the S1P analogon FTY720 in vitro and in vivo. A similar, yet more pronounced, block was observed upon preventing Cdc42/Rac and/or Rho activation by specific inhibitors. These results suggest that S1P-mediated signaling plays a pivotal role in the life cycle of DC. ***The Journal of Immunology,*** **2005, 175: 2960–2967.**

Dendritic cells (DC)[3] represent the most important class of APCs in the immune system, holding a key position in instructing adaptive immune responses or tolerance induction (1). Immature DC are present in nearly any tissue, where they continuously collect and process Ag. At a low, but constant, rate, these DC migrate to their corresponding draining lymph node (LN) to maintain peripheral tolerance by anergizing matching T cells (2). However, Ag uptake coupled to DC stimulation via danger signals will trigger their maturation and enhanced migration to the LN, wherein T cells will be activated to initiate adaptive immune responses. The transition from an immature to a mature DC is accompanied by up-regulation of activation markers such as CD40, CD83, and CD86. Commencing CCR7 expression allows the maturing DC to direct migration to the LN (3, 4), whereas up-regulation of MHC class II (MHCII) expression ensures efficient Ag presentation to T cells inside the LN. The maturation of DC is inevitably correlated with huge phenotypic changes. Thus, an immature DC residing in tissue such as skin is sessile and possesses multiple dendrites, enabling efficient Ag uptake. Upon triggering migration to the LN, the DC retract dendrites and become highly motile. Once inside the LN, DC rebuild dendrites and lose motility again (5, 6). Little is known about the mechanisms controlling these transitions. However, it was demonstrated that the small GTPases, Cdc42, Rac, and Rho, account for related phenotypic changes in various immune cells by manipulating actin skeleton reorganization (7–12).

Sphingosine-1-phosphate (S1P), a metabolite produced by many cell types, such as platelets, and present in high concentrations in serum (up to 1 μM), is increasingly recognized as a potent lipid mediator important in various physiological processes (13, 14). In the immune system, S1P was shown to possess chemoattractive properties on several cell types (15–18). S1P regulates the egress of B and T cells from LN, constituting one of the check points controlling lymphocyte homeostasis (18). In addition to this, S1P is required for correct positioning of marginal zone B cells inside the spleen (15). S1P exerts most of its effects by binding to five related G protein-coupled receptors, termed $S1P_{1–5}$, possessing distinct expression profiles and affinities toward S1P (19, 20). Interestingly, the five S1P receptors differ in their downstream effects (21, 22). Although $S1P_1$ predominantly activates the Rac/Cdc42 pathway, thereby sustaining cell migration, $S1P_2$ has been shown to inhibit locomotion via Rho activation. The range of $S1P_1$ and $S1P_2$ is extended by $S1P_3$, affecting Rac as well as Rho activity (23–26). The compound FTY720 represents an S1P analogon binding to four of the five S1P receptors, thereby diverting S1P signaling (19, 20). When administered in vivo, the immunomodulator FTY720 inhibits egress of lymphocytes from secondary lymphoid organs and, thus, recirculation of cells to peripheral inflammatory tissue. Therefore, the resulting lymphopenia substantially impairs immune responses, and FTY720 was assigned considerable potential as a therapeutic tool in organ transplantation (27, 28).

In vitro studies mimicking DC differentiation greatly facilitated elucidation of the steps in the DC maturation program (1). We studied the influence of S1P and its analogon FTY720 on DC migration using in vitro differentiated murine DC. Our results demonstrate that mature, but not immature, DC migrated to S1P. Inhibitors specifically preventing either Rac/Cdc42 or Rho activation hampered migration to a substantial degree, whereas blocking both types of small GTPases resulted in a complete failure to migrate to S1P. FTY720 did not trigger DC migration per se. Preincubation of mature DC compromised, yet did not abolish, their S1P-induced migration. In vitro as well as in vivo DC maturation/

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[3] Abbreviations used in this paper: DC, dendritic cell; DAPI, 4′,6-diamido-2-phenylindole hydrochloride; HPRT, hypoxanthine guanine phosphoribosyl transferase; LN, lymph node; MHCII, MHC class II; S1P, sphingosine-1-phosphate.

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migration correlated with a profound up-regulation of $S1P_1$ and $S1P_3$ expression. FTY720 interfered with DC migration to the LN after FITC painting, suggesting that part of the immune modulation accomplished by FTY720 may be caused by impaired DC migration.

Materials and Methods

Reagents

FTY720 was a gift from Volker Brinkmann (Novartis). S1P was purchased from Sigma-Aldrich. Chemokines were purchased from R&D Systems.

FACS analysis

Cells were analyzed on a FACSCalibur (BD Biosciences) after having been stained with the following mAbs: FITC-labeled anti-CD11c, biotinylated anti-MHCII (1Ab, 1Ad; BD Biosciences); PE-labeled anti-CD86 (Immunotech); and anti-CD207 mAb clone 929F3 (provided by S. Saeland, Schering-Plough, Dardilly, France). As secondary reagents, Cy5-labeled mouse anti-rat, Cy5-labeled goat anti-human IgG (Dianova), and PerCP-labeled streptavidin (BD Pharmingen) were used. CCR7 staining was performed using an ELC-human IgG1 fusion protein (provided by S. Krautwald, University of Kiel, Kiel, Germany). Cell sorting was performed on a FACSAria (BD Biosciences).

