The Origin and Maturity of Dendritic Cells Determine the Pattern of Sphingosine 1-Phosphate Receptors Expressed and Required for Efficient Migration

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Dendritic cells (DCs) represent an important interface between innate and adaptive immunity. They are specialized in the uptake, processing, and presentation of Ag, conferring them with a unique capacity to induce adaptive immune responses, as well as to generate and maintain tolerance (1). To accomplish this, DCs form a dense network settling lymphoid organs and extralymphatic tissue. The very distinct environments and niches occupied by these cells give rise to a bewildering heterogeneity of DCs with regard to their phenotype and functional capabilities (2, 3). For example, splenic CD8+CD205+ DCs residing in the T cells areas cross-present Ag inducing the differentiation of cytotoxic CD8+ T cells. Therefore, these cells play an important role in the elimination of viral infections (4). In contrast, CD8-33D1+ DCs settling the bridging channels and red pulp predominantly elicit Th2-type responses upon immunogenic challenge (5). In skin, epidermal CD11c+CD205+ Langerhans cells are efficient in mounting cellular immune response, whereas dermal interstitial CD11c+DC-SIGN+CD11b+ DCs tend to initiate humoral immunity (1). In GALT, CD103+ DCs of the mesenteric lymph nodes (MLNs) represent DCs that emigrated from the lamina propria (LP). Under steady-state conditions, they present soluble Ag captured in the LP and induce preferential polarization of CD4+Foxp3+ regulatory T cells along with a gut-homing phenotype (CCR9+e4B7+). In contrast, CD103- DCs in MLNs are derived from blood and present systemic Ag (6–10). CD103 also discriminates DC subsets of other origins, such as lung, where CD103+ DCs cross-present Ag to CD8+ T cells, whereas CD103- DCs prime CD4+ T cells under tolerogenic conditions (11).

To trigger T cell responses, tissue-resident DCs need to migrate to their draining lymph node (LN). This inherently necessitates at least some degree of maturation; DCs emigrating into LNs exert a more mature phenotype compared with local LNs or splenic DCs. The environmental conditions met at the time of their mobilization in tissue have a great impact on the maturation phenotype of the emigrating DCs, thereby determining the outcome of the subsequent T cell priming (1, 12–14). In vitro-generated DCs are frequently used as a model system to mimic the processes of DC maturation/migration and T cell priming. This greatly facilitated the investigation and understanding of molecular mechanisms underlying DC phenotype and functions. In vitro-derived mature DCs may resemble tissue-resident interstitial DCs that migrate upon stimulation. However, there might be no genuine in vivo correlate to these cells, nor do they reflect the complex variety of DC subtypes found in vivo (3).

Sphingosine-1 phosphate (SIP) represents a small bioactive metabolite that is virtually omnipresent throughout eukaryotes (15, 16). Although the pathways involving intracellular SIP remain largely obscure, the discovery of the five transmembrane receptors for SIP, SIP1–5, helped to reveal the multifaceted implications of extracellular SIP in a variety of fundamental biological activities of mammalian cells (17). Accordingly, SIP receptors are also
widely expressed in the immune system; however, such expression occurs in a cell-type–specific pattern (18). Thus, naïve CD4+ T cells predominantly express S1P1 and S1P3. The latter is mainly involved in T cell proliferation and cytokine secretion (19), whereas S1P1 activity is indispensable for T cell recirculation (20). Ligand-triggered receptor internalization, equivalent to the withdrawal of functionally available receptor, represents an important tool of the S1P/receptor control circuits (20, 21). This is illustrated by the prevention of T cell exit from thymus or LN s in S1P1-deficient cells (20) or under conditions of permanent receptor internalization that can be provoked by drugs, such as FTY720 (22). Therefore, FTY720 and other agonists/antagonists of S1P1, s were valuable tools for elucidating the function of these receptors. In addition to this, the establishment and analyses of mice deficient for all S1P receptors, with the exception of S1P3, have been reported (23). Altogether, the studies support the notion that the S1P/receptor system is of particular (nonredundant) importance for molecular mechanisms operating in the cardiovascular and immune systems.

The mobilization and migration of DCs also depend on S1P (24–29). We reported earlier that the intrasplenic positioning of 33D1+ DCs is controlled by S1P3, that is expressed by DCs, but at much lower levels compared with T cells (25). In addition, DCs stimulated to mature upregulate S1P1 and S1P3, and available evidence suggests that signaling via both receptors influences the migratory capacity of DCs (24, 28). In this report, we show that the presence of S1P1 and S1P3 is indispensable for the migration of DCs generated and matured in vitro. In vivo, the migration of skin-resident DCs is modulated by S1P1, but not by S1P3. This also applies to the intrasplenic positioning of immature DCs to the bridging channels. In contrast, LP DCs require S1P1 and S1P3 for efficient migration to the MLN. Based on these results, we hypothesize that the contribution of different S1P receptors to DC mobilization and migration is dependent on the particular DC subset under investigation. However, our findings also support the notion that S1P1 activity may be mandatory for the migration of maturing DCs from any peripheral tissue to the draining LNs.

