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Chemical modulators of sphingosine-1-phosphate receptors as barrier-oriented therapeutic molecules

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Abstract

Biological barriers regulate the passage of cells, pathogens, fluids, nutrients, ions and signalling molecules between anatomical compartments during homeostasis and disease. Yet strategies that allow for reversible therapeutic modulation of these barriers are still in their infancy. The enhancement or protection of natural barriers is desirable in conditions such as acute respiratory distress syndrome or ischaemia–reperfusion injuries, whereas a temporary disruption could facilitate the penetration of drugs across such barriers. This Review discusses the role of sphingosine-1-phosphate receptors in the regulation and protection of biological barriers, and the potential of therapeutic strategies that target this receptor family.

Tight regulation of molecular and cellular exchanges between organs and the bloodstream is central to the maintenance of homeostasis in the body, and the dysregulation of this dynamic process is known to cause pathology. A greater understanding of the mechanisms involved in these exchanges during homeostasis and disease might allow the identification of novel strategies to control cellular and molecular trafficking, or delivery of therapeutic molecules to specific anatomical compartments. Of key importance to the control of these exchanges is the barrier constituted by the network of contiguous endothelial cells that line the lumen of blood vessels, which is known as the vascular endothelium. Endothelia can regulate the passage of cells by modifying the cell surface expression of adhesion molecules, such as integrins and selectins, and regulate molecular exchanges through paracellular gaps by acting on intercellular adhesion molecules from the cadherin, occludin and junctional adhesion molecule families.

Endothelia are found throughout the body and are highly heterogeneous. For example, the blood–brain barrier (BBB) is a stringent endothelial interface specialized in the protection of

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Competing interests statement

The authors declare competing financial interests: see web version for details.

DATABASES

UniProtKB: http://www.uniprot.org

S1P1 | S1P2 | S1P3 | S1P4 | S1P5

FURTHER INFORMATION

Hugh Rosen's homepage: http://www.scripps.edu/chemphys/rosen/

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the central nervous system (CNS) from xenobiotics and toxins. The specificity of the BBB also hinders the delivery of pharmacological agents to the CNS, although the 600 km network of blood vessels within the CNS feeds virtually every neuron[1]. It is well established that almost 100% of large-molecule drugs and 98% of small-molecule drugs do not cross the BBB[2]. When the BBB is disrupted during meningitis or tumorigenesis, leakage of intravascular material to the extravascular tissue leads to increased pressure, vascular dysfunction and, in the case of meningitis, ionic imbalance and accumulation of inflammatory infiltrates, which leads to neuronal damage.

The integrity of the pulmonary endothelial barrier is crucial for the maintenance of gas exchange. When the pulmonary barrier is disrupted during pneumonia or sepsis, the accumulation of fluids and inflammatory cells in the extracellular space, and ultimately in the alveolar airspace, leads to impaired gas exchange. Various other organs are susceptible to barrier-mediated dysfunction, particularly during inflammatory conditions. During the reperfusion phase that occurs after a period of ischaemia — as in the case of myocardial infarction or after solid organ transplantation — the endothelium becomes activated and recruits inflammatory cells, which can irreversibly damage the organ. Consequently, enhancement, protection or even disruption of endothelial barriers could contribute to a range of useful therapeutic strategies for different pathologies.

Although the function of physiological barriers was the focus of scientific curiosity in the nineteenth century[3], when the organ distribution of intravenously delivered dyes was studied, few currently used therapeutics elicit their effects through barrier modulation. The biosynthetic pathway of the lysophospholipid sphingosine-1-phosphate (S1P) has been extensively characterized[4–8] (FIG. 1) and S1P has emerged as a potent modulator of barrier integrity owing to its ability to modulate a family of high-affinity G protein-coupled receptors (GPCrs)[7]. S1P receptors, previously called endothelial differentiation gene receptors, were discovered in the early 1990s, in parallel with the immunosuppressive myriocin analogue FTY720. Mandala *et al*.[9] showed that FTY720 was a prodrug that required *in vivo* phosphorylation to induce immunosuppression, by acting as an agonist on receptors of the structurally similar endogenous ligand, S1P. Five high-affinity receptors have been cloned: S1P1, S1P2, S1P3, S1P4 and S1P5.

Synthetic S1P receptor modulators have shown therapeutic efficacy in clinical trials for multiple sclerosis[21] and in a range of animal models, including acute respiratory distress syndrome (ARDS)[10,11], disseminated intravascular coagulation[12,13], ischaemia–reperfusion injury[14–16], solid graft transplantation[17,18] and experimental autoimmune encephalomyelitis (EAE)[19,20]. Here, we focus on the involvement of S1P receptors in the regulation and protection of the endothelial barrier in both physiological and pathophysiological conditions. On this basis, we elaborate on how the use of current investigative tools can help to refine our understanding of the involvement of S1P receptors in barrier modulation and protection, and how this may lead to the development of highly specific, barrier-oriented small-molecule-based therapeutics.

S1P receptor signalling

Sphingosine is a member of the sphingolipid family and consists of an aliphatic chain with 18 carbon atoms, with hydroxyl groups on carbon atoms 1 and 3, and an amine moiety on carbon atom 2. Sphingosine is generated from *N*-deacylation of ceramide by ceramidase, and can be phosphorylated by sphingosine kinases (SphKs) on its primary hydroxyl group to produce S1P (FIG. 1). Two major sphingosine kinase isoforms have been identified so far — SphK1 and SphK2 — and both possess a number of splice variants[22]. These two enzymes are differentially regulated and distributed within the cell and in different tissues. For example, in mice, SphK1 is mostly expressed in the lungs, spleen, kidneys and blood, whereas high expression of SphK2 occurs in the liver, kidneys, brain and heart[23,24]. SphK1 is present in the cytosol and translocates to the plasma membrane after phosphorylation, which is thought to account, at least in part, for the compartmentalization of S1P synthesis inside the cell (FIG. 2). moreover, SphK1 can be secreted and might contribute to plasma S1P levels, whereas SphK2 is only found intracellularly[25], mostly in the cytosol and nucleus. Notably, the SphK2 isoform efficiently phosphorylates synthetic sphingosine analogues, such as FTY720 and AAL-R. This is in contrast to SphK1 (REF. 26) (FIG. 1), which has slow enzyme kinetics for phosphorylation of FTY720. After phosphorylation to S1P, sphingosine can activate any of its five differentially distributed, high-affinity GPCRs[27]. These receptors regulate a range of biological processes, including cell adhesion, migration and endocytosis. Physiologically, S1P receptors modulate smooth-muscle cell contraction, vascular tone, heart rate, lymphocyte trafficking and barrier integrity (FIG. 3). The role of S1P as a potential ligand for the orphan receptors GPR3, GPR6 and GPR12 (REF. 28) remains controversial, and no compelling experimental evidence has been found.

S1P is present at high nanomolar concentrations in the plasma, although only low nanomolar concentrations of free S1P are available for activity as 98.5% is bound to high density lipoprotein (HDL) and albumin[29]. Although the exact sources of plasma S1P are still a matter of debate, S1P is thought to be produced by endothelial cells, platelets and red blood cells[30–32]. Different subsets of S1P receptors (S1P1, S1P2 and S1P3, discussed below) have different and sometimes opposing effects on the regulation of cellular functions, thereby providing a teleological basis for the use of specific molecular probes directed towards a unique receptor to achieve unidirectional modulation of barrier function.