Expression and purification of the cell-accessible C3-fusion toxin

Exoenzyme C3 from *Clostridium botulinum* was administered to mature DCs using the *C. botulinum* C2 toxin as a delivery system (29). In brief, GST fusion proteins were expressed by *Escherichia coli* harboring plasmid pGEX2T-C2II or pGEX2T-$C2I^{1-225}$-C3 and precipitated from the cell lysate using a 50% slurry of glutathione-Sepharose 4B in PBS. $C2I^{1-225}$-C3 was liberated from the agarose matrix by treatment with thrombin at room temperature for 1 h (3.25 National Institutes of Health units/ml bead suspension). GST-C2II was activated by treatment with trypsin at room temperature for 20 min. Trypsin was then inactivated by addition of trypsin inhibitor.

Purification of C. difficile *toxin B (toxin B) and* C. sordellii *lethal toxin (lethal toxin)*

Toxin B and lethal toxin were purified from culture supernatants of *C. difficile* strain VPI 10463 or *C. sordellii* strain 6018, respectively (30). In brief, a dialysis bag containing 900 ml of 0,9% NaCl in a total volume of 4 l brain heart infusion (Difco) was inoculated with 100 ml of an overnight culture of *C. difficile* or *C. sordellii* and grown under microaerophilic conditions at 37°C for 72 h. Proteins were precipitated from the culture supernatant by ammonium sulfate at 70% saturation. The precipitates were dialyzed against Tris-HCl, pH 7.5, buffer overnight and loaded onto a MonoQ columns (Amersham Biosciences). The toxins were subsequently eluted with Tris-HCl, pH 7.5, buffer containing 500 mM NaCl.

Treatment of cells with toxins

Cells were incubated with C3 fusion toxin (a mixture of 2.5 μg/ml qjC2I^{1-225}-C3 and 10 μg/ml C2IIa), toxin B (100 pM), lethal toxin (300 ng/ml), or pertussis toxin (100 ng/ml; Sigma-Aldrich) at 37°C overnight.

Mice

Eight- to 12-wk-old BALB/c and C57/BL6 mice were purchased from Charles River. CCR7-deficient mice (3) were backcrossed for seven generations to a C57BL/6 background and kept under specific pathogen-free conditions. Animal care and experiments were conducted in compliance with institutional guidelines and the German law for welfare of animals.

In vitro differentiation of DC

DC were prepared according to a modified protocol described by Lutz et al. (31). In brief, 2×10^6 bone marrow cells from tibia and femur of C57/BL6 mice were cultured in RPMI 1640, 10% FCS, β-ME, glutamine, and penicillin/streptomycin supplemented with 100–200 ng/ml GM-CSF produced by a recombinant cell line (4) for 7 or 8 days. Cells were matured for 2 additional days with 1 μg/ml PGE_2 (Sigma-Aldrich) and 30 ng/ml TNF-α (PeproTech).

FITC skin painting

BALB/c mice were kept for 4 days with a water supply containing 3.5 μg of FTY720/ml. The mice were then anesthetized, and the outer skin of the ears was painted twice with 0.1% FITC in acetone/dibutylphthalate (1/1) or vehicle alone (Sigma-Aldrich). After 24 h the mice were killed; the draining (facial) LN were collected, and single-cell suspensions were prepared. The proportions of cells positive for CD11c, MHCII (1Ad), langerin (CD207), and FITC were analyzed by flow cytometry.

Migration assays

Most migration assays were conducted using a 48-well Boyden chamber and polycarbonate membranes possessing 5 μm pores (NeuroProbe). The membranes were coated with 20 μg/ml murine collagen IV (BD Biosciences) in PBS overnight. Migration experiments were performed for 75–90 min, applying $1–2 \times 10^5$ cells in RPMI 1640/20 mM HEPES, pH 7.4, in the upper well and the corresponding chemoattractant, as indicated, in the lower well. After completion, membranes were removed, wiped on the side facing the upper well, and stained with DiffQuick (Dade Behring). Membranes were then scanned, and the colorimetric densities of the spots were determined using the Genetools software package from Syngene (VWR International). Colorimetric densities of spots corresponding to wells with no chemoattractant added were defined as a migration index of 1. Transwell migrations were performed with 3×10^5 cells/well for 2.5–3 h using the medium described above, applying collagen IV-coated Transwell inserts equipped with 5-μm pores (Corning).

RNA preparation and RT-PCR

Total RNA was prepared using the TRIzol method (Invitrogen Life Technologies) according to the manufacturer's protocol. RNA was reverse transcribed (SuperScript II reverse transcriptase; Invitrogen Life Technologies) using random hexamer primers. PCR was performed using Takara *Taq* (Takara Bio) polymerase/buffer system and the following primers. S1P1 sense, 5′-tct ctga cta tgg gaa cta tg-3′; S1P1 antisense, 5′-cca gga tga ggg aga tga c-3′; S1P2 sense, 5′-cct taa ctc act gct caa tcc-3′; S1P2- antisense, 5′-gct gga aga tag gac aga cag-3′; S1P3 sense, 5′-aca agg tcc ggg tgc tga g-3′; S1P3 antisense, 5′-gta atg ttc ccg gag agt gtc-3′; S1P4 sense, 5′-gct atg ccc att gtc cag tag-3′; S1P4 antisense, 5′-gga cca ggt act gat gtt cat g-3′; hypoxanthine guanine phosphoribosyl transferase (HPRT) sense, 5′-aag cca aat aca aag cct aag-3′; and HPRT antisense, 5′-tga aag tgg gaa aat aca gc-3′.

