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The emerging alliance of sphingosine-1-phosphate signalling and immune cells: from basic mechanisms to implications in hypertension

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The immune system plays a considerable role in hypertension. In particular, T-lymphocytes are recognized as important players in its pathogenesis. Despite substantial experimental efforts, the molecular mechanisms underlying the nature of T-cell activation contributing to an onset of hypertension or disease perpetuation are still elusive. Amongst other cell types, lymphocytes express distinct profiles of GPCRs for sphingosine-1-phosphate (S1P) – a bioactive phospholipid that is involved in many critical cell processes and most importantly majorly regulates T-cell development, lymphocyte recirculation, tissue-homing patterns and chemotactic responses. Recent findings have revealed a key role for S1P chemotaxis and T-cell mobilization for the onset of experimental hypertension, and elevated circulating S1P levels have been linked to several inflammation-associated diseases including hypertension in patients. In this article, we review the recent progress towards understanding how S1P and its receptors regulate immune cell trafficking and function and its potential relevance for the pathophysiology of hypertension.

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Abbreviations

AngII, angiotensin II; APC, antigen-presenting cell; CCR2, chemokine receptor type 2; CD, cluster of differentiation; DCs, dendritic cells; M1, M1-polarized macrophages; M2, M2-polarized macrophages; PAH, pulmonary arterial hypertension; S1P, sphingosine-1-phosphate; S1PL, S1P lyase; SPHK, sphingosine kinase; T_{reg}, T regulatory

Sphingosine-1-phosphate biosynthesis and degradation

Sphingosine 1-phosphate (S1P) is a bioactive phospholipid with a complex metabolism typically orchestrated by a number of different enzymes. Two distinct kinases [sphingosine kinase 1 (**SPHK1**) and **SPHK2**] generate S1P from **sphingosine**, which itself is produced by degradation of ceramide (reviewed in Spiegel and Milstien, 2003). Both SPHK isoforms exhibit different catalytic properties, subcellular locations and tissue distribution. While SPHK1 is highly specific for sphingosine and dihydrosphingosine, SPHK2 is able to phosphorylate a broader range of substrates, including sphingosine, dihydrosphingosine, phytosphingosine and **fingolimod** (reviewed in Spiegel and Milstien, 2003). In contrast to SPHK2 that is mostly found in the nucleus, SPHK1 resides in the cytosol and translocates to the plasma membrane upon activation (Lidington *et al.*, 2009). Most importantly, studies have shown that a fraction of SPHK1 is released to the extracellular space (Ancellin *et al.*, 2002; Rigogliuso *et al.*, 2010), which enables the local production of S1P in the vicinity of its cell-surface receptors.

Due to its pleiotropic effects, S1P levels need to be tightly controlled. Besides a finely tuned generation, S1P degradation is mediated by three types of enzymes: S1P phosphatase (SPP), S1P lyase (**S1PL**) and lipid phosphate phosphohydrolase (PPA; also known as LPP). In most cells, S1P is irreversibly degraded by S1PL. SPPs (SPP1 and SPP2), which are highly selective for sphingoid base-1-phosphates, dephosphorylate S1P to sphingosine (reviewed in Spiegel and Milstien, 2003). However, due to their intracellular localization, the degradation of extracellular S1P by S1PL and SPPs requires an import mechanism. Several studies have suggested that proteins of the ABC transporter family facilitate this S1P uptake (Boujaoude *et al.*, 2001; Meissner *et al.*, 2012). The only S1P-catabolizing enzymes with the ability to degrade extracellular S1P belong to the PPA family, which shows broad substrate specificity. In contrast to **PPA3A**, **PPA2A** and **PPA2B** are mainly localized to the plasma membrane and have been shown to degrade extracellular lipid phosphate substrates (reviewed in Spiegel and Milstien, 2003).

Many cell types supply the circulating S1P pool. Amongst them, erythrocytes serve as one of the main S1P sources due to their ability to import sphingosine from the surrounding environment, their lack of S1P degradation enzymes and their constitutive release of S1P to maintain plasma S1P concentrations (reviewed in Thuy *et al.*, 2014). Recently, MFSD2B was characterized as an important S1P transporter in erythroid cells (Vu *et al.*, 2017; Kobayashi *et al.*, 2018). Similarly, platelets possess low levels of active S1PL and can therefore store large amounts of S1P. However, their contribution to physiological homeostasis is minimal since S1P release from platelets is dependent on very specific stimuli, which might support a role for platelet-mediated S1P release in disease. Like platelets, mast cells release S1P in response to stimuli including allergic reactions. Leukocytes on the other hand only play a minor role in S1P release due to the highly effective S1P-degrading system in these cells (reviewed in Ksiazek *et al.*, 2015). In addition to haematopoietic cells, cells of non-haematopoietic origins, such as endothelial cells and hepatocytes, contribute to maintaining homeostatic S1P levels. Endothelial cells provide approximately 40% of the total S1P plasma concentration through exporting S1P *via* spinster homologue transporter 2 (SPNS2) (Hisano *et al.*, 2012) and by constitutively releasing SPHK1 for extracellular S1P production (Ancellin *et al.*, 2002). S1P release from hepatocytes is still controversial; however, hepatocytes are the major source of S1P carrier apolipoprotein M, which may play a contributory role in maintaining physiological S1P levels (reviewed in Ksiazek *et al.*, 2015).

The tight regulation of S1P levels is vital for the maintenance of S1P gradients between different tissue compartments and thus of significant importance for immune cell trafficking. Because recent investigations suggested a deregulation of S1P levels and its signalling axis during disease, this review commends the importance of S1P signalling in immune cell trafficking and activation and their potential implications in hypertension.

