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Cutting Edge: Suppression of T Cell Chemotaxis by Sphingosine 1-Phosphate

Markus Graeler,* Geetha Shankar, † and Edward J. Goetzl2* 

Murine CD4 and CD8 T cells express predominantly types 1 and 4 sphingosine 1-phosphate (SIP) G protein-coupled receptors (designated SIP1 and SIP4) or a previous endothelial differentiation gene-encoded 1 and 6 for SIP, which has a normal plasma concentration of 0.1-1 μM. SIP now is shown to enhance chemotaxis of CD4 T cells to CCL-21 and CCL-5 by up to 2.5-fold at 10 nM to 0.1 μM, whereas 0.3-3 μM SIP inhibits this chemotaxis by up to 70%. Chemotaxis of SIP1, but not SIP4, transfectants to CXCL1 and CXCL4 was similarly affected by SIP. Activation of CD4 T cells, which decreases SIP receptor expression, suppressed effects of SIP on chemotaxis. Pretreatment of labeled CD4 T cells with SIP before reintroduction into mice inhibited by a maximum of 75% their migration into chemokine-challenged s.c. air pouches. The SIP-SIP1 receptor axis thus controls recruitment of naive T cells by maintaining their response threshold to diverse lymphomafactor. The Journal of Immunology, 2002, 169: 4084–4087.

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The lyposphospholipid growth factors sphingosine 1-phosphate (SIP) and lysophosphatidic acid (LPA) are generated predominantly by stimulated platelets and leukocytes, resulting in up to micromolar concentrations in normal plasma and some other extracellular fluids (1–3). SIP and LPA evoke cellular proliferation and diverse functional responses by binding to members of a family of homologous G protein-coupled receptors (GPCRs), which originally were designated endothelial differentiation gene-encoded or Edg receptors (4–6). Recently, a nomenclature subcommittee of the International Union of Pharmacologists has renamed these receptors for their principal ligand and order of discovery, so that the LPA receptors are LPA1 (Edg-2), LPA2 (Edg-4), and LPA3 (Edg-7), and the SIP receptors are SIP1 (Edg-1), SIP2 (Edg-5), SIP3 (Edg-3), SIP4 (Edg-6), and SIP5 (Edg-8) (7).

Blood unstimulated CD4 T cells express high levels of LPA2 (Edg-4) receptors constitutively, whereas CD8 T cells from the same source show only traces of LPA1 (Edg-2) (8). In studies of regulation of expression of LPA receptors, mitogen activation of blood CD4 T cells down-regulated expression of LPA1 and up-regulated that of LPA4, but such activation of CD8 T cells did not alter expression of any LPA receptor (9). IL-2 production by blood CD4 T cells was suppressed through LPA2 and enhanced through LPA1. In contrast, LPA4 transduced LPA-evoked chemotaxis and chemokinesis of CD4 T cells, whereas LPA1 failed to signal chemokine-elicited chemotaxis of CD4 T cells (10). Thus, the effects of activation of blood CD4 T cells on LPA receptors converted LPA from a migration-enhancing and cytokine production-inhibiting factor to a migration-inhibiting and cytokine production-enhancing factor for effector T cells.

In the course of studies of mouse splenic T cell LPA and SIP receptors, SIP1 and SIP3 were found to be the vastly predominant SIP receptors of CD4 and CD8 T cells and LPA receptors were detected at only very low levels (11). SIP was noted to elicit T cell chemotactic responses at concentrations well below those typical of plasma (11). It was also discovered that the high level of expression of SIP1 and SIP4 receptors by mouse splenic CD4 and CD8 T cells is suppressed nearly completely by TCR-dependent activation (11). Chemotactic responses of both unstimulated CD4 and CD8 T cells were elicited by 1 nM SIP, attained a maximum at 0.1 μM SIP, and were reduced in parallel with suppression of the levels of SIP receptors by TCR-dependent activation (11). A pharmacological probe of SIP receptors (12, 13) also inhibited chemotactic responses of T cells to SIP in vitro and in vivo (11).

It is now reported that the major effect of plasma concentrations of SIP on T cells is suppression of their responses to other chemomafactor responses, such as chemokines, in a basement membrane transmigration assay. TCR-dependent activation of the T cells, resulting in decreased expression of SIP1 receptors, reduced the extent of suppression of chemomafactor responses by SIP. SIP1 receptor is the major transducer of SIP effects based on findings that SIP suppressed chemokine-evoked chemotaxis of transfectants expressing only SIP1 receptors, but not that of transfectants expressing only SIP4 receptors.