The expression of GAPDH, S1P1, S1P2, S1P3, and S1P4 was also analyzed using a LightCycler 2.0 (Roche) and the Fast Start DNA Master Plus SYBR Green Kit (Roche). Gene expression was standardized with serial dilutions of genomic DNA with calculated copy numbers, except for S1P3. Because the primer detecting this gene product encompasses two exons, genomic DNA could not be used as a template. Therefore, the S1P3 PCR product was purified, and the DNA concentration was quantified with the Nanodrop ND-1000 spectrophotometer (PegLab). Copy numbers were calculated, and a standard curve was computed. The relative gene expression (fold of GAPDH) of each gene was calculated by dividing copy numbers of the appropriate gene by copy numbers of GAPDH. Standard curves were derived analyzing triplicate determinations; samples were amplified in duplicate. The following primers were used (5′->3′): GAPDH forward, TGCACCACCAACTGCTTAG; GAPDH reverse, GGATGCAGGGAT GATGTTC; S1P1 forward, GGAGGTTAAAGCTCTCCGC; S1P1 reverse, CGCCCCGATGTTCAAC; S1P2 forward, GTGACGGGACGCA GAGGT; S1P2 reverse, AAATGTCGGTGATGTAGGCATATG; S1P3 forward, GGAGCCCCTAGACGGGAGT; S1P3 reverse, CCGACT GCGGGAAGAGTGT; S1P4 forward, CCACAGCCTCCTCATTGTC; and S1P4 reverse, TCAGCATCCCTAGCCCTC.

Isolation of DC from skin and LN

Ears of C57/BL6 mice were split into halves and incubated for 1 h at 4°C in PBS containing 20 mM EDTA. After washing with PBS/3% FCS, the skin was cut into small pieces and digested at 37°C for 1 h with 1 mg/ml collagenase/dispase (Roche) in RPMI 1640/10% FCS containing 20 mM HEPES according to the manufacturer's protocol. Skin pieces and cell suspension were processed through a cell strainer (BD Biosciences) and washed with PBS/3% FCS. The resulting cell suspension was stained with mAbs to CD11c, MHCII, CD80 (all from BD Biosciences), B220 (clone TIB146), and 4′,6-diamido-2-phenylindole hydrochloride (DAPI) nuclear dye. $CD11c^+MHCII^+CD80^+B220^-DAPI^-$ cells were sorted twice using a FACS-Aria (BD Biosciences). This resulted in population purities of ≥98%. The draining LN of FITC skin-painted mice were isolated 24 h after painting, cut into pieces, and digested at 37°C for 30–45 min with 0.5 mg/ml collagenase A and 50 U/ml DNase I in RPMI 1640/HEPES/10% FCS (Roche). A single-cell suspension was prepared, and the cells were stained with mAb against CD11c and MHCII. $CD11c^+MHCII^{high}FITC^+DAPI^-$ DC were sorted as described above for skin DC. After sorting, cells were lysed,

and RNA was prepared using the Stratagene RNA Microprep kit. RNA integrity was analyzed applying an Agilent bioanalyzer.

Results

In vitro-generated mature DC migrate toward S1P

We generated murine DC by cultivating bone marrow cells in the presence of GM-CSF. Unstimulated immature DC on day 7 of culture and matured DC after 2 days of permanent treatment with TNF-α/PGE_2 were used in Transwell as well as Boyden chamber experiments to analyze their migration to S1P. Mature DC were discriminated from immature DC by virtue of their expression profile (immature, $CCR7^-MHCII^-CD86^{low}$; mature, $CCR7^+MHCII^{high}CD86^{high}$; data not shown). In contrast to human DC, the established protocols used to generate bone marrow-derived murine DC tend to yield less homogeneous populations of immature vs mature DC. Thus, the fraction of cells with a mature phenotype varied between 50 and 70% after TNF-α/PGE_2-induced differentiation. Similarly, the culture of immature DC on day 7 contained 5–15% of cells with a mature phenotype, probably due to spontaneous maturation caused by endogenous production of TNF-α. As shown in Fig. 1*A* (dashed line), a mature population used in the migration assays contained phenotypically immature as well as mature cells, as evidenced by differential expression of CCR7. Notably, transmigrated cells were exclusively recruited from the mature fraction ($CCR7^+$) of loaded cells (Fig. 1*A*, solid line). Their mature phenotype was confirmed when analyzing CD86 or MHCII expression (data not shown). The maximal migration caused by S1P in our experimental setup is comparable to that elicited by 5–10 nM CCL21 (Figs. 1*B* and 2*C*), a ligand for CCR7 known as a very effective chemoattractant for mature DC (32). Our observations were corroborated by applying a range of S1P concentrations in the assays. Although mature DC displayed a concentration-dependent optimum of migration (500–1000 nM; Fig. 1*B* and data not shown), immature DC remained unresponsive to S1P over the entire concentration range (Fig. 1*B*). Immature DC did not lack inherently any migratory potential, because they were efficiently attracted by the CCR5 ligand CCL3 (Fig. 1*B*). The migration of mature DC to S1P was found to be independent of CCR7, because DC matured from CCR7 knockout mice possess a similar migration pattern toward S1P as wild-type cells (data not shown).