The S1P signalling axis – a busy multitasker

S1P acts as intracellular second messenger as well as an extracellular receptor ligand. Although the intracellular targets of S1P remain largely elusive, a few interesting findings have suggested a role for intracellular S1P in cell-cycle progression, DNA synthesis and control of apoptosis (reviewed in Payne *et al.*, 2002). S1P's extrinsic functions are mediated *via* a family of five GPCRs (**S1P receptors 1–5**). S1P receptors are widely studied entities with important functions in a variety of cells and tissues. With the exception of **$S1P_4$** and **$S1P_5$** receptors, S1P receptors are essentially ubiquitously expressed. Their expression patterns vary in tissues and change during development and ageing. To date, the **$S1P_1$** receptor is the most studied S1P receptor due to its distinct effects in the vasculature and the immune system (Takabe *et al.*, 2008; Maceyka *et al.*, 2009). In the vasculature, endothelial $S1P_1$ receptor activation promotes relaxation of resistance arteries (Cantalupo *et al.*, 2017), increases barrier function or mediates the release of pro-inflammatory factors from storage granules (Jang *et al.*, 2009). In the immune system, the same receptor fulfils similarly important functions through modulation of lymphocyte trafficking and development (Tarrason *et al.*, 2011). S1P receptor signalling has furthermore revealed a significant involvement in vascular tone regulation through apparent vascular bed-specific pro-constrictive function of **$S1P_2$** and **$S1P_3$** receptors (Hoefer *et al.*, 2010; Yang *et al.*, 2012). Interestingly, both S1P receptors have also been associated with the regulation of myeloid cell migration and activation (Michaud *et al.*, 2010). The $S1P_3$ receptor is further implicated in the regulation of heart rate (Sanna *et al.*, 2004) and, similar to $S1P_2$ receptors, also in vascular permeability (Sanchez *et al.*, 2007). Different S1P receptor-specific effects are summarized in Figure 1.

Besides the important role of S1P receptors in the regulation of critical vascular and immune cell functions, the involvement of S1P generating enzymes has also been shown: SPHK1 activity modulates Ca^{2+} sensitivity and thus myogenic

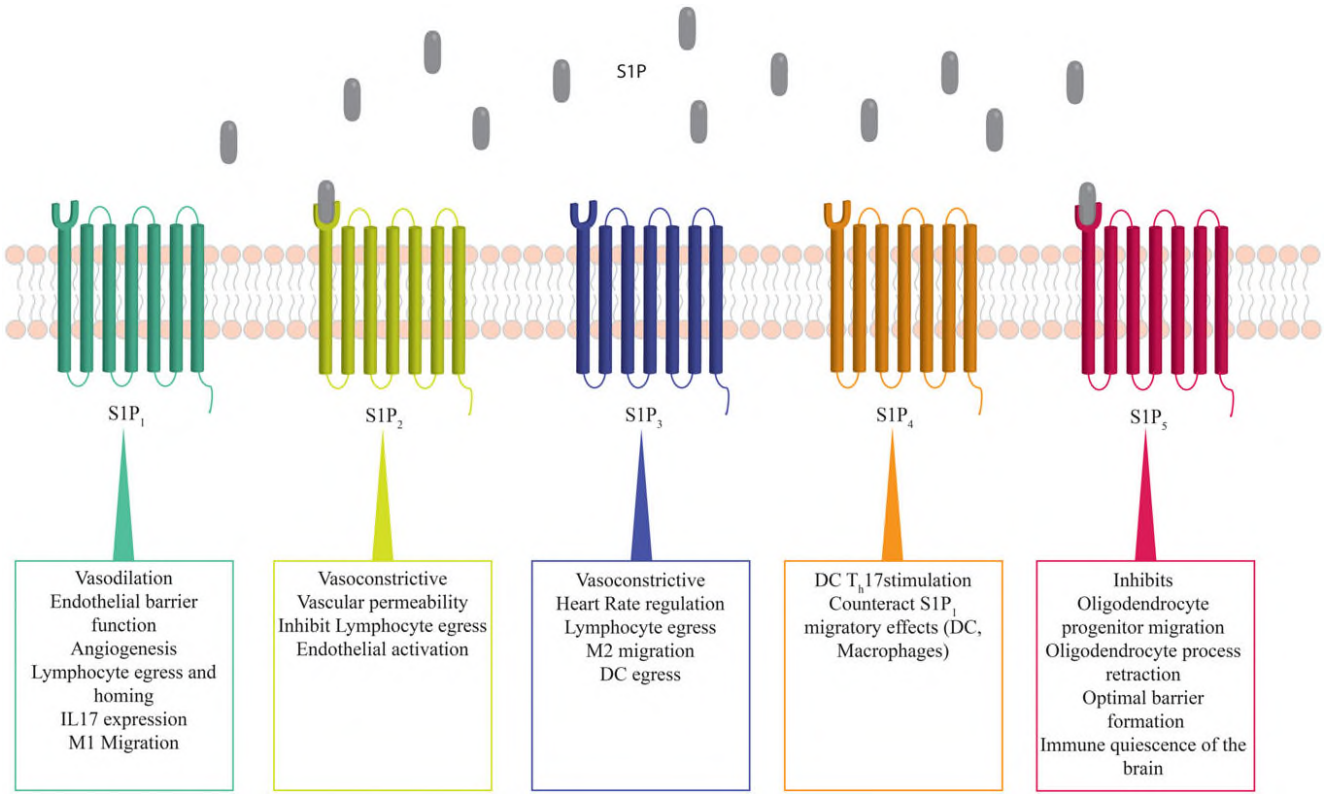

Figure 1

S1P mediates distinct effects *via* five GPCRs. Extracellular receptor binding facilitates receptor-specific and cell-specific effects of S1P. T_h17, T helper 17 cells.

responsiveness in resistance arteries (Lidington *et al.*, 2009), critically regulates vascular barrier integrity (Tauseef *et al.*, 2008) and participates in NF-κB activation (Hait *et al.*, 2009). SPHK2-mediated effects on the other hand are rather sparsely investigated. Thus far, SPHK2 has been shown to participate in the maintenance of the blood–brain barrier (Wacker *et al.*, 2012), histone acetylation and gene expression (Hait *et al.*, 2009) and the redistribution of blood-borne S1P into tissues (Sensken *et al.*, 2010).

S1P signalling in hypertension – a doubled-edged sword

Over the past few years, our understanding of S1P signalling in various cell types and tissues has steadily increased; yet the application of this knowledge to disease is still lagging behind. With respect to hypertension, the information regarding S1P's role in disease development and propagation is similarly sparse. Although several reports indicate enhanced sphingolipid metabolism and increased S1P tissue and plasma concentrations in different forms of experimental and human hypertension (Spijkers *et al.*, 2011; Chen *et al.*, 2014; Tabeling *et al.*, 2015; Zhao *et al.*, 2015; Gairhe *et al.*, 2016; Meissner *et al.*, 2017), the underlying molecular mechanisms are mostly elusive.

