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REVIEW

Sphingosine-1-phosphate signaling as a therapeutic target

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Abstract: Sphingosine 1-phosphate (S1P) is a small bioactive lipid molecule that is involved in several processes both intracellularly and extracellularly. It acts intracellularly to promote the survival and growth of the cell, through its interaction with molecules in different compartments of the cell. Extracellularly, it can exist at high concentrations in the blood plasma and lymph, further down inside the tissue. This causes an S1P gradient important for cell migration. S1P signals through five G protein-coupled receptors, S1PR1–S1PR5, whose expression varies in different types of cells and tissue. S1P signaling can be involved in physiological and pathophysiological conditions of the cardiovascular, nervous, and immune systems and diseases such as ischemia/reperfusion injury, autoimmunity, and cancer. In this review, we discuss this involvement and how it can be used to discover novel therapeutic targets.

Keywords: S1P, CD69, T-cell activation, lymph node, recirculation

Introduction

Spingosine 1-phosphate (S1P) is a bioactive lipid mediator metabolite of membrane sphingolipids. It was first identified in the early 1990s as a novel lipid involved in cell proliferation and signal transduction.[1,2] Since then, S1P has been implicated in several different biological functions both intracellularly and extracellularly. The identification of at least five G protein-coupled receptors (GPCRs) involved in S1P signaling, termed S1PR1–5, and their wide expression by different cell types, led to a better understanding of the complex signaling pathways S1P can mediate and its potential role in regulation of several pathophysiological processes. In this review, after an overview of S1P and its signaling, we summarize some identified roles of S1P signaling in disease and discuss its potential in therapeutic targeting.

S1P metabolism

S1P is a 379Da member of the lysophospholipid family. It is the direct metabolite of sphingosine through the action of two sphingosine kinases, SphK1 and SphK2. The main metabolic pathway starts with the hydrolysis of sphingomyelin, a membrane sphingolipid, into ceramide by the enzyme sphingomyelinase and the subsequent production of sphingosine by ceramidase (Figure 1). Ceramide can also be produced de novo in the endoplasmic reticulum (ER) from serine and palmitoyl coenzyme A through multiple intermediates. S1P production is regulated by various S1P-specific and general lipid phosphatases, as well as S1P lyase, which irreversibly degrades S1P into phosphoethanolamine and hexadecanal.[3] The balance between intracellular

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S1P and its metabolite ceramide can determine cellular fate. Ceramide promotes apoptosis, while S1P suppresses cell death and promotes cell survival.[4–7] This creates an S1P ceramide "rheostat" inside the cells. S1P lyase expression in tissue is higher than it is in erythrocytes and platelets, the main "suppliers" of S1P in blood.[8,9] This causes a tissue–blood gradient of S1P, which is important in many S1P-mediated responses, like the lymphocyte egress from lymphoid organs.[10,11]

S1P signaling overview

S1P is produced inside cells; however, it can also be found extracellularly, in a variety of different tissues. It is abundant in the blood, at concentrations of 0.4–1.5 μM, where it is mainly secreted by erythrocytes and platelets.[8,9] Blood S1P can be found separately, but mainly it exists in complexes with high-density lipoprotein (HDL) (~60%). Many of the cardioprotective effects of HDL are hypothesized to involve S1P.[12] Before 1996, S1P was thought to act mainly intracellularly as a second messenger. However, the identification of several GPCRs that bind S1P led to the initiation of many studies on extracellular S1P signaling through those receptors. In 2001, Tamama and colleagues showed that S1P-mediated regulation of DNA synthesis and migration of rat aortic smooth muscle cells does not involve intracellular S1P but extracellular S1P signaling through the Edg-5 receptor, now known as S1PR2.[13] There are five receptors that have been identified currently. These can be coupled with different G-proteins. Assuming that each receptor coupling with a G protein has a slightly different function, one can recognize the complexity of S1P receptor signaling. An overview of the S1P receptors follows.

