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Transduction of Multiple Effects of Sphingosine 1-Phosphate (S1P) on T Cell Functions by the S1P₁ G Protein-Coupled Receptor

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Sphingosine 1-phosphate (S1P) is one of the cell-derived lysophospholipid growth factors which signal many types of cells through a distinct subfamily of G protein-coupled receptors (GPCRs) (1–5). S1P GPCRs originally were termed endothelial differentiation gene-encoded receptors or Edg receptors and now are designated S1P₁ (Edg-1), S1P₂ (Edg-5), S1P₃ (Edg-3), S1P₄ (Edg-6), and S1P₅ (Edg-8) (5). In circulating blood and lymph, S1P is generated predominantly by mononuclear phagocytes and platelets, binds to albumin and some other proteins, and is distributed, but also initiation of early events of differentiation and tissue distribution. The Journal of Immunology, 2003, 171: 3500–3507.

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of APCs, washed twice and covered again with RPMI-C. Then 0.5 µg/ml OVA substitution peptide 323–339 (OVA323–339; AnaSpec, San Jose, CA) was added and incubation continued for 1 h. CD4 T cells were selected from splenocytes of DO11.10 αβ TCR transgenic mice (BALB/c, H-2b haplotype) (12) with anti-CD4 mAb-metallic beads and magnetic retention chromatography, as for splenic CD4 T cells of wild-type mice. The CD4 T cells then were suspended in RPMI-C containing 20 µg/ml anti-IL-4 mAb (clone 11B11; BD Pharmingen) and 40 ng/ml IL-12 (R&D Systems, Minneapolis, MN) to stimulate differentiation of Th1 cells and incubated for 72 h with APCs and OVA323–339 Ag at a 20:1 ratio of APC-Th. An equal volume of RPMI-C containing 10 U/ml mouse IL-2 (Peprotech, Rocky Hill, NJ) was added to the flask and culture continued for 96 h. The T cells then were washed, added to a fresh layer of APC (1:20) in RPMI-C with 0.5 µg/ml of OVA323–339, and further incubated for 72 h. Th1 cells were recovered, washed, and suspended in RPMI-C at 0.5–1.0 × 10^6/ml for repeated 7-day cycles of restimulation with irradiated APCs and OVA323–339, but without cytokines or anti-cytokines. Th1 cells were taken on day 4 or 5 for functional studies. A cDNA encoding human full-length S1P1, (Edg-1) was inserted into the pGC-IREs retroviral vector (obtained from G. L. Costa, Stanford University, Stanford, CA), which encodes yellow fluorescent protein (YFP), and introduced into some sets of mouse Th1 cells using the Ecopo2-pack-293 system with a HEK 293-based packaging cell line for production of rodent ecotropic retroviruses (BD Biosciences, Palo Alto, CA). Transduction efficiency was over 60%, as assessed by flow cytometric analyses of expression of YFP. Real-time PCR quantification of human S1P1 mRNA revealed levels 3- to 4-fold higher in day 4 Th1 transductants than mouse spleen naïve CD4 T cells. Western blots of proteins extracted from Th1 transductants and developed with rabbit anti-human S1P1, IgG Ab (ExAlpha, Beijing, MA) and rat anti-mouse S1P1 mAb showed levels of human S1P1, 5- to 10-fold higher than endogenous mouse S1P1, in corresponding untransduced Th1 cells.

**Real-time PCR quantification of mRNA encoding S1P1 GPCRs**

Total RNA was isolated with TRizol (Life Technologies, Grand Island, NY), treated with DNase I, and amplified quantitatively in 50-ng replicates derived from known amounts of RNA.

**Western blot analyses of S1P1s**

Immunoblots of proteins extracted from 5 × 10^6 mouse Th1 cells, before and after retroviral transduction with human S1P1, and HTTC4 rat hepatoma transfectants stably expressing S1P1 or S1P4 receptors were prepared as described (8) and developed with rabbit anti-S1P1 Ab or rabbit anti-S1P4 Ab (ExAlpha).