DC migration toward S1P is hampered by FTY720

The immunomodulating effects of FTY720 are most likely caused by the sequestration of lymphocytes into secondary lymphoid organs, thereby withdrawing immune surveillance from the periphery (33). Available evidence suggests that FTY720 binding triggers internalization of $S1P_1$ (18, 34, 35), evoking a strikingly diminished responsiveness of this receptor to its natural ligand S1P. Therefore, we studied the effects of this drug on DC migration. Over the range of concentrations investigated, mature DC remained refractory to migration to FTY720 (Fig. 1*B*). Intriguingly, preincubation of the cells overnight with increasing concentrations of FTY720 led to a concomitant decrease in migration toward S1P up to 60% compared with untreated DC (Fig. 2*A*). However, the cells did not become totally ignorant of S1P. Instead, FTY720 incubation caused a shift in the response curve toward higher concentrations of S1P. Dose-response profiles of untreated or FTY720-treated mature DC ran into identical saturation plateaus at S1P concentrations beyond 2 μM (Fig. 2*B*), yet the loss of sensitivity to S1P seemed most pronounced at those concentrations of S1P reported to be physiologically relevant. It has been suggested that FTY720 might induce apoptosis of the treated cells (36), explaining its immunomodulating effects. However, induction of apoptosis seems to require unphysiologically high concentrations of FTY720. We examined the viability of cells incubated in the presence of FTY720 by propidium iodide uptake or trypan blue staining. Noticeable differences between treated (up to 2 μM FTY720) and untreated DC were never observed by these criteria, indicating an absence of enhanced cell death or late phase apoptosis (data not shown). This idea was also supported by the finding that FTY720 did not reduce migration to the chemokine CCL21 (Fig. 2*C*). Furthermore, FTY720 administration exerted no influence on the expression level of DC markers such as CD11c, CD86, MHCII, or CCR7 (data not shown). These data suggest that FTY720 treatment did not generally inhibit migration or maturation of DC; instead, it induced an alteration in sensitivity to S1P. Therefore, our findings support the hypothesis that FTY720 acts through specific internalization of S1P receptors, rendering the cells less responsive to an S1P signal (18, 34).

Influence of FTY720 on DC migration in vivo

To test whether FTY720 shows an effect on in vivo migration of DC, we conducted FITC skin-painting experiments with untreated BALB/c mice and animals that received FTY720 for 4 days. In these experiments, the ears of the mice were painted with an organic solvent containing FITC. Those DC residing in the dermis and epidermis taking up the stain were induced to mature. The migration of DC was monitored 24 and 48 h later by analyzing the draining LN. Immigrated DC were traced as side $scatter^{high}$ and $FITC^+$ cells (Fig. 3, *A* and *B*) and were analyzed for expression of additional surface markers. Regardless of FTY720 treatment, all

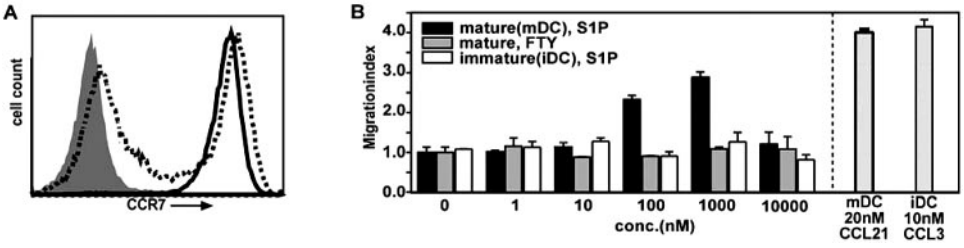

FIGURE 1. Migratory responses of in vitro-derived DC to S1P and FTY720. Bone marrow-derived DC were differentiated in the presence of GM-CSF for 7 days (immature DC) and induced to mature for 2 additional days by adding a mixture of TNF-α/PGE_2 (mature DC). *A*, Mature $CCR7^+$ cells migrate to S1P. In vitro-generated DC induced to mature for 2 days (day 9; dashed line), containing both phenotypically mature ($CCR7^+$) and immature ($CCR7^-$) DC, were subjected to Transwell migration toward 500 nM S1P. Reanalysis of the transmigrated cells (solid line) revealed their mature phenotype ($CCR7^+$). The isotype control of the cells used for migration is shown as a shaded area. *B*, Migration assays were performed in a Boyden chamber. Immature DC (□) were unresponsive to S1P over the tested range of concentrations, but migrated well to 10 nM CCL3 (▨). Mature DC migrated dose-dependently to S1P (■); in contrast, FTY720 itself did not evoke chemotaxis (▨). Migration of mature cells to 20 nM CCL21 is shown as a positive control (▨). Experiments were performed in duplicate, and one representative of at least three independent experiments is shown.

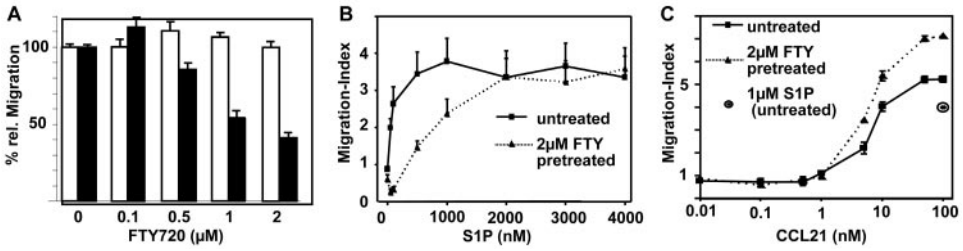

FIGURE 2. Influence of FTY720 on migratory responses of mature in vitro-generated DC. DC were generated and matured as described in Fig. 1 and used in Boyden chamber migration experiments. *A*, Mature DC were incubated overnight in the presence or the absence of distinct concentrations of FTY720. The subsequent migration toward 750 nM S1P (■) was found to be reduced dose-dependently down to 40% of the untreated control value, whereas the migration to 20 nM CCL21 (□) was slightly enhanced. *B*, Mature DC were incubated overnight with or without 2 μM FTY720. Subsequently, their migration to various concentrations of S1P was analyzed. Concentrations of up to 2 μM S1P, FTY720-treated cells (dotted line) were less sensitive to S1P than untreated cells (solid line). *C*, Untreated DC (solid line) or DC pretreated overnight with FTY720 (dashed line) were used in migration assays toward CCL21. Migration of untreated cells toward 1 μM S1P was included as a control (encircled asterisk). Experiments were performed at least in duplicate. *A* and *B*, Pooled data from three independent experiments are shown. *C*, One representative experiment of three independent experiments is shown.

