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Sphingosine 1-Phosphate and Its G Protein-Coupled Receptors Constitute a Multifunctional Immunoregulatory System

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Abstract The lysophospholipid growth factors sphingosine 1-phosphate (S1P) and lysophosphatidic acid (LPA) are generated by many cells involved in immunity, including macrophages, dendritic cells, mast cells, and platelets, with resultant lymph and plasma concentrations of 0.1–1 μM. All immune cells express distinctive profiles of G protein-coupled receptors (GPCRs) for S1P and LPA, which are regulated developmentally and by cellular activation. For T-cells, constitutive S1P signaling through their principal $S1P_1$ GPCR inhibits chemotactic responses to chemokines, with lesser suppression of proliferation and cytokine production. These S1P-$S1P_1$ GPCR signals tonically reduce T-cell chemotactic sensitivity to chemokines and thereby limit homing of blood and spleen T-cells to secondary lymphoid tissues. $S1P_1$ GPCR antagonists evoke lymphopenia by permitting blood T-cells to enter lymph nodes and blocking $S1P_1$ GPCR-dependent T-cell efflux from lymph nodes. Inversely, there is a longer than normal persistance in blood and a decrease in lymphoid transit time for T-cells overexpressing transgenic $S1P_1$ GPCRs. The immunotherapeutic potential of $S1P_1$ GPCR antagonists derives from their capacity to limit T-cell access to organ grafts and autoimmune antigens without reducing their other intrinsic functional capabilities. Lysophospholipids and their GPCRs thus constitute an immunoregulatory system of sufficient prominence for pharmacological targeting in transplantation, autoimmunity and immunodeficiency. J. Cell. Biochem. 92: 1104–1114, 2004. © 2004 Wiley-Liss, Inc.

Key words: lysophospholipids; T-cells; chemotaxis; chemokines; immunosuppression

Lysophosphatidic acid (LPA) and sphingosine 1-phosphate (S1P) are distinctive mediators of immune cellular proliferation, survival and functions, which are present at highly active concentrations in blood, lymph, and tissue corridors of lymphocyte traffic [Moolenaar et al., 1997; Spiegel et al., 1998]. In the immune system, dendritic cells, macrophages, mast cells, and platelets are the principal sources of LPA and S1P. Each type of immune cell expresses a characteristic profile of one or more of the nine Edg-family G protein-coupled receptors (GPCRs) selective for either S1P or LPA, which is controlled developmentally and by diverse stimuli (Table I) [Goetzl et al., 2000; Chun et al., 2002]. S1P and, often less effectively, LPA are chemotactic for many types of immune cells, including T-cells, B-cells, dendritic cells, and NK cells at their normal ambient tissue levels of 3–300 nM [Zheng et al., 2000, 2001; Graeler et al., 2002; Idzko et al., 2002; Kveberg et al., 2002]. At blood and lymph concentrations of over 0.3–3 μM, however, S1P but not LPA nearly completely inhibits lymphocyte migration to chemokines and other stimuli, and suppresses less profoundly lymphocyte proliferation and secretion of some cytokines [Graeler et al., 2002; Dorsam et al., 2003]. That expression of LPA and S1P GPCRs in thymus is restricted to the last phases of development indicates possible roles in terminal differentiation and emigration of CD4 and CD8 T-cells, which remain to be elucidated definitively. As there are numerous

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TABLE I. Immune Cell Expression of mRNAs Encoding LPA and S1P GPCRs*

LPA/S1P GPCR	CD4 T-cells	CD8 T-cells	B-cells	Macrophages	Dendritic cells	NK cells
$S1P_1$	+	+	+	+	+	+
$S1P_2$	0	0	0	+	+	0
$S1P_3$	0	0	0	±	+	0
$S1P_4$	+	+	+	±	+	+
$S1P_5$	0	+	0	0	0	+
LPA_1	0	0	0	+	0	0
LPA_2	+	+	+	0	0	0
LPA_3	0	0	0	0	0	0

*Expression levels are for newly isolated cells without any stimulation. ±, barely detectable.

and often redundant stimuli of T-cell migration and distinct immunological functions, but fewer specific suppressive influences, the immunoregulatory activities of S1P and its GPCRs are the principal focus of current interest. The potential importance of these immunoregulatory effects is emphasized by their magnitude and involvement in development, tissue distribution, and activities of lymphocytes. Some of the recent excitement in this field is also attributable to the strong possibility of discovering unique classes of immunoactive drugs that target S1P GPCRs. Newly established mouse genetic models of altered expression of T-cell S1P GPCRs and novel functional anti-S1P R monoclonal antibodies now have provided systems and reagents for analyzing further the separate immune contributions of each of the predominant T-cell S1P GPCRs. The results of such studies also may permit accurate predictions of the immune effects of individual S1P GPCR-selective agonists and antagonists.

IMMUNE CELL EXPRESSION, SIGNALS, AND FUNCTIONAL EFFECTS OF LPA AND S1P GPCRS

The earliest analyses of lymphocyte LPA and S1P GPCRs, by PCR and Western blot techniques, showed that lines of human T-cell tumors express similar levels of LPA_1, LPA_2, $S1P_2$, and $S1P_3$ GPCRs without significant alterations in these levels by numerous different stimuli [Goetzl et al., 1999]. In contrast, human blood and mouse spleen CD4 T-cells and B-cells express predominately $S1P_1$, $S1P_4$, and LPA_2 GPCRs, as assessed by the same methods (Table I) [Zheng et al., 2000; Graeler and Goetzl, 2002]. Mouse spleen CD8 T-cells express the same three major LPA and S1P GPCRs as CD4 T-cells, but initial studies of human blood CD8 T-cells showed only a low level of $S1P_1$ GPCRs. This was in part due to the high levels of heparin used to anticoagulate blood, as CD8 T-cells isolated from sodium citrate-anticoagulated blood had S1P and LPA GPCR profiles similar to CD4 T-cells with one exception. Human and mouse CD8 T-cells, but not CD4 T-cells, also express $S1P_5$. Activation of each of these sets of normal lymphocytes with a preferred stimulus transcriptionally downregulates the $S1P_1$, $S1P_4$, and LPA_2 GPCRs and concurrently upregulates LPA_1 GPCRs (Fig. 1) [Zheng et al., 2000; Graeler and Goetzl, 2002]. The downregulation of LPA and S1P GPCRs contrasts with upregulation of chemokine GPCRs observed after similar stimulation of T-cells. $S1P_1$ and $S1P_4$ GPCR downregulation in mouse CD4 T-cells was sufficient to suppress an array of biochemical and functional responses to S1P, which could be largely reconstituted by introduction of recombinant $S1P_1$, but not $S1P_4$ GPCRs. The mechanisms of principal expression of LPA_1, $S1P_1$, and $S1P_2$ by macrophages and other mononuclear phagocytes, $S1P_1$, $S1P_2$, $S1P_3$, and $S1P_4$ by diverse dendritic cells, and $S1P_1$, $S1P_4$, and $S1P_5$ by NK cells (Table I), and the optimal conditions for respective coupling of S1P GPCRs to immune activities of these other types of cells have only recently been examined.