**Quantification of migration, proliferation, and cytokine secretion by CD4 T cells and Th1 cell lines**

Migration of mouse purified CD4 T cells and Th1 cells, without and with recombinant S1P1, introduced by retroviral transduction, was analyzed in Transwell chambers (Costar, Cambridge, MA) with human type IV collagen (Sigma-Aldrich, St. Louis, MO)-coated 5-µm pore width polycarbonate filters and incubation for 4 h, as described (7). T cell suspensions were 1 × 10^6/ml in RPMI 1640 with 5% heated and charcoal-extracted FBS, from which 0.1-ml portions of each were loaded into top compartments of chemotactic chambers. CC chemokine ligand (CCL)-21 (Exodus-2) or CCL5 (RANTES) chemokines (Peprotech) were the positive chemotactic stimuli. The significance of differences between alternate and control migration was calculated with a two-sample t test.

For studies of T cell proliferation and cytokine generation, replicate 0.5-ml suspensions of 1.0–1.2 × 10^6 CD4 T cells, and S1P1-low and recombinant human S1P1-transduced DO11.10 Th1 cells in RPMI 1640 containing 5% heated and charcoal-extracted FBS and 50 µg/ml FAF-BSA were preincubated without and with a biochemical agonist or inhibitor and without or with lysophosphatidic acid (LPA) or S1P before addition to 24-well plates. Wells were precoated with 1 µg each of adherent anti-CD3 + anti-CD28 mAbs (BD Pharmingen) to activate through the TCR or anti-CD3 alone for stimulation of homeostatic proliferation by 10–7 to 10–8 M fluid-phase mouse recombinant IL-7 (Peprotech). After 24 h of incubation, 200-µl aliquots were removed from each well for ELISA quantification of cytokines and the T cells were incubated 16 h further in fresh medium reconstituted with the original biochemical inhibitor or other agent, LPA and/or S1P, and 1.5 uCi [3H]thymidine (New England Nuclear, Boston, MA) to assess proliferation. ELISAs for mouse IFN-γ, IL-2, and IL-4 were from Pierce-Endogen (Cambridge, MA) and the assays were conducted as suggested by the manufacturer. For some replicate suspensions, the T cells were recovered by centrifugation, washed and lysed after 30 min of the initial incubation for measurement of the intracellular concentration of cAMP, termed [cAMP]. Quantification of [cAMP], was by a solid-phase low pH immunosay after acetylation of standards and samples (R&D Systems). Statistical significance of the effects of stimuli and inhibitors was calculated as for migration assays.

**Lysophospholipids and biochemical inhibitors**

LPA and S1P (Sigma-Aldrich); 8-bromo-cAMP, dibutyryl-cAMP, the cell-permeant calcium chelator BAPTA-AM, the inhibitor of phosphatidylinositol-specific phospholipase C termed 1-O-octadecyl-2-O-methyl-sn-glycercyl-3-phosphorylcholine or ET-18-OCH3 (BioMol, Plymouth Meeting, PA); the adenylyl cyclase inhibitor MDL-12,330 A HCl, the protein kinase A (PKA)-selective inhibitor KT5720, and the cAMP-selective phosphodiesterase (IV) inhibitor Ro-20-1724 (Calbiochem, San Diego, CA) were obtained from the suppliers noted.

**Results**

SIP suppressed proliferation of CD4 T cells evoked by a TCR activation pathway, which was initiated by anti-CD3 plus anti-CD28, without substantially altering the minor uptake of [3H]thymidine observed in unstimulated T cells (Fig. 1). Proliferation was suppressed significantly by 10–7 to 10–6 M SIP up to a mean of 50% at the highest concentration. SIP also suppressed the cytokine-induced homeostatic proliferation of CD4 T cells stimulated with anti-CD3 plus IL-7 (13) (Fig. 1). The suppression of IL-7–elicited proliferation was significant at 10–9 to 10–6 M SIP and attained a mean maximum of 48% at 10–8 M SIP. In contrast, 10–6 M LPA attained a slight mean suppression of proliferation evoked by anti-CD3 plus anti-CD28 of only 16% and had no effect on T cell proliferation induced by anti-CD3 plus IL-7 or that observed without stimulation (Fig. 1). In parallel analyses of mouse splenic CD8 T cells, activated by exposure to anti-CD3 plus anti-CD28 Abs, mean suppression of proliferation by 10–8 to 10–7, and 10–6 M SIP was 31, 42, and 56%, respectively (n = 2), and by 10–8 and 10–6 M LPA, 3 and 11%.