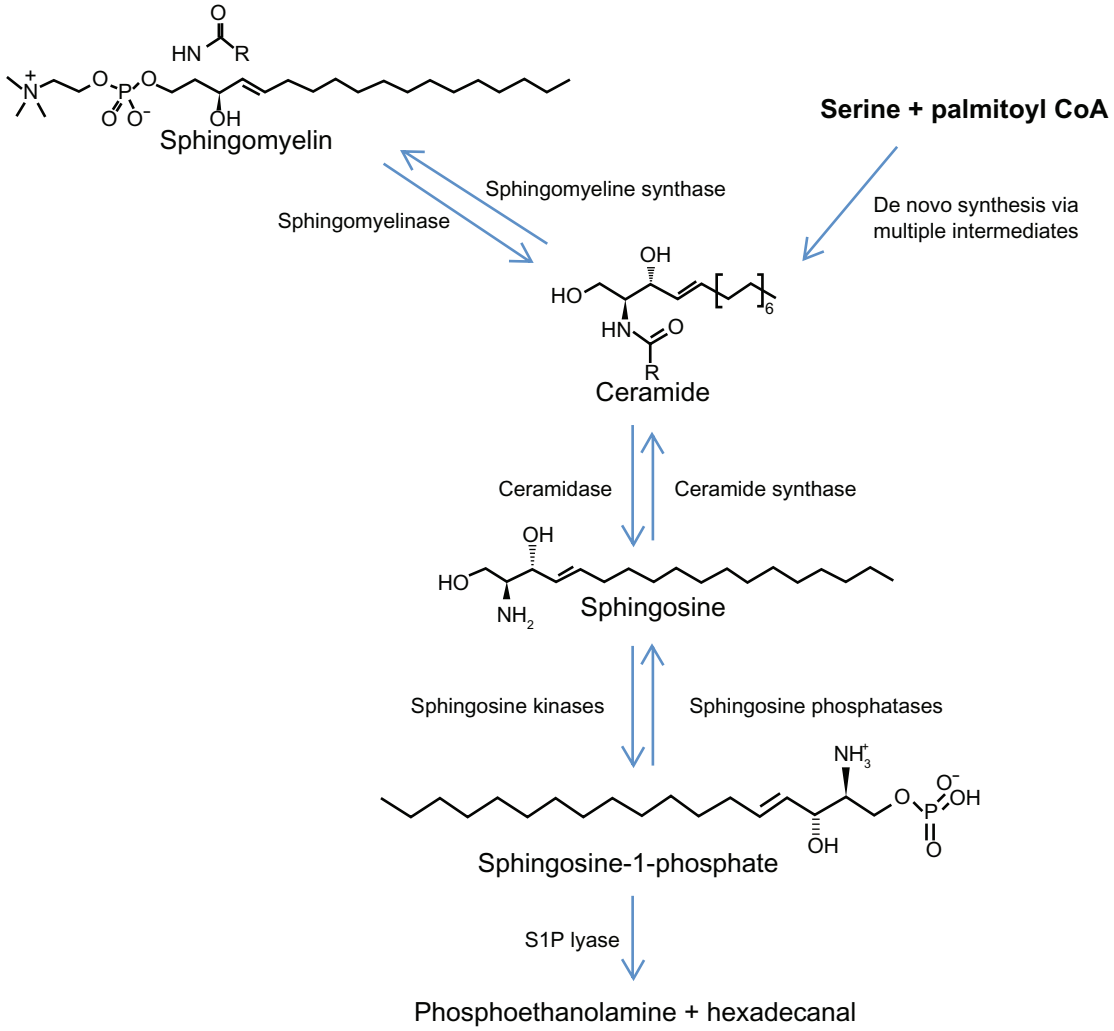

Figure 1 Sphingosine-1-phosphate (S1P) metabolism.
Notes: Ceramide produced either de novo or from sphingomyelin, is hydrolyzed into sphingosine, which is then phosphorylated to become S1P. S1P can be irreversibly degraded by S1P lyase to phosphoethanolamine and hexadecanal.
Abbreviation: CoA, coenzyme A.

