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Human Naive and Memory T Cells Display Opposite Migratory Responses to Sphingosine-1 Phosphate

Annabelle Drouillard,* Antoinette Neyra,* Anne-Laure Mathieu,* Alexandre Belot,* and Thierry Walzer*

The role of sphingosine-1 phosphate (S1P) in leukocyte trafficking has been well deciphered in mice but remains largely unaddressed in humans. In this study, we assessed the ex vivo response to S1P of primary human T cell subsets. We found that tonsil but not blood leukocytes were responsive to S1P gradients, suggesting that T cell responsiveness is regulated during their recirculation in vivo. Tonsil naive T cells were readily chemoattracted by S1P in an FTY720-sensitive, S1PR1-dependent manner. Surprisingly, S1P had the opposite effect on effector memory T cells, resident memory T cells, and recently activated T cells, inhibiting their spontaneous or chemokine-induced migration. This inhibition was also more pronounced for CD4 T cells than for CD8 T cell subsets, and was dependent on S1PR2, as shown using the S1PR2 antagonist JTE-013. S1PR1 was progressively downregulated during T cell differentiation whereas S1PR2 expression remained stable. Our results suggest that the ratio between S1PR1 and S1PR2 governs the migratory behavior of T cell subsets. They also challenge previous models of the role of S1P in lymphocyte recirculation and suggest that S1P promotes retention of memory T cell subsets in secondary lymphoid organs, via S1PR2. The Journal of Immunology, 2018, 200: 000-000.

Memory T cell subsets have complementary functions and distinct tissue distributions. Central memory T cells (TCM) defined as CCR7+CD45RA\(^2\) in humans have superior proliferative capacity and recirculate through secondary lymphoid organs (SLO) via blood and lymph. Effector memory T cells (TEM), defined as CCR7+CD45RA\(^-\)CD45RO+ in humans, have important immediate effector functions and recirculate through the blood and spleen (1). In humans, a high frequency of T cells found in nonreactive lymph nodes (LN) is of TEM phenotype, even in infants, suggesting that TEM may recirculate via nonlymphoid tissues to the afferent lymph, as previously proposed in mice. This phenomenon is, however, transient as S1PR1 levels increase during differentiation into effector and memory T cells, which is believed to permit egress from SLO by restoring responsiveness to S1P (14, 15). S1P upregulates CD69 expression. CD69 binds surface S1PR1 and increases responsiveness to S1P via Rho instead of Rac-like G-protein-coupled receptors (7). This property may explain how S1PR2 mediates chemorepulsion and antagonizes S1PR1 signaling in mouse osteoclast precursors (8) or germinle center B cells (9), a process important for their proper localization. S1PR2 is also known to be insensitive to the effect of FTY720, a supra agonist for all other S1P receptors (16). S1P responsiveness is regulated during T cell activation, as shown in mouse studies. Recently activated T cells (act-T cells) upregulate CD69 expression. CD69 binds surface S1PR1 and induces its internalization, trapping act-T cells in inflamed LNs (10) or in nonlymphoid tissues such as the skin (13). Upon TCR stimulation, S1PR1 is also downregulated transcriptionally, which may contribute to T cell sequestration in LN. This downregulation is, however, transient as S1PR1 levels increase during differentiation into effector and memory T cells, which is believed to permit egress from SLO by restoring responsiveness to S1P (14, 15). S1P responsiveness is also cyclically modulated during lymphocyte recirculation. In particular, lymphocyte S1PR1 is downregulated...
in the blood, upregulated in lymphoid organs, and downregulated again in the lymph, in a manner dependent on local S1P concentrations (16) and on the GRK2 kinase (17).

Few studies have addressed the role of S1P receptors in primary human lymphocytes. It was reported that human thymocytes displayed a strong response to S1P in migration assays (18) and that tonsil B cell subsets respond to S1P (19). Moreover, numerous studies have documented the important lymphopenia induced by the treatment with FTY720, a Food and Drug Administration–approved treatment for relapsing multiple sclerosis (20–23). FTY720 binds with a higher affinity to S1PR1 and, although the mechanism of FTY720 action is still debated, it is likely that much of its action comes from its ability to induce S1PR1 internalization in lymphocytes. In treated patients, FTY720 induces a quick decrease in peripheral naïve cells and TCM but it does not affect peripheral TEM and NK cells (20, 21, 24), which is attributed to a low S1PR1 expression on these cell types and a lower sensitivity to FTY720-induced internalization of S1P5 (25). Hence, most of our knowledge of the role of S1P receptors in lymphocyte trafficking results from the study of loss-of-function mutant mouse models or the study of the impact of FTY720 in patients, and the role and regulation of S1P receptors in human leukocytes remains mostly unexplored. In this study, we revisited this point and measured the response of human memory T cell subsets to S1P, and assessed the role of the different S1P receptors in this response.