Profiles of T-cell LPA GPCRs, based initially on radioactive semi-quantitative PCR and Western blots, showed that human unactivated CD4 T-cells and CD8 T-cells from blood collected in non-heparin anticoagulants expressed predominately LPA_2 Rs, a level of LPA_1 Rs less than 10% that of LPA_2 Rs, and no detectable LPA_3 Rs. The levels of mRNA encoding $LPA_{1\text{-}3}$ Rs were requantified subsequently by TaqMan real-time PCR, which confirmed the original results for human blood T-cells and demonstrated the same levels for mouse spleen CD4 and CD8 T-cells. After activation of CD4 or CD8 T-cells by mitogen or adherent monoclonal antibodies (MoAbs) to the T-cell receptor (CD3) plus a

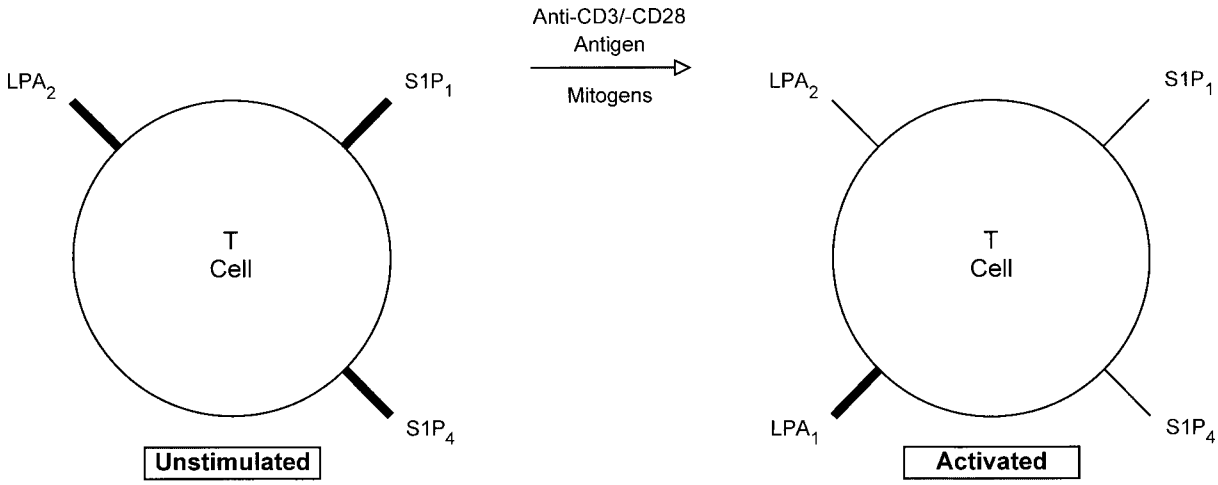

Fig. 1. Immune activation-regulated expression of LPA and S1P GPCRs by human and mouse T-cells. The thickness of each line depicting a GPCR represents its relative level of expression and functional contributions.

co-receptor (CD28), the level of LPA_2 Rs decreased 30–50% and the level of LPA_1 Rs increased to a mean of 50–100% of that of the LPA_2 Rs [Zheng et al., 2000]. The isolated expression of LPA_2 Rs in unactivated CD4 T-cells and the co-dominant expression of LPA_2 Rs and LPA_1 Rs in activated CD4 T-cells provided the first opportunity to examine their separate effects on T-cell functions. Acute induction of IL-2 secretion from naïve unactivated human blood or mouse spleen CD4 T-cells over 24 h by anti-CD3 plus anti-CD28 MoAbs was suppressed up to 60% by 10^{-10}–10^{-6} M LPA [Zheng et al., 2000]. That the predominant LPA_2 Rs transduced suppression of IL-2 secretion by LPA was confirmed by a similar inhibitory effect of anti-LPA_2 R mouse MoAb, but not of anti-LPA_1 R MoAb. When LPA_2 Rs were reduced and LPA_1 Rs upregulated by preactivation of CD4 T-cells, IL-2 secretion evoked by anti-CD3 plus anti-CD28 MoAb stimulation was enhanced up to twofold by 10^{-10}–10^{-6} M LPA and by anti-LPA_1 R MoAb, but not by anti-LPA_2 R MoAb [Zheng et al., 2000]. The two major LPA Rs of CD4 T-cells transduced opposite effects on chemotaxis as well, when assessed in Transwell chambers with Matrigel-coated 5 μm pore filters [Zheng et al., 2000]. LPA elicited chemotaxis of unactivated CD4 T-cells, with a peak at 10^{-9}–10^{-7} M LPA, but not of CD4 T-cells after activation. Chemotaxis of unactivated CD4 T-cells was also induced by anti-LPA_2 R MoAb, but not by anti-LPA_1 R MoAb. LPA inhibition of chemokine-elicited chemotaxis of preactivated CD4 T-cells suggested transduction of a suppressive signal by LPA_1 Rs, but the results were not sufficiently consistent. Thus a Jurkat human T-cell model was developed to permit studies of the chemotactic regulatory signals from LPA_1 Rs in isolation [Zheng et al., 2001]. Jurkat T-cells express endogenous LPA_1 Rs and LPA_2 Rs at levels lower than those of activated blood CD4 T-cells. Co-transfection with a mixture of expression plasmids encoding sense message for LPA_1 Rs and antisense messages for the other endogenous LPA/S1P Rs plus selection yielded Jurkat–T-L1 cells, in which LPA_1 Rs were vastly predominant. A similar transfection procedure with a mixture of expression plasmids amplifying LPA_2 Rs plus selection yielded Jurkat-T-L2 cells. Chemotaxis of Jurkat-T-L2 cells, but not Jurkat-T-L1 cells, was stimulated significantly by 10^{-9}–10^{-6} M LPA and anti-LPA_2 R MoAbs. Although Jurkat-T-L1 cells did not respond directly to LPA or anti-LPA_1 R MoAbs, chemokine-evoked chemotaxis was normal and was inhibited by 10^{-8}–10^{-6} M LPA and anti-LPA_1 R MoAbs [Zheng et al., 2001]. Thus LPA_1 Rs do not mediate a T-cell chemotactic response to LPA, but alone transduce LPA suppression of chemokine-evoked chemotaxis, which may occur in activated T-cells.