S1PR1

S1PR1 (formerly Edg-1) is widely expressed by cells of the immune system, brain, heart, lung, kidney, spleen, and vasculature. It is coupled with G_i only – as pertussis toxin sensitivity suggests – so it acts through the extracellular signal-regulated kinases (ERK), phosphatidylinositol 3-kinase/protein kinase B (Akt), Ras/mitogen-activated protein, Rac, and endothelial nitric oxide synthase pathways.[14] *S1PR1* knockout in mice is lethal, mainly due to severe vascular disruption.[15] Experiments with partial inactivation suggest that S1PR1 plays key roles in angiogenesis, neurogenesis, immune cell trafficking, endothelial barrier integrity, and regulation of vascular tone.[16–19] S1PR1 seems to be important for lymphocyte egress from the thymus, as well as T-cell migration through the lymph nodes and other lymphoid organs.[17,20] It seems that upon S1P ligation, S1PR1 is internalized and can be degraded. In micromolar saturating concentrations of S1P, as in blood and lymph, this effect is more persistent. In the thymus and other tissues, however, with nanomolar concentrations of S1P there is a mechanism that recycles S1PR1 on the cell surface, resulting in a dynamic receptor stabilization. However, there are cases where lymphocyte egress from lymphoid organs is inhibited, either for accumulation of cells before their release or in immunosuppressive situations. In those cases, there seems to be a cross-linking of S1PR1 with CD69, a T-cell activation antigen, causing greater levels of internalization and degradation and disrupting the balance.[21,22] Data from our group suggest that, 24 hours following T-cell activation, CD69 expression increases, causing a disruption to S1PR1 signaling. After 3 days of activation, though, subsequent T-cell mitosis causes a decrease in the surface levels of CD69, allowing the reacquisition of S1PR1 responsiveness and the egress of mature effector T cells from the lymph nodes.[23] Recent evidence suggests that S1PR1 is also involved in immature B-cell egress from the bone marrow into the blood.[24,25]

S1PR2

Unlike S1PR1, S1PR2 (formerly Edg-5) couples with several different types of G protein, including G_i, G_q and $G_{12/13}$. This means that it signals through all the pathways S1PR1 does, with the addition of phospholipase C (PLC) and Rho pathways. *S1PR2* knockout mice do not have an apparent phenotype, but there are reports of epileptic seizure cases[26] as well as deafness.[27,28] This S1P receptor is also widely expressed. Recently, S1PR2 has been suggested to regulate macrophage recruitment and inflammatory cytokine production.[29] S1PR2 signaling is proposed to promote smooth muscle cell differentiation through Rho A activation.[30] S1PR2 is also induced in microvascular endothelial cells and skin mast cells by various inflammatory factors.[31,32]

S1PR3

Like the previous receptors, S1PR3 (formerly Edg-3) is ubiquitously expressed by immune cells, as well as cells in the heart, lung, spleen, kidney, intestine, diaphragm, and cartilage. Both vascular endothelial cells and smooth muscle cells express it and S1PR3 can mediate vasoconstriction or vasodilation depending on the vascular bed and the G protein signaling pathway that is activated. S1PR3 couples with G_i, G_q, and $G_{12/13}$ leading to activation of distinct pathways with contradictory effects. It can activate Akt and endothelial nitric oxide synthase through G_i or Rho through $G_{12/13}$ as well as PLC and Ca^{2+} mobilization through G_q. The cardioprotective effects of HDL are suggested to involve S1PR3 signaling.[33] Alternatively, S1PR3 could cause endothelial barrier disruption, in contrast to S1PR1, which helps maintain barrier integrity.[34] Further, S1PR3 could promote inflammatory recruitment of monocytes and macrophages.[35] *S1PR3* knockout mice do not seem to have an obvious phenotype, although they lack several S1P effects on the cardiovascular system.[36]

