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Review Article

The role of sphingosine 1-phosphate in immunity and sepsis

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Abstract: Sphingosine 1-phosphate (S1P) is a lipid metabolite with intra- and extracellular signalling properties. It activates five G protein-coupled cell surface receptors designated S1P-receptors type 1-5 ($S1P_{1-5}$) that transmit extracellular signals into cells, and it modulates intracellular signalling as a cofactor. The analysis of sphingosine kinases (SphK) type 1 and 2, the key enzymes for S1P production, in different infection models point to an important role for the activation of different immune cells like macrophages, mast cells, and dendritic cells. S1P additionally influences local and systemic lymphocyte circulation and positioning, the vascular tone, and blood pressure. Modulation of S1P-mediated signalling pathways therefore results either in local immune cell activation or systemic immune suppression, or both. Pharmacological approaches that modulate certain S1P-mediated signalling pathways while leaving others untouched appear to be promising new avenues for next generation pharmaceuticals. This review summarizes current strategies to modulate S1P signalling for immune intervention with the clear focus on the specificity of the different principles applied. Known local and systemic effects of S1P on immunity are discussed as potential pharmaceutical targets to combat immune and autoimmune diseases and sepsis.

Keywords: Lymphocyte egress, sphingosine kinase, S1P-lyase, sphingolipid metabolism, lymphopenia, FTY720, rheumatoid arthritis, multiple sclerosis, asthma, anaphylaxis

Introduction

S1P is a signalling molecule with an extraordinary broad functional repertoire [1, 2]. Surprisingly the importance of this molecule in immunity was barely recognized until a small molecule called FTY720 was shown to induce profound immune suppression in rodents by a totally new mechanism at that time (**Figure 1**) [3]. Although early investigations reported that this substance induced apoptosis [3-6], subsequent studies revealed disruption of lymphocyte emigration from thymus and lymph nodes as its predominant mode of action [7-9]. FTY720 shared structural similarities with sphingosine, the unphosphorylated precursor of S1P (**Figure 1**). While FTY720 itself was a prodrug and not inhibiting lymphocyte emigration, it was phosphorylated in vivo by sphingosine kinases (SphK), the major S1P producing enzymes, to the respective phosphate FTY-P (**Figure 1**), which turned out to be the active compound that binds to four out of five S1P receptors except $S1P_2$ [10, 11]. Of the two known sphingosine kinases, SphK2 was the predominant one involved in FTY720 phosphorylation [12-16]. At first activation of S1P receptors was considered to establish an insuperable barrier for exiting lymphocytes [17, 18]. The analysis of several different S1P receptor deficient mice however demonstrated that loss-of-function of $S1P_1$ in T and B cells was critical to interrupt their circulation, emphasizing activation-induced $S1P_1$ receptor internalization as the most important function of FTY-P in order to block lymphocyte emigration from thymus and lymph nodes [19-21]. Consequently S1P in blood and lymph was established as the predominant exit signal for emigrating lymphocytes [22]. Further studies have shown that locally produced S1P in the microenvironment of thymus and lymph nodes is also important for efficient lymphocyte emigration [23-25], and the expression of different S1P receptors in various

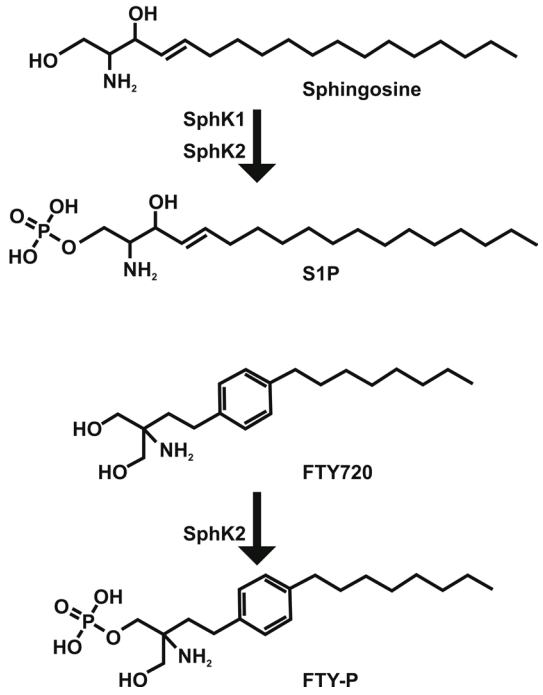

Figure 1. Phosphorylation and structure of sphingosine and FTY720. While sphingosine is phosphorylated by SphK1 and SphK2 to S1P, FTY720 is predominantly phosphorylated by SphK2 in vivo.