Systematic studies of T-cell S1P GPCRs were initiated when preliminary results suggested that these were more important than LPA Rs in regulating several aspects of T-cell movements required for thymic differentiation, lymphoid tissue distribution, and diverse responses to complex antigenic challenges. The quantitatively predominant S1P Rs of T-cells are $S1P_1$

and $S1P_4$, as assessed by TaqMan real-time PCR and Western blots, and the former appears to transduce most functional signals from S1P to T-cells. In contrast to LPA Rs, both $S1P_1$ and $S1P_4$ are downregulated by all T-cell stimuli that have been examined, including many mitogens, anti-CD3 plus anti-CD28 MoAbs, superantigens, and phorbol esters [Graeler and Goetzl, 2002]. CD4 T-cell levels of both $S1P_1$ and $S1P_4$ are higher than those of CD8 T-cells and B-cells, but the basic profiles are similar. The major in vitro effects of S1P on T-cell migration are highly S1P concentration-dependent (Fig. 2) [Graeler and Goetzl, 2002; Graeler et al., 2002]. S1P evokes direct chemotaxis and enhances chemotaxis to chemokines optimally at concentrations of 1–100 nM, which are found in tissues. In contrast, S1P inhibits chemokine-elicited chemotaxis by up to 90% at blood and lymph concentrations of 0.3–3.0 μM (Fig. 2) [Graeler et al., 2002]. In the context of a vast array of known lymphocyte chemotactic factors, the capacity of S1P but not LPA to inhibit chemotactic responses of naïve and possibly memory T-cells to chemokines is the most unique and quantitatively striking effect. The chemotactic inhibitory activity of blood and lymph concentrations of S1P is one basis for a new conceptual model of the T-cell regulatory activities of S1P and explains the mechanism of action of an immunosuppressive drug designated FTY720 [Fujita et al., 1994; Brinkmann and Lynch, 2002], which also acts on many S1P GPCRs.

In contrast to the LPA GPCRs, which act solely on T-cell production of IL-2, the S1P GPCRs have no affect on IL-2 production but instead suppress both generation of specialized functional cytokines, such as IFN-gamma and IL-4, and proliferation of T-cells (Fig. 2) [Dorsam et al., 2003]. Proliferation of mouse spleen CD4 T-cells, assessed by uptake of ^{3}H-thymidine and cell counts, was suppressed by 10^{-9}–10^{-6} M S1P, with mean maximal suppression at the highest concentration of 48–50% when the stimuli were anti-CD3 MoAb plus anti-CD28 MoAb or IL-7. In contrast, 10^{-6} M LPA only suppressed proliferation by a mean of

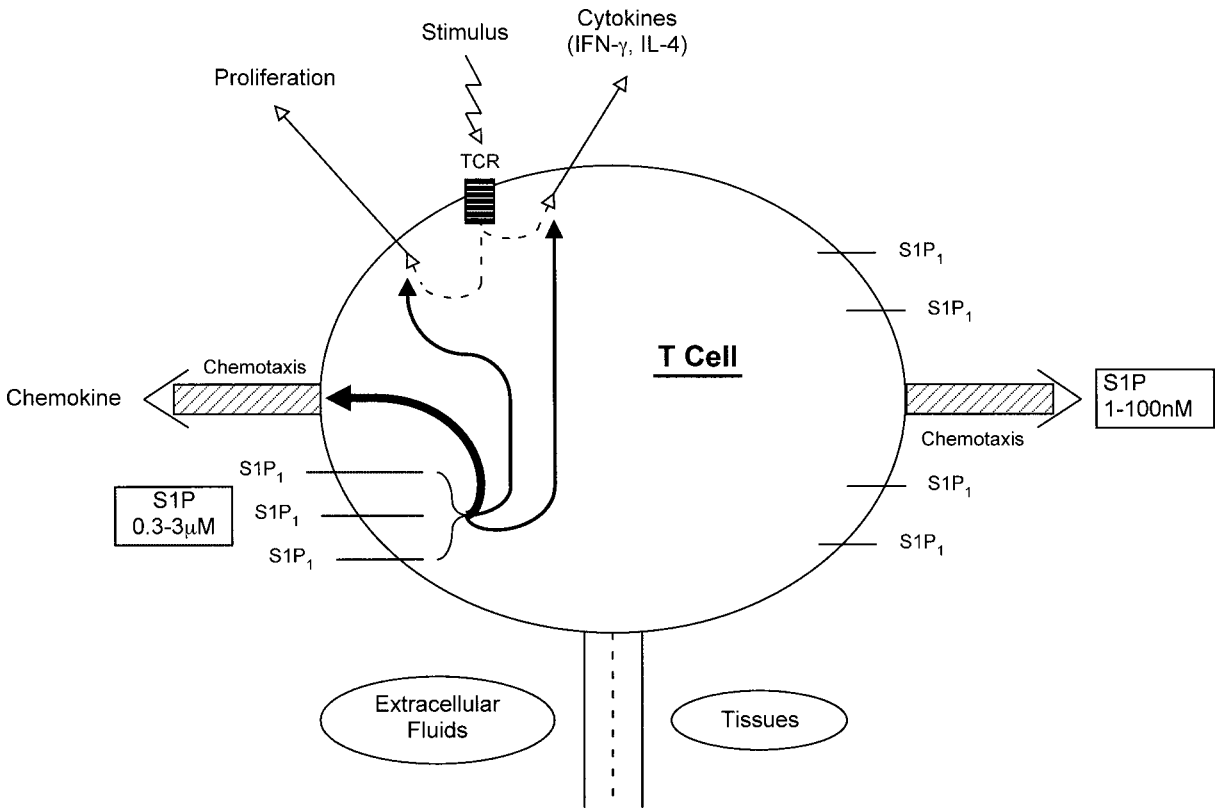

Fig. 2. Regulation of CD4 T-cell functions by the S1P-$S1P_1$ GPCR axis. At tissue levels (right-hand side), S1P through $S1P_1$ GPCRs elicit T-cell chemotaxis (larger diagonally striped arrow). At plasma and lymph levels (left-hand side), S1P through $S1P_1$ GPCRs exerts predominately inhibitory effects on chemotaxis to chemokines (heaviest solid arrow), but also suppresses to a lesser degree (light solid arrows) T-cell antigen receptor (TCR)-evoked other functional responses.