In vitro model systems involving receptor overexpression or gene silencing were employed to dissect the function of S1P1, which was shown to be involved in cytoskeletal rearrangement and the formation of intercellular adherens junctions[33,34]. Genetic deletion in mice subsequently proved that S1P1 is essential for complete vascular maturation during embryogenesis[35], and resulted in embryonic lethality. Genetic deletion of S1P2 leads to deafness[36,37], whereas S1P3 knockout mice show no obvious phenotypic abnormalities[38]. Perinatal mortality is observed in S1P2–S1P3 double-knockout mice[38]. Furthermore, a number of small-molecule chemical probes have been generated (TABLE 1) to study the S1P receptor system. For example, probes that are selective for S1P1 revealed that expression of this receptor was sufficient to induce lymphocyte arrest in the lymph nodes in rodents, and that tonic activation of S1P1, caused by constant exposure of the endothelial S1P1 receptor to blood-borne S1P, was required for the maintenance of blood-barrier

integrity in specific vascular beds (FIG. 4), including the lungs and kidneys, in the adult mouse[39–41].

In vitro model systems proved that S1P can rescue endothelial cell monolayers from thrombin or lipopolysaccharide (LPS)-induced disruption[42,43], and that S1P, FTY720-phosphate (FTY720-P) and AFD-R, which are nonspecific agonists of S1P receptors[7], can inhibit vascular endothelial growth factor (VEGF)-induced permeability in embryonic endothelial cells *in vitro*[44]. Enhancement of endothelial barriers proceeds through a pathway that has been shown to include S1P1 receptor engagement, activation of Rac1, recruitment to membrane rafts of anchoring substrates for polymerized actin[45], an increase in the amount of phosphorylated myosin light chain kinase and cortactin at the cell periphery, cortical rearrangement of polymerized actin[46] and cortical relocalization of focal adhesions[47] (FIG. 2). S1P2 and S1P3 have opposite effects to S1P1 regarding the regulation of endothelial barrier permeability (FIG. 2). Whereas S1P1 couples solely to Gi/o, S1P2 and S1P3 couple to Gi/o, Gq, and G12 and G13. Activation of G12 and G13 leads to activation of the small GTPase, Rho, which induces destabilization of cortical actin, favours stress-fibre formation and induces endothelial barrier disruption[48,49]. Sanchez *et al*.[50] observed that overexpression of S1P2 in human umbilical vein endothelial cell (HUVEC) monolayers increased both basal and S1P-triggered permeability. In accordance with these results, the S1P2 antagonist JTE-013 diminishes basal permeability of HUVEC monolayers and synergizes with the barrier-enhancing effect of S1P. S1P3 gene silencing inhibited thrombin- and low-molecular-weight hyaluronan-induced disruption of endothelial monolayers[51,52], suggesting that some stimuli need S1P3 transactivation to induce barrier disruption. After exposure of endothelial cells to various barrier-disrupting agents, the S1P3 receptor is phosphorylated on threonine residues, which seems to depend on the activity of rhoA and of the serine–threonine kinases rOCK1 and rOCK2 (REF. 52). The importance of S1P3 phosphorylation remains to be determined.

The combination of *in vitro* and *in vivo* data suggests that the tonic activation of the different S1P receptor subtypes at the surface of endothelial cells favours barrier maintenance under basal conditions, and that this equilibrium is changed in favour of barrier disruption in pathological states. This change might be due to modification of the relative expression of S1P1 and S1P3 and/ or S1P2 combined with variable G protein coupling of S1P3 (in contrast to the uniform Gi/o coupling of S1P1). Adding to the complexity of S1P receptor signalling, cognate S1P receptors homodimerize and heterodimerize, not only with each other[53,54] but also with members of other receptor families, such as chemo kine receptors[55]. Furthermore, S1P receptor expression varies among different endothelia[34], cell types and pathophysiological conditions[50,56,57]. The involvement of the S1P receptors in the dysregulated steps that lead to barrier dysfunction has also been addressed in several animal models[10,11,15,16,58], and acute lung injury is perhaps the model that has provided the largest amount of information regarding the barrier-modulating capacity of the S1P system.

Pulmonary barrier regulation by S1P receptors

Activation and disruption of the pulmonary endothelium reaches its apotheosis in ARDS, in which increased barrier permeability results in leakage of protein-rich fluid from the

vascular compartment to the interstitium and the alveolar airspaces, leading to a life-threatening impairment of gas exchange[59]. Supportive strategies, such as extracorporeal membrane oxygenation, adoption of the prone position, ventilator-assisted strategies and pharmacological interventions (such as corticosteroid administration and intravenous infusion of activated protein C) were shown to be ineffective, to have a modest therapeutic effect or produced inconclusive outcomes[60–63]. Pharmacological treatment remains an important unmet need for ARDS.

The involvement of S1P receptors in the regulation of pulmonary barrier integrity was addressed in various models, including intradermal VEGF injection and intratracheal instillation of barrier-disrupting agents, such as bacterial LPS. The sphingosine analogues FTY720 and AAL-R, but not the enantiomer AAL-S, which cannot be phosphorylated, reduced VEGF-induced leakage in mouse ears[44]. S1P infusion protected the lungs from excessive protein-rich fluid accumulation in the pulmonary parenchyma and/or alveolar airspace in ARDS models; for example in dogs[10] and rodents[10,11]. Furthermore, intraperitoneal injection of FTY720 alleviated features of ARDS 24 hours post LPS instillation in mice; however, this might not only result from pulmonary barrier modulation, but also reflect alterations in leukocyte trafficking[64]. So far, little or no documentation is available regarding the respective contribution of S1P receptors to ARDS *in vivo*, which could speculatively be explained, in the case of genetic knockdown models, by adaptive mechanisms, receptor redundancy and the opposing effects of S1P receptors on different effector cells involved in the exacerbation of pulmonary leakage. The lack of publications on positive effects of S1P1 mono-selective agonists in models of ARDS, in spite of their availability for more than 3 years, suggests that multiple receptors are involved in pulmonary endothelial barrier protection in pathological conditions. The exact function of the different S1P receptors during the course of ARDS *in vivo* needs to be further investigated.

S1P signalling in inflammatory conditions

An increasing body of evidence suggests that S1P could function as a modulator of the innate immune response. Activation of the endothelium is characterized by increased permeability and stimulation of cell surface expression of adhesion molecules, leading to recruitment and accumulation of inflammatory cells that can exacerbate disease by further promoting barrier damage. This paradigm is not only relevant to ARDS, but also to other pathologies, such as myocardial infarction, transplantation-associated ischaemia and multiple sclerosis. Essentially, in response to an insult, sentinel cells, such as fibroblasts[65], macrophages[66], epithelial cells[67] and dendritic cells[68], release a plethora of proinflammatory and chemotactic mediators (FIG. 5a). The activated endothelium expresses adhesion molecules, including selectins and integrins, allowing for rolling and adhesion of leukocytes to the vascular wall[69]. Adherent and infiltrating leukocytes release proteases and toxic molecules for migration and defence, which induce host cell death and tissue damage. This exacerbates endothelial dysfunction and tissue injury, with dramatic consequences for highly differentiated cells and organs that have limited regenerative capacity[70]. For these clinical phenomena, in which activation of sentinel cells and endothelial cells leads to excessive leukocyte accumulation and immunopathological damage, animal models have highlighted

the potential usefulness of a range of molecules. These include anti-inflammatory molecules, pro-survival factors, neutralizers of pro-inflammatory mediators and adhesion molecule blockers[71,72]. All have resulted in a disappointing translation to human diseases[73,74], increasing the need for the identification of new therapeutic targets.