leukocyte subsets led to the rapid expansion of immune functions mediated by S1P and FTY-P [1, 2], why the latter is now called immunomodulator rather than immunosuppressant. Different functions of the five known S1P receptors together with the presence of local and systemic pools of S1P and a vivid regulation of S1P receptor surface expression and S1P metabolism attracted a lot of attention from pharmacologists to modulate S1P receptor function and S1P metabolism for targeted immune intervention [26, 27]. This review discusses the principles of S1P and S1P receptor signalling in different immune conditions and disease states.

Immune suppression

FTY720 was first tested in clinical trials as an immunosuppressant after renal transplantation [28-31]. Although FTY720 was able to induce apoptosis in lymphocytes [3-6], it turned out that concentrations reached in patients were not high enough to substantially eradicate lymphocytes [8, 32]. The predominant mode of action was subsequently attributed to disrupted lymphocyte circulation due to prevention of their egress from thymus and lymph nodes [7-9]. The finding that FTY720 had to be phosphorylated in order to be active in vivo and that it was structurally similar to the naturally occurring lipid metabolite sphingosine suddenly attracted notice to S1P and its receptors as potential target molecules of FTY720 [10, 11]. Efficient activation of four out of five S1P receptors except $S1P_2$ by FTY-P led to the hypothesis that S1P receptor activation prevented lymphocyte exit by establishing endothelial cell barriers [17, 18]. Parallel investigations however demonstrated that FTY720 inhibited T and B cell chemotaxis to S1P even in the absence of endothelial cell barriers simply by internalization and degradation of the $S1P_1$ receptor in lymphocytes [20, 33, 34]. The analysis of $S1P_1$ deficient fetal liver chimeric and T cell-specific conditional knockout mice supported the notion that $S1P_1$ expression in T and B cells was required in order to exit thymus and lymph nodes [19, 21]. Additional support provided the analysis of SphK2 deficient mice with an inducible deletion of SphK1 preferentially in hematopoietic and vascular endothelial cells (VEC), resulting in almost complete depletion of circulating S1P and therefore referred to as "S1P-less mice" [22]. The absence of circulating S1P in these mice induced lymphopenia due to a block of T and B cell emigration from thymus and lymph nodes and resembled the phenotype of lymphocyte-specific $S1P_1$ receptor deletion [22]. These results constituted a system where S1P in blood and lymph served as an exit signal for T and B cells in thymus and lymph nodes. The latter expressed the $S1P_1$ receptor on their cell surface in order to sense S1P for exiting [19-21, 35, 36]. This general picture was further specified by studies indicating that locally produced S1P within thymus and lymph nodes supported lymphocyte egress most likely via establishment of local S1P gradients at the respective exit sites [23-25], although the existence of such gradients was never directly shown due to analytical constraints. Main sources for S1P were shown to be red blood cells (RBC) and VEC in blood [22, 37, 38], lymphatic endothelial cells (LEC) in lymph [39], and pericytes in thymus [25]. Regulation of $S1P_1$ receptor surface expression on lymphocytes turned out to be critical for maintaining lymphocyte circulation [36]. Surprisingly not only sur-

face expression of $S1P_1$ in thymus and lymph nodes was important for T and B cell emigration, but also its internalization and desensitization to re-enter lymphoid organs [40]. Prevention of $S1P_1$ receptor desensitization trapped lymphocytes in circulation [40]. While $S1P_1$ was the predominant exit signal-sensing receptor on T and B cells, natural killer (NK) cells were shown to emigrate from lymph nodes and bone marrow mainly via activation of the $S1P_5$ receptor [41, 42]. An interesting finding was that $S1P_5$ signalling on NK cells was not influenced by CD69 expression [41], while $S1P_1$ was inhibited upon CD69 upregulation through direct interaction [43, 44]. This could be an important functional characteristic of NK cells to escape CD69-mediated retardation of lymphocytes in lymph nodes in order to enable their participation in the early immune defense against infections [45].