Materials and Methods

Patients and preparation of human lymphocyte suspensions

All material was used after obtaining informed consent, and the research was conducted in accordance with the Helsinki Declaration. Human peripheral blood was obtained from healthy donors. Cells were ficollized and then washed several times in complete medium before resuspension in chemotaxis medium. Pediatric tonsils were obtained from patients undergoing tonsillectomy and were cut into pieces using a scalpel and then passed through a 70 μm cell strainer to produce a cell suspension. Cells were then ficollized and washed before migration studies.

Chemotaxis assays

Tonsil lymphocytes or PBMC were suspended in RPMI 1640 supplemented with 4 mg/ml fatty acid–free bovine albumin (Sigma, St. Louis, MO). The same medium was used to prepare S1P (Sigma) or CXCL12 (R&D Systems, Minneapolis, MN) at the indicated concentrations. Cell migration was analyzed in Transwell chambers (Costar, Cambridge, MA) with 5 μm pore-width polycarbonate filters. Pharmacological modulators of S1P receptors FTY720 (Novartis, Basel, Switzerland), JTE-013 (Tocris, Bristol, U.K.), SEW2871 (Sigma), and CYM50358 (Tocris) were used at the indicated concentrations. PBMC or tonsil cells were added to the top chambers of the Transwell systems in the presence or absence of the pharmacological inhibitors and incubated at 37°C for 2 h. The chemoattractants were then added in the lower chamber and the cells were allowed to migrate for 2 h. Transmigrated cells were stained for CCR7 (G043H7), CD69 (FN50), CD103 (Ber-act8), CD38 (HIT32), and CD3 (UCHT1), CD4 (RPA-T4), CD8 (RPA-T8), CD45RO (UCHL1), and HLA-DR (LN3) and analyzed/counted by flow cytometry (MACSQuant; Beckman-Coulter (Miami, FL), or BioLegend (San Diego, CA)). The purity of sorted cell populations was over 98% as checked by flow cytometry. Sorted cells were lysed using Trizol reagent (Invitrogen) and RNA was extracted according to the manufacturer’s instructions.

Quantitative RT-PCR

We used a high-capacity RNA-to-cDNA kit (Applied Biosystems, Carlsbad, CA) to generate cDNA for RT-PCR. PCR was carried out with a SybrGreen-based kit (FastStart Universal SYBR Green Master, Roche, Basel, Switzerland) or Sensifast SYBR No-ROX kit (Bioline) on a StepOne plus instrument (Applied Biosystems) or a LightCycler 480 system (Roche). Primers were designed using the Roche software. The following primers were used for quantitative PCR: S1PR1 (forward) 5′-AAGCCTCGCTGTGAGCA-3′, S1PR1 (reverse) 5′-TCAAGAATGCTTTTGTGTGTTG-3′, S1PR2 (forward) 5′-CCACTCGCCATACTCTC-3′, S1PR2 (reverse) 5′-ACGCCGCGCTTTCATGCTG-3′, S1PR3 (forward) 5′-GCGGCGCACTCTTCTCTTC-3′, S1PR3 (reverse) 5′-GAGGCTGTCCTCCTGCTCCTA-3′, S1PR4 (forward) 5′-GAATGCGGCCTACCATGTC-3′, S1PR4 (reverse) 5′-CAAGGAGCTCGTCTGCTGCTGC-3′, KLF2 (forward) 5′-CATCTGAGGCGCATCG-3′, KLF2 (reverse) 5′-CAGGTCTCCGAGTACTGG-3′, OAZ1 (forward) 5′-GAGGAAAACGACAGGTGCAATGTC-3′, OAZ1 (reverse) 5′-TACAGCACTGGAGGGAGAC-3′.

Statistics

Statistical analyses were performed using parametric or nonparametric tests (t test or ANOVA) run on the Prism software (GraphPad). Levels of significance are expressed as p values (*p < 0.05, **p < 0.01, ***p < 0.001).