Materials and Methods

Mice

Eight- to twelve-week-old C57BL/6 mice were purchased from Charles River Laboratories (Sulzdorf, Germany). S1P1−/− mice were kindly provided by B. Levkau (University Hospital, Essen, Germany) with the permission of J. Chun (The Scripps Research Institute, La Jolla, CA). S1P1−/− mice (obtained from Mutant Mouse Regional Resource Centers, Bar Harbor, ME) were back-crossed onto the C57BL/6 Ly5.1 background for at least eight generations. Mice were maintained in specific pathogen-free conditions in the animal facility of Hannover Medical School. Fetal liver cells of embryos at day E13.5 from S1P1−/−Ly5.1 breeders were genotyped; the S1P1−/− fetal liver cells were injected into C57BL/6 Ly5.2 recipients. Two-cell (WT) DCs and S1P1−/−/Ly5.2−/− fetal liver cells were injected into C57BL/6 Ly5.2 recipients. All chimeras were analyzed 8 wk later. All experiments including animals were conducted according to the regulations of the local government and institutional guidelines.

Abs and flow cytometry

Bone marrow–derived DCs (BMDCs) or cells isolated form the lymphoid organs of mice were analyzed by flow cytometry using an LSRII (BD Biosciences, Erembebedge, Belgium). Sorting of cells was performed using a FACSAria (BD Biosciences), The following mAbs (all from BD Biosciences, unless stated otherwise) were used: allopseudocyanin-labeled anti-CD11c (eBioscience, Frankfurt, Germany), FITC-labeled/biotinylated (eBioscience, Billendon, Belgium, anti-MHCII, PE-labeled anti-CD103, PE-labeled CD86 (BioLegend), PE (BioLegend)/FITC-labeled anti-CD45.1 (eBioscience), PerCP Cy5.5-labeled anti-CD45.2 (eBioscience), A405-labeled anti-CD44 (Caltag Laboratories, Hamburg, Germany), allopseudocyanin Cy7/PE-labeled anti-CD8α, biotinylated anti-TCR Vβ5.15.2, PE-labeled anti-TCR αβ, Cy5 (home-made)/FITC (Caltag Laboratories)-labeled anti-CD4, Pacific orange-labeled anti-B220 (home-made), allopseudocyanin-labeled anti-hamster IgG1 isotype control, FITC-labeled anti-rat IgG2a isotype control, PE-labeled anti-rat IgG2a isotype control, PerCP-labeled anti-rat IgG2a isotype control, and PerCP (BD Biosciences)/Pacific orange (Invitrogen, Karlsruhe, Germany)-labeled streptavidin were used to detect biotinylated primary Abs.

In vitro differentiation of DCs

DCs were prepared according to the protocol described previously (24). A total of 2 × 106 bone marrow cells were grown in RPMI 1640/10% FCS/50 μM β-ME and 100–200 ng/ml GM-CSF for 8 d and stimulated with 1 μg/ml LPS (Sigma-Aldrich, Taufkirchen, Germany) or 30 ng/ml TNF-α (PeproTech, Hamburg, Germany), together with 1 μg/ml PGE2 (Sigma-Aldrich) for 48 h.

FITC skin painting

Mice were gavaged with 40 μg FTY720 (LC Laboratories, Woburn, MA) at ~24 and ~12 h. Four hundred micrograms of SEW2871 (BIOMOL, Hamburg, Germany) in PBS/10% Tween 20% ethanol was administered via gavage at ~6, +4, and +14 h. Painting of FITC was done at 0 h. Mice were anesthetized (20% ketamin, 10% Rompun in PBS), and the ears were painted with 0.1% FITC (Sigma-Aldrich) in acetone/dibutylphthalate (1:1). Mice were sacrificed after 24 h, and the draining facial LN was collected and treated with 0.5 mg/ml collagenase A (Roche, Basel, Switzerland) and 50 U/ml DNase 1 (Roche) in RPMI 1640/10% FCS for 30 min to obtain single-cell suspensions. The number of FITC+ DCs migrating from the epidermis expressing MHC class II and CD11c was determined by flow cytometry.

In vitro migration assays

Transwells (5-μm pore size, Corning, Corning, Bremen, Germany) were coated with 10 μg/ml murine collagen IV (BD Biosciences) in 0.1 M HCl. A total of 3–4 × 105 in vitro-differentiated mature DCs in RPMI 1640/20 mM HEPES (pH 7.4) were added to the upper well. The chemoattractants S1P (Sigma-Aldrich) and CCL21 (R&D Systems, Wiesbaden, Germany) were added to the lower well. The assay was performed for 4 h, and the numbers of DCs that migrated to the lower wells were determined.