Interfering with the outlined regulation of lymphocyte egress from lymphoid organs has evolved as a new concept for immune suppression [46]. A major advantage of this approach is the complimentary mode of action in combination with classical immunosuppressants like cyclosporine or tacrolimus, which would theoretically allow for better efficacy and lower toxicity in combinatorial treatment. But there are some obstacles that turned out to be counteractive. One major problem was the rather ubiquitous expression of $S1P_1$ which resulted in various side effects like transient bradycardia, impaired renal function, and the development of macula edema [28]. More specific agonists and antagonists for $S1P_1$ were developed to decrease the list of unwanted side effects. But while FTY720 was predominantly activated via phosphorylation by SphK2 in tissues, which led to more than 100-fold higher FTY-P concentrations in tissues than in blood [47], direct agonists and antagonists were present in blood at high concentrations and interfered with the maintenance of the vascular tone by blood-borne S1P and $S1P_1$ receptor stimulation on VEC [48]. Thus application of FTY720 as a prodrug turned out to be beneficial to prevent increased vascular leak. Novel strategies need to be established to circumvent these pitfalls of currently available small molecules in order to be regarded as competitive immunosuppressive candidates in the current pharmaceutical market.

Multiple sclerosis

Multiple sclerosis (MS) is an autoimmune disease of the central nervous system. Although the cause of this disease remains unknown, it is evident that lymphocytes cross the blood-brain-barrier and cause inflammation around the axons of the brain and the spinal cord, leading to demyelination, neuroaxonal injury, astrogliosis, and finally neurodegeneration [49]. The initial finding that FTY720 had a therapeutic effect on experimental autoimmune encephalomyelitis (EAE) in mice [50-53], an established animal model for MS, led to the exploration of its therapeutic potential in clinical trials for MS [54, 55]. FTY720 was approved in the United States in 2010 and in Europe in 2011 for treatment of the relapsing form of MS under the brandname Gilenya [56]. It was the first orally available treatment for MS. The initial finding that FTY720 induced lymphopenia shaped the hypothesis that lymphocytes did not reach the inflammatory sites in the brain anymore, which would result in decreased inflammation and potentially lower destruction of neuronal tissue [50]. More recent studies documented that predominantly naïve and central memory T cells including interleukin 17 producing (Th17) T cells were reduced in peripheral blood whereas effector memory T cell counts were normal [57, 58]. It was suggested that effector memory T cells were not trapped in lymph nodes by the mechanism outlined in “immune suppression” because they did not circulate through lymph nodes due to the lack of the chemokine receptor CCR7, which was involved in redirecting T cells from peripheral blood into lymph nodes [57, 58]. While the predominant deletion of Th17 cells in peripheral blood was considered as the most important event for its therapeutic efficacy in MS [58], FTY720 may also modulate the function of astrocytes in the brain [59, 60]. Mice carrying a genetic deficiency of the $S1P_1$ gene in glial fibrillary acidic protein (GFAP) expressing astrocytes not only showed attenuated EAE, but FTY720 had also no additional effect on EAE progression [59]. These data suggest that the efficacy of FTY720 in MS treatment may at least partially depend on its local activity on astrocytes in the brain rather than immune cell trafficking. Notably the conditional genetic deletion of $S1P_1$ in GFAP expressing astrocytes also showed diminished lymphocyte infiltration without alterations of peripheral lym-

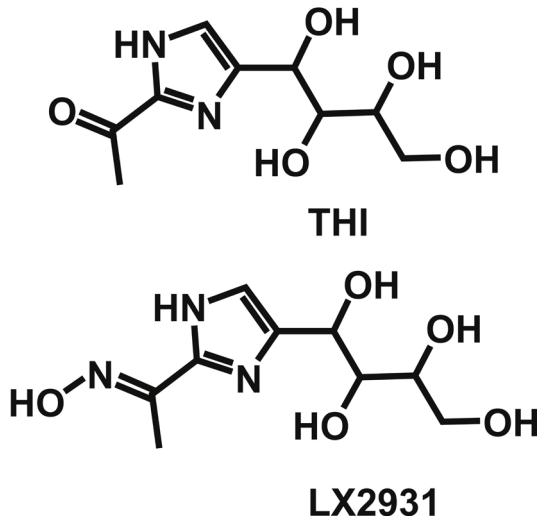

Figure 2. Structure of THI and LX2931. THI is a component of caramel colour III (E150c), while LX2931 was developed by Lexicon Pharmaceuticals for treatment of RA.