16% ($P < 0.05$) when the stimulus was anti-CD3 plus anti-CD28 MoAbs and had no effect when CD4 T-cells were activated by anti-CD3 MoAb plus IL-7 [Dorsam et al., 2003]. Similar results were obtained with CD8 T-cells. Optimal inhibitory activity required that S1P be present just before or at the same time as the interaction of T-cells with the activating factor. An increase in $[Ca^{++}]i$ is an obligatory event in the pathway by which S1P suppresses CD4 T-cell proliferation as both the intracellular Ca^{++} chelator BAPTA-AM and the phosphoinositol-specific phospholipase C inhibitor Et-18-OCH3 completely prevented suppression of proliferation by S1P, whereas inhibitors of PKA, adenylyl cyclase, and other signals had no effect. Increases in [cAMP]i attained by additions of 8-bromo-cAMP, dibutyryl-cAMP, or the cAMP phosphodiesterase-specific inhibitor Ro-20-1724, significantly reversed S1P suppression of T-cell proliferation [Dorsam et al., 2003]. Studies then were extended to cytokine generation by activated CD4 T-cells. Mean maximal inhibition of IL-2 secretion by 10^{-8}–10^{-6} M LPA exceeded 50%, but 10^{-10}–10^{-6} M S1P had no effect on IL-2 secretion [Zheng et al., 2000; Dorsam et al., 2003]. Although levels of secretion of IL-4 and IFN-gamma were much lower after 6–24 h than at 72 h, partial persistence of the S1P Rs during the 24 h after stimulation permitted modulation by S1P. In contrast to the profile for IL-2, 10^{-8}–10^{-6} M S1P, but not LPA, suppressed secretion of IFN-gamma by stimulated CD4 cells up to 70%. S1P inhibition of IL-4 secretion by CD4 T-cells depended on the stimulus. With anti-CD3 MoAb plus IL-7, T-cell secretion of IL-4 was suppressed significantly and progressively by 10^{-8}–10^{-6} M S1P, but with anti-CD3 plus anti-CD28 MoAbs inhibition was only significant at 10^{-6} M S1P [Dorsam et al., 2003]. Only IL-4 secretion elicited by the former stimulus was suppressed by LPA and only at 10^{-6} M.

Although some differences between the T-cell immunoregulatory signals from S1P and LPA GPCRs have been elucidated, many critical questions remain unanswered. Certain specialized cellular mechanisms controlling downregulation and re-expression of T-cell S1P GPCRs, but not LPA GPCRs, have been identified in vitro. The individual contributions of each T-cell LPA and S1P GPCR to transduction of the respective net effects of LPA and S1P on T-cell functions also have been only partially delineated in vitro. Little is known about regulation of expression or functional effects of any T-cell S1P or LPA GPCR in vivo during immune responses. Nonetheless, the weight of present evidence favors a dominant role for T-cell $S1P_1$ GPCRs in S1P control of migration and specific functions of T-cells [Graeler and Goetzl, 2002; Dorsam et al., 2003].

S1P AND $S1P_1$ GPCR IMMUNOREGULATORY AXIS: CONTROL OF T-CELL MIGRATION

That $S1P_1$ GPCRs are the principal transducers of S1P effects on T-cell migration was suggested initially by findings that prior activation of T-cells sufficient to nearly completely downregulate $S1P_1$ Rs and $S1P_4$ Rs ablated functional responses to S1P and that selective re-introduction of $S1P_1$ but not $S1P_4$ restored such T-cell responses to S1P [Graeler and Goetzl, 2002; Dorsam et al., 2003]. Further support for the hypothesis came from the observation that S1P induced migration of S1P R-null hepatoma cells transfected with $S1P_1$ Rs but not $S1P_4$ Rs. Confirmation also came from limited applications of functional MoAbs and S1P R-selective antagonists, where those specific for $S1P_1$ Rs but not that directed to $S1P_4$ Rs suppressed both chemotactic and chemotactic-inhibitory effects of S1P in vitro and in vivo. The first definitive set of studies of T-cells employed a line of Th1 cells, generated by cytokine-directed deviation of splenic CD4 T-cells of D011.10 ovalbumin peptide-specific TCR transgenic mice, in which the expression of $S1P_1$ Rs and $S1P_4$ Rs was suppressed more than 90% by pulses of stimulation with ovalbumin peptide antigen and antigen-presenting cells every 5–6 days. In these functionally S1P GPCR-null Th1 cells, retrovirally mediated introduction of human $S1P_1$ Rs led to far greater direct chemotactic responses to S1P, S1P inhibition of CCL-5-induced chemotaxis, and S1P inhibition of anti-TCR MoAb-evoked proliferation and cytokine production than in sham $S1P_1$ R transductants or $S1P_4$ R transductants [Dorsam et al., 2003]. These results all implicated $S1P_1$ GPCRs as the major T-cell transducers of signals from S1P and supported multifunctional immunoregulatory roles for the S1P-$S1P_1$ GPCR axis (Fig. 2).