FITC$^+$ DC were distinguished by high expression levels of MHCII and were positive for CD11c (Fig. 3*D*). A minority of these cells coexpressed CD207 (langerin), which classified them as Langerhans cells (37) that emerged from the epidermis (Fig. 3*C*). Comparing the expression of costimulatory molecules on DC in skin draining lymph node 24 h after FITC painting, we identified recently immigrated, FITC-positive DC as fully matured because they are distinguished by high levels of CD80 and CD86. In contrast, FITC$^-$ DC apparently possess a semimature phenotype expressing intermediate levels of CD40, CD80, and CD86 (Fig. 3*E*). The expression level of any of these markers on FITC$^-$ or FITC$^+$ DC remained unaffected by FTY720 treatment (data not shown).

In line with the in vitro data presented above, we observed that the number of cells that migrated into the LN was significantly reduced after FTY720 application (Fig. 3*F*; ~60% of the level found in untreated mice). This effect was even more pronounced after 48 h, because only 40% of the DC found in untreated mice reached the lymph node in mice treated with FTY720 (Fig. 3*F*; note the cumulative effect of time on DC number). Interestingly, the proportion of Langerhans cells in the migrated FITC$^+$ DC population was not altered in the presence of this drug (untreated mice, 8.6 ± 0.4%; FTY720-treated mice, 8.9 ± 0.4%; mean ± SEM; 10 lymph nodes/group).

FTY720 treatment might trigger an unspecific exodus of skin-resident DC, thereby diminishing the pool of migration-competent cells in the FITC skin-painting experiments. Therefore, the number of DC residing in the epidermis after FITC skin painting in wild-type mice and those fed with FTY720 for 4 days was analyzed by means of MHCII-positive cells. It was obvious that the number of epidermal DC in both groups remained unaltered. The mean number of DC settling the epidermis added up to ~870 cells/mm^2 (untreated, 872 ± 113 (n = 11); treated, 869 ± 91 (n = 9); mean ± SEM) in both groups.

S1P receptor expression on in vitro differentiated DC

Although S1P also acts as a cell internal second messenger (38), most of its effects are transmitted via extracellular binding to S1P receptors formerly addressed as endothelial differentiation gene receptors. Hence, the expression profile of S1P receptors was determined in immature as well as mature DC. Because Abs that can detect the single S1P receptor are not available, we first established a semiquantitative PCR to study the expression of $S1P_{1-5}$. RNA was prepared from FACS-sorted, in vitro-generated DC populations. Unstimulated immature DC on day 7 of culture were stained for CD11c, MHCII, and CCR7, and then sorted. Immature cells that expressed CD11c, but no CCR7, and low to intermediate levels of MHCII were collected (purity of fraction, >95%). Mature DC were sorted on day 9 of culture after stimulation with TNF-α/PGE_2 on day 7. More than 90% of the sorted cells were CD11c$^+$, MHC II^{high}, and CCR7$^+$. The PCR products shown in Fig. 4 reflect the relative expression levels of each S1P receptor obtained with serially diluted cDNA templates representing either immature or mature DC. To ensure that roughly equal amounts of RNA-derived template DNA were used, the transcript of the murine HPRT gene was titrated simultaneously. Most strikingly, an $S1P_1$-specific transcript was undetectable in immature DC at the given experimental conditions, whereas its induction in mature DC gave rise to a readily visible PCR product. Both immature and mature DC express $S1P_2$, $S1P_3$, and $S1P_4$, whereas $S1P_5$ could not be detected in any case (data not shown). $S1P_2$ and $S1P_4$ appeared unaltered or slightly down-regulated due to maturation. $S1P_3$, already expressed in immature cells at detectable levels, was observed to be substantially up-regulated upon maturation. Given that $S1P_2$ and $S1P_4$ mRNA levels remained almost unaffected by maturation, $S1P_3$ and particularly $S1P_1$ expression on mature DC seemed to enable in vitro migration of mature DC toward S1P.

Induction of $S1P_1$ after activation of skin DC in vivo

To further investigate the importance of S1P signaling for DC migration and maturation in vivo, we compared the expression of S1P receptors 1, 2, 3, and 4 of DC isolated from untreated skin and of FITC$^+$ LN DCs isolated 24 h after FITC skin painting. Skin DC (CD11c$^+$MHCII$^+$CD80$^+$B220$^-$) and LN DC (FITC$^+$MHCII^{high}CD11c$^+$) were sorted twice by flow cytometry to obtain pools of >98% homogeneity (Fig. 5*A* and data not shown). mRNA expression of the S1P receptors of the given DC populations was analyzed by quantitative RT-PCR and referred to that of GAPDH. Compared with GAPDH, we found weak to moderate expression of all receptors on DC isolated from skin. In contrast, once migrated to the draining LN, $S1P_1$ expression was massively induced in the DC (~38-fold), whereas a slight up-regulation (2.4-fold) was observed for $S1P_3$. In contrast, the expression of $S1P_2$ and $S1P_4$ remained at a constant level. Taken together, these data correspond strikingly to the findings obtained for in vitro differentiated DC (see Fig. 4) and indicate that $S1P_1$ along with $S1P_3$ play important roles in the migration of DC from the periphery to lymphoid organs.

Influence of toxins on DC migration

To gain further insight into the signal transduction caused by S1P receptor engagement, we tested several specific inhibitors of cell internal signal transduction molecules for their influence in migration assays. Migration to the CXCR4 ligand CXCL12 was included in the assays as a control, because it is known that CXCL12 represents a potent chemoattractant for mature DC (32).