In line with that, the expression and activity of several key players of the S1P signalling axis seem to be critically associated with elevated BP levels in different rodent models. In particular, enhanced S1P production through SPHK1 has been linked to unfavourable prognosis in pulmonary arterial hypertension (PAH) in mice and men (Chen *et al.*, 2014). Genetic depletion of SPHK1 protected from the development of experimental PAH (Chen *et al.*, 2014), reduced S1P levels and attenuated the occlusive pulmonary arteriopathy associated with PAH (Gairhe *et al.*, 2016). Interestingly, genetic depletion of the S1P's catabolizing enzyme S1PL promoted hypoxia-induced hypertension in mice (Chen *et al.*, 2014), pointing towards an important involvement of the S1P signalling axis in PAH. However, exact mechanistic insights are still to be established. Transcriptome profiling recently identified SPHK1 as one of the key modulators of **angiotensin II** (AngII)-induced hypertension (Siedlinski *et al.*, 2017). This notion has been strengthened in experimental studies that show blunted BP responses to AngII in mice deficient in SPHK1 (Meissner *et al.*, 2017; Siedlinski *et al.*, 2017). Moreover, genetic network-based analyses established a central role for components of the ceramide-S1P rheostat in BP regulation and hypertension (Fenger *et al.*, 2011; Fenger *et al.*, 2015).

Beside S1P production and degradation, S1P receptor activation has been intensively studied with respect to its involvement in the regulation of vascular tone and BP. Several studies showed apparent smooth muscle-specific effects of $S1P_2$ and $S1P_3$ receptor-associated signalling (Hoefer *et al.*, 2010; Yang *et al.*, 2012; Cantalupo *et al.*, 2017) where specific

$S1P_2$ receptor-goverened activation of myosin light chain kinase (**MLCK**) leads to enhancements of myogenic tone in small resistance arteries (Hoefer *et al*., 2010) and is associated with reduced tissue perfusion (Yang *et al*., 2012). Cantalupo *et al*. (2017) assigned $S1P_3$ receptors a prevalent role in vascular smooth muscle-mediated tone regulation in response to S1P and intraluminal pressure without apparent effects on BP. In the same investigation, the group described an endothelial-specific offset mechanism to S1P's vasopressor-mediated signalling through $S1P_{2/3}$ receptor-**RhoA** or $S1P_{2/3}$ receptor-MLCK: endothelial S1P-$S1P_1$ receptor-**NO** signalling regulates vascular relaxation in response to flow and hence BP under physiological conditions and in hypertension (Cantalupo *et al*., 2017). Earlier, the same group identified the endoplasmic reticulum membrane protein **reticulon** (Nogo-B) as an important negative regulator of endothelial sphingolipid biosynthesis that critically affects vascular function and BP through an autocrine $S1P_1$ receptor-NO signalling loop (Cantalupo *et al*., 2015). Recently, an engineered S1P chaperone (apolipoprotein M-Fc) was shown to selectively activate the same S1P receptor in the endothelium and thereby attenuated hypertension (Swendeman *et al*., 2017). Besides its vascular-specific effects, $S1P_1$ receptor surface expression on T-cells critically modulates their migration along the S1P gradient (Matloubian *et al*., 2004) between tissue and blood and is responsible for lymphocyte egress and homing (Rivera *et al*., 2008). Recent findings also revealed a key role for SPHK2-driven S1P chemotaxis and T-cell mobilization for the onset of AngII-induced hypertension in mice (Meissner *et al*., 2017).

S1P – conductor in immune cell trafficking

Chemotaxis represents a critical mechanism for proper immune responses, which is influenced by a number of signalling molecules. Amongst them, S1P gradients play a major role in determining egress and homing of immune cells (Rivera *et al*., 2008). To date, the exact molecular mechanisms underlying tissue-specific S1P concentrations and thus the development of distinct S1P gradients are not very well understood. However, the regulation of the S1P gradient seems to involve a specific spatial contribution of different S1P signalling components: while SPHKs synthesize S1P in lymphatic endothelium (Pham *et al*., 2010), in platelets (Fukuda *et al*., 2003; Urtz *et al*., 2015) and red blood cells (Ochi *et al*., 2004), S1P-catabolizing enzymes such as S1PL, SPPs and PPAs control S1P levels within the primary and secondary lymphoid organs (Schwab *et al*., 2005; Breart *et al*., 2011; Park *et al*., 2014). In the thymus, the PPA2B-mediated regulation of S1P levels critically controls the S1P gradient and thus thymic egress (Breart *et al*., 2011). Furthermore, the involvement of sphingolipid transporters (SPNS) in the maintenance of S1P gradients has been reported. In particular, SPNS2 was shown to mediate the release of S1P from the vascular endothelium rather than platelets or red blood cells (Fukuhara *et al*., 2012; Hisano *et al*., 2012). Mice deficient in SPNS2 exhibit reduced S1P plasma levels and lymphopaenia, suggesting a vital role for SPNS in lymphocyte egress from the thymus (Fukuhara *et al*., 2012; Hisano *et al*., 2012). Hisano *et al*. (2012) furthermore conclude that SPNS2 deficiency has no effect on secondary lymphoid organ S1P concentrations. However, in the lymph of these animals, S1P levels are severely affected by SPNS2 depletion with implications for lymphocyte egress from lymph nodes and hence the number of circulating lymphocytes (Mendoza *et al*., 2012). The same group recently described a critical role for S1P, secreted from lymphatic endothelial cells by a SPNS2-dependent mechanism, in $S1P_1$ receptor-mediated naïve T-cell viability (Mendoza *et al*., 2017).

Additionally, different S1P chaperones are thought to deliver different biological functions of S1P: unlike albumin-bound S1P, S1P bound to apolipoprotein M restrains lymphopoiesis in the bone marrow and suppresses lymphocyte progenitor generation *via* $S1P_1$ receptor activation (Blaho *et al*., 2015).

Of significance to S1P-governed chemotaxis is the expression of specific S1P receptors on immune cells. Different subtypes of immune cells rely on different S1P receptors to migrate towards higher S1P concentrations. In the innate immune system, a reliance on all of the S1P receptors besides $S1P_5$ has previously been demonstrated (Maeda *et al*., 2007; Schulze *et al*., 2011; Awojoodu *et al*., 2013; Weichand *et al*., 2013), while cells of the adaptive immune system require mostly $S1P_1$ receptors to migrate along the S1P gradient (reviewed in Park and Im, 2017). T-cell trafficking along the S1P gradient has been of significant interest to scientists and the pharmaceutical industry. The ability of T-cells to migrate towards S1P has been successfully exploited for clinical applications in multiple sclerosis therapy (**fingolimod**). Fingolimod induces lymphopaenia *via* agonistic activation and subsequent internalization of $S1P_1$ receptors on lymphocytes. A deficiency in $S1P_1$ receptor surface expression blocks lymphocytes egress from secondary lymphoid organs along the S1P gradient (reviewed in Park and Im, 2017). Most T-cells down-regulate their surface $S1P_1$ receptor S1PR1 expression upon activation and hence lose their chemotactic response to S1P. This in turn can increase their responsiveness to chemokines responsible for T-cell homing to the spleen or lymph node. In line with that, a permanent down-regulation of $S1P_1$ receptors is required for the establishment of tissue-resident memory T-cells (Skon *et al*., 2013).