S1PR4

Unlike the previous receptors, S1PR4 (formerly Edg-6) expression is mainly restricted to cells of the immune system, especially lymphocytes and hematopoietic cells. A *S1PR4* knockout mouse model has only recently been investigated.[37] It seems that S1PR4 deficiency does not affect lymphoid organ structure and lymphocyte numbers in blood, but dendritic cell function is severely altered, leading to reduced Th17 T-cell differentiation.[37] S1PR4 mainly couples with G_i and $G_{12/13}$, so it acts through ERK, PLC, and Rho signaling pathways. S1PR4 is upregulated during megakaryocyte development and plays a role in their differentiation into pro-platelets.[38] There are also claims that S1PR4 signaling can inhibit T-cell proliferation and cytokine secretion.[39]

S1PR5

Like S1PR4, S1PR5 (formerly Edg-8) is more specifically expressed by cells in the central nervous system (CNS). It can similarly couple to G_i and $G_{12/13}$, acting through ERK, Akt, or Rho signaling pathways. S1PR5 signaling plays a dual role: it can promote the survival of mature oligodendrocytes, through an Akt-dependent pathway, or lead to process

retraction of pre-oligodendrocytes, through Rho activation.[40] Moreover, S1PR5 is expressed in natural killer cells, where it acts, similarly to S1PR1 on lymphocytes, to regulate cell trafficking of mature cells through the bone marrow and from blood to the lymph nodes.[41,42] *S1PR5* knockout in mice leads in absence of observed oligodendrocyte retraction[40] and deficient natural killer cell trafficking.[42]

S1P as a second messenger

S1P is involved in many cellular processes through its GPCR signaling; however, there are studies[47–50] demonstrating that S1P also acts at an intracellular level.[43] As previously mentioned, intracellular S1P plays a role in maintaining the balance of cell survival signal toward apoptotic signals, creating a cell "rheostat" between S1P and its precursor ceramide. Important evidence that S1P can act intracellularly as a second messenger came from yeast (*Saccharomyces cerevisiae*) and plant (*Arabidopsis thaliana*) cells. Yeast cells do not express any S1P receptors, although they can be affected by S1P during heat-shock responses.[44,45] Similarly, *Arabidopsis* has only one GPCR-like protein, termed "GCR1," which does not bind S1P, although S1P regulates stomata closure during drought.[46]

In mammals, the sphingosine kinases have been found to localize in different cell compartments, being responsible for the accumulation of S1P in those compartments to give intracellular signals. In mitochondria, for instance, S1P was recently found to interact with prohibitin 2, a conserved protein that maintains mitochondria assembly and function.[47] According to the same study, SphK2 is the major producer of S1P in mitochondria and the knockout of its gene can cause disruption of mitochondrial respiration and cytochrome c oxidase function. SphK2 is also present in the nucleus of many cells and has been implicated to cause cell cycle arrest.[48] Further, it causes S1P accumulation in the nucleus.[49] It seems that nuclear S1P is affiliated with the histone deacetylases HDAC1 and HDAC2, inhibiting their activity, thus having an indirect effect in epigenetic regulation of gene expression.[49] In the ER, SphK2 has been identified to translocate during stress, and promote apoptosis. It seems that S1P has specific targets in the ER that cause apoptosis, probably through calcium mobilization signals.[50]

The involvement of S1P signaling in disease

It is clear that S1P signaling plays a part in several important processes involving different kinds of cells, tissues, and systems. Several studies[33,51–61] show either how S1P signaling can be vital in pathophysiological conditions or how its deficiency can be the cause of such conditions. Here, we will try to analyze a few of the effects of S1P signaling in disease.