Convincing evidence for the role of $S1P_1$ GPCRs (Rs) as the principal transducers of S1P effects on T-cells has raised several questions about distinctive cellular characteristics

of the S1P-$S1P_1$ R axis. S1P downregulation of epitope-tagged or fluorescent protein-containing $S1P_1$ Rs in several types of transfectants involved rapid phosphorylation, internalization, and translocation to caveolae with caveolin-1 by a GPCR kinase 2-dependent and N-linked glycan-facilitated process (Fig. 3) [Igarashi and Michel, 2000; Kohno et al., 2002; Watterson et al., 2002]. However, T-cells isolated from blood or lymph, where S1P concentrations assure complete saturation of $S1P_1$ Rs, normally express a full complement of functional $S1P_1$ Rs. Thus it was assumed that distinct mechanisms assure the recovery, re-expression, and cell-surface stabilization of T-cell $S1P_1$ Rs. Initial studies of S1P desensitization of both T-cell chemotactic responses to nanomolar concentrations of S1P and inhibition of chemokine-elicited T-cell chemotaxis by micromolar levels of S1P showed suppression of both responses within 1 h of exposure of S1P-deprived CD4 T-cells to 0.1 μM S1P [Graeler et al., 2003]. Within 12 h and for up to 48 h after re-exposure of CD4 T-cells to S1P, both chemotaxis to nanomolar S1P and micromolar S1P inhibition of chemokine-evoked chemotaxis returned to baseline control levels despite maintenance of S1P at the desensitizing concentration. Application of inhibitors of signals with potential relevance to recovery of downregulated $S1P_1$ Rs showed that those directed to PKC and PKCepsilon, but not numerous other enzymatic targets, significantly suppressed recovery of both effects of S1P on T-cell migration (Fig. 3) [Graeler et al., 2003]. Neither PKC nor PKCepsilon inhibitors affected the rapid downregulation of T-cell $S1P_1$ Rs. Consistently, $S1P_1$ Rs of splenic CD4 T-cells from PKCepsilon-null mice showed normal rapid downregulation by 0.1 μM S1P, but failed to recover after 24 h.

The involvement of PKCepsilon suggested that a known downstream event, such as AP-1 activation, might also be invoked by S1P and be required for recovery of downregulated $S1P_1$ Rs. S1P increased nuclear contents of the AP-1 substituents c-Fos and phosphorylated c-Jun/JunD at 24 h, but not at 1 h, and the increases were blocked by PKCepsilon inhibitors [Graeler et al., 2003]. To establish a relevant role for the recruited AP-1, concentrations of antisense oligonucleotides (AS) for both c-Fos and c-Jun known to decrease AP-1 activity were introduced into CD4 T-cells prior to S1P treatment [Graeler et al., 2003]. Recovery of $S1P_1$ R-mediated effects on T-cell migration at 24 h was significantly suppressed by the AP-1-specific AS, without any effect of control sense oligonucleotides and the initial downregulation

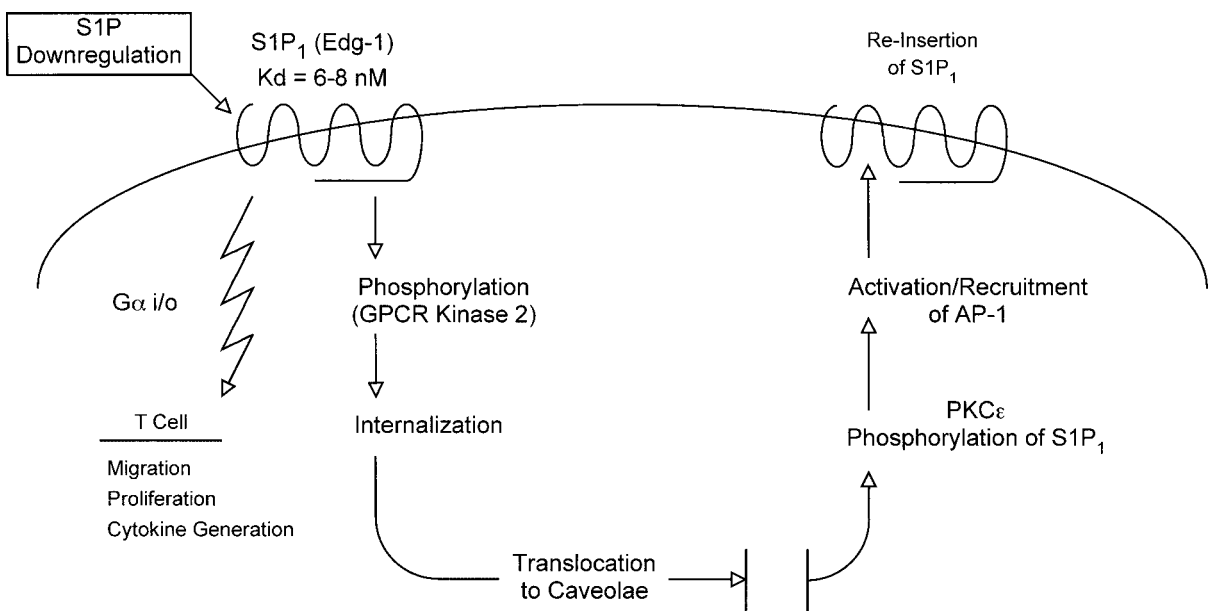

Fig. 3. Mechanisms of S1P-evoked acute downregulation, recovery, and persistent re-expression of T-cell $S1P_1$ GPCRs. The jagged line arrow in the left-hand side of the T-cell depicts $S1P_1$ GPCR-transduced suppressive effect of micromolar levels of S1P on multiple CD4 T-cell functional responses to diverse stimuli. The straight line arrows represent segments in the intracellular course of traffic of $S1P_1$ GPCRs, which at their downregulated destination associate with caveolin-1 in the caveolae. AP-1, activator protein 1.

at 1 h was not altered by AS compared to the controls. To examine $S1P_1$ R phosphorylation in relation to S1P-elicited downregulation, rat S1P R-null HTC4 hepatoma cells were transduced with hemagglutinin (HA) epitope-tagged human $S1P_1$ Rs and stimulated with 0.1 μM S1P for 1 and 24 h prior to dissolution, $S1P_1$(HA) R immunoadsorption on anti-HA MoAb-agarose, electrophoresis, and detection with anti-phospho-threonine MoAbs. Selective inhibition of PKCepsilon suppressed S1P-evoked threonine phosphorylation at 24 h, but not after 1 h, which follows the same course as PKCepsilon-dependent recruitment of AP-1 and recovery of downregulated $S1P_1$ Rs. Although the interrelationships of transcriptional events and protein phosphorylation in $S1P_1$ R recovery from downregulation are not fully established, it is clear that $S1P_1$ R downregulation and recovery differ in biochemical requisites and therefore will be pharmacologically distinguishable in relation to normal cellular physiology and effects of numerous diseases (Fig. 3).