The S1P–S1P receptor regulatory axis has also emerged as a modulator of leukocyte infiltration. Neutrophils isolated from healthy humans express S1P1, S1P4 and S1P5 (REF. 55), whereas monocytes express S1P1, S1P2 and S1P3 (REF. 75). S1P modulates key cellular events involved in leukocyte migration, such as store-operated calcium entry[76], cytoskeletal organization[42,77] and chemokine receptor expression[78]. moreover, S1P receptor expression is modulated by receptor stimulation. For example, S1P3 expression increases in the neutrophils of patients suffering from pneumonia[55] and S1P3 heterodimerizes with the interleukin-8 (IL-8) receptor IL-8RA (also known as CXCR1), although the functional corollary of this association remains to be determined. As tumour necrosis factor-α (TNFα) has been shown to induce SphK1 expression and S1P release from human monocytes[79], and pharmacological inhibition of SphK1 impairs neutrophil migration *in vitro* by decreasing surface expression of the adhesion molecule CD11b[80], S1P biosynthetic enzymes also seem to influence leukocyte migration. The exact mechanism of SphK1-mediated regulation of leukocyte migration remains to be determined, but seems to occur through autocrine S1P receptor activation. Paradoxically, the inflammatory response seems to be intact in SphK1-deficient mice[81], which might result from adaptive genetic compensation. S1P1 expression was also increased in the kidneys of mice after ischaemia–reperfusion injury[15]. FTY720 diminished the extent of ischaemia-induced injury by downregulating neutrophil accumulation, which was reversed by the S1P1 antagonist VPC-44116 (REF. 15). This suggests that S1P1 plays a crucial part in the mediation of leukocyte infiltration to the injured site. Accordingly, SEW2871, a specific agonist of S1P1, decreased expression of intracellular adhesion molecule 1 (ICAM1), P selectin, vascular cell adhesion molecule 1 (VCAM1) and TNFα after renal ischaemia[16]. These effects might be due to the upstream downregulation of pro-inflammatory TNFα[16] or due to S1P1 activation interfering with signalling cascades downstream of TNFα that would normally lead to upregulation of adhesion molecules[82]. moreover, S1P and SEW2871 impaired TNFα-induced monocyte–endothelial cell interactions, which was reversed by the inhibition of S1P1 and/or S1P3 with the dual antagonist VPC23019 (REF. 82). In a similar model system, S1P1 but not S1P3 modulation downregulated endothelial activation, as measured by monocyte adhesion and VCAM1 expression[83]. Aoki *et al*.[84] showed that dual blockade of S1P1 and S1P3 reversed S1P-mediated inhibition of basal U937 monocyte adhesion to endothelial cells *in vitro*, not by causing VCAM1 down-modulation, but rather by affecting α5β1 and αvβ3 integrin relocalization. Surprisingly, *in vitro* stimulation of HUVECs with S1P induced IL-8 and monocyte chemoattractant protein 1 release in an IL-1-dependent manner[85]. Although the data described above are scarce and sometimes contradictory, they suggest that S1P receptors, especially S1P1, might constitute feasible targets for therapeutic chemical modulation by interfering at multiple steps. For example, S1P receptor activation could lead to alleviation of endothelial barrier injury by decreasing the release of pro-inflammatory mediators and reducing activation of the endothelium (FIG. 5).

S1P and the modulation of the BBB

The BBB is the most stringent endothelial interface of the human body, and its dysregulation is associated with a range of devastating diseases with no satisfactory therapies. For example, the aetiology of multiple sclerosis is still incompletely understood but is known to involve BBB endothelium activation and disruption, as well as accumulation of autoreactive lymphocytes and macrophages in the CNS[86,87]. So far, there is no direct evidence that S1P receptors can regulate the BBB. recently, FTY720 was shown to ameliorate clinical scores during the relapsing phase in the EAE rodent model of multiple sclerosis[20,88], as well as in human clinical trials[19]. The effect of FTY720 is likely to result, at least in part, from protection of the BBB. Indeed, FTY720 was recently shown to reduce the expression of pro-inflammatory genes and metalloproteases, and to favour the expression of tissue inhibitors of metallo proteases[19] in the brain of EAE rats. The design of the study did not allow any direct modulation of S1P receptors to be determined. However, other studies have shown that, although FTY720 was protective in an EAE model in rats, an analogue that cannot be phosphorylated did not confer any protection[89], suggesting that phosphorylation of sphingosine analogues and subsequent activation of S1P receptors are required. moreover, FTY720 and FTY720-P levels are increased by more than tenfold in the CNS when compared with blood content[90], and both species were present in blood at an approximate 1:2 FTY720 to FTY720-P ratio. S1P receptors are widely and differentially expressed on various cells of the CNS (reviewed in REF. 90), and were shown to contribute to astrocyte, oligodendrocyte and neuronal fate and functions (reviewed in REFS. 6, 86). S1P1 and S1P3 are expressed on BBB endothelial cells and astrocytes[86,91] that work cooperatively to coordinate exchanges between the blood and brain. Nonetheless, little is known regarding the regulation of the BBB by S1P receptors during EAE or in other models of CNS inflammation. By contrast, modulation of S1P receptors is better characterized in the periphery and could explain, at least partially, the beneficial effect of FTY720 in EAE models.

Administration of S1P1 receptor agonists can disrupt the chemokine–chemokine receptor system[92] and enhance inter-endothelial junctions in the lymphatic sinus of lymph nodes[93], leading to an accumulation of lymphocytes on the abluminal side of the sinus-lining endothelial cells and ultimately to impaired egress of lymphocytes to the efferent lymphatic vessels, causing blood lymphopaenia[9,39]. FTY720-P was shown to prevent the development of EAE in naive mice adoptively transferred with congenic T cells autoreactive for myelin[94] and to decrease activated lymphocyte accumulation in the CNS, which is likely to result from S1P1-dependent impairment of T-lymphocyte recirculation[94,95] (FIG. 6).

In addition to protecting the BBB, modulating cell fate in the CNS and influencing lymphocyte recirculation, some evidence suggests that modulating the S1P pathway might allow direct manipulation of the BBB, extending therapeutic possibilities to other non-inflammatory diseases, such as brain cancer. For example, it was recently suggested that the disease-associated increase in hydrostatic pressure in the CNS might reduce the efficacy of drug delivery strategies that involve BBB disruption[96], which could explain the unsuccessful delivery of chemotherapeutic agents to the CNS in several such trials[97,98]. A combination of barrier-enhancing agents and small lipophilic molecules that readily cross the BBB or

strategies involving active drug transport might constitute a better approach to deliver drugs to the CNS[1,2,78]. For example, FTY720 accumulates in the CNS, where it is phosphorylated and can act on multiple S1P receptors and cellular targets. results obtained using immortalized rat brain endothelial cells showed that S1P functioned through S1P1 and S1P3 to upregulate the expression of ABCB1, a glycoprotein that is highly expressed in brain capillaries and can mediate efflux of xenobiotics from endothelial cells, ultimately protecting the CNS[91] (FIG. 6). Hypothetically, antagonism of S1P3 might favour not only an enhancement of the BBB, but also the accumulation of therapeutic compounds in the CNS. Clearly, sphingosine analogues and the S1P regulatory axis show therapeutic potential for diseases of the CNS, such as multiple sclerosis and even brain cancer. However, the regulation and regulatory activities of the S1P receptors in the specialized BBB endothelium remain poorly understood. moreover, adverse effects that are related to S1P receptor activation, such as bradycardia[99], arrhythmia[100] and dyspnoea, as well as non-mechanistic adverse effects, such as upper respiratory tract infections and liver enzyme elevations[21], have been associated with FTY720. Refining our knowledge of the minimal signalling requirement for each pathological process modulated by FTY720 is the next step towards the generation of drugs with higher specificity and fewer side effects.