In a mouse model of myocardial ischemia-reperfusion injury (IRI), S1P and its carrier, HDL, can help protect myocardial tissue and decrease the infarct size.[33] It seems they reduce cardiomyocyte apoptosis and neutrophil recruitment to the ischemic tissue and may decrease leukocyte adhesion to the endothelium. This effect appears to be S1PR3 mediated, since in *S1PR3* knockout mice it is alleviated.[33] Fortunately for therapeutic potential, this cardioprotective effect of S1P can be produced not only by administering it before ischemia (preconditioning) but also during reperfusion (post-conditioning). Ischemia activates SphK1, which is then translocated to the plasma membrane.[51] This leads to an increase of intracellular S1P, helping to promote cardiomyocyte survival against apoptosis, induced by ceramide.[52] *SphK1* knockout mice cannot be preconditioned against IRI,[53] whereas *SphK1* gene induction in the heart protects it from IRI.[54] Interestingly, a recent study shows SphK2 may also play a role, since its knockout reduces the cardioprotective effects of preconditioning.[55] Further, administration of S1P or sphingosine during reperfusion results in better recovery and attenuation of damage to cardiomyocytes.[56] As with preconditioning, SphK1 deficiency also affects post-conditioning of mouse hearts after ischemia reperfusion (IR).[57]

S1P does not only protect the heart from IRI. During intestinal IR, multiple organs can be damaged, including the lungs. S1P treatment of mice during intestinal IR seems to have a protective effect on lung injury, probably due to suppression of iNOS-induced nitric oxide generation.[58] In renal IRI, SphK1 seems to be important, since its deficiency increased the damage in kidney tissue, whereas the lentiviral overexpression of the *SphK1* gene protected from injury.[59] Another study suggests that, after IRI, apoptotic renal cells release S1P, which recruits macrophages through S1PR3 activation and might contribute to kidney regeneration and restoration of renal epithelium.[60] However, SphK2 is negatively implicated in hepatic IRI, its inhibition helping protect hepatocytes and restoring mitochondrial function.[61]

Further studies are implicating S1P signaling or sphingosine kinases in several kinds of cancer as well as autoimmune diseases. The S1P antagonist FTY720 has been approved by the US Food and Drug Administration to be used as a drug against multiple sclerosis (MS). FTY720 is in fact a prodrug, since it is phosphorylated in vivo by SphK2 into FTY720-P, an S1P structural analog, which can

activate S1PR1, 3, 4, and 5. FTY720-P binding to S1PR1 causes internalization of the receptor, as does S1P – but instead of recycling it back to the cell surface, it promotes its ubiquitination and degradation at the proteasome.[62] This has a direct effect on lymphocyte trafficking through the lymph nodes, since it relies on S1PR1 signaling and S1P gradient (Figure 2). In MS, it stops migrating lymphocytes into the brain, but it may also have direct effects on the CNS through neuroprotection. FTY720 can pass the blood–brain barrier and it could be phosphorylated by local sphingosine kinases to act through S1PR1 and S1PR3 receptors that are mainly expressed in the CNS. In MS lesions, astrocytes upregulate those two receptors and it has been shown that FTY720-P treatment in vitro inhibits astrocyte production of inflammatory cytokines.[63] A recent study confirms the importance of S1PR3 signaling on activated astrocytes, as well as SphK1, that are upregulated and promote the secretion of the potentially neuroprotective cytokine CXCL-1.[64]

The first indication that S1P signaling might be involved in cancer came with the evidence that *SphK1* overexpression in NIH 3T3 fibroblasts transforms them into cancer-like cells – in the sense that they proliferate in serum-free conditions, have increased growth in vitro, and form fibrosarcomas in vivo.[65] Although there is no proof that *SphK1* is an oncogene, cancer cell lines overexpress it and depend on it for growth and survival.[66] Further, in various human cancers, such as stomach,[67] lung,[68] brain[69] and breast,[70] increased levels of SphK1 mRNA and/or protein have been identified.[71] There are several studies[72–75] implicating the intracellular S1P ceramide rheostat to cancer cell survival or apoptosis and resistance to chemotherapy or irradiation in vitro.[71] Studies with SphK1 inhibition in pancreatic,[72] prostate cancers,[73,74] and leukemia,[75] show increased ceramide/S1P ratio and induction of apoptosis. However, S1P receptor signaling plays conflicting roles in cancer cell migration and metastasis. Generally, it seems that S1PR1 and S1PR3 activation stimulates cancer cell migration, whereas S1PR2 inhibits cancer cell motility.[76–80]