PHARMACOLOGICAL INSIGHTS FROM THE S1P GPCR ANTAGONIST FTY720

An immunosuppressive compound termed ISP-1 or Myriocin was isolated from the ascomycete Isaria sinclairii, which was long used as an "eternal youth" nostrum in China [Fujita et al., 1994]. Substantial and limiting gastrointestinal side-effects of Myriocin led to a series of chemical modifications. The most active derivative was 2-amino-2[2-(4-octylphenyl) ethyl]propane-1,3-diol hydrochloride, designated FTY720, which is immunosuppressive in numerous T-cell-dependent assays [Brinkmann and Lynch, 2002]. Intravenous or oral FTY720 induced profound lymphopenia and suppressed the levels of re-circulating lymphocytes in lymphatics of mice within hours by shifting the lymphocyte contents of blood and spleen to secondary lymphoid organs [Brinkmann et al., 2002; Mandala et al., 2002]. Egress of lymphocytes from lymph nodes also was inhibited by FTY720. The major immune functional consequence of lymphocyte re-distribution by FTY720 was immunosuppression in rodent models of transplantation and autoimmune diseases. Application of FTY720 in human renal transplantation, usually with co-administration of low-dose cyclosporine, afforded excellent protection from rejection without increasing the risk of infections [Brinkmann et al., 2001]. FTY720 subsequently was found to interact with S1P GPCRs, which mediates its immunosuppressive effects, but the specific mechanisms of action have not been fully elucidated and are a source of current controversy.

The first hypothesis is that FTY720 is phosphorylated, principally by sphingosine kinases and perhaps other intracellular kinases. FTY720-phosphate then acts as an agonist for several lymphocyte S1P GPCRs to both stimulate movement of blood and spleen lymphocytes into lymph nodes and maintain their intranodal sequestration, presumably by inducing a state of unresponsiveness to S1P or other chemotactic factors [Brinkmann et al., 2002; Mandala et al., 2002]. These effects require micromolar concentrations of FTY720 for optimal phosphorylation and lymphocyte stimulation. Most supporting data are from in vivo studies and few analyses of cellular mechanisms of action of FTY720-phosphate have been conducted with lymphocytes. The second hypothesis is that unaltered FTY720 acts at the nanomolar concentrations attained in patients on treatment to inhibit lymphocyte $S1P_1$ GPCRs selectively, without any agonist or direct antagonist activity. FTY720 has been shown recently to induce internalization and consequently loss of signaling activity of $S1P_1$ GPCRs, but not $S1P_4$ GPCRs (Fig. 4). By the second hypothesis, FTY720 is tentatively designated a non-competitive inhibitor of $S1P_1$, $S1P_2$, and $S1P_5$, but not $S1P_3$ or $S1P_4$ GPCRs in some model cell transductants and of $S1P_1$ GPCRs alone in lymphocytes (Fig. 4). FTY720 thereby suppresses S1P inhibition of lymphocyte chemotaxis to lymph node chemokines and thus accelerates lymphocyte migration from blood and spleen into lymph nodes (Fig. 5). Concurrently, FTY720 also blocks the lymph node lymphocyte chemotactic response to S1P required for their return into lymph and then blood (Fig. 5). Many aspects of the FTY720 effects on S1P regulation of lymphocyte traffic, which account for its highly selective and relatively safe immunosuppressive profile, have not been fully delineated. Further, it is also likely that the mechanisms of the second hypothesis are exercised with cellular selectivity, as there is evidence against their applicability to some endothelial cell responses to FTY720. The relative contributions of different mechanisms of action of FTY720 thus may depend on the type of target cell. In addition, it