Results

Lymphocyte response to S1P is regulated during recirculation across lymphoid organs

We measured the response of freshly isolated human lymphocyte subsets to S1P using Transwell migration systems. We first tested the response of PBMC obtained from healthy donors, taking naïve CD4 and CD8 T cells as the prototypic cell types responsive to S1P. T cell subsets were identified by flow cytometry as shown in Fig. 1A. However, as previously demonstrated in mice, human naïve T cells did not respond to S1P (Supplemental Fig. 1A). A prior incubation in culture medium supplemented (or not) with serum or cytokines did not change this response (data not shown). This unresponsiveness was specific to S1P as blood T cells were highly responsive to chemokines such as CXCL12 (Supplemental Fig. 1B). Assuming that S1P receptors were desensitized in blood leukocytes as a result of high S1P concentrations in this compartment as described in mice, we measured the response of freshly isolated tonsil lymphocytes to S1P gradients. As shown in Fig. 1B, 1C, tonsil naïve T cell chemotaxis was strongly increased when S1P gradients were applied, and compared with the control condition without S1P. Maximum migration was obtained with 30–60 nM S1P, in the range of previously published values for mouse T cells. Higher S1P concentrations inhibited this migration, a response typically observed with other G protein–coupled receptors. Both naïve CD4 and CD8 T cells migrated in response to S1P with naïve CD8 T cells displaying the strongest response.

These data show that, like in the mouse (16), human lymphocyte response to S1P fluctuates during recirculation, presumably because of receptor internalization in blood lymphocytes.

S1P inhibit spontaneous migration of memory T cells

Next, we measured the migration of memory T cell subsets in response to S1P or CXCL12. Blood memory T cells did not respond to S1P but were strongly reactive to CXCL12 (Supplemental Fig. 1C–F). Surprisingly, when analyzing the response of tonsil lymphocytes, S1P did not attract TCM and TEM toward the lower chamber but rather inhibited their spontaneous migration.
especially for memory CD4 T cells. TCM had an intermediate migratory response between naive and TEM, suggesting that the response to S1P was mediated by two different receptors whose expressions were regulated in opposite ways during T cell differentiation. When expressing the same migration results as a percentage of input, we noticed that tonsil naive T cells were rather stationary in the absence of chemotactic signals. Reciprocally, memory T cells of both subsets were constitutively motile, but this migration could be inhibited in a dose-dependent manner by S1P added in the lower chamber (Fig. 1D–G). Similar results were obtained with LN lymphocytes (data not shown), suggesting that our conclusions on the response of memory T cells to S1P likely apply to those of all SLO. Finally, we also tested the capacity of tonsil T cells to respond to a physiological source of S1P, i.e., FCS, diluted at different concentrations. As shown in Supplemental Fig. 2, naive and memory T cells displayed opposite responses to FCS, naive T cells being attracted and memory T cell migration being inhibited by S1P.

Altogether, these results show that T cell response to S1P is highly regulated during differentiation and that naive and TEM subsets have opposite responses to S1P. These data also suggest that TEM are retained by S1P within SLO whereas naive T cells can exit in response to the same signal. S1PR1 and S1PR2 are respectively involved in naive and memory T cell response to S1P

To gain insight into the mechanism underlying the different behaviors of naive and memory T cell subsets in the presence of S1P gradients, we first measured the expression of S1P receptors by semiquantitative RT-PCR in sorted T cell subsets. As shown in Fig. 2A and 2B, S1PR1, S1PR2, and S1PR4 were expressed at high levels in T cells, whereas S1PR3 was barely detected and S1PR5 was only expressed at low levels in CD8 TEM. Of note, S1PR1 expression was progressively downregulated upon T cell differentiation into memory cells, whereas S1PR2 and S1PR4 expression levels remained constitutively expressed. S1PR1 downregulation correlated with KLF2 being strongly downregulated in TEM (Fig. 2C). KLF2 is known to induce S1PR1 expression in T cells (26).

We then tested the effect of different pharmacological inhibitors of these receptors on the capacity of T cell subsets to respond to S1P. Tonsil naive T cell migration was strongly inhibited by S1PR1 inhibitors FTY720 and SEW2871 (Fig. 2D, 2E) but insensitive to the S1PR4 inhibitor CYM50358. The S1PR2 inhibitor JTE-013 abrogated the inhibitory effect of S1P on TCM and TEM migration (Fig. 2F–I). This inhibitor had, however, no effect on spon-
taneous T cell migration (data not shown). Of note, FTY720 and SEW2871 had a negative impact on memory T cell migration in the presence of S1P whereas JTE-013 treatment tended to increase naive T cell migration to S1P, suggesting that S1PR1 and S1PR2 are active in all T cell subsets but that the relative level of each receptor conditions the migratory behavior in response to S1P. Interestingly, when plotting the maximum migration index as a function of S1PR1 expression in the various T cell subsets, we found a very tight linear correlation between both factors (Supplemental Fig. 3), suggesting that S1PR1 expression is the limiting rate factor in S1P-induced migration and that the apparent repulsion mediated by S1PR2 only occurs when S1PR1 is expressed at very low levels, i.e., in memory T cells, especially of the CD4 subset.