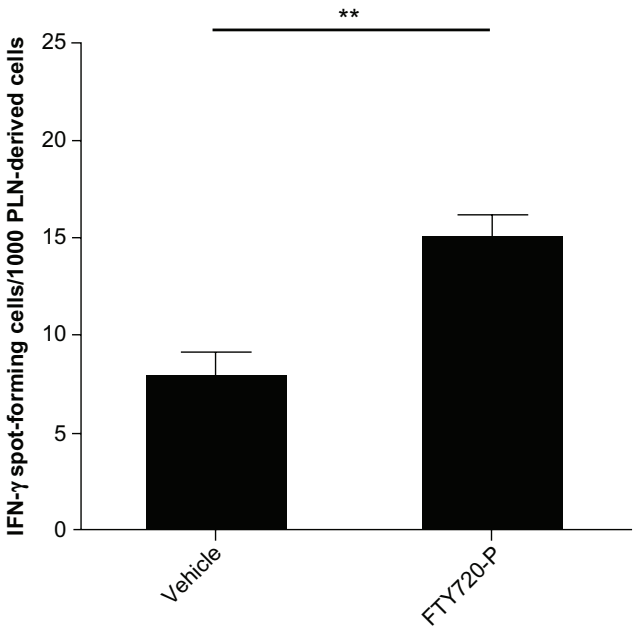

Figure 2 FTY720-P causes retention of T cells in the lymph nodes.
Notes: C57BL/6 mice were injected with BALB/c splenocytes in the footpad to create an allogenic response then treated with FTY720-P or vehicle every day on days 2 to 5. On day 6, the popliteal lymph nodes were removed. Popliteal node-derived cells were mixed with BALB/c splenocytes in interferon gamma (IFN-γ) cultured enzyme-linked immunosorbent spot reactions. Bars represent the mean number of IFN-γ spot-forming cells per 1000 popliteal node-derived cells, from six mice treated with vehicle and seven with FTY720-P. $^{**}P < 0.01$.
Abbreviation: PLN, popliteal lymph node.

Modulation of S1P signaling: therapeutic potential

As discussed, S1P signaling can be involved in many pathophysiological conditions. This means that we could look for therapeutic targets in all the molecules taking part in S1P signaling and production, most importantly the S1P receptors and the sphingosine kinases. S1P agonists and antagonists could also be used to modulate S1P signaling during pathological conditions. Here we will discuss some of potential therapies based on the S1P signaling axis.

S1P can have direct effects on the cardiovascular system. As previously discussed, during IRI, intracellular S1P can protect the cardiomyocytes and promote their survival. Pre- or post-conditioning of the heart with S1P could be used as a treatment, but upregulation of sphingosine kinases could also increase intracellular S1P bioavailability. However, S1P could also have effects on endothelial cells and neutrophil trafficking. Vascular endothelial cells mainly express S1PR1 and S1PR3; only a few types express S1PR2.[81,82] S1PR1 and S1PR3 activation on these cells has been shown to enhance their chemotactic migration, probably through direct phosphorylation of S1PR1 by Akt,[83] in a phosphatidylinositol 3-kinase and Rac1-dependent signaling pathway.[84,85] Moreover, it stimulates endothelial cell proliferation through an ERK pathway.[85,86] S1PR2 activation, however, inhibits endothelial cell migration, morphogenesis, and angiogenesis,[87–89] most likely through Rho-dependent inhibition of Rac signaling pathway, as Inoki et al showed in mouse cells with the use of S1PR1 and S1PR3 specific antagonists.[87]Apart from endothelial cells, S1PR2 has an inhibitory effect on vascular smooth muscle cell migration and proliferation

as well. These cells mainly express S1PR2 and S1PR3 and it seems that their activation has similar contradictory effects as in endothelial cells through the activation of equivalent pathways.[81,86,90–92]