Materials and Methods

Isolation, activation, and fluorescent labeling of mouse spleen CD4 T cells

Mouse CD4 T cells were isolated from splenocytes of 6- to 8-wk-old C57BL/6 female mice at a minimum purity of 97% using metallic microbeads bearing anti-CD4 mAbs and two cycles of magnetic retention chromatography (Miltenyi Biotec, Auburn, CA), as described elsewhere (8, 9). For some studies, 1-ml suspensions of purified T cells in RPMI 1640-50 μg/ml fatty acid-free BSA (FAF-BSA) (Calbiochem, La Jolla, CA) with
100 U/ml penicillin G and 50 μg/ml streptomycin (RPMI 1640-BSA) were activated by incubation for 24 h on 2 μg each of adherent anti-CD3 plus anti-CD28 mAbs (BD PharMingen, San Diego, CA) in six-well plates. Stock solutions of 50 μg of methylbenzamido-1,1’-di-octadecyl-3,3’,3’-tetramethylindo-diacarbocyanine (FM-DiI; Molecular Probes, Eugene, OR) in 50 μl of DMSO were prepared immediately before labeling of CD4 T cells. Suspensions of 2 × 10^6 CD4 T cells from normal C57BL/6 mice/ml Ca^{2+}− and Mg^{2+}-free PBS were incubated with 2 μg/ml FM-DiI for 5 min at 37°C and 15 min at 4°C, washed three times in PBS, and resuspended in 1 ml of RPMI 1640-FAF-BSA for incubation with S1P for 1 h at 37°C before i.p. injection into test mice. The fluorescent labeling did not affect T cell viability or chemotactic responses in vitro nor was any fluorescent label transferred to mouse adherent peritoneal macrophages by labeled T cells, at a cellular ratio of 1:5, after 6 h of incubation at 37°C.

**Generation of HTC4 cell-S1P receptor transfectants**

The cDNAs encoding human S1P1 and S1P4 were subcloned into pLXSN and introduced into the EcoHEK-293 packaging cell line (Clontech Laboratories, Palo Alto, CA). Viral particles secreted by this line were filtered, treated with polybrene, and substituted for culture medium over HTC4 rat hepatoma cells. HTC4 cells resistant to 400 μg/ml genetinic acid were subcloned, washed, and resuspended in MEM with 10% FBS and 4 mM L-glutamine, and then incubated for 1–4 h. The mRNAs encoding S1P1 and S1P4, extracted from the respective HTC4 cell lines were quantified by real-time PCR.

**Quantification of mouse T cell and HTC4 cell-S1P receptor transfectant chemotaxis**

Migration of mouse purified CD4 T cells was analyzed in Transwell chambers (Costar, Cambridge, MA) with 8-μm pore width polycarbonate filters, as described previously (14), except that the layer of growth factor-depleted Matrigel was reduced from 15 to 8 μg/ml as described previously (14), except that the layer of growth factor-depleted Matrigel was reduced from 15 to 8 μg/ml as described previously (14). The cDNAs encoding human S1P1 and S1P4 were subcloned into pLXSN and introduced into the EcoHEK-293 packaging cell line (Clontech Laboratories, Palo Alto, CA). Viral particles secreted by this line were filtered, treated with polybrene, and substituted for culture medium over HTC4 rat hepatoma cells. HTC4 cells resistant to 400 μg/ml genetinic acid were subcloned, washed, and resuspended in MEM with 10% FBS and 4 mM L-glutamine, and then incubated for 1–4 h. The mRNAs encoding S1P1 and S1P4, extracted from the respective HTC4 cell lines were quantified by real-time PCR.

**Assessment of effects of S1P on mouse CD4 T cell recruitment to dorsal s.c. air pouches**

Air pouches were established on the back of each mouse by s.c. injection of 5 ml of micropore-filtered air. Two days later, pouch airspaces wereirrigated twice with 1 ml of PBS and then received 1.0 ml of 0.5 μM CCL-21 (Exodus-2) or CCL-5 (RANTES) (RPMI 1640-BSA) as in vivo chemotactic stimuli 15 min before i.p. injection of FM-DiI fluorescently labeled CD4 cells that had been preincubated with 0.3 or 1 μM S1P, or with medium alone. Pouch cells were harvested in 2 ml of PBS and peritoneal cells in two washes of 2 ml of PBS each after 24 h for performance of microscopic counts and determination of fluorescence.