Spontaneous migration of TRM and act-T cells is inhibited by S1P in an S1PR2-dependent way

As our results suggested that S1P could be an important tissue-retention signal in humans, we next studied the S1P response of TRM and recently act-T cells. In mice, TRM are believed to be retained in tissues by default of a response to S1P. Indeed, TRM express very low levels of KLF2 and S1PR1 (27). Moreover, CD69 expression induces S1PR1 internalization, further inhibiting migration toward S1P (12). To test this point in humans, we measured the S1P response of human tonsil TRM defined as CD69 positive T cells coexpressing (or not) CD103. As shown in Fig. 3A and 3B, S1P inhibited spontaneous migration of CD4+ TRM, irrespective of their CD103 expression. Again, the S1PR2 antagonist JTE-013 abrogated this inhibitory effect (Fig. 3C). For CD8+ TRM, S1P inhibited spontaneous migration of CD69+CD103- cells, and JTE-013 abrogated this effect.

Upon Ag-mediated activation, T cells are retained within SLOs, presumably to sustain their activation and differentiation through serial interactions with Ag presenting cells. Mechanistically, it was previously reported that mouse T cells lose their reactivity during this phase by down regulating S1PR1 expression (14). We addressed this point in humans, exploiting the fact that tonsils contain act-T cells in a variable proportion, classically defined as...
CD38⁺HLA-DR⁺. As shown in Fig. 3A and 3B, the spontaneous migration of act-CD4⁺ and act-CD8⁺ T cells was strongly inhibited by S1P, an effect that could be again curbed by the S1PR2 antagonist JTE-013 (Fig. 3C). We also measured the expression of S1P receptors in TRM CD69⁺ and act-T cells. S1PR1 was found to be highly downregulated in act-T cells compared with naive T cells and undetectable in TRM whereas the level of S1PR2 was similar to that of other T cell subsets (Fig. 3D).

**S1PR2 engagement inhibits chemokine-induced migration of memory T cell subsets**

Altogether, our results demonstrate the major role of S1PR2 in human memory T cell response to S1P and suggest a functional antagonism between S1PR1 and S1PR2 in T cell subsets. As lymphocytes are constantly exposed to opposing signals influencing their mobility, we also wanted to test the impact of S1P on the capacity of naive and memory T cells to respond to chemokine gradients. We therefore measured the migration of tonsil T cells in response to various concentrations of CXCL12, the ligand for CXCR4, a receptor involved in T cell homeostasis (28) and SLO organization (29), in the presence or absence of S1P given at an optimal concentration (37 nM). As shown in Fig. 4, the addition of S1P in the lower chamber slightly increased the migration of naive CD4 and CD8 T cells but decreased that of TEM and to a lesser extent that of TCM induced by CXCL12. The latter effect was abrogated by JTE-013 in all conditions, demonstrating that S1PR2

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**FIGURE 3.** S1PR2 inhibits spontaneous migration of TRM and act-T cells. Transwell assays of the migration of the indicated tonsil T cell subsets assessing movement toward different concentrations of S1P as indicated. Results are shown as (A) migration index; (B) percentage of input. Lines show biological replicates (n = 6 donors). Asterisks indicate statistical significance when comparing cell migration in the control condition (medium) with the conditions with S1P. *p < 0.05, **p < 0.01, ***p < 0.001 (ANOVA test with Dunnett posttest). (C) Prior to the addition of S1P in the lower chamber, cells were treated for 2 h in the top chamber with the JTE-013 at the indicated concentrations (nanomolars). Results show the mean ± SD of six independent experiments with n = 6 donors. Asterisks indicate statistical significance when comparing cell migration in the control condition (S1P only) with the conditions with JTE-013. *p < 0.05, **p < 0.01 (Mann–Whitney tests). (D) Quantitative real-time PCR analysis of S1PR expression in the indicated T cell subsets sorted by flow cytometry from human tonsil lymphocyte preparations. n = 3 sorts from three different donors.
engagement can oppose chemokine-induced migration in memory T cell subsets.