phocyte counts [59]. Besides general immune suppression by impairment of lymphocyte circulation, disruption of local $S1P_1$ signalling in astrocytes could be an alternative treatment option for MS, which may also be effective for primary progressive forms of MS that are insensitive for current immune therapies. A correspondent phase III clinical trial for FTY720 (INFORMS) is ongoing [56]. While $S1P_1$ inhibition in astrocytes could be an effective treatment option for MS, the endogenous role of S1P and $S1P_1$ receptor signalling in astrocytes and other cells of the central nervous system (CNS) remains enigmatic. A better understanding of the basic functions of S1P in the CNS may not only explain the unforeseen efficacy of FTY720 for MS treatment, but may offer additional and more CNS-specific target molecules for better treatment options.

Rheumatoid arthritis

Rheumatoid arthritis (RA) is a chronic disease, characterized by an inflammatory synovitis that can lead to functional impairment and destruction of joints. Although the cause of this disease is unknown, autoimmunity plays a pivotal role in RA pathology [61]. Targeting S1P-driven lymphocyte circulation seems to be a promising strategy to combat this disease. In fact application of FTY720 in SKG mice, which spontaneously develop T cell-mediated chronic autoimmune arthritis due to a mutation in ZAP-70, proved to have therapeutic potential [62]. While FTY720 has not yet been tested for RA treatment in the clinical setting, a different strategy evolved that targets the metabolism of S1P. Inhibition of the retro-aldolase S1P-lyase (SGPL1), which irreversibly cleaves S1P into hexadecenal and phosphoethanolamine, resulted in more than 100-fold accumulation of S1P in lymphoid organs [63-65]. Consequently the S1P-gradient between blood and lymph with high S1P levels and lymphoid organs with low S1P levels was annulled, and surface expression of $S1P_1$ on lymphocytes was prevented in thymus and lymph nodes. Exit-mediating $S1P_1$ signalling was ultimately abrogated in these cells, and lymphopenia was developing similar to that seen after FTY720 treatment [63-65]. The SGPL1 inhibitor LX2931 was developed by Lexicon Pharmaceuticals and is currently tested in phase II clinical trials for RA treatment [66]. First results demonstrated low efficacy of this compound compared to placebo controls, why an additional dose escalation trial was initiated (Lexicon Pharmaceuticals). LX2931 is structurally similar to 2-acetyl-4-tetrahydroxybutylimidazole (THI), a compound found in the food additive caramel colour III (E150c, **Figure 2**) [67]. Inhibition of SGPL1 by THI was evident in mice and could be antagonized by vitamin B_6 supplementation, indicating that THI blocked the functionally important interaction of SGPL1 with its prosthetic group pyridoxal phosphate (PLP) [63, 67-70]. It is very likely that LX2931 also functions as an antagonist of PLP, which implicates that vitamin B_6 consumption may partially neutralize the pharmacological effect of LX2931 in patients. Thus, although inhibition of SGPL1 proved to be a promising approach for RA treatment, PLP-independent direct inhibitors for this metabolic enzyme are still missing. Compared to FTY720 treatment, SGPL1 inhibition had at least one remarkable advantage: S1P accumulation was predominantly seen in lymphoid organs, and less or no accumulation of S1P was observed in most other peripheral organs like heart, eye, or liver [66]. This profile decisively increased the specificity to lymphoid compartments, which was probably a major reason for the favourable safety of LX2931 demonstrated in clinical trials.