An optimal inhibitory effect required the presence of SIP before or at the time of interaction of the T cells with anti-CD3 plus anti-CD28, presumably because TCR-mediated activation of T cells down-regulates expression of SIP1 and SIP4 GPCRs nearly completely by 24 h (7). When SIP was added 12 h after exposure to anti-CD3 plus anti-CD28, the extent of suppression of proliferation was reduced for all concentrations of SIP examined relative to their effects when added at the time of stimulation (Fig. 2). When SIP was added at 24 h after stimulation, when SIP GPCR protein is just barely detectable by Western blot, there was only a modest suppression of proliferation at the highest concentration of 10–6 M.

SIP1 (Edg-1), which is the functionally dominant SIP GPCR of T cells, signals predominantly or exclusively through Gαq, with resultant decreases in basal and evoked [cAMP]. As SIP1 couples inefficiently or not at all to Gαq, Gβγ dimers or other signaling units coupled to SIP1 are presumed to activate phospholipase C (PLC) and thereby evoke increases in intracellular Ca2+ concentration ([Ca2+]i). To better delineate these signaling pathways, several selective inhibitors were examined for their capacity to alter SIP suppression of T cell proliferation. The intracellular Ca2+ chelator BAPTA-AM and the phosphatidylinositol-specific PLC inhibitor Et-18-OCH3 both completely prevented suppression of T cell proliferation by an optimal level of SIP, suggesting an obligatory role for increases in [Ca2+]i (Fig. 3). Neither inhibition
of adenylyl cyclase by MDL-12,330A nor of PKA by KT5720 had any effect on S1P suppression of T cell proliferation. In contrast, the cAMP phosphodiesterase-specific inhibitor Ro-20-1724 significantly reversed the S1P suppressive effect at a concentration which substantially elevated [cAMP], (Fig. 3, values above columns). The level of [cAMP], in T cells stimulated for 30 min with anti-CD3 plus anti-CD28 without and with 10^{-6} M S1P was not detectable at the standard T cell density (Fig. 3). In the presence of 2 and 5 μM Ro-20-1724, however, T cell [cAMP], rose to respective mean values of 0.24 and 0.72 pmol/ml (value above column, Fig. 3). The increase in [cAMP], attained by the lower concentration of Ro-20-1724 had no significant effect on S1P suppression of T cell proliferation (not shown). However, the higher concentration of Ro-20-1724, which elevated [cAMP], 3-fold more, significantly reversed S1P suppression of T cell proliferation (Fig. 3). To assess the effect of cAMP directly, replicate suspensions of T cells were preincubated for 15 min with 5 μM 8-bromo-cAMP and 5 μM dibutyryl-cAMP before addition of S1P and exposure to anti-

FIGURE 1. S1P suppression of mouse splenic CD4 T cell proliferation evoked by adherent anti-CD3 plus anti-CD28 mAbs (left frame) or anti-CD3 plus 10^{-6} M mouse IL-7 (middle frame), and on unstimulated proliferation (right frame). Each column and bar depicts the mean ± SD of the results of three different studies of the effects of 10^{-11}–10^{-6} M S1P (□) and 10^{-8} M and 10^{-6} M LPA (□). The range of control baseline values (0%) were 156,700–202,153 dpm and control stimulated values (100%) were 80,228–231,964 dpm. The levels of statistical significance are: + = p < 0.05, * = p < 0.01, ** = p < 0.0025. The mean levels of S1P/R mRNA relative to GAPDH were 301 without stimulation, 50 at 24 h after anti-CD3 plus anti-CD28 mAbs, and 64 at 24 h after anti-CD3 plus 10^{-6} M mouse IL-7.