Competitive DC migration assay

In vitro-differentiated DCs stimulated with LPS for 48 h were used. Up to 107 cells in 5 ml RPMI 1640/20 mM HEPES (pH 7.4) were incubated at 37°C for 20 min. Wild type (WT) DCs and S1P1−/− DCs were labeled with 1.8 μM CFSE or 10 μM TAMRA for 10 min at 37°C and washed with PBS/3% FCS three times. A total of 5 × 105 WT DCs and S1P1−/− DCs were mixed and injected s.c. (30 μl) into the footpads, as well as into the trachea of mice (60 μl). The draining popliteal LN and bronchial LN were analyzed 48 h later. The isolated LNs were treated for DC isolation, as described above. The cells were stained for MHC class II and CD11c, and the numbers of CFSE and TAMRA+ DCs were determined by flow cytometry. WT DCs and S1P1−/− DC were labeled with TAMRA and subjected to in vivo migration, as described, and the numbers of migrated DC were analyzed using the congenic markers CD45.1 and CD45.2.

Endogenous DC migration assay

Sixty micrograms of LPS was administered to WT/S1P1−/− mixed bone marrow chimeric mice. Mice were sacrificed 12 h later, and DCs were isolated from the MLN. LP DCs were isolated as described by Carlens et al. (30). The numbers of MLN and LP CD103+ migratory DCs were determined by flow cytometry.

Immunohistology

Spleens of WT, S1P1−/−, S1P1−/− fetal liver chimeric mice, and mock-treated FTY720- or SEW2871-treated mice were embedded in Tissue-Tek (Sakura, Zoeterwoude, The Netherlands), and 8-μm-thick cryosections were prepared. Immunohistochemistry was done as described (25). Biotinylated ICAM-1 and allopseudocyanin-labeled CD11c were used for staining, Cy3-labeled streptavidin (Dianova, Hamburg, Germany) was used to detect the biotinylated Abs. Cryosections of the small intestine of control animals or mice injected i.p. with 60 μg LPS with/without prior FTY720, SEW2871 application were prepared 12–14 h following LPS treatment, as described earlier (31), and were incubated with Abs recognizing CD103 and CD11c, respectively, followed by DAPI staining. The numbers of CD11c+CD103+ DCs present in the LP of 10 randomly selected villi per mouse were counted; concomitantly, the area

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containing the counted cells was determined. Fluorescent images were taken using an Olympus BX61 microscope (Olympus, Hamburg, Germany) at a magnification of ×20. Photo documentation was performed using CellP software.

RNA isolation and real-time PCR

Total RNA was isolated using the Absolutely RNA Microprep kit (Stratagene, La Jolla, CA), according to the manufacturer’s protocol. RNA was reverse transcribed using Superscript II reverse transcriptase (Invitrogen). SYBR Green PCR Master Mix (Applied Biosystems, Darmstadt, Germany) was used, and the PCR reaction was performed in an ABI Prism 7500 cycler (Applied Biosystems). Amplifications of the target genes were done using primers, as described (24). Absolute quantification of the gene expression as copy numbers was done by referring to standards curves using plasmid cloned target amplicons. S1P2 gene expression was analyzed using the Quantitect primer assay (Mm_S1pr5_1_SG QT00282744, Qiagen, Hilden, Germany), and absolute copy numbers were calculated with standard graphs generated with genomic DNA.

Results

Expression of S1P receptors on immature and mature in vitro-derived DCs

It is well documented that S1P has a profound influence on the capacity of maturing DCs to migrate to their draining LNs. The levels of S1P increase upon inflammation (32), probably helping to mobilize DCs that reside in the afflicted tissue. By investigating S1P3-deficient cells or the impact of drugs interfering with S1P receptor activity, it was concluded that the effects of S1P on DCs are mainly mediated by coupling onto S1P1 and/or S1P3. Notably, the expression of the latter two receptors, but not that of S1P2, S1P4, or S1P5, was found to be substantially upregulated by DCs undergoing maturation. However, it is known that DC maturation is largely influenced by the particular circumstances accompanying their stimulation. Therefore, we investigated the regulation of S1P

![Figure 1: Determination of S1P receptor expression by real-time PCR in in vitro-matured DCs. BMDCs were generated in culture, as described in Materials and Methods. The resulting immature DCs were sorted for RNA preparation or stimulated with LPS or TNF-α/PGE2 for 1–2 d before the cells were harvested and sorted by flow cytometry. Subsequently, RNA was prepared and subjected to real-time PCR to detect S1P receptor levels. The amount of mRNA specific for each receptor was calculated in relation to that detected for GAPDH-specific message. Results are shown from two independent experiments; each dot represents one PCR data point. Note that a different scale was used for S1P1.](http://www.jimmunol.org/ .)
receptors S1P<sub>1-5</sub> in more detail by monitoring the mRNA levels by real-time PCR. To this end, in vitro-generated immature DCs were stimulated with LPS or TNF-α/PGE<sub>2</sub> for 1 or 2 d. The quality of the DCs obtained by such procedures usually suffers from impurities because the pool of immature DCs contains a fraction of spontaneously matured DCs. Likewise, not all immature DCs respond properly to the stimuli and fail to mature fully. To restrict the S1P receptor analysis to well-defined DC populations, immature CD11c<sup>+</sup>MHCII<sup>int</sup> DC and the mature CD11c<sup>+</sup>MHCII<sup>hi</sup> DC fractions were sorted by flow cytometry prior to RNA isolation and real-time PCR quantification (Fig. 1). Nonstimulated CD11c<sup>+</sup>MHCII<sup>int</sup> DCs express S1P<sub>2</sub> but very low levels of S1P<sub>1</sub>, S1P<sub>3</sub>, and S1P<sub>4</sub>. The expression of all S1P receptors is sensitive to stimulation with LPS or TNF-α/PGE<sub>2</sub>; however, the extent of regulation depends on the