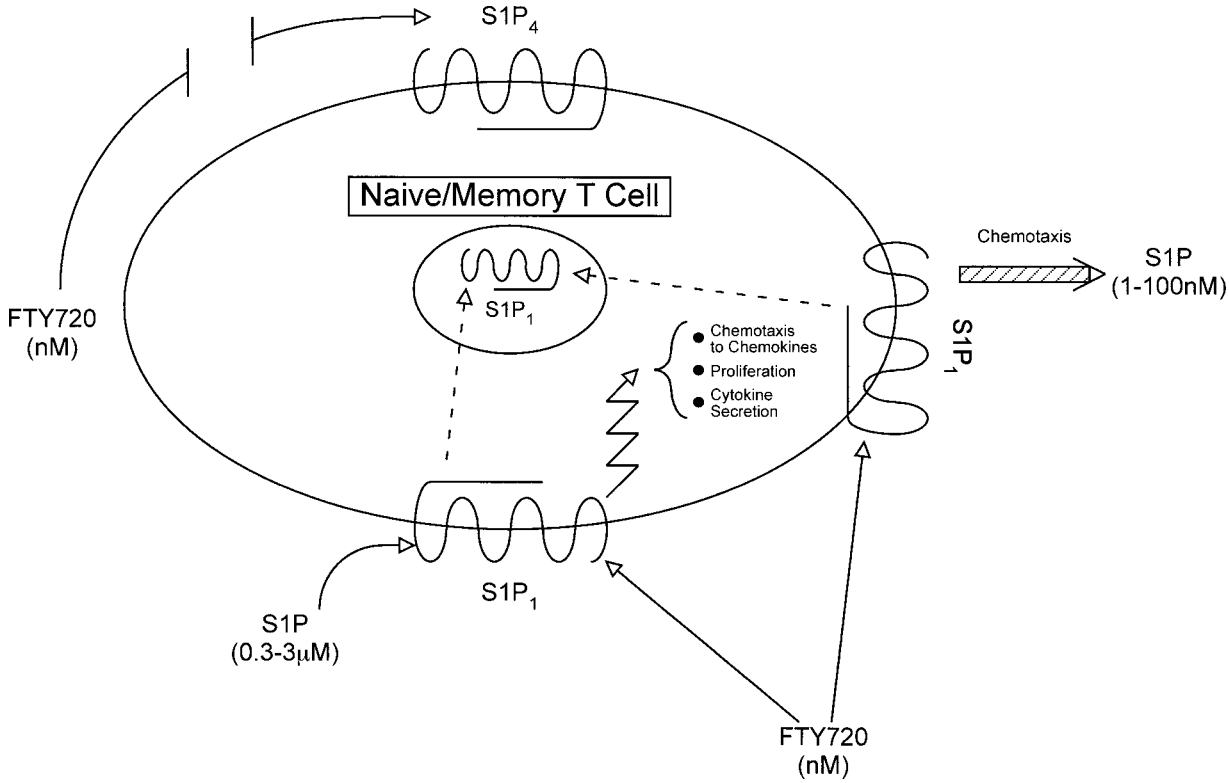

Fig. 4. FTY720 mediation of immunosuppression by downregulation of T-cell $S1P_1$ GPCRs. Blood and lymph concentrations of FTY720 evoke internalization of T-cell $S1P_1$ GPCRs, but not of $S1P_4$ GPCRs, with resultant loss of both stimulatory and inhibitory chemotactic signals. The dashed lines depict internalization of S1P-occupied $S1P_1$ GPCRs.

remains to be established how TCR stimulation downregulates S1P GPCRs and what natural factors in lymph downregulate $S1P_1$ Rs of the majority of T-cells in HEV, which do not have activating interactions with antigen or exposure to a drug like FTY720. Finally, investigations of the ability of FTY720 to influence activated effector T-cells, which have much lower levels of S1P Rs than naïve and memory cells, have yielded conflicting results and more studies will be required.

CONSEQUENCES OF ALTERED T-CELL EXPRESSION OF $S1P_1$ GPCRS IN MOUSE MODELS

S1P Rs of T-cells in blood, lymph, and lymphoid organs normally are fully saturated with endogenous S1P, but this does not decrease expression of the $S1P_1$ Rs persistently (Fig. 5). The predominately inhibitory native signal from S1P-occupied $S1P_1$ Rs thus continuously diminishes T-cell chemotactic responses to chemokines and other stimuli in lymph nodes and maintains the level of T-cells in blood, lymph, and spleen. Maintenance of these steady-state levels of T-cells is mediated by mechanisms which are disrupted when the $S1P_1$ Rs are downregulated by T-cell activation, forced intracellularly by FTY720, or blocked by an $S1P_1$ R-selective antagonist (Fig. 5). Consequent release of T-cells from trapping by the $S1P/S1P_1$ R axis evokes their movement from blood and spleen to secondary lymphoid organs, limits egress from lymph nodes, and thereby prevents responses to tissue antigenic challenges, which may explain inhibition of transplant rejection by FTY720.

Mouse genetic models of different levels of expression of the lymphocyte S1P-$S1P_1$ GPCR axis are being generated to examine further the mechanisms by which signals from $S1P_1$ GPCRs modify T-cell movement, tissue distribution, and access to foreign antigens. A high level of constitutive expression of $S1P_1$ GPCRs selectively on T-cells and only a moderate decrease in this level after their immune activation has been attained in a transgenic (TG) mouse model recently by targeted overexpression of human $S1P_1$ GPCRs under control of the CD2 promoter.

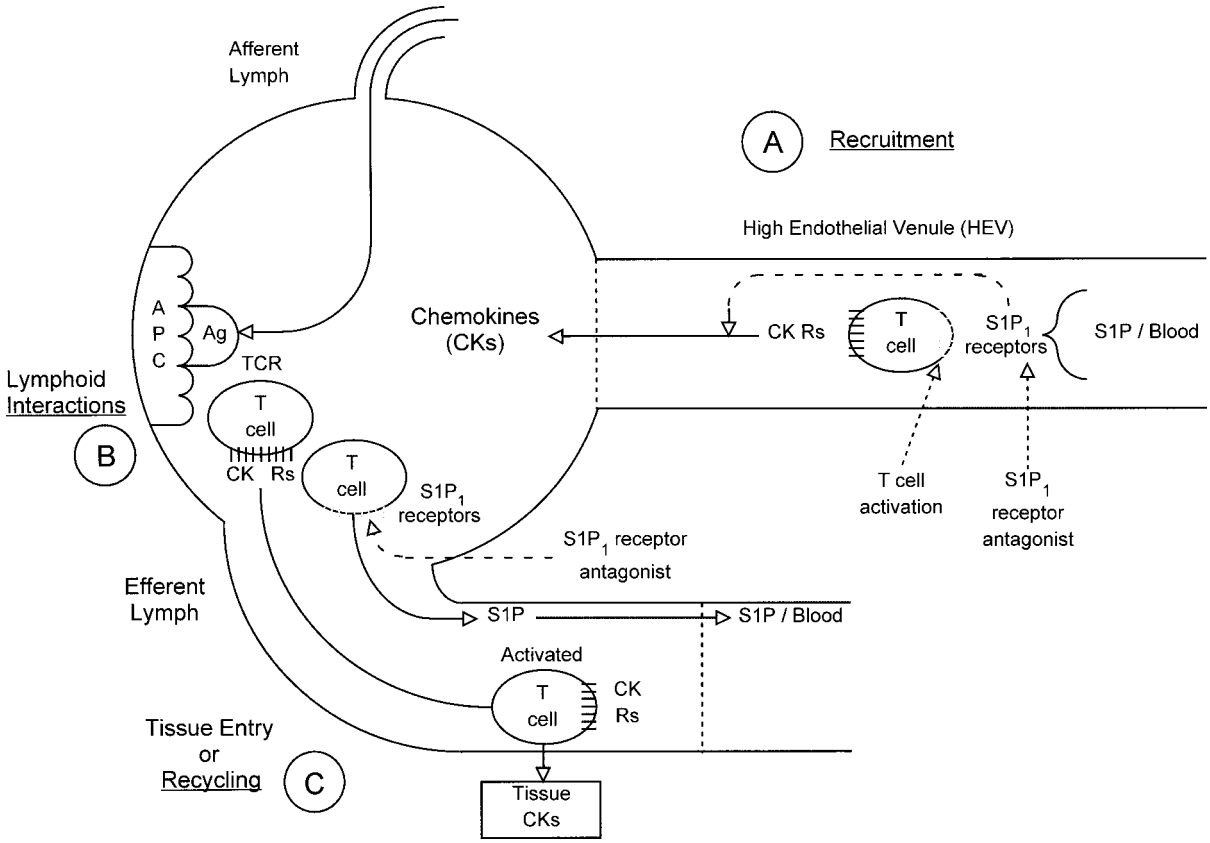

Fig. 5. Model of control of T-cell entry into secondary lymphoid organs and of T-cell recirculation by the S1P-$S1P_1$ GPCR axis. CK, chemokine; R, receptor; APC, antigen-presenting cell; HEV, high endothelial venule. **A**: Recruitment of T-cells from blood to lymph nodes after T-cell activation or introduction of an $S1P_1$ GPCR antagonist. **B**: Generation of small subsets of effector T-cells unresponsive to S1P and recovery of expression of $S1P_1$ GPCRs by the other T-cells. **C**: Recycling of T-cells into efferent lymph under control of the S1P-$S1P_1$ GPCR axis.