Outlook

The discovery of FTY720 has facilitated new insights into the immunoregulatory system, and FTY720 can be viewed as a prototypic compound for further drug development. FTY720 has already led to the identification of other broad S1P receptor modulators, such as KRP-203 and AAL-R, and to the generation of structurally related and unrelated chemical probes, including monoselective S1P1 agonists, such as SEW2871, CYM-5442 and AUY954, and antagonists, such as W146 (REFS. 40,101–103) (TABLE 1).

In the quest to develop monoselective receptor modulators, numerous compounds are discarded because of their non-selective activities. Based on the opposing effects of the different S1P receptors in endothelial barrier regulation, these compounds — especially antagonists of similar potencies for different S1P receptors — would in fact be important proof-of-principle tools to address the contribution of S1P receptor interactions during disease. Indeed, S1P is present at high nanomolar concentrations in the plasma, which makes it difficult to determine whether the effect on outcome measures obtained after inhibition of a single receptor isoform results from intrinsic receptor inhibition or from relative alteration of S1P signalling tone between this receptor isoform and its opposing homologues. Availability of an array of monoselective antagonists, or of single molecules with multiple antagonistic activities on different S1P receptor subsets, could therefore be key to the study of this tonic receptor balance in different organs during homeostasis and disease.

In addition to the opposing activities of the various S1P receptors, the diversity of the receptor partners also has to beconsidered to develop clinically useful drugs. G protein-coupled receptors act as large signalling units and trigger numerous distinct signalling cascades. An increasing body of evidence suggests that different synthetic ligands can differentially affect receptor activity, for example by influencing their pairing with other

receptors[101,104], and S1P receptors seem to form distinct ‘signalplexes’ that vary according to the biological system studied. Genetic models need to be used to identify the involvement of specific receptors during disease and to determine cell-specific receptor repertoires and interactions, as exemplified by the recent identification of S1P3 on dendritic cells as a major exacerbating factor for mortality during sepsis[12]. After the identification of a disease-specific cellular target, mouse models with functional tagged receptors knocked in would allow cell-based proteomic analysis of physically interacting receptor partners during experimental disease. A similar approach has successfully been used to identify the content of lipid rafts after cell stimulation with S1P[45]. Using these findings to construct fluorescence–bioluminescence resonance energy transfer experiments or reporter-based assays, for example, along with the availability of public high-throughput screening facilities, would generate proof-of-concept molecules for the dissection of disease-specific receptor interactions and functions. Therefore, the design of assays for high-throughput screening should not be only based on feasibility, but should also be dictated by disease-specific signalling pathways of a single receptor isoform.

So far, the combined use of nonspecific and specific chemical probes supports the potential of S1P receptors as therapeutic modulators of endothelial barriers. A deeper understanding of the specific activities of S1P receptors in a cell- and disease-specific manner could facilitate the design of therapeutics that target S1P signalling for a range of conditions. Our experience suggests that specific-tool generation in both the pharmaceutical industry and academia, in combination with genetic approaches, is leading to the identification and validation of new points of chemical intervention that can modulate these complex pathophysiological interactions and ultimately benefit human health.

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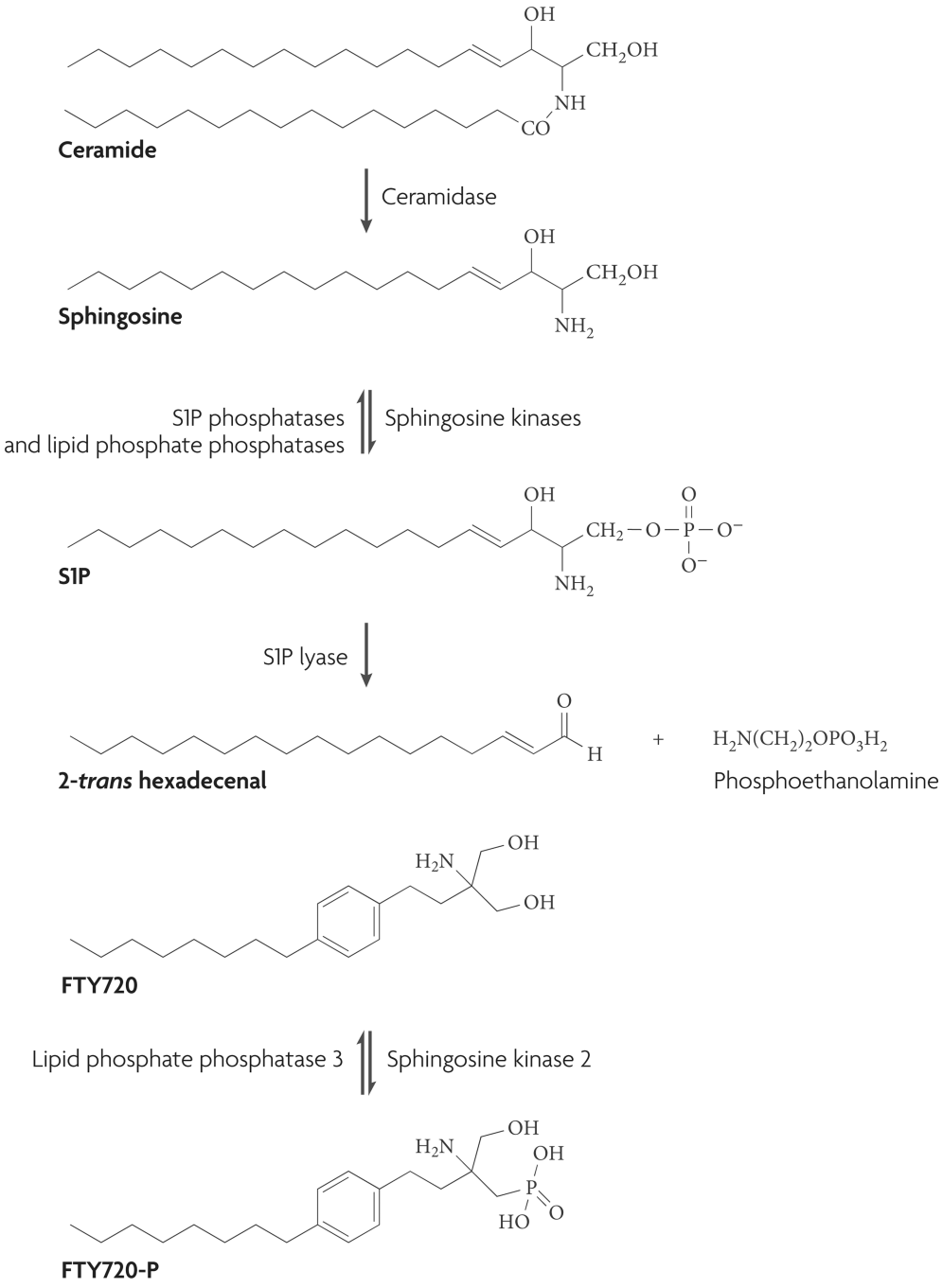