Preincubation of mature DC with pertussis toxin, a selective inhibitor of the heterotrimeric G protein $G_{\alpha i}$, resulted in complete abrogation of migration to S1P and CXCL12 (see Table I), demonstrating that both agents signal via $G_{\alpha i}$ to initiate migration. The small GTPases of the Rho family, Rac, Cdc42, and Rho, integrate upstream signals, converting them into cytoskeleton remodeling activity, which, in turn, regulates dynamic cellular processes such as motility, morphology, and endocytosis (7, 39). Incubation of mature DC with *Clostridium difficile* toxin B (toxin B), which inhibits Cdc42, Rac, and Rho (40), entirely blocked responses to CXCL12 and S1P, confirming that migration depends on integral signal transmission by small GTPases. Exoenzyme C3 from *C. botulinum* (C3 exoenzyme) specifically ADP-ribosylates RhoA/B/C (40) and was found to hamper the migration toward S1P and CXCL12 significantly (>80%). A comparable effect was observed when cells were treated with *Clostridium sordellii* lethal toxin (lethal toxin), which prevents activation of Rac and Cdc42, but not that of Rho (41). These results indicate that the migration of mature DC is crucially dependent on 1) intact $G_{\alpha i}$ signaling and 2) the simultaneous activity of both types of GTPases, Rac/Cdc42 and Rho, respectively.

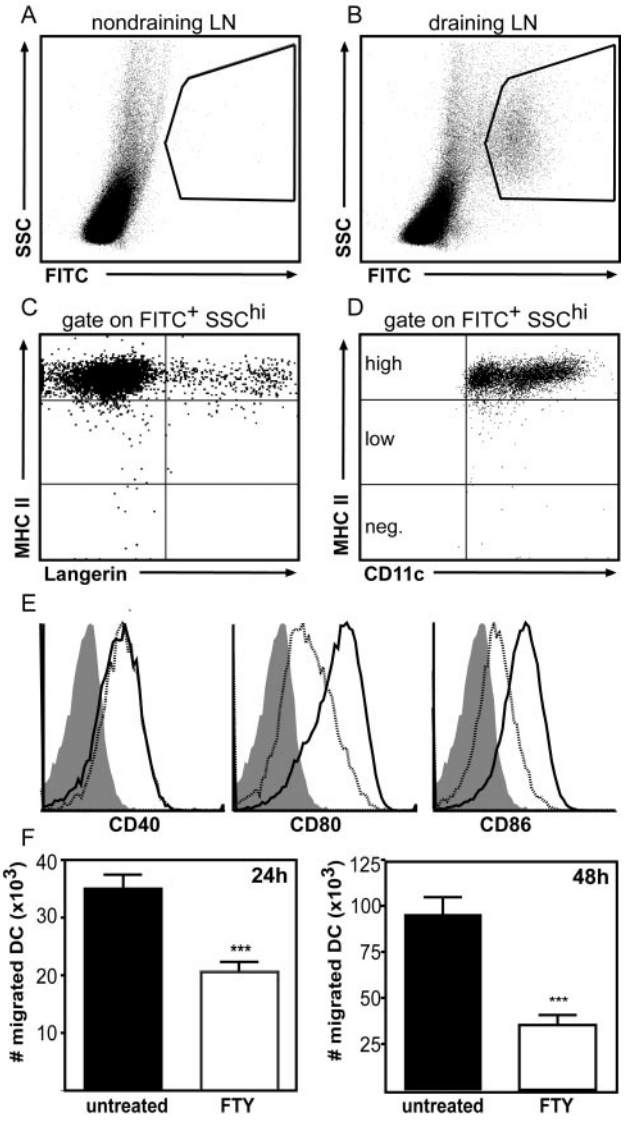

FIGURE 3. Influence of FTY720 on in vivo migration of DC in FITC skin-painting experiments. BALB/c mice were painted at the outer side of the ears with 0.1% FITC in dibutylpthalate/acetone. Twenty-four hours later the draining (facial) LN was analyzed. Migrated DC appear as side scatter^{high}FITC^{+} cells in the draining (*B*), but not in nondraining, inguinal LN (*A*). A small proportion of the FITC^{+} DC are Langerhans cells, as evidenced by the coexpression of MHCII and Langerin/CD207 (*C*), whereas all migrated cells show expression of MHCII and CD11c (*D*). *E*, Expression of CD40, CD80, and CD86 on immigrated FITC^{+} DC (solid line) and resident FITC^{-} DC (dashed line) from draining LN of FTY720-treated, skin-painted animals. *F*, Number of FITC^{+} DC isolated from the draining LN 24 and 48 h after FITC skin painting of untreated animals (■) or of animals treated with FTY720 (□; data from two or three independent experiments with eight to 15 animals/group for each time point; mean ± SEM). ***, $p < 0.0001$.