Discussion

In this study, we demonstrate that human T cell responsiveness to S1P is regulated at multiple levels. First, as previously demonstrated in mice (16), S1P receptors are desensitized in blood circulating T cells. This is likely the consequence of ligand-induced receptor internalization. All our attempts to restore T cell sensitivity to S1P by preincubation in various conditions failed, suggesting that the recycling of S1P receptors on the cell surface requires active signals, more complex than the mere deprivation of S1P. Second, the expression of S1P receptors is highly regulated during human T cell differentiation. S1PR1 expression is indeed high in human naive T cells, likely induced by the transcription factor KLF2 (26) but decreases upon differentiation in TEM and even more in TEM, TRM and act-T cells. By contrast, S1PR2 expression remains stable during differentiation. Hence the ratio between S1PR1 and S1PR2 progressively decreases during T cell differentiation into memory subtypes. This correlated with the migratory behavior of naive versus memory T cells in the presence of S1P; S1PR1 mediated attraction of naive T cells whereas S1PR2 inhibited spontaneous or chemokine-induced memory T cell migration. The affinity of S1P to S1PR2 appears substantially lower than its affinity to S1PR1 (Kd of 27 nM versus Kd of 8 nM for review, see Ref. 30). This suggests that T cells are attracted toward high S1P concentrations as long as S1PR1 expression is sufficient to overcome S1PR2 activity. Our results suggest that TEM, TRM, act-T cells, and to a lesser extent TCM are retained within SLO through S1PR2 signaling. These data challenge the classical view that S1P is a major exit signal for T lymphocytes. S1P-mediated retention of memory and act-T cells within SLO may favor the re-encounter of Ag presented by dendritic cells. These data may also explain why, as recently shown, TEM are very abundant within SLO (2). Importantly, memory T cell subsets were found to display higher spontaneous migration than naive T cells, possibly through constitutive activation of integrins. This intrinsically high mobility may be important for their entry into SLO and to more efficiently scan APCs. It has been postulated that egress structures in SLO like LN may be relatively permissive to T lymphocytes, possibly through egress portals (15). In this context, S1PR2 may be important to override the constitutive mobility of memory T cells and promote their retention within SLO. How memory T cell subsets reach the blood circulation remains to be determined. S1P-induced S1PR2 desensitization and attraction by other chemotactic signals such as proinflammatory or homeostatic chemokines may allow the egress from SLO. Similar to S1PR1, S1PR2 can be indeed internalized upon stimulation with agonists (31). In zebrafish, the miles apart mutant, S1PR2 R150H alters the migration of cardiac precursor cells to the midline, a phenomenon due to constitutive desensitization and internalization of S1PR2 (32). CXCL12 is highly expressed in the medullar region of LN (29) and may also contribute to promote entry of memory T cells into lymphatic vessels.

S1PR2 has been previously shown to contribute to accumulation of germinal center B cells in the central region of the mouse follicle [Wang et al. (6); Green et al. (9)]. Sic et al. (19) also showed that human tonsil germinal center and plasma B cells spontaneous migration was inhibited by S1P. As these cells express high levels of S1PR2, this was likely to be mediated by S1PR2, although this point required formal testing. Likewise, S1PR2 was shown to be critical for mouse follicular helper T cell retention in germinal centers (33) and in the proper localization of osteoclast precursors in the bone (8). S1PR2 also inhibits migration in many nonhematopoietic cell types, including vascular endothelial and smooth muscle cells as well as tumor cells (7). Our findings therefore corroborate prior reports suggesting counterbalancing roles of S1PR1 and S1PR2, and suggest that the antagonism between S1PR1 and S1PR2 is also important to control the distribution of naive and memory T cells in human. Previous studies suggest that S1PR2 usually inhibits S1PR1 signaling by activating Rho and inhibiting Rac (34–36). S1PR1 is also known to signal through Akt (36), an event that has been coupled to S1P responsiveness and actin polymerization in human T cells (37).

The S1PR2 receptor plays important roles in the physiology of several tissues and organs, and thus, a therapeutic application of S1PR2 antagonists or allosteric modulators will inevitably cause adverse effects if given systemically. However, local targeting of S1PR2 may alleviate symptoms induced by overt T cell reactions, in the context of various inflammatory, allergic, or autoimmune syndromes.
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Disclosures
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