FIGURE 2. Temporal requirement for suppression of T cell proliferation by S1P. Each column and bar shows the mean ± SD of the results of three different studies. The range of control anti-CD3 plus anti-CD28 mAb-stimulated values (100%) were 156,700–202,153 dpm. The levels of statistical significance of the differences between control and S1P-treated samples are: + = p < 0.05 and * = p < 0.005.
CD3 plus anti-CD28 mAbs. The resultant mean [cAMP], 30 min after mAb stimulation was significantly increased at 2.07 and 2.83 pmol/ml, respectively. S1P suppression of T cell proliferation was prevented completely by both forms of cell-permeant exogenous cAMP (+). The numbers in parentheses above the S1P control and four right columns are the mean values for [cAMP], in picomoles per milliliter.

The studies of S1P effects on T cell functions were extended to cytokine generation in the first 24 h after stimulation, when the concentrations of IFN-γ and IL-4 are only 5–20% of their peak levels on days 4 to 6 but S1PRs are not yet fully down-regulated by TCR signals. As for analyses of proliferation, replicate suspensions of mouse splenic T cells were stimulated with anti-CD3 plus anti-CD28 mAbs, which initiates a TCR-coreceptor pathway, and anti-CD3 mAb plus IL-7, which induces homeostatic proliferation of the T cells. LPA had previously been shown to suppress IL-2 secretion by stimulated naive human blood T cells (14). Inhibition of IL-2 secretion by LPA was confirmed in mouse splenic CD4 T cells stimulated by both mechanisms, where mean maximal inhibition by 10^{-6} M LPA exceeded 50% (Figs. 4 and 5). In contrast, 10^{-10}–10^{-8} M S1P had no effect on IL-2 secretion evoked by either signal, with the exception of marginally significant 12%
suppression of that elicited by IL-7 at $10^{-6}$ M S1P (Fig. 5). Inversely, $10^{-8} - 10^{-6}$ M S1P but not LPA suppressed secretion of IFN-γ from T cells stimulated by both pathways to the same extent as inhibition of proliferation by identical concentrations of S1P (Figs. 4 and 5). The susceptibility of T cell secretion of IL-4 to inhibition by S1P and LPA differed for the two stimulatory pathways. The secretion of IL-4 by T cells exposed to TCR-coreceptor stimulation was inhibited significantly only by $10^{-6}$ M S1P or LPA (Fig. 4). In contrast, IL-4 secretion by T cells exposed to TCR-cytokine stimulation was inhibited significantly and progressively by $10^{-8} - 10^{-6}$ M S1P, but not by $10^{-6}$ M LPA (Fig. 5).