Figure 1. The sphingosine-1-phosphate (s1P) biosynthetic pathway

Degradation of ceramide to sphingosine by ceramidase and subsequent phosphorylation by sphingosine kinase 1 or sphingosine kinase 2 produces S1P. S1P can be reversibly degraded to sphingosine by S1P phosphatase and lipid phosphate phosphatases or irreversibly degraded by S1P lyase to 2-*trans* hexadecenal and phosphoethanolamine[4,22]. Notably, FTY720 and some of its analogues are preferentially phosphorylated by sphingosine kinase 2 (REF. 26) and were shown to be degraded by lipid phosphate phosphatase 3 *in vitro*[105].

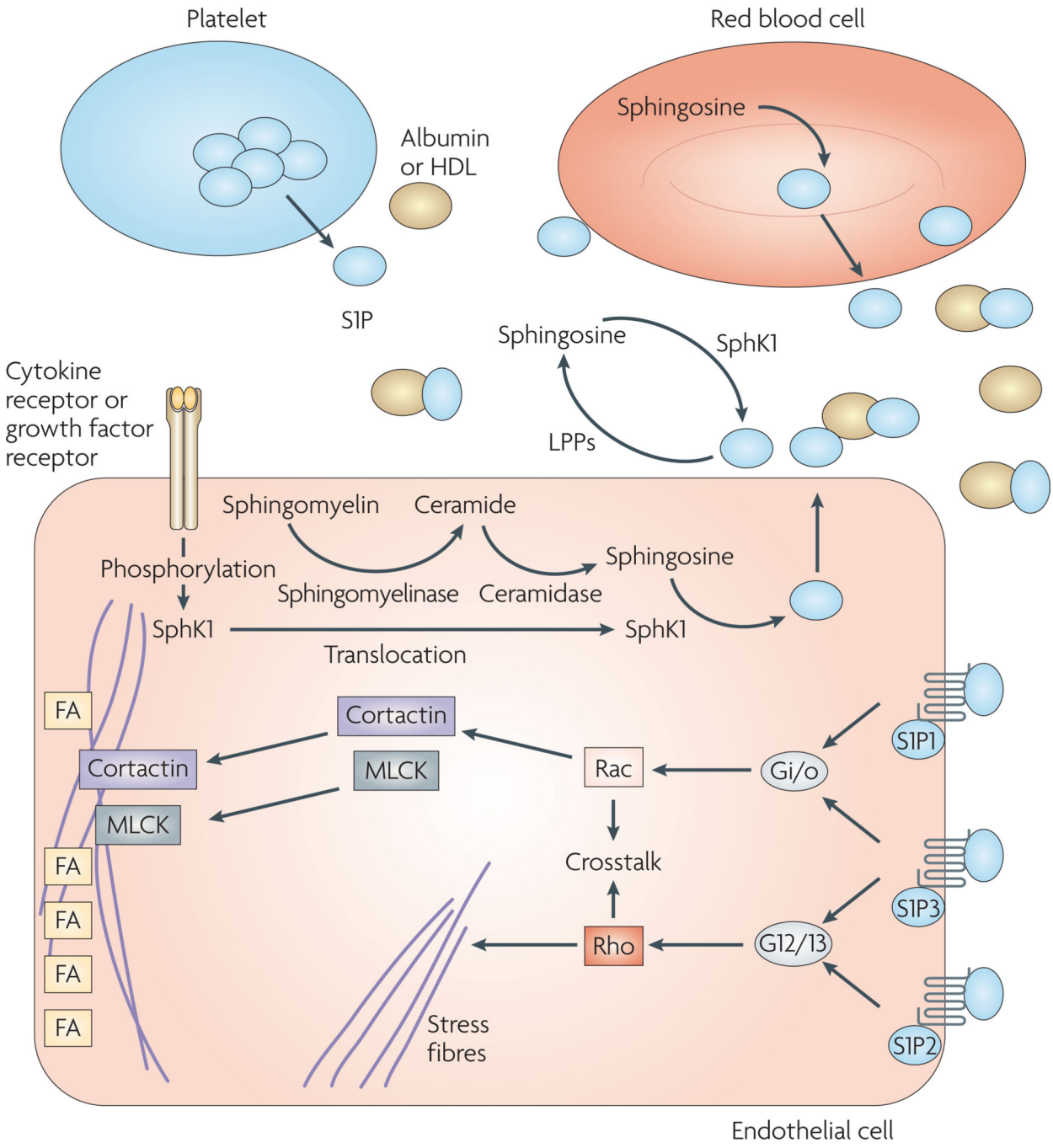


Figure 2. sphingosine-1-phosphate (s1P) receptor signalling

S1P is present at high nanomolar concentrations in plasma; 98.5% is bound to albumin and other transporters such as high density lipoproteins (HDLs)[29]. S1P is released to the blood from various sources. It was originally thought to be mainly produced by platelets[106], but recent evidence suggests that red blood cells are also a source of S1P in mice[30–32] and that secreted sphingosine kinase 1 (SphK1) could also help regulate plasma S1P levels[25]. S1P can act intracellularly (not shown) or be transported to the extracellular space by ATP-binding-cassette transporters[107]. Another S1P transporter was recently identified in the

zebrafish[108]. Once in the extracellular space, S1P is susceptible to degradation to sphingosine by different classes of lipid phosphate phosphatases (LPPs). In turn, sphingosine might also be phosphorylated by secreted SphK1 in the extracellular space. S1P1 and S1P3 are known to couple to Gi/o, which is associated with endothelial barrier enhancement. Signalling through Gi/o leads to Rac activation, which is coupled to the translocation of cortactin to the periphery of the cell, where it co-localizes with myosin light chain kinase (MLCK) and leads to the formation of the cortical polymerized actin ring. Moreover, cortactin is also thought to contribute to the organization of tight junctions. The cortical actin ring is stabilized by the assembly of focal adhesions (FAs) at the periphery of the cell, leading to tightening of the endothelial barrier. In an apposite manner, S1P3 and S1P2 couple to G12 and G13. G12 and G13 activation leads to Rho activation, stress fibre formation and disruption of the endothelial barrier. This figure was inspired by various working models[4,10,49,109].