S1P-governed trafficking is somewhat more complex for cells of the myeloid lineage [dendritic cells (DCs) and monocytes/macrophages] as they are typically tissue residents and only upon activation do they migrate into the circulation.Specifically, their responsiveness to S1P is regulated by activation-associated S1P receptor expression. Prior to maturation, DCs mainly express $S1P_2$ receptors, which is thought to counteract the pro-migratory role of $S1P_1$ receptors. During maturation, the expression of $S1P_1$ and $S1P_3$ receptors is stimulated, thus enabling DC egress into the lymphatic system (Czeloth *et al*., 2005). Likewise, macrophages require specific S1P receptor expression patterns to facilitate S1P-guided migration to and from sites of inflammation (Weichand *et al*., 2013).

Despite the recent advancements in the field, further research is needed to underpin the current concepts and to understand the molecular regulators of pathogenic S1P shifts

and S1P-governed immune cell trafficking. In hypertension research, evidence has accumulated showing elevated levels of circulating and tissue-specific S1P (Spijkers *et al.*, 2011; Chen *et al.*, 2014; Tabeling *et al.*, 2015; Zhao *et al.*, 2015; Gairhe *et al.*, 2016; Meissner *et al.*, 2017), which posits S1P chemotaxis as a potential link between inflammation and high BP.

S1P signalling in adaptive and innate immune responses

Adaptive immunity

Aberrant activation of adaptive immunity appears to play a key role in the pathogenesis of hypertension; conversely, its suppression has been shown to attenuate experimental and human hypertension. Despite numerous research efforts, the exact mechanisms underlying the activation of adaptive immune responses during hypertension are still elusive. A growing body of evidence demonstrating S1P's apparent role in immune cell trafficking, T-cell differentiation and cytokine production positions S1P signalling as an important player in the control of immune responses that might well be critical in the pathogenesis of hypertension. The following section describes S1P involvement in adaptive immune responses of relevance to hypertension.

T-lymphocytes. In various experimental models, T-cell mobilization and activation crucially promote the development of sustained hypertension in a feedforward fashion (Marvar *et al.*, 2010; Meissner *et al.*, 2017). T-cell invasion in critical end-organ tissues in response to hypertension was shown in various rodent models as well as humanized mouse models (Guzik *et al.*, 2007; Itani *et al.*, 2016; Meissner *et al.*, 2017). A major determinant for T-cell mobilization is the existing S1P gradient between tissue and blood that drives lymphocyte egress and homing (Rivera *et al.*, 2008) and is governed by $S1P_1$ receptor surface expression on T-cells (Matloubian *et al.*, 2004). This gradient is devoid in conditional-knockout mice that lack both S1P-generating enzymes; consequently, T-cell exit from lymphoid organs is absent in these mice (Pappu *et al.*, 2007). Recently, our group showed that haematopoietic SPHK2 activity majorly contributes to a greater S1P gradient between blood and lymphoid tissue in AngII-induced hypertension (Meissner *et al.*, 2017). Systemic as well as haematopoietic depletion of SPHK2 protected from AngII-induced elevation of BP by preventing increases in plasma S1P and thus diminishing S1P-driven T-cell migration into the blood stream. At the same time, blocking the development of a sufficient S1P gradient responsible for the augmented T-cell egress in response to AngII led to the accumulation of T-cells in lymphoid tissue (concept is illustrated in Figure 2). Our data furthermore support the possibility that chronic AngII treatment not only enhances T-cell mobilization from secondary lymphoid organs but also regulates T-cell trafficking from blood to the lymphatic tissue (Meissner *et al.*, 2017). Although its involvement in BP regulation has not been demonstrated yet, the inhibition of S1P's catabolizing enzyme S1PL results in an increase in thymus, spleen and lymph node S1P concentrations, thus causing a loss of circulating lymphocytes (Schwab *et al.*, 2005; Vogel *et al.*, 2009; Weiler *et al.*, 2014). Interestingly, interfering with S1P catabolism showed no effect on plasma S1P concentrations (Schwab *et al.*, 2005; Harris *et al.*, 2016). As previously mentioned, the spatial contribution of different S1P signalling components to creating S1P gradients is highly dependent on tissue and cell type. Although a growing body of publications has generated initial basic mechanistic insight into this complex system with respect to immune cell trafficking (Schwab *et al.*, 2005; Breart *et al.*, 2011; Fukuhara *et al.*, 2012; Hisano *et al.*, 2012; Mendoza *et al.*, 2012; Harris *et al.*, 2016), hypertension-