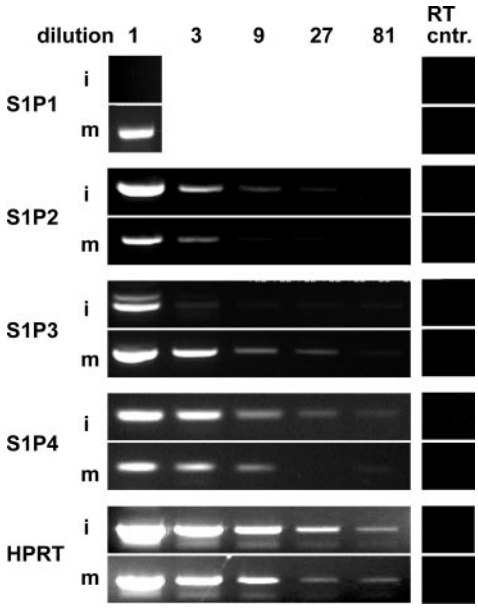

FIGURE 4. Expression of S1P receptors and HPRT in immature and mature in vitro-generated DC. RNA was prepared from in vitro-differentiated, bone marrow-derived, FACS-sorted immature DC (i; CD11c^{+}MHCII^{low}CCR7^{-}) and mature DC (m; CD11c^{+}MHCII^{high}CCR7^{+}) and reverse transcribed. The cDNA was used for RT-PCR in 3-fold serial dilutions. In the RT controls, undiluted mock cDNA templates derived from parallel RT reactions lacking reverse transcriptase were used.

Discussion

Recent observations suggested that the lipid mediator S1P is critically involved in trafficking of immune cells (15, 18). Lymphocyte homeostasis is continuously maintained to warrant quick responses of the immune system to both potentially harmful and innoxious Ags. This circuit is interrupted by the action of the semisynthetic compound FTY720 interfering with proper signaling via S1P (19, 34). As a consequence, regular adaptive immune reactions are compromised (42, 43). This immunomodulating trait of FTY720 is currently exploited therapeutically in trials to enhance survival of incompletely MHC-matched organ grafts (44). Induction of adaptive immunity is crucially dependent on proper presentation of Ag to T cells. Among the family of APCs, DC are of primordial interest due to their exquisite efficacy in Ag sampling

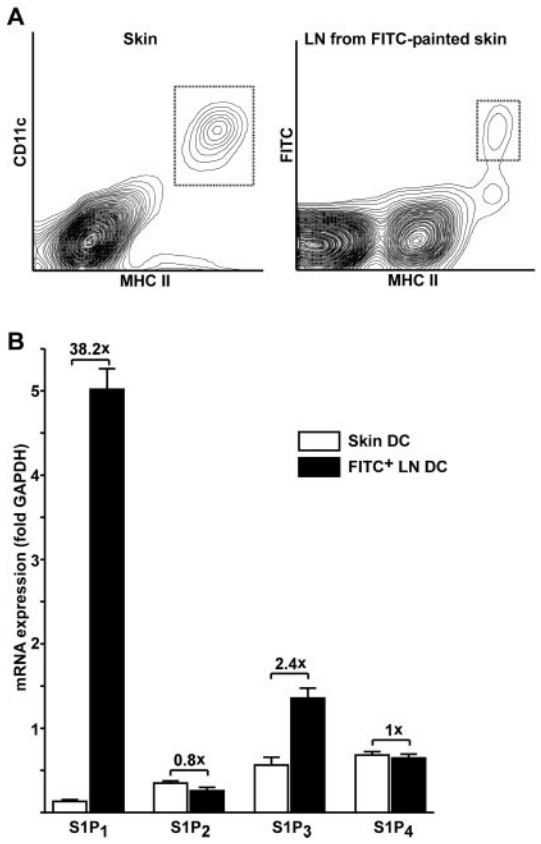

FIGURE 5. Expression of S1P receptors in resident skin DC and FITC$^+$ LN DC after FITC skin painting. *A*, DC were isolated from untreated skin or from the draining LN 24 h after FITC skin painting. According to the gates depicted as doted lines, CD11c$^+$MHCII$^+$CD80$^+$B220$^-$ skin DC or FITC$^+$CD11c$^+$MHCII^{high} DC from the draining LN were FACS sorted, and RNA was prepared. *B*, Expression of S1P receptors in sorted resident skin DC and FITC$^+$ DC immigrated into the draining LN. The relative mRNA expression of S1P receptors 1–4 are shown referred to GAPDH expression. □, mRNA expression in resident skin DC; ■, expression in FITC$^+$ skin-derived DC in the draining LN. Numbers above bars represent the fold induction of gene expression between the two DC populations (mean ± SEM). Two independent experiments with duplicate determinations were performed; in both experiments, DC were pooled from four to eight animals per group.

and presentation. Like lymphocytes, DC are required to travel to accomplish their mission. For these reasons, we intended to elucidate in more detail the effects of S1P and its analogon FTY720 on DC traffic in vitro and in vivo. Furthermore, we investigated the expression of the known S1P receptors and the anticipated involvement of intracellular mediators of S1P signaling in DC migration. In assays performed with in vitro differentiated murine DC, we found that mature cells migrated to S1P, whereas immature DC remained unresponsive over a wide range of S1P (Fig. 1, *A* and *B*). These observations are in conflict with those reported by Renkl et al. (45), suggesting that both immature as well as mature murine DC are S1P responsive. The causes of this discrepancy may be ascribed to the mixed composition of in vitro-generated immature/mature DC populations if used as a whole in the migration assays. Interestingly, human blood monocyte-derived DC migrate to S1P only in the immature state, whereas mature cells lose this ability (46). It remains an unresolved issue at present whether this suggests an inborn divergence between mouse and human DC or simply reflects the different source of progenitor cells (bone marrow in mouse vs blood in human) and/or variations in the differentiation protocols in use.