Regarding permeability of the vascular endothelium and endothelial barrier integrity, S1P receptors can have different effects. S1PR1 activation enhances endothelial barrier integrity by stimulation of cellular adhesion and upregulation of adhesion molecules.[18,93] However, S1PR2 and S1PR3 have been shown to have barrier-disrupting effects in vitro, and vascular permeability increasing effects in vivo.[34,94] All the effects S1P can have on vascular endothelium and smooth muscle cells suggest that activation of S1PR2, not S1PR1 and S1PR3, signaling, perhaps with the use of S1PR2 specific agonists, could be used therapeutically to inhibit angiogenesis and disrupt vasculature, suppressing tumor growth and progression. Yet, in several other situations, including transplantation and IRI, S1PR1 activation and inhibition of S1PR2 could have positive effects in promoting angiogenesis and barrier function for recovery of the transplant or injured tissue, respectively.

An important aspect of S1P signaling that is being already therapeutically targeted, but could be further investigated, is immune cell trafficking. Attempts have already been made to regulate lymphocyte cell migration with the use of the drug FTY720, whose phosphorylated form can inhibit the cells' S1PR1-dependent egress from the lymph nodes, causing lymphopenia. FTY720 is used as an immunosuppressant for MS but is also being investigated for other autoimmune conditions and for transplantation. Unfortunately, Phase II and III clinical trials for the prevention of kidney graft rejection have not shown an advantage over standard therapies.[113–117] Moreover, FTY720 can have some adverse cardiac effects, such as bradycardia. However, there are other S1PR1 antagonists that could be considered instead, including KRP-203,[95] AUY954,[96] and SEW2871.[97] KRP-203 in particular has been shown to prolong rat skin and heart allograft survival and attenuate chronic rejection without causing bradycardia,[95] especially when combined with other immunomodulators.[98,99] Moreover, it seems to have positive effects in mouse and rat models of autoimmune conditions, such as chronic colitis,[100] autoimmune myocarditis,[101] and autoimmune kidney disease.[102] KRP-203 is currently undergoing Phase II clinical trials for cutaneous lupus erythematosus.

The effects of S1P on neutrophil trafficking, though, have not been thoroughly investigated. Neutrophils predominantly express S1PR1, S1PR4, and S1PR5, although S1PR3 expression seems to be induced in pneumonia.[103] Quantitative real time polymerase chain reaction experiments on neutrophils by our group show that S1PR1 and S1PR4 mRNA is primarily detected, although there is a low-level expression of S1PR5 and even less of S1PR3 (Figure 3). There are studies that argue S1P pretreatment has a negative effect on neutrophil chemotaxis toward the chemokine CXCL-8 (interleukin-8) or the potent chemoattractant formyl-methionyl-leucyl-phenylalanine.[103,104] S1P pretreatment might also inhibit trans-endothelial migration of neutrophils, without affecting their adhesion to the endothelium.[104] However, different S1P concentrations in blood and tissue, and the activation state in which the neutrophils are, could have various effects on neutrophil trafficking in vivo. So, we need to consider everything if we want to assess the effect S1P has on neutrophil trafficking during inflammatory conditions. S1P effects on neutrophil migration toward CXCL-8 might be the result of S1PRs cross-linking with the CXCL-8 receptors in neutrophils, CXCR-1 and CXCR-2. Indeed, there is evidence suggesting S1PR4 and S1PR3 form heterodimers with CXCR-1 in neutrophils.[103] However, there are several studies showing S1P can increase CXCL-8 expression and/or secretion by several types of cells, including human bronchial epithelial cells,[105] alveolar epithelial cells,[106] gingival epithelial cells,[107] umbilical vein endothelial cells,[108] and immature dendritic cells.[109]