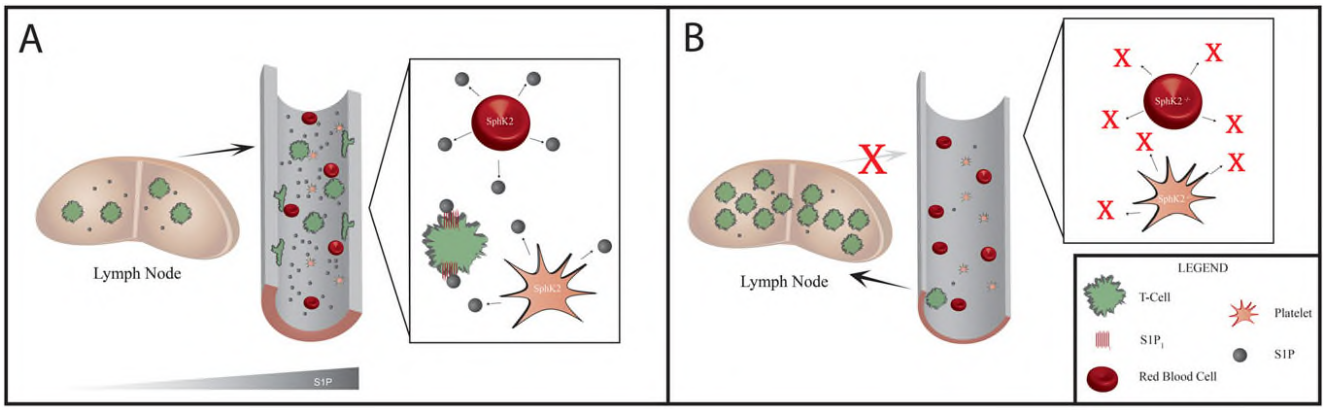

Figure 2

SPHK2-mediated T-cell trafficking in AngII-induced hypertension according to Meissner *et al.* (2017). (A) The SPHK2-mediated increase in plasma S1P levels governs T-cell mobilization from secondary lymphoid tissue in AngII-induced hypertension. S1P further facilitates endothelial activation and the expression of molecules necessary for T-cell adhesion. The resulting infiltration and accumulation of T-cells within the vascular wall critically alters vascular structure and function. (B) The inhibition of SPHK2 in haematopoietic cells prevents the AngII-induced S1P generation. The lack of S1P response flattens the S1P gradient and hinders T-cells egress into the blood in response to AngII. Subsequently, T-cells accumulate within the secondary lymphoid tissue.

associated shifts in compartment-specific S1P levels and associated immune cell function still need to be elucidated.

The synthetic S1P receptor modulator fingolimod circumvents the S1P gradient by antagonizing $S1P_1$ receptors on T-lymphocytes. By triggering the internalization of the S1P sensor in T-cells, fingolimod inhibits T-cell egress from lymphoid tissue and the recruitment of T-cells to sites of local inflammation (Mandala *et al.*, 2002). Fingolimod has proven efficacy in preventing the onset of experimental hypertension; however, its administration at times of already established hypertension failed to reduce BP levels despite inducing profound lymphopaenia (Meissner *et al.*, 2017). This may be due to its inability to reduce the number of memory T-cells (Hofmann *et al.*, 2006) or to its disadvantageous effects on endothelial NO production (Cantalupo *et al.*, 2017) with deleterious consequences for tissue inflammation and vascular function, respectively. The loss of $S1P_1$ receptor surface expression on mature lymphocytes has also been described after inhibition of S1PL using the food colourant 2-acetyl-4-tetrahydroxylbutylimidazole (**THI**). This might result from the elevation of S1P concentration in lymphatic organs observed after THI-induced S1PL inhibition (Schwab *et al.*, 2005). Together, this might account for the defective lymphocyte egress from lymphoid organs after THI treatment (Schwab *et al.*, 2005).

With respect to hypertension, T-cells are the most studied immune cells. Still, most underlying mechanisms contributing to disease onset or progression remain elusive. Emerging evidence supporting a critical involvement of S1P signalling in T-cell homeostasis encourages further research to achieve satisfying answers to burning open questions. In particular, future research needs to address the nature of S1P gradients between lymphoid and non-lymphoid organs and how they change during hypertension, or how T-cell-specific S1P receptor activity promotes T-cell viability, egress and tissue retention. In line with this, wherther the modulation of $S1P_1$ receptors impacts immunity either directly through signalling pathways or secondary to trafficking-dependent effects needs to be determined.

Th17 cells. In hypertensive patients, the increased percentage of circulating T-cells has been linked to the production of large amounts of pro-inflammatory cytokines (Youn *et al.*, 2013; Itani *et al.*, 2016). Elevated circulating **IL-17A** producing $CD4^+$ T-cells as observed in hypertensive patients (Itani *et al.*, 2016) might critically contribute to the exacerbation of tissue damage and disease progression. In murine models, genetic deletion of IL-17A as well as the administration of monoclonal antibodies to IL-17A has been shown to protect from hypertension (Madhur *et al.*, 2010), while the infusion of recombinant IL-17A elevated BP (Nguyen *et al.*, 2013).

Remarkably, S1P is suggested to be involved in augmented Th17 cell development, migration and IL-17 generation (Liao *et al.*, 2007; Garris *et al.*, 2013; Eken *et al.*, 2017). The mitigation of IL-17 generation mediated by the unspecific S1P receptor modulator fingolimod implicates substantial involvement of S1P receptor activation in these processes (Liao *et al.*, 2007; Maeda *et al.*, 2015). Experimental approaches specifically targeting $S1P_1$ receptors revealed an important role for this particular S1P receptor; $S1P_1$ receptor agonism significantly augmented IL-17 generation by $CD4^+$ T-cells (Liao *et al.*, 2007). A direct involvement of the $S1P_1$ receptor in IL-17 generation is furthermore supported by findings that show elevated IL-17 production in primary T-cell or antigen-presenting cell (APC) cultures isolated from mice expressing $S1P_1$ receptors with a phosphorylation deficiency in a residue that is crucial for receptor internalization (Garris *et al.*, 2013). The researchers concluded that impaired $S1P_1$ receptor phosphorylation and thus compromised $S1P_1$ receptor internalization enhances Th17 polarization and tissue infiltration, which might also bear significance in the pathogenesis of hypertension. Th17-specific $S1P_1$ receptor depletion indicated proliferation and differentiation defects as well as an involvement of the $S1P_1$ receptor in Th17 distribution (Eken *et al.*, 2017). The same report indicates an accumulation of IL-17A producing $CD4^+$ cells in lymph nodes after acute $S1P_1$ receptor deletion, suggesting the involvement of $S1P_1$ receptors not only in the generation and peripheral distribution of Th17 cells but also in Th17 cell homing to peripheral organs. Interestingly, the same group presented ongoing work where they used specific $S1P_1$ receptor agonism to inhibit human Th17 development from naïve T-cells and IL-17 production *ex vivo*. It remains to be determined whether blocking the generation of APC-derived cytokines is involved in Th17 polarization of T-cells, or direct effects on T-cells might be causative to this inhibition.

Although experimental evidence supports an important involvement of the S1P- $S1P_1$ receptor axis in Th17 development, polarization and migration and its contribution to tissue inflammation, the connection to Th17-driven immunity in hypertension is yet to be determined. Th17 priming is further complicated by evidence that beside S1Ps direct action, multiple immune cell subtypes, possibly *via* S1P, contribute to Th17 activation. An elucidation of the exact mechanisms involved in increased Th17 signalling in different models of hypertension is therefore needed. In this context, the specific elucidation of $S1P_1$ receptor-related signalling leading to Th17 priming would be of particular importance.