**FIGURE 2.** S1P<sub>1</sub> and S1P<sub>3</sub> are not essential for maturation of BMDCs but are for in vitro migration toward S1P. Immature WT DCs or DCs lacking S1P<sub>1</sub> or S1P<sub>3</sub> were matured in the presence of LPS for 2 d. A. The degree of maturation was determined by flow cytometry. Upper panels, Representative stainings comparing batches of WT and S1P<sub>3</sub><sup>−/−</sup> or WT and S1P<sub>1</sub><sup>−/−</sup> cells that were grown and stimulated in parallel experiments. The numbers given in the plots depict the percentages of CD11c<sup>+</sup>MHCII<sup>hi</sup> DCs present in the indicated gate. Lower panels, Graphs showing the extent of MHC class II, CD40, CD80, and CD86 expression in samples matured at the same time under identical conditions. Shaded area: isotype control; black line: WT; dotted line: S1P<sub>3</sub><sup>−/−</sup>(upper panels) and S1P<sub>1</sub><sup>−/−</sup>(lower panels). B and C, In vitro migration of LPS-matured DCs performed in Transwell assays. S1P was added to the lower chamber at the concentrations indicated. As a control, migration to 20 nM CCL21 is given. Migration indices for S1P<sub>3</sub><sup>−/−</sup> DCs (B) and S1P<sub>1</sub><sup>−/−</sup> DCs (C) compared with WT cells matured in parallel for each experiment. Three experiments were performed in duplicate. Data are shown as mean ± SD.
nature of the stimulant, the duration of the maturation time, and the particular receptor examined. Thus, S1P1 levels increase upon LPS addition only after 48 h, whereas TNF-α/PGE2 already elicited upregulation after 24 h and to a greater extent after 48 h. S1P2 mRNA levels decreased substantially following stimulation with LPS, causing a more pronounced decrease compared with TNF-α/PGE2-driven stimulation. However, after 48 h, S1P2 levels recovered slightly. Inversely, a low level of S1P1 distinguishes immature DCs and, depending on the stimulation, the amount of S1P3-specific message increased 24 h later. Although DCs treated with LPS for 48 h maintained increased levels of S1P3, those of DCs induced to mature in the presence of TNF-α/PGE2 decreased nearly to the level found in immature DCs. In contrast to S1P3, changes in S1P2 and S1P5 mRNA amounts were modest under all conditions tested.

Migration of mature in vitro-generated DCs depends on S1P1 and S1P3

It was reported that migration of mature DCs depends on S1P1 and/or S1P3, an assumption that is supported by the finding that the expression of both receptors increased upon maturational stimuli. Therefore, we tested in vitro-generated DCs lacking S1P1 or S1P3 in migration assays with S1P as attractant. Because S1P3-deficient mice are not viable postembryonic day 14.5 (33), we established fetal liver chimeras. The successful reconstitution of these mice with hematopoietic cells missing S1P1 was controlled (Supplemental Fig. 1). The bone marrow of these mice was subsequently used as a source for in vitro-derived DCs. When comparing the generation and maturation kinetics of DCs deficient for S1P1 or S1P3 with that of WT cells, we did not notice any difference with regard to the expression characteristics of diagnostic markers, indicating that neither receptor is essential for DC differentiation in vitro (Fig. 2A). Immature WT DCs did not migrate to S1P, as was found earlier (24, 28) (data not shown). However, although matured WT DCs migrated to S1P in a dose-dependent manner, DCs lacking S1P1 or S1P3 were unresponsive (Fig. 2B, 2C). In contrast, mutant DCs migrated well to CCL21, confirming that the loss of S1P1 or S1P3 did not interfere with the general capacity of the cells to respond to a chemotactic stimulus.

To test the motility/migration of mature DCs deficient in S1P1 and S1P3 in a more physiological setting, DCs matured in vitro in the presence of LPS were injected into the footpad of WT mice. To minimize experimental fluctuations, a 1:1 mixture consisting of WT and S1P1−/−DCs that were labeled with TAMRA or CFSE, respectively, was applied. A switch in dye labeling of the WT and S1P3−/−DCs had no impact on the results (data not shown). In the case of S1P1, we used the Ly5 congenic marker to prepare TAMRA-labeled cell mixtures (WT:Ly5.2, S1P1−/−:Ly5.1). The next day, the draining LN was analyzed by flow cytometry to detect and count the migrated cells (Fig. 3A, 3B). Reflecting the in vitro data presented above, both receptors are essential for efficient migration of mature DCs into the LN. However, there is no absolute block in migration in vivo as observed in vitro, because a considerable amount of knockout DCs migrated to the draining LN. Moreover, this “quenching” effect is more pronounced for S1P3-deficient cells. This is illustrated by the deviation factor that reflects the shift in the ratio among the migrated DCs from the originally applied 1:1 mixture of cells. Thus, a factor of 1 indicates that WT and knockout cells arrived in equal numbers in the LN. When averaging all individual LNs analyzed, this factor was 5.44 for S1P1 deficiency and 1.76 when the DCs lacked S1P3. This suggests that in contrast to the well-defined in vitro migration conditions, other migration mechanisms may compensate, in part, for the deficiency of S1P receptors. Alternatively, the minimalist setup for testing in vitro migration may impose nonphysiological challenges to the migrating cells that may provoke a complete.