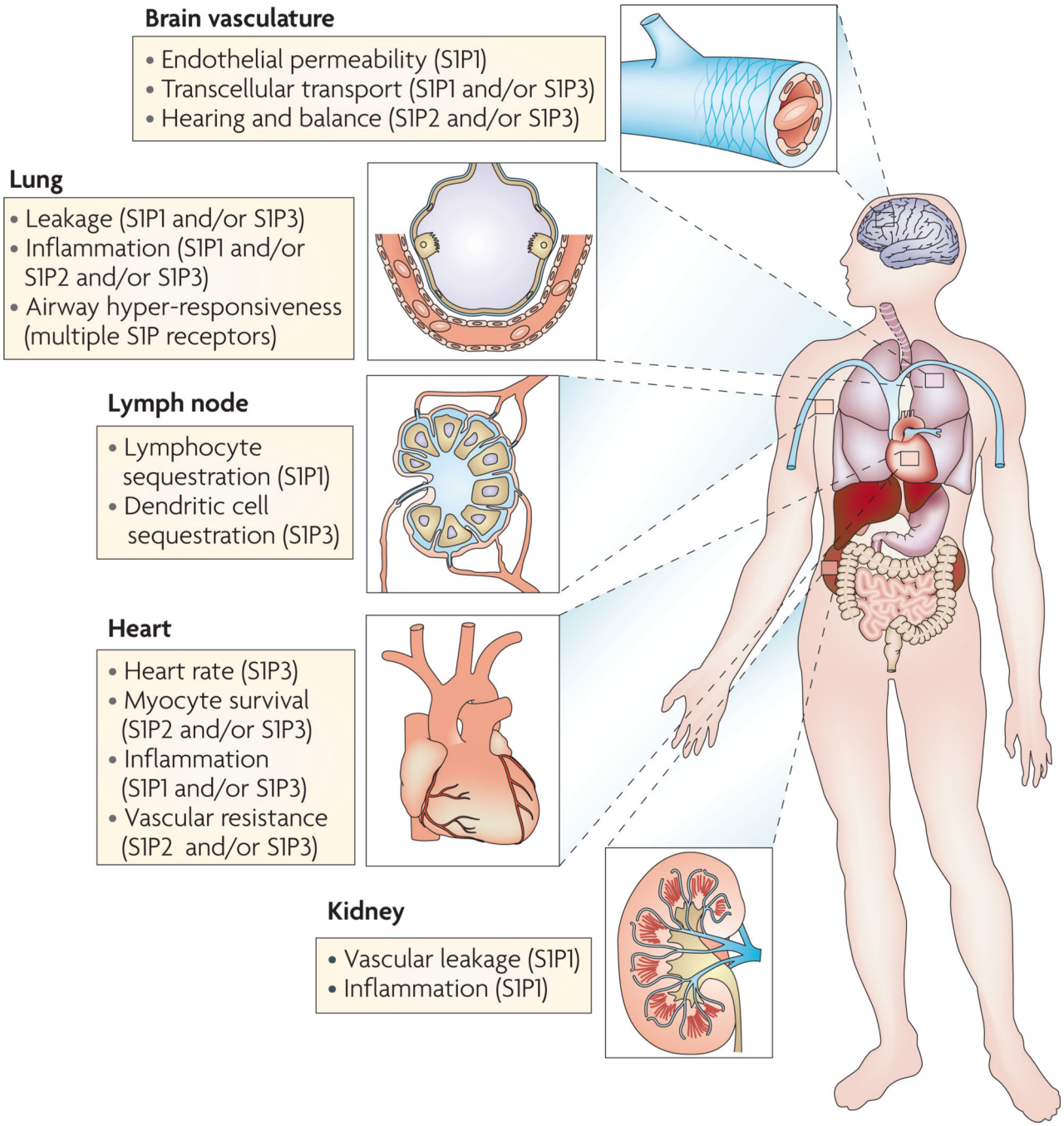


Figure 3. involvement of sphingosine-1-phosphate (s1P) receptors in the regulation of physiological and pathophysiological phenomena

S1P receptors are involved in the regulation of various physiological and pathophysiological phenomena, including hearing[36,37], vasodilation and vasoconstriction[110], heart rate[99], airway hyper-responsiveness[111] and lymphoid tissue function[9]. Accumulating literature supports the use of small molecules that target the S1P immunoregulatory pathway to modulate barrier activity in different organs. For example, S1P receptor activation was shown to favour pulmonary barrier integrity in models of acute lung injury and acute respiratory distress syndrome[10,11,48], and in the kidneys and myocardium after ischaemia–

reperfusion stresses[15,112], and to favour blood–brain barrier protection during experimental autoimmune encephalomyelitis[19]. Moreover, the S1P pathway was shown to enhance the lymphatic endothelial barrier integrity in lymph nodes, leading to sequestration of T cells in the lymph nodes[9].

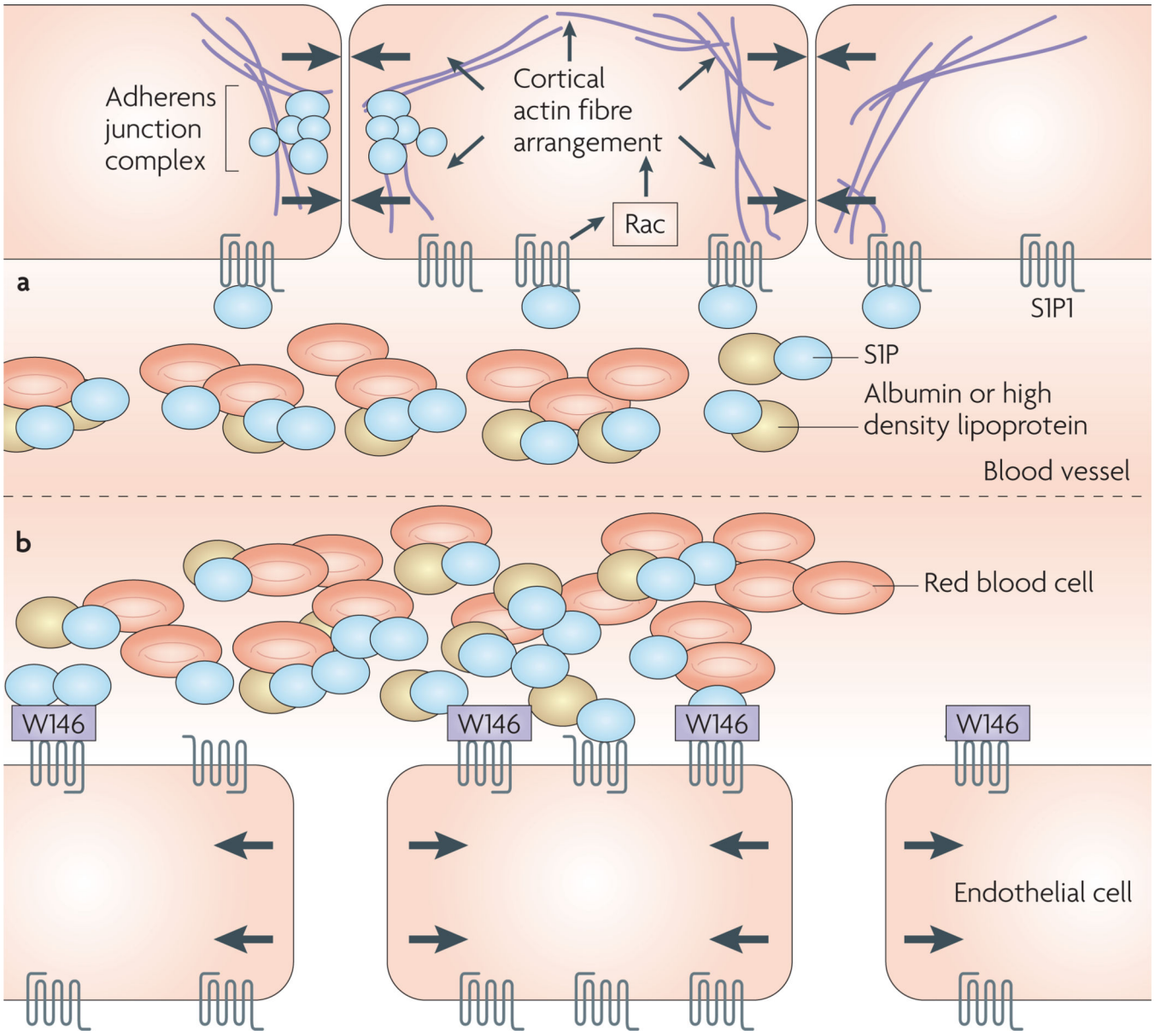


Figure 4. effects of sphingosine-1-phosphate (s1P)–s1P receptor 1 (s1P1) tone on the endothelium of blood vessels