**Results and Discussion**

S1P stimulates CD4 T cell migration across a Matrigel model membrane with concentration-dependent responses which are significant at 10−9 M and maximal at 10−7 M (11). The trans-Matrigel chemotactic responses of CD4 T cells to S1P at upper normal plasma levels of 3 × 10−7–10−6 M, however, are far below the maximum. Furthermore, direct exposure of CD4 T cells to 3 × 10−7–10−6 M S1P decreased chemotactic responses to 10−8 M through 10−7 M S1P with Matrigel-coated and type IV collagen-coated filters. To determine whether the chemotactic inhibitory effect of plasma concentrations of S1P extended to chemotactic responses of the T cells to other factors, replicate suspensions of CD4 T cells were preincubated with 10−10–3 × 10−8 M S1P before quantification of their responses to the chemokines CCL-21 (Exodus-2) and CCL-5 (RANTES) (Fig. 1). At the subplasma level of 10−8 M and at 10−7 M, S1P enhanced by up to 2.5-fold the chemotactic responses of the T cells to both CCL-21, at two concentrations, and CCL-5. At higher normal concentrations in plasma and up to those in some disease states, S1P suppressed significantly and by up to 70% the chemotactic responses of CD4 T cells to both chemokines. The inhibitory effect of S1P was not attributable to cytotoxicity and was fully reversed by washing the T cells before introduction into the chemotactic chambers.

**FIGURE 1.** S1P regulation of CD4 T cell chemotactic responses to chemokines. Each bar depicts the mean ± SD of the results of three studies performed in duplicate. Mean control chemotactic responses (100%) were 3.2, 4.1, and 4.2% of the CD4 T cells initially added to each chamber for 50 nM CCL-21, 12.9, 9.2, and 16.5% for 200 nM CCL-21, and 5.4, 10.4, and 7.8% for 100 nM CCL-5. The symbols for statistical significance of differences between a set of values and the concurrent controls are: +, p < 0.05 and *, p < 0.01.

**FIGURE 2.** Suppression of the regulatory effects of S1P on chemokine-induced chemotactic responses by activation of CD4 T cells. The derivation of data depicted in the bars and the meaning of statistical symbols are the same as for Fig. 1. Mean control chemotactic responses (100%) for unstimulated T cells were 9.1, 15.4, and 11.3% with 200 nM CCL-21 and 12.7 and 12.3, and 20.8% with 200 nM CCL-5, and for activated T cells were 23.5, 31.0, and 23.8% with 200 nM CCL-21 and 21.2, 30.9, and 27.5% with 200 nM CCL-5.
The distinctive role of S1P in regulating T cell traffic is a function of its sustained micromolar concentrations in plasma and some other fluids, and its predominantly suppressive effect on T cell trans-tissue chemotactic responses to highly active chemokines at these concentrations. Submicromolar levels of S1P found in some tissues may be directly chemotactic for T cells (11) and enhance T cell chemotactic responses to chemokines (Figs. 1 and 2). These potential contributions of S1P will be better understood after its levels in such tissues have been determined accurately. However, at micromolar concentrations typical of plasma, S1P is principally an inhibitory factor capable of dampening or preventing T cell responses to chemokines (Figs. 1 and 4). This may effectively be an adjustment of the threshold of responsiveness or complete suppression, depending on the relative concentrations of endogenous S1P and the primary chemotactic stimulus. The immunosuppressive agent FTY720 and phosphorylated FTY720, which are structurally similar to S1P and interact with S1P receptors (12, 13), also may modify T cell responses to chemokines directly or by altering S1P effects.