**FIGURE 3.** S1P1 and S1P3 are essential for in vivo migration of BMDCs. Mixtures (1:1) of in vitro-generated and LPS-matured DCs (BMDCs) of WT and S1P1−/−or WT and S1P3−/−origin were labeled with dye and injected into the footpad of WT mice (A, B) or applied intratracheally (C, D). Cells migrating into the draining LN were recovered by flow cytometry and counted. Each coupled pair of data points is derived from the analysis of one animal. All data were normalized (i.e., the average number of WT BMDCs counted in the draining LN of all animals was set to 1, and the cell counts of each individual evaluation were calculated in relation to this averaged value), ***p < 0.01; ****p < 0.001, paired, two-tailed t test.
collapse upon S1P receptor deficiency that is otherwise not met in vivo.

Moreover, footpad injection is influenced by the physical damage caused by the injection itself, lending uncertainty to what extent cells may directly gain access to the lymph drainage connecting to the downstream LN. Therefore, we chose intratracheal application of the DCs as an alternative route to investigate the cell’s abilities to migrate to the draining LN (Fig. 3C, 3D). Although the absence of S1P1 or S1P2 had a less pronounced effect on DC migration, their contribution is still highly significant in both cases. Similar to the footpad injections described previously, it seemed that S1P1 deficiency exerted more profound effects on migration compared with S1P2 (average deviation factor: 6.85 for S1P1−/− and 2.43 for S1P3−/−).

Migration of mature skin-derived DCs requires S1P1 but is independent of S1P3

We showed earlier that the mobilization/migration of skin-resident DCs is modulated by S1P (24). DCs stimulated by FITC skin painting migrated less efficiently into the draining LN when mice were pretreated with FTY720. The skin-derived DCs upregulate S1P1 and S1P3, leaving the levels of S1P2 and S1P4 almost unchanged. Notably, S1P3 was affected much more by the stimulus-driven upregulation than was S1P3 (24). In a continuation of these experiments, we treated mice with SEW2871 prior to skin painting and monitored the appearance of FITC+ DCs in the draining LN 1 d later. SEW2871 specifically interferes with S1P1 function, whereas FTY720 affects all S1P receptors, with the exception of S1P2. However, SEW2871 hampered DC migration, suggesting that S1P1 is primarily involved in regulating the migration of skin-derived DCs (Fig. 4A, 4B). Surprisingly, when S1P3-deficient mice were subjected to FITC skin painting, we did not observe any difference in the number of DCs migrating into the draining LN, even when the migration period was extended to 48 h (Fig. 4C). Although this result does not exclude the possibility that S1P3 is also involved in the migration of maturing DCs into the draining LN, its contribution seems at least redundant and was not detectable under the experimental conditions that we used. Thus, S1P1, and not S1P3, represents the most important mediator of S1P-driven effects in this process.

LP DCs require S1P1 and S1P3 for efficient migration into the MLNs

In the LP, CD11c+ MHCII+ DCs express CD103 (Fig. 5A). DCs migrating to the MLN retain a high level of CD103 (Fig. 5B); thus, this marker can be used to identify DCs of LP origin in the MLN. To investigate whether S1P3-mediated signaling is important for this type of DC migration, bone marrow mice chimeric for WT and S1P3-deficient DCs (mixed chimera) were analyzed before and after treatment with LPS for 12 h. As a result of the LPS stimulus, WT CD103+ DCs emigrated from the LP, as evidenced by their decreased numbers compared with PBS control-treated mice (Fig. 5C). Accordingly, increased numbers of CD11c+ MHCII+ CD103hi DCs were found in the MLN (Fig. 5D). In contrast, a corresponding redistribution of CD103− DCs between LP and MLN was not observed in S1P3-deficient mice treated with LPS. Although there was a tendency toward a loss in the LP, these changes were not statistically significant. To corroborate this finding, mice deficient for S1P3 were treated with LPS, and the small intestines were analyzed by immunohistology 12 h later. Following staining (Fig. 5E), CD11c+CD103+ DCs present in given areas of villi were counted to determine their frequency (Fig. 5F). Compared with mice receiving PBS as a control, there was a substantial loss of DCs in LPS-treated animals. The absence of S1P3 mostly prevented DCs from leaving the LP.

To address the importance of S1P3 in DC traffic out of the LP, WT mice were treated with SEW2871 prior to LPS application. Microscopic evaluation of the DC frequency revealed that the drug was able to significantly block DC migration. However, this block was not as complete as observed in the small intestines of mice pretreated with FTY720. This suggests that S1P1 and S1P3 are important determinants governing the migration of maturing DCs from the LP to the MLN.