a | During homeostasis, blood-borne S1P can activate S1P1, which induces Rac activation. This leads to the arrangement of the cortical actin ring and tightening of adherens junctions, favouring the maintenance of barrier integrity. **b** | The essential requirement of tonic S1P1 activation for the maintenance of endothelial barrier integrity can be shown by *in vivo* blockade of S1P1 by a chemical probe, such as W146, which leads to disruption of the endothelial barrier and plasma leakage into the interstitium.

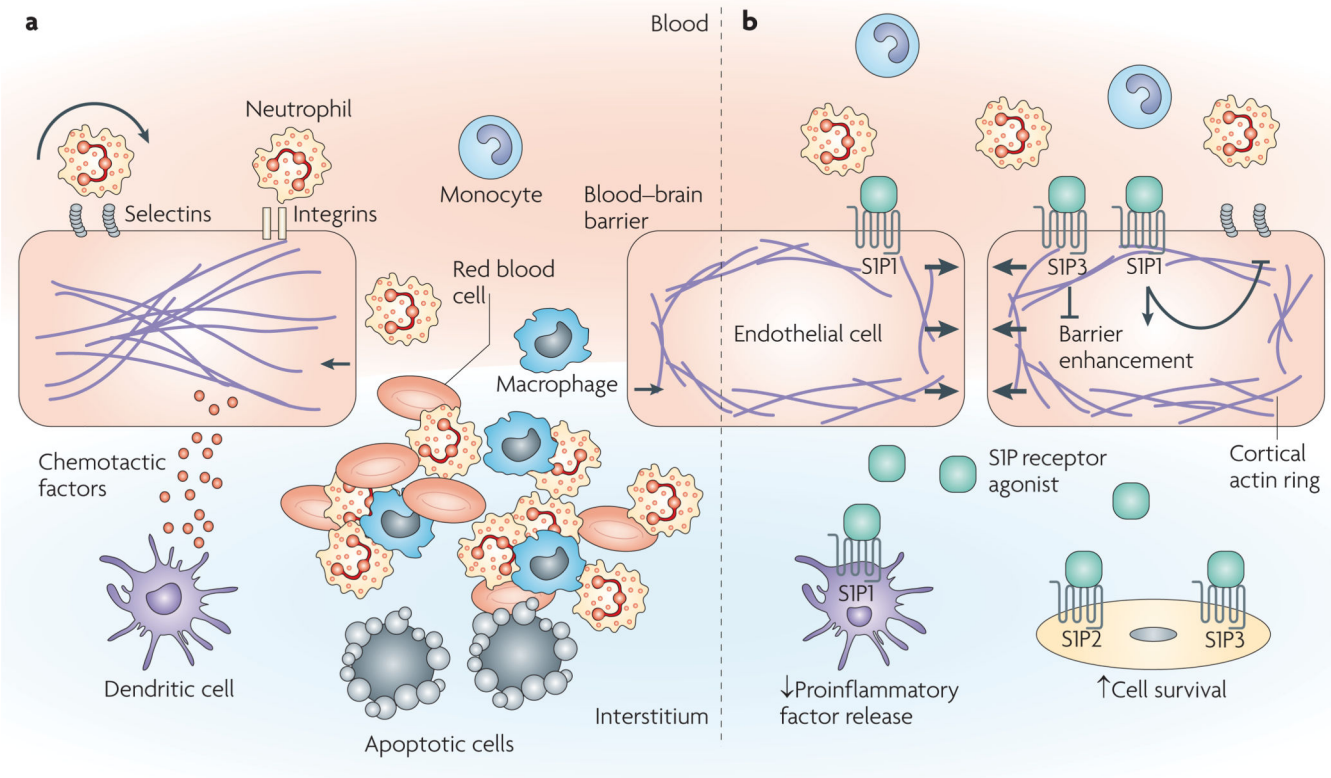


Figure 5. Targets for endothelial barrier protection during inflammation

a | After tissue insult, tissue-homing cells, such as macrophages or dendritic cells, release proinflammatory and chemotactic factors, which leads to activation of the endothelium. The endothelium expresses adhesion molecules, such as selectins and integrins, at its surface, thereby inducing tethering, rolling, firm adhesion and infiltration of leukocytes. Pro-inflammatory mediators, vasodilatory agents and infiltrating cells induce barrier disruption and injury, resulting in leakage of plasma into the interstitium and infiltration of inflammatory cells that can release toxic molecules and induce cell death. **b** | Sphingosine-1-phosphate (S1P) and sphingosine analogues were shown to impair the release of pro-inflammatory factors in various models, such as ischaemia–reperfusion injuries or viral infections[16,58,102]. Although S1P3 activation can disrupt barrier integrity, it seems that broad S1P receptor agonists, such as FTY720, favour barrier integrity. S1P1 activation was also shown to reduce expression of adhesion molecules, to impair interactions between leukocytes and the endothelium and to reduce inflammatory-cell infiltration[15,82–84,113]. Moreover, S1P2 and S1P3 activation might favour cell survival by activating the pro-survival Akt (also known as protein kinase B) pathway[14].