Another important variable in this regulatory formula is the level of expression of the S1P1 (Edg-1) and/or S1P4 (Edg-6) GPCRs and the importance of each as transducers of effects of S1P on T cells (11). TCR-dependent activation of T cells reduces substantially the levels of expression of both S1P1 (Edg-1) and S1P4 (Edg-6) with concomitant reduction in S1P signaling of T cell migration (11) (Fig. 2). Thus, it is not surprising that such activation of CD4 T cells eliminates both the augmentation by subplasma S1P levels and the suppression by plasma S1P levels of T cell responses to chemokines (Fig. 2). The relative contributions of signaling by S1P1 (Edg-1) and S1P4 (Edg-6) to the observed effects of S1P on T cell chemotaxis cannot be easily dissected with the presently available tools. It was assumed that S1P1 (Edg-1) would predominate at concentrations of S1P below 3 μM as the affinity for S1P and resultant transductional efficiency is nearly 30-fold higher than for S1P4 (Edg-6) (15). Transfectants expressing solely S1P1 (Edg-1) are susceptible both to S1P stimulation of their chemotaxis and chemokinesis and to S1P inhibition of their chemotactic responses to chemokines, whereas otherwise identical transfectants solely expressing S1P4 (Edg-6) exhibited neither response (Fig. 3). These data suggest that Edg-1 is the principal unit for cellular transmission of S1P signals to T cells.

The findings now reported answer for immunity a fundamental question in the field of lysosphospholipid mediators. Polypeptide and all other lipid chemotactic factors, whose signals are transduced by high-affinity GPCRs, are generated and degraded rapidly in immune responses, such that their optimally active picomolar to nanomolar concentrations are maintained most often for only very brief periods. So why then are the fluid-phase concentrations of lysosphospholipid T cell migration-directed factors, which act

line which lacks endogenous S1P receptors and responds chemotactically to the CXCL-4 (PF-4) and CXCL-1 (GRO/KC) chemokines. The two lines of HTC4 transfectants responded similarly to an optimal concentration of CXCL-4, but only the S1P1 receptor-HTC4 cells responded chemotactically and chemokinetically to S1P (Fig. 3A). Similarly, only the chemotactic responses of S1P1 receptor-HTC4 cells to CXCL-4 were enhanced by 10\(^{-8}\) and 10\(^{-7}\) M S1P and suppressed by 10\(^{-6}\) and 3 \times 10\(^{-6}\) M S1P (Fig. 3B). In more limited studies of rat CXCL-1 (GRO/KC) at an optimal concentration of 10\(^{-8}\) M, equivalent chemotactic responses of S1P1 receptor-HTC4 cells and S1P4 receptor-HTC4 cells were 15.2 and 18.0% of initial transfectants (mean, n = 2), respectively. As for chemotactic responses to CXCL-4, those of S1P1 receptor-HTC4 cells to CXCL-1 were increased significantly by 10\(^{-8}\) M S1P to 146% of the control value and decreased significantly by 10\(^{-9}\) M S1P to 37% of the control value (mean, n = 2). No effects of 10\(^{-8}\) and 10\(^{-9}\) M S1P were observed on chemotactic responses of S1P4 receptor-HTC4 cells to CXCL-1. These results support a primary role for S1P1 receptors as the transducer of S1P signals in CD4 T cells and suggest that S1P4 does not fulfill any function in T cell migration.

The investigation of S1P inhibition of T cell chemotaxis was extended to an in vivo model. CD4 T cells labeled with a fluorescent tracking dye were preincubated ex vivo with plasma concentrations of S1P before i.p. reintroduction into mice for assessment of their responses to CCL-21 (Exodus-2) in a dorsal s.c. air pouch. Approximately 10% of the total labeled control CD4 T cells were recovered from the peritoneal cavity and air pouch of normal mice (Fig. 4). S1P pretreatment of the labeled cells significantly increased recovery from the peritoneum and decreased that found in the air pouch, with a greater effect at 1 than 0.3 μM S1P.

In Figs. 1-4, symbols are the same as for Fig. 1.
through similarly high-affinity GPCRs, sustained at near micromolar concentrations in physiological fluids? It appears for S1P that its high concentrations in T cell corridors serve the primary homeostatic function of regulating the threshold of responsiveness of naive and memory T cells to other chemotactic factors, such as chemokines, and efficiently preventing responses to minimal perturbations of these powerful effector systems. Many other aspects of the question remain to be addressed, including the immune implications of fluctuations in the fluid-phase concentrations of S1P and the involvement of many other possible regulators of expression of S1P receptors on T cells. More than ever, we will need S1P receptor pharmacological agonists and antagonists of useful potency and single receptor selectivity to elucidate the range of regulatory immune activities of S1P and related lysophospholipid mediators.

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References