FIGURE 4. Skin-resident DCs require S1P1, but not S1P3, for efficient migration into the draining LN. The ears of mice were painted with FITC solution, as described in Materials and Methods. A, DCs that arrived in the draining LN were identified as FITC+ cells (left panel) and identified as mature CD11c+ MHCII+ cells (right panel). Representative plots are shown. B, Mature DCs identified in the draining LN following skin painting of mice pretreated with SEW2871 or mock-treated animals. C, In a separate set of experiments, WT and S1P3−/− mice were subjected to FITC skin painting. Each dot represents one mouse. Two independent experiments were performed. Cell counts were normalized as described in Fig. 3. *p < 0.05, unpaired, two-tailed t test. ns, not significant.
Localization of splenic DCs to the bridging channels is independent of S1P3

We showed earlier that FTY720 and SEW2871 treatment caused a redistribution of immature splenic 33D1+ DCs to the marginal zone, suggesting that S1P1 is essential to retain these cells in their original location, the bridging channels (25). This was confirmed when investigating the localization of splenic DCs in S1P1−/− bone marrow chimeric mice by immunohistology (Fig. 6). The CD11c/ICAM-1 stain revealed that DCs reside, to a large extent, in the marginal zone instead of the bridging channels. However, no information is available as to whether S1P3 is also important for localization to the bridging channels that is apparently actively maintained by S1P/receptor signaling. Therefore, spleens of S1P3−/− mice were investigated by immunohistology (Fig. 6). The CD11c/ICAM-1 stain revealed that DCs reside, to a large extent, in the marginal zone instead of the bridging channels. However, no information is available as to whether S1P3 is also important for localization to the bridging channels that is apparently actively maintained by S1P/receptor signaling. Therefore, spleens of S1P3−/− mice were investigated by immunohistology (Fig. 6). The CD11c/ICAM-1 stain revealed that DCs reside, to a large extent, in the marginal zone instead of the bridging channels.

FIGURE 5. LP-resident DCs require S1P1 and S1P3 for efficient migration into the MLN. A, Gut-resident CD11c+MHCII+ DCs (left panel, shaded graph = isotype control). B, In the MLN, the DCs constantly migrating from the LP (gated as CD11c+MHCII+, left panel) retain a high level of CD103 expression (black line) compared with CD11c− MHCII− cells (dotted line) that most likely represent B cells. Shaded graph, isotype control. Quantitative assessment of CD11c+MHCII+CD103+ DCs isolated from the LP of the small intestine (C) or the MLN (D) of animals treated as indicated. Mice were injected i.p. with LPS or PBS as control 12 h before sacrifice. Each dot represents one animal. Cell counts were normalized and expressed relative to the average number of DCs (set to 1) identified in WT LP (C) or WT MLN (D) of mice treated with PBS. Data are summarized from three independent experiments. E, Representative cryosection of a WT villus of the small intestine showing stains for DAPI, CD11c, and CD103. Arrowheads denote CD11c+CD103+ nucleated cells. The dashed lines define the area determined. Original magnification ×20; scale bar, 200 μm. F, Summary of histological data. Ten villi per mouse small intestine were chosen and evaluated by determining the number of CD11c+CD103+ nucleated cells/mm². Each dot represents a mouse. Data from two independent experiments are shown. *p < 0.05; **p < 0.01; ***p < 0.001, unpaired, two-tailed t test. ns, not significant.
deficient mice were investigated (Fig. 6). DCs were distributed in a fashion indistinguishable from WT mice, suggesting that S1P3 is dispensable for a proper migration of DCs to the bridging channels. This was also observed in spleens of mice that were reconstituted with mixed S1P3−/−/WT bone marrow (data not shown). Along with the results obtained from the analyses of the S1P1−/−/chimeric mice, these observations suggest that accumulation of DCs in the bridging channels depends on the S1P responsiveness of the cells themselves and does not rely on an S1P-mediated effect of an undetermined nonhematopoietic bystander cell.

Discussion

By subjecting immature DCs generated in vitro to different maturation protocols using LPS or TNF-α/PGE2 as stimulant, we observed that the duration of the stimulus and its nature have a profound impact on the regulation of S1P receptor expression. DC maturation in the presence of LPS or TNF-α/PGE2 mimics conditions favoring a subsequent Th1- or Th2-dominated T cell response, respectively. Thus, the differences observed in the expression profile of S1P receptors following these stimuli may well represent part of the information that is imprinted, for example, on tissue-resident DCs mobilized by distinct environmental conditions. Remarkably, however, in nonstimulated immature DCs, we already noted a S1P receptor pattern that diverged from that published by other investigators. Maeda et al. (28) found a predominant S1P2 expression coupled to a lower level of S1P3 and even more decreased amounts of mRNA coding for S1P2 and S1P3 (compare with Fig. 1). Although in vitro-differentiated DCs were generated in both laboratories according to the most common standard protocol using GM-CSF, subtle differences in the handling or usage of growth media and supplements are apparently sufficient to elicit the observed differences. This assumption is supported by the fact that we also determined S1P receptor-specific mRNA levels in freshly isolated naïve CD4+ T cells that revealed an expression pattern that was identical to those observed by Maeda et al. (28) and Wang et al. (19) (Supplemental Fig. 2). These findings illustrate that it is difficult to assess divergent results unless the conditions used for the generation and maturation of the DCs are well specified. To this end, it may also be essential to analyze only sorted DC populations that are highly pure and uniform with regard to the DC markers that are able to discriminate between mature, immature, and semimature (spontaneously matured) cells.