Regulatory T-cells. T regulatory (T_{reg}) cells represent an important immune suppressor subtype that is thought to be critical for the regulation of arterial BP. Decreases in T_{reg} populations have been associated with the pathogenesis of hypertension in several experimental models (Barhoumi *et al.*, 2011; Mian *et al.*, 2016). In line with this, adoptive transfer of T_{reg}s isolated from normotensive mice reduced AngII-induced BP levels (Barhoumi *et al.*, 2011). Recently, an elegant study showed that forkhead box P3 (FOXP3)-deficient T_{reg}s exacerbates AngII-induced hypertension by enhancing innate and adaptive immune responses (Mian *et al.*, 2016).

The role of S1P and specifically that of $S1P_1$ receptor activation seems most controversial in this specific T-cell subset. Differentiation of pro-inflammatory Th1 cells and anti-inflammatory T_{reg} cells was reported to be reciprocally regulated by $S1P_1$ receptor signalling (Liu *et al.*, 2010). While deletion of $S1P_1$ receptors in T-cells using a $CD4^{cre}$ system significantly improved T_{reg} generation and function, $S1P_1$ receptor overexpression in $CD4^+$ T-cells reduced their differentiation into T_{reg} cells (Liu *et al.*, 2009). Interestingly, the T_{reg} cells found in $S1P_1$ receptor-deficient mice revealed an

activated phenotype and were more prone to apoptosis, thus converted to effector T_{reg} (Eken *et al*., 2017). Thus, $S1P_1$ receptors have been shown to not only regulate phenotypic diversity but also T_{reg} egress cells from lymphoid organs and subsequent non-lymphoid tissue distribution (Eken *et al*., 2017). These somewhat contradictory findings raise more questions than answers regarding the role of the S1P signalling axis during T_{reg}-mediated inflammatory responses, necessitating further investigation. Of particular interest in this context would be the elucidation of $S1P_1$ receptor-dependent T_{reg} cell generation in the thymus and its role in their distribution to secondary lymphoid organs. Moreover, potential $S1P_1$ receptor-mediated migratory properties of T_{reg} cells to non-lymphoid tissue might be of significance in the evaluation of treatment options involving the modulation of S1P receptors for hypertension and associated target organ damage.

B-lymphocytes. In both experimental and human hypertension, the elevation of several antibodies produced by B-cells has been reported (Chan *et al*., 2014; Chan *et al*., 2015). Because B-cell activation can be dependent or independent of T-cells, the absence of an AngII response in Rag1-deficient mice after adoptive B-cell transfer is not surprising (Guzik *et al*., 2007). Recently, experimental evidence revealed that a genetic deficiency of B-cell-activating factor receptors, or pharmacological depletion of B-cells, protects against the BP elevation and organ damage induced by AngII (Chan *et al*., 2015).

Similar to T-lymphocytes, B-cells migrate along the S1P gradient (Pereira *et al*., 2010). Like in T-cells, the $S1P_1$ receptor promotes marginal zone B-cell migration (Pereira *et al*., 2010) towards the bone marrow vascular compartment and the peripheral blood. Comparable with T-cells (Shiow *et al*., 2006), this $S1P_1$ receptor-mediated egress is inhibited by the expression of CD69 (*Sic et al*., 2014). In contrast to T-cells, the activation of $S1P_2$ and $S1P_4$ receptors in B-lymphocytes also reduces $S1P_1$ receptor-mediated B-cell migration (*Sic et al*., 2014). However, most interestingly and of potential significance for the pathology of hypertension is the finding that B-cells migrated towards elevated pulmonary S1P concentrations in the inflamed lung of S1P-treated mice and these S1P stimulated B-cells release **TNF-α** and **granzyme B** (Sorrentino *et al*., 2015).

Future research needs to address the open questions regarding the role of B-cells in hypertension and the potential involvement of S1P-governed B-cell distributions. Specifically, an increase of knowledge regarding a potential role of the S1P signalling axis in T-cell dependent B-cell activation might provide valuable insights into hypertension onset and maintenance.

Innate immunity

Several experimental and clinical studies have tackled the involvement of adaptive immunity in respect to the pathophysiology of hypertension; however, the role of innate immune responses during hypertension is not completely understood. Besides adaptive immune responses, innate immunity may be an important mediator of chronic inflammation in hypertension, which can be facilitated by DCs and monocytes/macrophages. During hypertension, the innate immune system contributes to inflammation either directly or indirectly by activating adaptive immune responses mediated mainly by T-lymphocytes. Several studies have identified an essential role for S1P and its receptors in a variety of innate immune responses.

Dendritic cells. DCs represent a major class of APCs that form a critical link between the adaptive and innate immune system. Although DC function in hypertension is not yet clearly understood, experimental evidence suggests a critical role for DCs in the stimulation of hypertension-associated inflammation, as the inhibition of DC maturation blunted BP responses to AngII (Vinh *et al*., 2010). Likewise, the transfer of mature DCs from hypertensive into normotensive mice was associated with increased activation of T-cells and hypertension (Kirabo *et al*., 2014).

Due to the relatively sparse knowledge regarding the role of DCs in the pathogenesis of hypertension, investigations exploring the involvement of DC-related S1P signalling in hypertension are lacking. However, various reports show a close connection between S1P signalling and DC chemotaxis, cytokine production and maturation, which may have implications in hypertension. Besides mediating DC trafficking along an S1P gradient, S1P receptor expression has been implicated in the regulation of DC maturation and cytokine production: DC-specific depletion of $S1P_4$ receptors critically reduced Th17 differentiation and shifted immune responses towards Th2-dominated responses (Schulze *et al*., 2011). Similarly, $S1P_1$-deficient and $S1P_3$-deficient DCs promoted a shift towards an anti-inflammatory environment and Th2/**IL-4** responses respectively (Bajwa *et al*., 2012). DC-specific $S1P_4$ receptor-dependent cytokine production appears to be most influential during chronic inflammation as it can promote for instance, Th17 differentiation of T-cells. This may render the $S1P_4$ receptor in DCs an attractive target in chronic inflammatory disease.

The modulation of S1P degradation has also been suggested to play a role in DC-associated inflammation. Specific genetic deletion of S1PL in DCs was linked to impaired T-cell egress from the thymus, pointing to a significant involvement of DCs in the generation of S1P gradients and thus the homeostatic regulation of thymic T-cell export (Zamora-Pineda *et al*., 2016).