The consistent susceptibility of IFN-γ generation to suppression by S1P, but not LPA, irrespective of the CD4 T cell stimulus suggested that more detailed studies be conducted with Ag-stimulated Th1 lines derived from CD4 T cells of TCR transgenic mice. Profiles of mouse S1P GPCRs in the OVA peptide Ag-stimulated and expanding Th1 line by real-time PCR showed mean levels of S1P$_1$ R mRNA relative to GAPDH of 5.1 on day 4 and 2.7 on day 7 after Ag stimulation, as contrasted with 334 for unstimulated mixed splenic CD4 T cells. In contrast, S1P$_4$ receptor mRNA mean levels relative to GAPDH were similar for T cells of all types at 27 on day 4 and 41 on day 7 after Ag stimulation, as contrasted with 69 for unstimulated mixed splenic CD4 T cells. To further define the regulatory roles of S1P$_1$ GPCRs in functional responses of this Th1 cell line, a subline was generated by retroviral transduction with mRNA encoding human S1P$_1$ and YFP. Flow cytometric analyses of Th1-S1P$_1$ transductants showed that approximately 80% expressed YFP and thus presumably high levels of S1P$_1$. Real-time PCR quantification of human S1P$_1$ mRNA revealed levels in Th1-S1P$_1$ transductants at least 30-fold higher than that of the PCR reagent background and at least 2-fold higher than mouse S1P$_1$ mRNA in normal splenic naïve CD4 T cells. Western blots confirmed that human S1P$_1$ R protein was present at much higher levels in transductants than the sham-transduced parent Th1 line (Fig. 6). These differences in S1P$_1$ expression were sufficient to predict distinctive responses of S1P$_1$ transductants to S1P. Indeed, S1P$_1$-high transductants of Th1 cells exhibited significantly greater chemotactic responses to S1P than the S1P$_1$-low parent Th1 line (Fig. 7A), whereas chemotactic responses to CCL5 (RANTES) were the same for both groups. Similarly, S1P inhibited more significantly the CCL-5-induced chemotactic responses of Th1-S1P$_1$ transductants than control Th1 sham-transductants (Fig. 7B). The results were the same with CCL-21 (Exodus-2) as the chemotactic stimulus. S1P inhibition of proliferation evoked by OVA peptide Ag and APCs also was greater for the S1P$_1$-high transductants than the S1P$_1$-low parent Th1 line (Fig. 7C). S1P inhibition of IFN-γ secretion by Th1 cells stimulated with OVA peptide and APC was
much greater for the S1P₁-high transductants than the S1P₁-low parent line (Fig. 7D). The marginal chemotactic, chemotactic inhibitory, and proliferation inhibitory responses of Th₁ sham-transductants to the higher concentration of S1P may be transduced by very low levels of S1P₁ not detected in Western blots or by an S1P₁, R-independent effect of S1P on store-operated calcium entry.

**Discussion**

S1P and some immunomodulatory drugs which act through S1PRs have striking effects on lymphocyte migration, tissue distribution and homing in the absence of apparent alterations in host defense. These absorbing phenomena have diverted attention from the possibilities of other lymphocyte activities of S1P. Concentrations of S1P similar to those in plasma and lymph, which inhibit T cell chemotactic responses to several different chemokines (8), also suppress T cell proliferative responses to immunologically different stimuli (Fig. 1). Suppression of T cell proliferation was highly significant at 10 nM to 1 μM S1P, reaching levels up to 50% at 1 μM S1P, and required the presence of S1P at the time of stimulation for an optimal effect (Fig. 2). One other laboratory has just reported suppression of T cell proliferation also ex-

**FIGURE 7.** Predominant role of S1P₁ in mediating effects of S1P on Th₁ cells. Each column and bar depict the mean ± SD of the results of four determinations for Th₁ cells either transduced with an S1P₁ (Edg-1) receptor-encoding retroviral vector (♂) or sham-transduced with the vector alone (□). The levels of statistical significance are the same as in Fig. 4. A. Chemotaxis. Unstimulated control values (100%) ranged from 4.3 to 6.8% of the total number of Th₁ cells for sham transductants and 5.7 to 9.5% for Th₁ S1P₁ transductants. B. Inhibition of chemotaxis. Chemotaxis stimulated by 200 nM CCL-5 (RANTES) without S1P in the cell suspensions (100%) ranged from 26 to 30% of the total number of Th₁ cells for sham transductants and 28 to 34% for Th₁ S1P₁ transductants. C. Proliferation. Uptake of [3H]thymidine without S1P (100%) ranged from 74,448–193,512 dpm for sham transductants and 76,688–204,580 dpm for Th₁ S1P₁ transductants. D. Generation of IFN-γ. Secretion of IFN-γ without S1P (100%) ranged from 21.7–29.2 ng/ml for sham transductants and 19.4–25.5 ng/ml for Th₁ S1P₁ transductants.