Asthma

The role of S1P in allergic responses is probably best demonstrated for asthma, which is a chronic inflammatory disease of the airways

[71]. Airway smooth muscle cells are thought to play a key role in asthmatic attacks, and the finding that S1P was not only increased in airways of asthmatic patients, but also induced contraction of human airway smooth muscle cells prompted researchers to take a closer look into the regulation of S1P and its signalling in allergic asthma [72]. Notably administration of S1P increased bronchial hyperresponsiveness in mice [73], while inhalation of FTY720 and sphingosine kinase inhibitors abrogated experimental asthma [74, 75]. The main S1P receptor involved in the induction of airway hyperreactivity was $S1P_3$ [76]. Besides the contraction of smooth muscle cells, different immune cells were modulated by S1P as well, including dendritic cells (DC) [74], eosinophils [77, 78], and mast cells [79]. Activated mast cells produced S1P [80], and human eosinophils upregulated expression of S1P receptors type 1-3, the chemokine receptor CCR3, and its ligand CCL5 upon S1P stimulation, and supported their recruitment to inflamed sites [77]. Inhalation of S1P and FTY720 did not result in systemic lymphopenia and immune suppression, but inhibited the migration of lung DC to mediastinal lymph nodes, which abrogated the development of allergen-specific Th2 cells in respective lymph nodes even during ongoing allergen challenge [74]. Notably FTY720 also reduced the capability of DC to form an immunological synapse with naïve and effector Th2 cells, which additionally impaired the allergen-specific immune response [74]. While the mechanistic details of this impairment remained unknown, activation of $S1P_3$ downstream of the protease-activated receptor 1 (PAR1) in DC also promoted inflammation, although in a very different setting of systemic inflammatory response syndromes [81]. The involvement of $S1P_3$ in the promotion of other DC functions like the above mentioned activation of Th2 cells may be possible. Importantly S1P signalling not only modulated DC migration, but also improved DC function [74, 81]. All different cell types involved seemed to have one characteristic in common: They all signal via S1P. Abrogation of S1P signalling in the local environment of the lung could therefore be a promising approach to combat asthma. Antagonists for $S1P_1$ and/or $S1P_3$ seem to be attractive candidates, which could be delivered locally by inhalation. Although this concept has not yet been evaluated in the clinical setting, it might be a reasonable future approach.

Anaphylaxis

Anaphylaxis is a serious allergic response with a rapid onset. It typically starts upon allergen exposure with Immunoglobulin E (IgE) binding to an antigen of the allergen, which then activates FcεRI receptors on mast cells and basophils, although IgE-independent mechanisms also exist [82]. The release of inflammatory mediators like histamine subsequently impairs the function of multiple organ systems and induces e.g. vasodilation, vascular leak, bronchial smooth muscle contraction, and heart muscle depression [83]. As mentioned above, mast cells released S1P upon activation [80], and S1P in turn enhanced degranulation and histamine release via activation of $S1P_2$ on mast cells in an autocrine manner, resulting in increased anaphylaxis [84]. SphK2 deficient mast cells revealed impaired effector functions like degranulation, suggesting that S1P production in mast cells predominantly occurred via SphK2 [80]. But in contrast to the impaired effector function of SphK2 deficient mast cells, SphK2 deficient mice had higher histamine concentrations in blood after induction of passive anaphylaxis than wild type control mice and SphK1 deficient mice [80]. It turned out that histamine concentrations in blood were not only dependent on the ability of mast cells to produce S1P via SphK2, but also on the amount of S1P in blood circulation, which may additionally activate mast cells in the event of an allergic response [80]. SphK1 deficient mice had lower S1P concentrations in blood compared to wild-type mice due to lower S1P production [12]. SphK2 deficient mice however exhibited higher S1P-levels in blood due to defective distribution and SGPL1-dependent degradation in peripheral tissues [85]. Further investigations demonstrated that increased S1P concentrations in blood of SphK2 deficient mice resulted in a faster recovery from an anaphylactic shock due to enhanced histamine clearance in blood [86]. The latter was mediated by an $S1P_2$-dependent increase in blood pressure and pulse distension [86]. Furthermore blood-borne S1P regulated vascular integrity via stimulation of $S1P_1$ signalling, most likely in endothelial cells [48, 87]. Depletion of blood-borne S1P in the above mentioned S1P-less mice or abrogation of $S1P_1$ signalling by activation-induced receptor internalization entailed increased vascular leak, a common phenotype of anaphylaxis [48]. Because of the counteract-

ing roles of local (enhancing) and systemic (attenuating) S1P in early (enhancing) and late-stage (attenuating) anaphylaxis, S1P signalling pathways turned out to be rather difficult therapeutic targets for the treatment of anaphylaxis.