The real-time PCR analyses provided valuable information. This is particularly true with regard to altered steady-state levels of the mRNAs coding for the S1P receptors when comparing immature and mature DCs. However, such data do not indicate whether an altered mRNA level was due to a different rate of transcriptional activity or a modified RNA half-life. It also remains uncertain whether the altered mRNA levels translate into a more or less efficient presentation of the functionally relevant receptor on the cell surface because anti-S1P receptor Abs suitable for flow cytometry are not available. This problem can be bypassed, in part, by functional approaches, such as assessing migration of DCs in vitro or in vivo. Despite the divergences in the S1P receptor-expression profile, we (24; this study) and Maeda et al. (28) did not observe migration of immature DCs toward S1P. This is in conflict with the results of Renkl et al. (29), who showed that already immature in vitro-generated DCs migrated in response to S1P. However, they differentiated their DCs in the presence of GM-CSF and IL-4, a mixture that is usually used to generate human DCs from blood monocytes. In this context, it is interesting to note that human in vitro-generated DCs responded to S1P when immature, but they lost S1P-driven migration upon maturation. Unfortunately, the expression pattern of S1P receptors was not investigated in that study, making it difficult to draw additional conclusions (29).

With regard to mature in vitro-derived murine DCs, Maeda et al. (28) showed that S1P3 is required to migrate to S1P in standard Transwell assays. However, because they were unable to block migration in the presence of SEW2871, they concluded that S1P1 is not essential for in vitro migration. We also failed to block in vitro migration of mature DCs (data not shown), despite the proven efficiency of SEW2871 in diverse in vivo settings (25, 28, 34; this study). However, using gene-deficient BMDCs, we found that S1P1 and S1P3 are required for in vitro and in vivo migration (footpad injection and intratracheal application). This unequivocally stresses the notion that S1P-driven migration of mature BMDCs depends on simultaneous S1P1- and S1P3-based signaling and proves that S1P1 and S1P3 cannot replace each other in this particular receptor function. Therefore, the failure of SEW2871 to inhibit migration in vitro requires another explanation. Indeed, mature DCs do not migrate in vitro when treated with FTY720, which interferes with all S1P receptors except for S1P2 (24). However, in contrast to FTY720, which triggers S1P1 internalization, thus generating a desensitized S1P1-deficient cell, SEW2871 may not be as efficient in this aspect (28, 34). Therefore, a contribution of S1P1 signaling triggered by the agonist SEW2871 may prove sufficient to allow migration to an S1P gradient that is recognized by S1P3, a receptor that is not affected by this drug. Because SEW2871 is unable to interfere with DC migration in vitro, how does it execute inhibition in vivo, as observed in our experiments? A comparison of in vitro and in vivo DC migration must take into consideration the fact that the in vivo experiments did not test only for migration. Analyzing the traffic of LP DCs to the MLN or skin-resident DCs to the draining LN also encompassed the in situ maturation of the DCs, including their mobilization and polarization. Without these initial steps, a DC does not gain the ability to migrate. Therefore, it is conceivable that in vivo SEW2871 successfully blocks an earlier step in the cascade of events that usually results in the arrival of DCs in the target LN.
Moreover, in vivo, the cells reside in particular microenvironments that modulate the input of external signals affecting the stimulation of the DCs. Apart from external circumstances, individual characteristics acquired during the differentiation of DCs from precursors may dominate their responsiveness to S1P-mediated signals. The effects of such coming may extend into stages beyond induction of maturation. The role of cell-intrinsic predispositions is particularly interesting in light of a recent report describing the migration of DCs as a process of flowing and squeezing (35). This mode of migration differs from that of nonleukocyte cell types. It is fueled mainly by a propulsive expansion of the actin network building the leading edge of a migrating DC and works efficiently, even in the absence of classical integrin-mediated attachments to the extracellular matrix. The possibility of using a migration modus that is largely independent from the presence of a scaffold of distinct adhesive ligands confers a considerable autonomy from the tissue context through which the DC is migrating (35). These observations emphasize the extraordinary importance of cell-intrinsic predispositions, which may explain why some DC subsets, but not others, require input from S1P1 in addition to S1P3.