S1P₁ suppression of T cell proliferation also exhibits stringent Ca²⁺-dependence, as demonstrated by elimination of this suppressive effect when PI-specific PLC activity is reduced by the selective inhibitor Et-18-OCH₃ or downstream increases in [Ca²⁺], are blunted by BAPTA-AM (Fig. 3). Similar dependence on [Ca²⁺], was observed in limited investigations of the inhibition of IFN-γ generation by S1P. In two such studies, secretion of IFN-γ by Th₁ cells stimulated with anti-CD3 and anti-CD28 mAbs was suppressed a mean (±range) of 44 ± 13% by 10⁻⁶ M S1P. The PI-specific PLC inhibitor Et-18-OCH₃ at 1 μM reduced 10⁻⁶ M S1P suppression of IFN-γ secretion to 6 ± 5% of control levels, and calcium buffering by 0.2 μM BAPTA-AM similarly reduced 10⁻⁶ M S1P suppression of IFN-γ release to 3 ± 2%. Most S1P GPCRs also interact with G_{i1} to lower [cAMP], but pharmacologically induced increases in endogenous [cAMP], or introduction of exogenous stable and cell-permeant forms of cAMP may prevent or inhibit cellular responses to S1P. The >70-fold increase in [cAMP], induced by selective inhibition of phosphodiesterase activity and the over 200-fold increase in [cAMP], attributable to bioconversion of stable exogenous analogues significantly and nearly totally prevented S1P suppression of T cell proliferation (Fig. 3).

S1P prevention of cytokine production by T cells stimulated with anti-CD3 mAb and either the TCR costimulator anti-CD28 mAb or the IL-7 stimulator of homeostatic proliferation did not extend substantially to IL-2 (Figs. 4 and 5). Thus both the naive-memory and activated effector T cell regulatory mechanisms of S1P spare the IL-2 growth and survival functions, to focus control selectively on differentiation-inducing cytokines capable of evoking and maintaining Th₁ or Th₂ subsets. The relative effectiveness of S1P in preferentially controlling Th₁ and Th₂ cytokines depends both on the CD4 T cell stimulus, which determines the magnitude of the cytokine response, and the concentration of S1P. Generation of IFN-γ and IL-4 apparently were both more sensitive to inhibition by S1P when the T cell stimulus was anti-CD3 and IL-7, but the unregulated levels of both cytokines produced were lower in this homeostatic model than in TCR-coreceptor activation of effector T cells (Figs. 4 and 5).

The results of previous analyses of contributions of individual S1PRs to T cell migration relied on overexpression of each S1PR in the same non-T migratory cell line and on partial antagonism by...
polyspecific drugs and low-affinity Abs. The decreased expression of S1P1 and other S1PRs in spleen-derived and cytokine-deviated Th1 cells compared with freshly isolated splenic CD4 T cells is attributed to persistent TCR stimulation, that also down-regulates S1PRs in balanced mixtures of CD4 T cells (7). This state of depressed expression of all S1PRs in otherwise normal Th cells also provided an opportunity for selectively up-regulating the S1P1R, which had been implicated as the principal transducer of T cell migration.

Retroviral transduction of the mouse Th1 subline with human S1P1R resulted in a striking increment in S1P1R protein in the transductants compared with sham-transduced controls (Fig. 6). As a result, the functional responses of the Th1 cell transductants to S1P were markedly different from those of sham transductants (Fig. 7). The recombinant S1P1R in Th1 cells was expressed in Th1 cells, which had been implicated as the principal transducer of T cell migration.

As S1P1R of T cells are nearly fully occupied by ligand at concentrations of S1P characteristic of blood and lymph, regulation of immune effects of the system is dependent predominantly on alterations in expression of the receptors and the presence of natural and pharmacological antagonists (Fig. 8). Diverse cellular events, but not persistent exposure to S1P, determine the level of expression of S1P1R. It is thus predicted that genetic alterations in expression of T cell S1P1Rs in mice and the application of bioavailable S1P agonists and antagonists will have major effects on lymphocyte migration, homing and tissue distribution, but lesser or no effects on their other intrinsic cellular and immune functions.

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References