Another indication that S1P plays a role in neutrophil trafficking is a recent paper on S1P lyase deficiency, a deficiency that impairs neutrophil migration from blood to tissue in knockout mice.[110] Little is known of the mechanism by which S1P lyase acts, although S1PR4 deficiency seems to

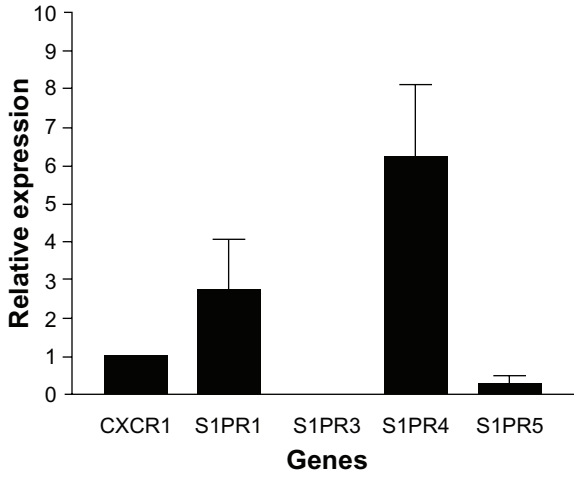

Figure 3 Relative mRNA expression of sphingosine-1-phosphate (S1P) receptors by neutrophils.
Notes: mRNA expression was detected using quantitative real time polymerase chain reaction. Data are normalized for the housekeeping gene *18S* and presented as relative expression in comparison with *CXCR1* gene expression.

alleviate the phenomenon, implicating S1P receptor signaling in neutrophil trafficking.[110] These findings suggest S1P lyase and S1PRs in neutrophils may be new therapeutic targets against IRI and inflammatory conditions in general. Consistent with these results, another study has shown that inhibition of S1P lyase can have a protective effect on the heart after IRI and this effect is alleviated when pretreated with an S1PR1 and S1PR3 antagonist.[111] Inhibition was achieved with a US Food and Drug Administration-approved food additive, 2-acetyl-4-tetrahydroxybutylimidazole, providing a possible new drug perspective.[10,111] Another S1P lyase inhibitor, LX2931, a synthetic analog of 2-acetyl-4-tetrahydroxybutylimidazole, has been shown to cause peripheral lymphopenia when administered in mice, providing a potential treatment for autoimmune diseases and prevention of graft rejection in transplantation.[112] This molecule is currently under Phase II clinical trials in rheumatoid arthritis patients.

Conclusion and future perspectives

S1P signaling research has the potential to discover novel therapeutic targets. S1P signaling is involved in many physiological and pathological processes. However, the complexity of S1P signaling makes it necessary to consider every possible pathway, either through its GPCRs, or intracellularly, with S1P as a second messenger. Where the activation of one S1P receptor may lead to the desired outcome, the simultaneous activation of another S1P receptor may lead to the opposite outcome. Thus, if we are to target a specific signaling pathway, we might need specific agonists for S1P receptors to activate one S1P receptor pathway, while, at the same time, we might need to inhibit another through S1P receptor antagonists. If we need to increase S1P bioavailability inside the cells – for instance, to promote their survival and protection – it might not be enough to give exogenous S1P to the patient. In this case, we might need to consider sphingosine kinases or S1P lyase as targets, or other enzymes in the S1P metabolic pathway, whose overexpression or downregulation can cause an in vivo accumulation of S1P intracellularly. Other factors also need to be considered during drug development. For example, in addition to the opposing effects of the various S1P receptors, the diversity of receptor partners must be taken into account. The activation of an S1P receptor when coupling with a specific G protein could have opposing results to the activation of the same receptor, coupling with a different G protein.

It is clear that S1P research is a promising field; however, much work is required before we can develop therapies that have the desired effects, without any potentially dangerous adverse effects.

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Disclosure

The authors report no conflicts of interest in this work.

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