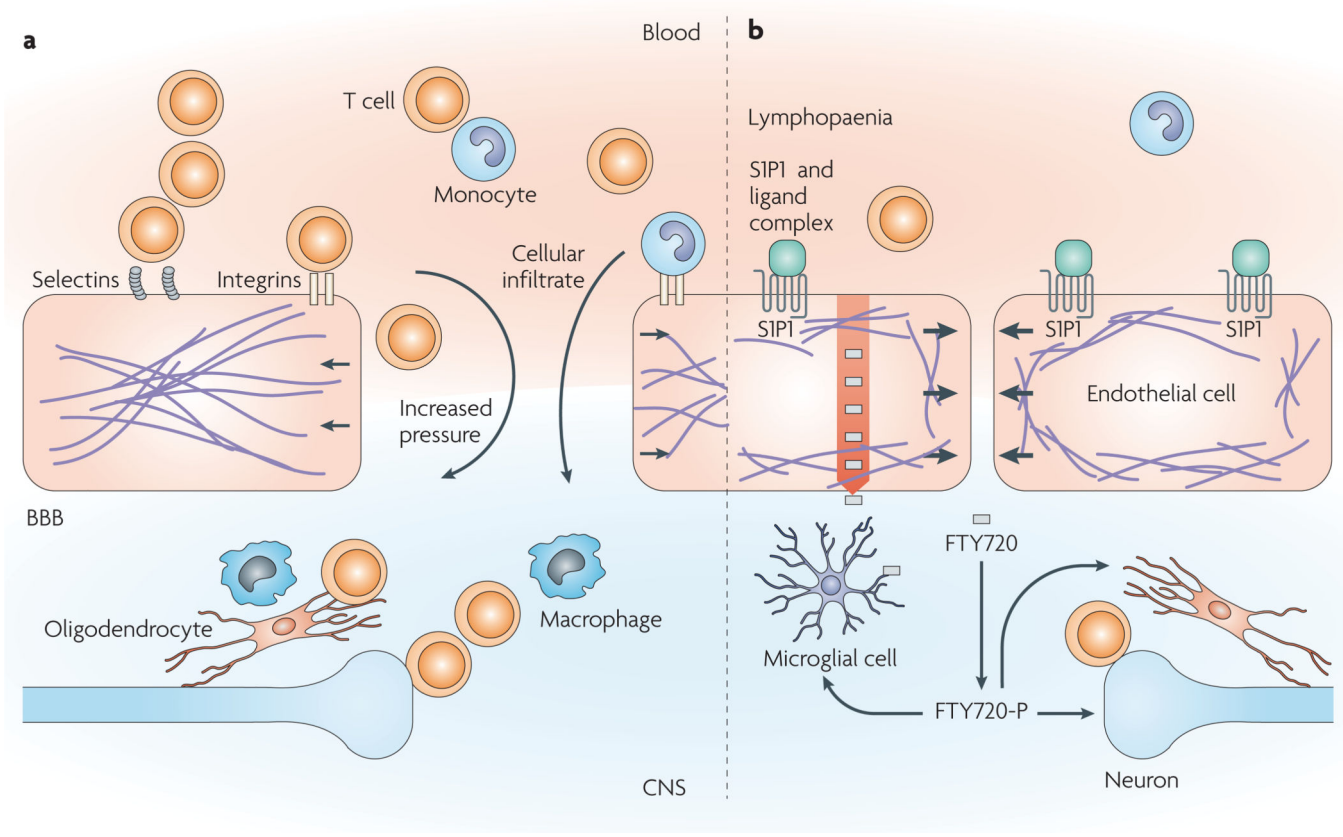
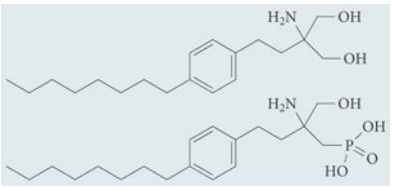
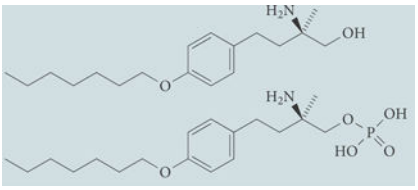
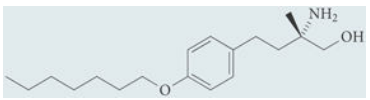
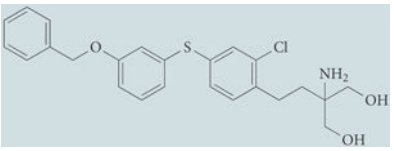
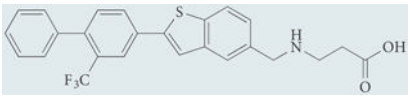
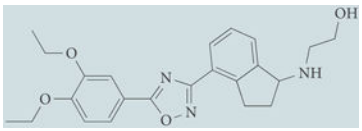
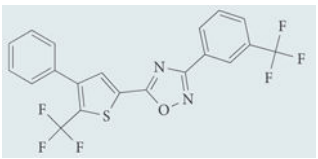
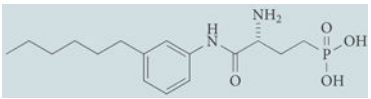


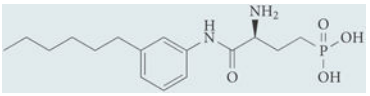
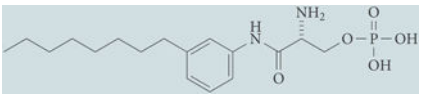
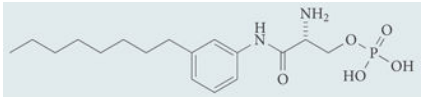
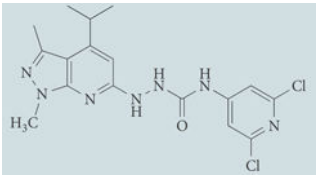
Figure 6. Putative strategies for modulating the immune response in the central nervous system (cNs)

a | The blood–brain barrier (BBB) is central to the pathogenesis of various CNS diseases, as it controls the passage of molecules and cells, and can be damaged in the course of disease. During autoimmune disease, autoreactive lymphocytes access the brain through the bloodstream. Like the endothelium of acutely injured tissues, the BBB expresses adhesion molecules and becomes disrupted, leading to accumulation of fluids, macrophages and autoreactive T cells in the CNS. **b** | The sphingosine-1-phosphate (S1P) analogue FTY720 (grey rectangles) and S1P receptor 1-specific agonists (shown in green) induce sequestration of lymphocytes in secondary lymphoid organs owing to lymphatic barrier tightening, which might decrease the capacity of these lymphocytes to reach the CNS[9]. In addition to reducing leukocyte infiltration, BBB enhancement with S1P1-specific agonists could lead to decreased fluid accumulation in the CNS, which is an aggravating factor in brain cancer and might interfere with drug delivery. Decreased accumulation of fluids and activated lymphocytes in the CNS could translate into alleviation of tissue damage. Blood-borne small, lipophilic molecules, such as FTY720, can accumulate in the CNS (indicated by a red arrow)[90]. FTY720 is then phosphorylated (FTY720-P) in the CNS, where it can act on widely distributed S1P receptors.

Table 1

Synthetic chemical modulators of sphingosine-1-phosphate (S1P) receptors

Compound	Activity and usage	Structure	Ref.
FTY720 (prodrug; phosphorylated to FTY720-P *in vivo*)	Agonist for S1P1, S1P3, S1P4,and S1P5; in Phase III clinical trials for multiple sclerosis		9
AAL-R (prodrug; phosphorylated to AFD-R *in vivo*)	Agonist for S1P1, S1P3, S1P4 and, S1P5; chemical probe*		89
AAL-S (chiral enantiomer of AAL-R that cannot be phosphorylated)	Inactive on S1P receptors; chemical probe*		89
KRP-203 (prodrug)	Agonist with greater affinity for S1P1 than S1P3 or S1P4; in Phase I clinical trials for multiple sclerosis		114
AUY954	Agonist for S1P1; chemical probe*		17
CYM-5442	Agonist for S1P1; chemical probe*		101
SEW2871	Agonist for S1P1; chemical probe*		99
W146	Antagonist for S1P1; chemical probe*		40

Compound	Activity and usage	Structure	Ref.
W140	Control enantiomer for W146; chemical probe*		40
VPC44116	Antagonist for S1P1 and S1P3; chemical probe*		41
VPC23019	Antagonist for S1P1 and S1P3; chemical probe*		115
JTE-013	Antagonist for S1P2; chemical probe*		116

*The term chemical probe describes compounds with usefulness for fundamental research that may not have potential for use in humans.