In contrast to S1P, FTY720 failed to induce migration of mature murine DC (Fig. 1*B*). Remarkably, pretreatment of mature DC with this drug dose-dependently impaired migration to S1P, but not to CCL21 (Fig. 2*A*). An efficient block of DC migration provoked by FTY720 pretreatment apparently requires relatively high concentrations of the drug. Therefore, it was critical to validate this effect in vivo by investigating FITC-induced migration of skin DC to the draining LN. As in the in vitro setting, a substantial, yet incomplete, block of migration was observed (Fig. 3*F*). Considering the key position taken by DC in inducing immune responses, this finding would suggest that the immunomodulating effects of FTY720 may in part be caused by impairing proper T cell priming in the secondary lymphoid organs. However, this observation may also be taken into account when evaluating side effects of therapeutic long-term FTY720 treatments because of their potentially harmful influence on maintaining peripheral tolerance. Yet a drastic failure of DC-based immune functions seems unlikely, because S1P-mediated migration is not revoked, but is rendered less sensitive (Fig. 2*B*). In addition, migrations controlled by chemokines remained unaffected (Fig. 2*A* and data not shown).

The most striking differences in S1P receptor expression linked to DC maturation in vitro and in vivo relate to the up-regulation of $S1P_1$ and $S1P_3$. As a consequence, cells obviously acquire the capability to migrate to S1P. Alternatively, general cellular predispositions for migration may be given only in mature cells. However, immature DC were found to migrate well to CCL3. Our observations are in line with other reports exploring S1P receptor function in migration; the prevalence of $S1P_1$- and $S1P_3$-mediated signaling promoted migration, whereas that of $S1P_2$ antagonized it (see review by Sanchez and Hla (21)). Tracking the signaling paths used by S1P receptors, evidence accumulated that signals relevant for migration are transmitted via the heterotrimeric G protein $G_{\alpha i}$ to Cdc42 and Rac (23, 25, 26). At the level of small GTPases, a predominating Cdc42/Rac activation favors migration, whereas constantly active Rho results in arrest. This is substantiated by the finding that Rac-deficient mature murine DC are neither motile nor able to migrate into LN (47). Migration is a complex cellular process requiring the integration of diverse counteracting events, each for itself paradigmatic for a sessile (e.g., formation of cell/cell or cell/matrix contacts) or a motile phenotype (e.g., assembly of lamellipodia and filopodia). Dissected in consecutive steps such as attachment, moving the cell body, and detachment, it is conceivable to assume that migration requires both signals conducted by Cdc42/Rac and Rho. This is reflected by our finding that inhibitors for both pathways are effective in hampering DC migration. Such a scenario also explains the necessity for the presence of different S1P receptors on the cell surface. Even though Rac/Cdc42 signaling will prevail during initial activation of a DC due to up-regulation of $S1P_1$ and in part by $S1P_3$, an ongoing Rho activity transmitted by $S1P_2$ and probably $S1P_3$ remains a prerequisite for

Table I. *Influence of toxins on the migration of mature DC*[a]

Percent Inhibition	PTX	Toxin B	C3-Fusion Toxin	Lethal Toxin
S1P	98 ± 1.8	97 ± 0.2	84 ± 5.6	80 ± 6.7
CXCL12	94 ± 6.6	96 ± 0.4	80 ± 1.5	87 ± 6.8

[a] Mature DC were used in Transwell migrations to 750 nM S1P or 20 nM CXCL12. Cells were incubated overnight with either 100 ng/ml PTX, 100 pM toxin B, C3 exoenzyme (2.5 μg/ml fusion toxin C2IN-C3, 10 μg/ml binding component C2IIa), or 300 ng/ml lethal toxin. The degree of inhibition was calculated in relation to the migration of untreated cells. (Mean ± SD from two to three independent experiments).

migration. Studies investigating the dynamics in DC dendrite formation/retraction in human immature DC came to similar conclusions by claiming a sequential activity of Rac and Rho in these processes (12). Notably, the retraction of dendrites from surrounding keratinocytes in skin represents the first step in mobilizing a skin-resident DC destined to migrate to its draining LN (6). We hypothesize that S1P-mediated signaling is already effective at this early stage, where a shift in receptor expression occurs, resulting in changed intracellular Cdc42/Rac/Rho activities now favoring the motile over the sessile phenotype. This particular switch in the Cdc42/Rac/Rho activity profile might be prevented by FTY720, which is known to bind with high affinity to $S1P_1$ and $S1P_3$, leaving the Rho-coupled receptor $S1P_2$ untouched (19). Therefore, the correct counterbalance of Cdc42/Rac vs Rho activity is the most critical event in triggering DC migration. We tend to interpret S1P-induced motility as short pulsating, yet undirectional, cell polarizations. In conjunction with incoming chemokine signals, the transient polarization is rendered stable, resulting in a directed locomotion toward and into lymphatic vessels. Activated skin DC deficient in CCR7 cannot migrate into the nearby lymphatics (4) even though S1P-induced migration was unaffected in our in vitro assays. Vice versa manipulation of the S1P signaling path by FTY720 pretreatment fully retains the capacity of DC to migrate toward chemokine stimuli, indicating that signalings through S1P and chemokines are independent from each other in this scenario. In contrast, both types of signals are required simultaneously for triggering migration of maturing DC into the draining LN.

All in all, our results unequivocally demonstrate that mature DC depend on proper S1P signaling for migration, as reflected by their ability to migrate to this lipid mediator. Migration can be severely inhibited by FTY720 pretreatment in vitro and in vivo. Even though the implications of this effect on DC-borne immune reactions remain to be elucidated, FTY720 seems to suppress Cdc42/Rac activation by blocking signaling via $S1P_1$ and $S1P_3$. Tuning an S1P signal is accomplished by the concerted activities of several S1P receptors, thereby explaining the existing variety of S1P receptors frequently expressed at the same time on the same cell. A switch of the S1P receptor expression profile, as demonstrated in this study for the transition from immature to mature DC, may then convert an incoming S1P signal to another direction.

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Disclosures

The authors have no financial conflict of interest.

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