Sepsis

Sepsis is a systemic inflammatory response with an underlying infection that can cause organ dysfunction in its severe state [88]. Uncontrolled inflammation was typically regarded as the main cause of this disease. Current research however indicates that sepsis is much more complex than just being a deregulated immune response. Frequently a systemic inflammatory response is accompanied by an anergic phase, and both immune states are typically not severe enough to fully account for the observed high lethality rate [88]. Many factors contribute to the severity of this disease, and S1P might be a good candidate for therapeutic target molecules since it is involved in many processes that are relevant for sepsis onset and progression like regulation of vascular integrity [48, 87], lymphocyte circulation [20, 21], blood-borne antigen presentation [89, 90], and cytokine secretion [91, 92]. Probably the most challenging difficulty of targeting S1P signalling for sepsis treatment is to gain the required specificity for certain pathways at the right time. $S1P_1$ signalling in endothelial cells for example was shown to be critically involved in cytokine amplification during influenza virus infection [91, 92]. It may therefore also play a role in the release of cytokines during sepsis, which needs to be proven. Increased vascular permeability is a known complication during sepsis, and S1P in blood was shown to be an important regulator for the vascular tone [48, 87]. Apolipoprotein M (ApoM), which is a binding molecule for S1P in plasma [93], was decreased in sepsis patients and may not only serve as a new diagnostic biomarker, but could also have therapeutically relevant functional consequences, e.g. by providing less S1P in plasma, which may increase vascular permeability and contribute to disease severity [94]. Lymphopenia is also frequently observed in sepsis patients [95]. Although lymphocyte apoptosis was attributed as the cause for lymphopenia [96], the involvement of other mechanisms like deregulation of S1P-mediated lymphocyte egress from lymphoid organs as outlined above cannot be excluded. Disruption of S1P gradients may also interfere with the presentation of blood-borne antigens by marginal zone B cells to follicular DC in the B cell zone of the spleen [89, 90], which again needs to be investigated. Importantly $S1P_3$ signalling in DC was a crucial event in the signalling cascade of PAR1, which sustained an inflammatory response [81]. Application of $S1P_3$ antagonists could therefore be a first approach to interfere with sepsis progression.

Conclusion and outlook

S1P signalling evolved as a clinically relevant therapeutic target for immune suppression, MS, and RA. Further applications may include asthma, anaphylaxis, and sepsis in the future. S1P was shown to modulate lymphocyte egress from lymphoid organs [20, 21], vascular integrity [48, 87], blood pressure [86], pulse distension [86], DC function [74, 81], mast cell activation [80, 84], eosinophil recruitment [77, 78], antigen presentation [89, 90], and cytokine secretion [91, 92]. While some of its activities were relevant for normal operation (lymphocyte egress, vascular integrity, antigen presentation), others were only observed in certain disease states (blood pressure, pulse distension, DC function, mast cell activation, eosinophil recruitment, cytokine secretion). Current pharmacological target molecules include S1P receptors (agonists/antagonists like FTY720) [56], the degrading enzyme SGPL1 (inhibitors like LX2931) [66], the S1P producing enzymes SphK1 and SphK2 (inhibitors), the S1P transporter Spns2 (inhibitors) [97-100], and S1P itself (anti-S1P antibodies like iSONEP) [101, 102]. FTY720 was already approved for MS treatment (Gilenya, Novartis), and LX2931 (Lexicon Pharmaceuticals) and iSONEP (LPath, Inc.) are currently tested in phase II clinical trials for treatment of RA and age-related macular degeneration, respectively. Further research is needed to decipher the network of S1P signalling in order to support the development of more selective and efficient pharmaceutical compounds for clinical use in immunity and sepsis.

Abbreviations

S1P, sphingosine 1-phosphate; SGPL1, S1P-lyase; SphK, sphingosine kinase; RBC, red blood cells; DC, dendritic cells; MS, multiple sclerosis; RA, rheumatoid arthritis.

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