DC dependence on S1P is still not clearly understood, and there remains some disagreement as to which S1P receptors are involved in pro-inflammatory versus anti-inflammatory effects. Nonetheless, DC-associated S1P signalling might present a promising target to blunting the immune response during hypertension. In particular, understanding the role of S1P-S1P receptor signalling in Th17 and Th2 polarization might prove important in hypertension-associated immune responses. The notion that DCs potentially contribute to the generation of S1P gradients could furthermore strengthen the link between innate and adaptive immune responses during the pathogenesis of hypertension.

Monocytes and macrophages. Monocytes and macrophages have been shown to play a role in various experimental models as well as in human hypertension (Chan *et al*., 2012; Moore *et al*., 2015; Nosalski *et al*., 2017). During hypertension, macrophages and their circulating precursor

monocytes preferentially accumulate in the vasculature, the heart and the kidneys (Chan *et al.*, 2012). The release of pro-inflammatory mediators such as **monocyte chemotactic protein 1 (MCP-1/CCL2)** , C-C chemokine receptor type 2 (**CCR2**) and free radicals by infiltrating macrophages drastically affects vascular function and augments hypertension. Respectively, leukocyte-selective CCR2 deficiency and pharmacological blockade of macrophage CCR2 receptors abolished AngII-associated inflammation, vascular remodelling and blunted BP responses (Chan *et al.*, 2012; Moore *et al.*, 2015).

With respect to monocyte/macrophage populations, the role of S1P has mainly been studied in the vascular endothelium where S1P-S1P receptor-mediated endothelial activation increases the adhesion of monocytes. Here, endothelial-specific S1P receptor modulation for instance $S1P_1$ receptor agonism critically reduced monocytes' interaction with the endothelial cell layer. Additionally, several investigations also confirmed a critical role for monocyte/macrophage-specific S1P receptor expression in the regulation of their migration and activation. In general, monocytes' migration is mainly mediated through $S1P_1$ and $S1P_3$ receptors (Yang *et al.*, 2015), while the $S1P_2$ receptor acts as a negative regulator of monocyte migration (Michaud *et al.*, 2010). Macrophage migration along the S1P gradient seems to be subtype-dependent with apparent S1P receptor specificity: while the anti-inflammatory M2 migration is driven by $S1P_3$ receptors (Awojoodu *et al.*, 2013), the migration of pro-inflammatory M1 is mediated *via* $S1P_1$ receptors (Weichand *et al.*, 2013). $S1P_1$ receptor, highly expressed on naïve macrophages, is decreased in both M1- and M2-polarized macrophages, while $S1P_4$ receptor expression is only reduced in M1-polarized cells. Because their migration potential was shown to be higher compared with M2 macrophages, it has been suggested that the ratio between $S1P_1$/$S1P_4$ receptors orchestrates macrophage migration (Muller *et al.*, 2017).

Controversial findings have painted a blurry picture of S1P receptor involvement in monocyte/macrophage activation and chemotaxis. While fingolimod treatment resulted in a reduction of circulating monocytes levels, the same modulator reduces monocyte activation and egress from secondary lymphoid tissue (Lewis *et al.*, 2013) and enhanced the recruitment of anti-inflammatory, pro-angiogenic monocytes (Awojoodu *et al.*, 2013). Further work is needed not only to elucidate the role of S1P in monocytes/macrophages activation and migration but also to dissect their contribution to the pathophysiology of hypertension. Specifically, elucidation of the involvement of S1P signalling in monocyte differentiation, cytokine and chemokine expression and the generation of ROS from activated monocytes/macrophages might help to generate novel insights to immune system activation in hypertension and associated target organ damage. Moreover, further *in vivo* experiments specifically targeting the different S1P receptors are required to corroborate the physiological relevance of the differential S1P profiles in macrophage populations in health and disease.

Inflammasome activation. Experimental evidence strongly implicates an involvement of inflammasome activation in the initiation or progression of chronic diseases, including hypertension (Krishnan *et al.*, 2016). Inflammasomes are multimeric protein complexes that orchestrate the cleavage of the pro-inflammatory **IL-1**β or **IL-18**. After sensing pathogen-associated molecular patterns or danger-associated molecular patterns, the inflammasome complex recruits pro-caspase 1, which after auto-cleavage converts inactive pro-ILs into their mature forms. Currently, the most studied inflammasome is the **NOD-like receptor family** pyrin domain-containing protein (**NLRP3**) complex. Blocking NLRP3 assembly protected from DOCA salt-induced hypertension (Krishnan *et al.*, 2016). Similarly, a NLRP3 deficiency prevents BP responses to AngII (Shirasuna *et al.*, 2015).

Due to the scarcity of mechanistic studies regarding the role of inflammasome activation during hypertension, not much is known about a potential involvement of S1P signalling in hypertension-related inflammasome activation. Nonetheless, exciting studies showing a NLRP3-dependent production of IL-1β in response to sphingosine and after SPHK1 activation in differentiated macrophages (Barbour *et al.*, 2017) suggest that S1P could also be an interesting target in hypertension-associated inflammasome activation. Particularly, macrophage surface expression of $S1P_1$ receptors evolves as a key mediator in NLRP3 activation and hence IL-1β generation: in $S1P_1$ receptor-deficient mice, NLRP3 expression and associated IL-1β production is significantly reduced in tumour-associated macrophages. Likewise, **caspase-1** inhibition resulted in a decrease of $S1P_1$ receptor surface expression (Barbour *et al.*, 2017). These exciting findings might stimulate further research to establishing a link between inflammasome activation and S1P signalling in hypertension.

Targeting S1P signalling in hypertension

The mounting evidence showing important immune-modulatory functions for S1P has spurred an interest in identifying pharmacological targets within its signalling axis. Currently, several selective S1P receptor modulators are being subjected to clinical trials, but only one rather unspecific modulator has been approved for clinically use: fingolimod reduces lymphocyte egress from secondary lymphoid organ, thymus and bone marrow and thus induces lymphopaenia. *In vivo*, fingolimod is phosphorylated by SPHK2 and modulates four out of five S1P receptors ($S1P_1$, $S1P_3$, $S1P_4$ and $S1P_5$). While acting mainly as an agonist, chronic fingolimod treatment leads to initial $S1P_1$ receptor internalization and eventual degradation of the receptor. In hypertension, fingolimod has been shown to protect from AngII-induced hypertension when preventatively administered but failed to reduce already established hypertension (Meissner *et al.*, 2017). Similarly, fingolimod exerted BP-lowering properties in an acute setting likely mediated through the activation of the $S1P_1$ receptor-NO pathway (Cantalupo *et al.*, 2015). Chronic fingolimod treatment, however, drastically exacerbated hypertension (Spijkers *et al.*, 2012; Cantalupo *et al.*, 2017; Meissner, 2017). Due to reported adverse side effects exerted by fingolimod, the focus has shifted towards developing more specific S1P receptor modulators. Similar to fingolimod, the specific $S1P_1$ agonist **SEW2871** showed efficacy in lowering circulating lymphocyte levels (Park and Im, 2017) and BP in an acute setting (Cantalupo *et al.*, 2015;

Cantalupo *et al.*, 2017). Besides $S1P_1$ receptors, emerging evidence suggests that the other four S1P receptors are similarly viable targets for modulating immune responses and BP. A large number of S1P receptor agonists and antagonists are currently being explored; an overview of their effects on immunity and BP is summarized in Table 1.