With regard to the latter, a conundrum arises when considering that a cell may simultaneously express S1P receptor subtypes that drive, in part, opposing effects inside the cell upon S1P stimulation. Thus, S1P1 evokes Rac/Cdc42 signaling that facilitates formation of filopodia and lamellipodia, thereby fostering a cell’s motility. S1P2 triggers Rho-mediated effects, such as formation of focal adhesions and stress fibers. Consequently, a constant Rho activity sustains a sessile phenotype (36, 37). S1P3 is able to activate both pathways: Rac/Cdc42 and Rho (37). Migration results from a high degree of motility that is tightly linked to polarity, enabling a direction of travel. This is driven by cell-internal rearrangements of the cytoskeleton, thereby explaining the central role of the small GTPases Rac, Cdc42, and Rho in these processes (24, 38). Indeed, we showed earlier that DCs require signaling inputs via Rac, Cdc42, and Rho simultaneously to accomplish migration (24). Other receptor classes, such as the chemokine receptors or adhesion molecules that are expressed by DCs, are able to address these distinct S1P receptors. It will be interesting to learn whether such coining may extend into performing migration. However, they cannot replace each other, and we favor the view that S1P1 (and S1P3) signaling predominantly initiates and maintains a high degree of motility, whereas its function as a chemotaxant is of inferior importance in vivo. This role is filled by potent guidance systems, such as the chemokine/receptor network, which imposes directionality on the cells competent to migrate. However, a heterogeneous composition of the migration machinery available may add to the explanation of why DCs of divergent developmental origin and maturation history differ from each other in their requirements for S1P receptor activities to travel.

This is exemplified by the results reported in this paper: skin-derived DCs require S1P3-driven, but not S1P1-driven, activities to effectively migrate to their draining LN. In contrast, LP DCs show an additional dependence on S1P1 for efficient migration to the MLN. But again, the localization of splenic DCs accumulating in bridging channels was unaltered in S1P3-deficient mice, whereas their dislocation in S1P1−/− spleens confirmed our earlier finding that this receptor is essential to achieve retention of 33D1+ DCs in the bridging channels. The absence of S1P1 also cannot prevent the migration of DCs from the bridging channels to the T cell area following LPS-induced maturation (data not shown). This suggests that relocation of DCs maturing inside secondary lymphoid tissue may not require any S1P receptor activity. In support of this, we showed earlier that assembly of mature DCs in the T cell zone occurs, even if S1P receptor signaling is interrupted by FTY720 treatment (25). Thus, the dependence of maturing DCs on S1P-triggered signaling to reach their final destination may differ, in general, among cells that are already present in the lymphoid organ during stimulation and those that first need to emigrate from the periphery. This necessitates additional steps, as illustrated by Langerhans cells that make E-cadherin–based contacts to surrounding keratinocytes. Therefore, upon stimulation, many tissue-resident DCs must first disengage from firm adhesions to neighboring cells or extracellular matrix to get mobilized (42). Following polarization and interstitial migration to reach a nearby lymph vessel, the DC enters the lymphatic system and travels to the draining LN. As outlined above, our results corroborate a function of S1P receptors in migration, per se, but point to a crucial requirement of the S1P receptor system in mobilizing or polarizing DCs that are anchored in peripheral tissues and commence a maturation/migration program. For example, this is supported by the observation that the presence of SEW2871 already prevents displacement of most CD11c+CD103+ DCs from their original location in the LP. Apparently, these DCs do not reach a state of active and recordable migration. Our observations also suggest that S1P1 is always essential for peripheral DCs to accomplish migration efficiently, whereas the contribution of S1P3 varies, depending on the origin of the tissue-resident DCs. The pivotal role of S1P1 in these processes is substantiated by the finding that virtually all migratory cells of the hematopoietic lineage express this S1P receptor (18, 43).

Based on the observations presented in this paper, we conclude that, apart from the kind of stimulus, the provenance of the DC represents an important factor that determines the impact of a given S1P receptor on migration. It remains to be determined which particular mechanisms and environmental cues during their differentiation and maturation equip DCs to migrate or deprive them from migrating in response to S1P-driven signals that are mediated by distinct S1P receptors. It will be interesting to learn whether such scenarios can be mimicked by in vitro settings. This probably paves the way for a better understanding of why different in vivo subsets of DC show a varying dependence on S1P1 and S1P3 to move or, as is the case for immature 33D1+ splenic DCs, not to move.

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Disclosures
The authors have no financial conflicts of interest.

References

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Supplementary Figure S1. Successful reconstitution of mice chimeric for S1P1<sup>−/−</sup>. Fetal liver chimeras were produced as described in Materials and Methods. (A) The percentage of CD4<sup>+</sup> cells, CD8<sup>+</sup> cells, B220<sup>+</sup>MHCII<sup>+</sup> B cells and CD11c<sup>+</sup>MHCII<sup>+</sup> DC present in the indicated compartments that are of recipient (Ly5.2) or donor (Ly5.1) origin are shown. The known effect of blood lymphopenia triggered by S1P<sub>1</sub> absence on T cells is documented in (B). Data shown were derived from analyses of at least two mice and are representative for the animals used in the experiments.

Supplementary Figure S2. CD4<sup>+</sup> T cells express S1P<sub>1</sub> and S1P<sub>4</sub>. To control for proper amplification conditions in the real time studies, the expression pattern of S1P receptors in sorted splenic CD4<sup>+</sup> T cells was determined. Each dot represents one PCR sample. The results were set into relation to the GAPDH levels detected in each sample.
SUPPLEMENTARY FIGURE S1

1A

1B