These TG mice exhibit altered blood lymphocyte kinetics lymphocytosis consistent with increased trapping of circulating T-cells by the enhanced $S1P_1$ GPCR-S1P axis. There are no major abnormalities in lymph node or splenic architecture. Cutaneous delayed-type hypersensitivity (DTH) of TG mice was very significantly inhibited in several types of challenge models, which supports the capacity of the S1P-$S1P_1$ GPCR axis to block migration of T-cells into sites of antigenic or other immune stimulation. In contrast, IgE antibody responses and cutaneous immediate-type hypersensitivity were enhanced in the TG mice. The next stage in this series of investigations will be generation and characterization of several types of conditional deletions of $S1P_1$ at different stages of T-cell development. Investigations of the immunological phenotypes of each such $S1P_1$-null mouse will permit identification of additional roles of the S1P-$S1P_1$ GPCR axis in immune effector responses and examination of any requirements for the S1P-$S1P_1$ R axis at each stage of differentiation of T-cells. Predictions for the $S1P_1$ GPCR-null phenotype include lymphopenia and enlarged, hypercellular lymph nodes.

CLINICAL IMPLICATIONS

The capacity of antagonists and agonists of the $S1P_1$ GPCR-S1P axis to modify tissue distribution and effector chemotactic responses of T-cells, without altering substantially their intrinsic immune activities, represents a novel and potentially safer approach to immunotherapy in transplantation rejection and autoimmunity (Table II). The multi-S1P GPCR-directed agent FTY720, which acts as a non-competitive inhibitor at nanomolar concentrations and an agonist after phosphorylation, suppresses both immune rejection of transplanted organs and a wide range of different forms of autoimmunity in animal models of

TABLE II. Therapeutic Possibilities for $S1P_1$ GPCR-Specific Agonists and Antagonists

(1)	$S1P_1$ (Edg-1) antagonists suppress overactive or misdirected T-cell-dependent immune responses with only minor effects on host self-defense
	Transplantation
	Rheumatoid arthritis
	Multiple sclerosis
	Type 1 diabetes mellitus
(2)	$S1P_1$ (Edg-1) agonists act as dendritic cell and T-cell stimuli in recruitment and cytokine regulation
	Immunostimulation in deficiency states
	Adjuvant for vaccines

multiple sclerosis, rheumatoid arthritis, type 1 diabetes mellitus, and other organ-specific reactions [Matsuura et al., 2000; Yagi et al., 2000; Maki et al., 2002; Fujino et al., 2003]. FTY720 also has been beneficial for treatment of human renal graft rejection, especially in combination with low-dose cyclosporine. All of these successful applications of FTY720 in immunoregulation have been in conditions where T-cell involvement is a critical requirement. The predicted lack of susceptibility to infections in FTY720 recipients also has held true. Preliminary results of studies of several non-lipid small organic compounds with $S1P_1$ GPCR-selective antagonistic activity also have shown immunoregulatory effects similar to those of FTY720. Early characterization of lymphocyte-targeted $S1P_1$ transgenic mice and conditionally $S1P_1$-null mice reveal phenotypes with major immune abnormalities, which center on lymphocyte migration and tissue distribution.

Numerous lines of evidence support a necessary role for S1P in T-cell recruitment and effector activation. However, these data do not all convincingly implicate $S1P_1$ GPCRs as the sole transducer of S1P signals and some suggest possible contributions of $S1P_4$ GPCRs and of LPA GPCRs. Involvement of the S1P GPCR-S1P systems in functions of other immune cells are less well defined and implications for human biology and diseases are consequently less clear. Mononuclear phagocytes express a profile of LPA and S1P GPCRs distinct from that of lymphocytes and demonstrate both migration and cytokine responses, but differences among monocytes and tissue-specific sets of macrophages remain to be elucidated. Developing and mature dendritic cells express not only the predominant $S1P_1$ and $S1P_4$ GPCRs of lymphocytes, but similar levels of $S1P_2$ and $S1P_3$ as well. Currently available data do not permit assignment of any of the S1P-evoked migratory or cytokine responses of dendritic cells to individual S1P GPCRs. Although it is currently impossible to accurately predict how S1P GPCR-selective agonists or antagonists will affect dendritic cell recruitment and activation, one or more such agents alone or in combination with other immunostimulants are expected to enhance dendritic cell mobilization and differentially alter their capacity to induce Th1 and Th2 responses to vaccines.

FUTURE DIRECTIONS

Future research designed to elucidate functions of lysophospholipids and their GPCRs in natural immunity and in immune therapeutic applications should proceed with the knowledge of elements which distinguish these systems from all previously described immune cytokines and mediators. First, many of the regulatory roles of lysophospholipids and their GPCRs in normal immunity result from constitutive signaling of lymphocytes and other immune cells by $S1P_1$ and perhaps other S1P GPCRs which are fully occupied at usual plasma and lymph concentrations of S1P. Second, effects of these systems during active immune responses are almost solely mediated by changes in the levels of expression of S1P GPCRs by lymphocytes and other immune cells. Although fluid concentrations of S1P and LPA may also change in these responses, such alterations in ligands have relatively little influence on immunity. Third, all major immunoregulatory activities of lysophospholipids and their GPCRs are attributable directly or indirectly to effects on immune cell responses to antigens, cytokines, or other immune mediators. Thus these systems and agents which act on them should be characterized in their immune context and to the extent possible with complete knowledge of other relevant immune factors.

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