In addition to the S1P receptors, drugs targeting S1P generating enzymes have been developed and tested in various experimental models. SPHK1 inhibition using SLP7111228 showed an overall lowering of plasma S1P levels and lower occurrence of occlusive arteriopathy but failed to reduce BP in PAH (Gairhe *et al.*, 2016). While the SPHK1 inhibitor DMS failed to reduce elevated BP in a murine model of AngII-induced hypertension, the SPHK2-specific inhibitors **ABC294640** and K-145 diminished hypertension in these mice (Meissner *et al.*, 2017). Of note, the SPHK2-specific inhibitor ABC294640 has successfully undergone phase 1 clinical trials for treatment of solid tumours.

Recently, the food colourant THI was discovered to cause lymphopaenia due to its potent inhibitory effects on the

Table 1

Summary of drugs targeting the S1P pathway and their known effects in hypertension and on the immune system

Drug	Target (function)	Effect in hypertension	Effect on immune system
FTY720 (fingolimod)	$S1P_1$ R (agonist, functional antagonist)	Acute: reduction in BP Chronic: elevated BP	Induces lymphopaenia, phosphorylated by SPHK2, and leads to internalization and degradation of $S1P_1$ receptors
SEW2871	$S1P_1$ R (agonist)	Reduces BP	Induces lymphopaenia by $S1P_1$ R internalization
KRP-203	$S1P_1$ and $S1P_5$ R (agonist)	No effect on BP	Regulates lymphocyte homing, inhibits macrophage and $CD4^+$ T-cell migration, inhibits T-cell activation, decreases T-cell proliferation and IL-2 and IFN-γ production in splenocytes and reduces macrophage expression of activation markers
AUY954	$S1P_1$ R (agonist)	NT	Suppresses infiltration of T-cells, B-cell and macrophages in sciatic nerves in EAN
APD334	$S1P_1$ R (agonist)	NT	Lowers the number of absolute number of B-cells, NK cells and T-cells
BAF312	$S1P_1$ and $S1P_5$ R (agonist)	No effect on BP	Induces lymphopaenia and reduces T-cell and B-cell numbers in blood of healthy individuals in 4–6 h
VPC23153	$S1P_1$ and $S1P_4$ R (agonist)	Enhanced contraction of isolated pulmonary arteries	NT
NIBR-0213	$S1P_1$ R (antagonist)	NT	Reduces peripheral blood lymphocytes and induces expression of CD69
W146	$S1P_1$ R (antagonist)	Reduces vasodilatation	Induces lymphopaenia *via* internalization and degradation of the S1PR1 and decreases the number of $CD4^+$, $CD8^+$ and $CD19^+$ cells in the blood
JTE013	$S1P_2$ R (antagonist)	Decreased risk of HPH	Reduces pro-inflammatory cytokines *via* inhibition of CCL3 production
SKI2	SPHK1 and S1PR2 (inhibitor)	Prevented HPH	Blocks neutrophil-induced endothelial cell permeability
THI	S1P lyase inhibitor	NT	Induces lymphopaenia by disrupting the S1P gradient between lymphoid organs and blood
ABC294640	SPHK2 (inhibitor)	Reduces BP	Inhibits NF-κB, STAT3 and AKT activation
K145	SPHK2 (inhibitor)	Reduces BP	Shows efficacy against myeloma and lymphoma cell lines, leading to cell death
SLP7111228	SPHK1 (inhibitor)	Decreases plasma S1P and decreases occlusive lesions in PAH	Decreases level of S1P in histiocytic lymphoma cells
PF-543	SPHK1 (inhibitor)	Abolished AngII-induced mesenteric artery contraction	Increases $CD4^+$ T-cell, monocyte and macrophage in blood

R, receptor; **CCL3**, chemokine (C–C motif) ligand 3; EAN, experimental autoimmune neuritis; HPH, hypoxia-induced pulmonary hypertension; NT, not tested.

S1P-degrading enzyme S1PL (Schwab *et al.*, 2005). *In vitro* studies have had difficulties replicating this inhibitory effect, which spurred the interest in developing better-understood S1PL inhibitors. Specifically, findings that show the importance of S1PL for normal development suggest that long term targeting of S1PL may be toxic (Kumar *et al.*, 2017).

Due to S1P's pleiotropic effect and the proven involvement in immune cell regulation and vascular function, the S1P pathway presents a viable target for a multitude of diseases. But problems regarding specificity, toxicity and understanding of the mechanism of actions are hindering current drug development. Prior to successful exploitation of S1P signalling modulation in disease, a better understanding of the role of the S1P pathway in disease progression is needed.

Concluding remarks and future directions

Central to the exploitation of S1P signalling as an immune target in hypertension is the elucidation of how the diversity of S1P-mediated effects may be integrated into a coordinated immune response. The key to resolving this is an expansion of our mechanistic understanding about the actual role of specific immune cells in hypertension, how S1P levels control the function of various immune cells beyond trafficking and which of the diverse effects of S1P on the immune system are relevant to the pathogenesis of hypertension. Above all, further investigations concerning the role of S1P signalling in immune-cell differentiation might help to isolate more specific therapeutic targets.

Nomenclature of targets and ligands

Key protein targets and ligands in this article are hyperlinked to corresponding entries in http://www.guidetopharmacology.org, the common portal for data from the IUPHAR/BPS Guide to PHARMACOLOGY (Harding *et al.*, 2018), and are permanently archived in the Concise Guide to PHARMACOLOGY 2017/18 (Alexander *et al.*, 2017a,b,c).

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Conflict of interest

The authors declare no conflicts of interest.

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