Sphingosine 1–phosphate (S1P), a multifunctional lipid mediator that signals via the S1P family of G protein–coupled receptors (S1PR), regulates vascular maturation, permeability, and angiogenesis. In this study, we explored the role of S1P 2 receptor (S1P2R) in normal vascularization and hypoxia-triggered pathological angiogenesis of the mouse retina. S1P2R is strongly induced in ECs during hypoxic stress. When neonatal mice were subjected to ischemia-driven retinopathy, pathologic neovascularization in the vitreous chamber was suppressed in S1p2–/– mice concomitant with reduction in endothelial gaps and inflammatory cell infiltration. In addition, EC patterning and normal revascularization into the avascular zones of the retina were augmented. Reduced expression of the proinflammatory enzyme cyclooxygenase-2 (COX-2) and increased expression of eNOS were observed in the S1p2–/– mouse retina. S1P2R activation in ECs induced COX-2 expression and suppressed the expression of eNOS. These data identify the S1P2R-driven inflammatory process as an important molecular event in pathological retinal angiogenesis. We propose that antagonism of the S1P2R may be a novel therapeutic approach for the prevention and/or treatment of pathologic ocular neovascularization.
signaling by the S1P3 receptor as a novel target for the prevention and/or treatment of vision-threatening retinopathies.

Results

S1P3 receptor expression during ischemia-induced retinal angiogenesis. In order to evaluate whether S1P receptors play a role in retina neovascularization, we investigated the expression of S1P receptors in a mouse model of retinal ischemia (20). After pups and their nursing mothers had been exposed to 75% oxygen (hyperoxia) for 5 days (P7–P12), the capillary network of the central retina regressed (vascular obliteration). At P12, pups and their nursing mothers were returned back to room air (“hypoxia”). Resultant retinal ischemia initiated rapid vessel growth; however, pathologic angiogenesis occurred in the vitreous, reaching a maximum at P17 (Figure 1A).

The expression level of ubiquitously expressed S1P receptors, namely S1P1, S1P2, and S1P3, was measured by quantitative real-time RT-PCR assay (Figure 1B). Expression of all 3 receptors was detected at P12 before the onset of relative hypoxia. Interestingly, at P13 (24 hours of relative hypoxia) S1P3 mRNA level was increased 3-fold ($P < 0.035; n = 3$). The receptor expression increased further to 5-fold at P17 (5 days of relative hypoxia; $P < 0.035; n = 3$) at the growth phase of pathologic angiogenesis. However, mRNA levels of S1P1 and S1P2 receptors increased modestly during the course of relative hypoxia and returned to baseline levels by P17 (Figure 1B). As expected, ischemia enhanced retinal expression of VEGF mRNA by more than 2.5-fold ($P < 0.0015; n = 3$) and angiopoietin-2 (Ang-2) expression by 14-fold ($P < 0.015; n = 3$), which is consistent with previous reports that describe VEGF and Ang-2 as hypoxia-induced regulators of retinal angiogenesis (Figure 1C) (21, 22). In contrast, during the course of normal retina development (normoxia), S1P3 receptor expression sharply declined during the first week of vascular development (P5–P10) and remained at low levels (P15 and P28) (Supplemental Figure 1A; supplemental material available online with this article; doi:10.1172/JCI31123DS1).

Prompted by the observation that S1P3 mRNA expression is significantly increased during the course of relative hypoxia, we localized the cells that express this receptor in the retina. The S1P3 antibody detected an appropriately sized (~40-kDa) molecule in Western blot analysis of protein extracts of VSMCs and mouse embryonic fibroblasts that endogenously express S1P3 receptor, as well as of HEK293 cells transfected with the S1P3 receptor (Supplemental Figure 2). These observations suggest that the antibody is specific in the detection of S1P3 antigen. S1P3 receptor was detected by immunohistochemistry in retinal cross sections around the optic nerve area at P17. S1P3 staining exhibited vessel-like distribution in the ganglion cell layer (GCL; arrows) and in the inner nuclear layer (INL; arrowheads) of hypoxic retinas. However, there was no immunoreactivity in the avascular outer nuclear layer (Figure 1D). At a higher magnification, it was evident that S1P3 receptor is expressed in ECs of INL as well as in the primary vasculature of GCL, where S1P3 expression highlighted vascular tuft-like structures that abnormally sprout at the interface between vitreous and retina (Figure 1D). Although these data do not rule out nonvascular expression of S1P3, these observations suggest that S1P3 receptor expression during ischemia-induced retinal angiogenesis can be used as a novel target for the prevention and/or treatment of vision-threatening retinopathies.
is induced in ischemic retinal endothelium and underscore the possibility that its signaling in the endothelium is important in hypoxia-driven neovascularization.

Enhanced intraretinal revascularization in S1p2<sup>−/−</sup> mouse retina. We next examined the phenotypes of S1p2<sup>+/+</sup> and S1p2<sup>−/−</sup> retinal whole mounts stained en face with *Griffonia simplicifolia* lectin (GS-lectin) at the peak of neovascularization (P17) (Figure 2D). Interestingly, we observed that S1p2<sup>−/−</sup> retinas developed enhanced intraretinal revascularization, whereas the S1p2<sup>+/+</sup> littersmates showed increased avascular areas and formation of pathologic neovascular tufts, which is the expected phenotype of the ROP model (Figure 2D). To further study the role of S1P-R in intraretinal revascularization, we imaged GS-lectin–stained retinal whole mounts at different stages during the course of the ROP model. In particular, we measured avascular areas as percentage of total retinal area in S1p2<sup>+/+</sup>, S1p2<sup>−/−</sup>, and S1p2<sup>+/−</sup> mice. At P12, vascular obliteration occupied approximately 35% of the total retinal surface in S1p2<sup>+/+</sup> (34.2% ± 2.14%) as well as in S1p2<sup>−/−</sup> (31.2% ± 0.9%) and S1p2<sup>+/−</sup> littermates (31.9% ± 3.6%; *P* = 0.37; Figure 2, A and E). At P14 (2 days of hypoxia), no significant difference was observed in the avascular area of S1p2<sup>−/−</sup> (34.19% ± 0.74%; *n* = 3) and S1p2<sup>+/+</sup> mice (32.49% ± 2.4%; *n* = 6; **P* = 0.1) stained for GS-lectin. (B and D) At P14, vascular obliteration was also similar in S1p2<sup>−/−</sup> (34.19% ± 0.74%; *n* = 3) and S1p2<sup>+/−</sup> (32.49% ± 2.4%; *n* = 6; **P* = 0.1) retinas. (C and E) At P16, S1p2<sup>−/−</sup> retinas developed abnormal buds (arrowhead), and the capillary-free area was 16.6% ± 1.9% (*n* = 3); S1p2<sup>−/−</sup> retinas had improved vascular morphology (arrow) and reduced capillary-free area (8.6% ± 1.7%; *n* = 7; ***P* < 0.001). (D and E) At P17, S1p2<sup>−/−</sup> (*n* = 2) and S1p2<sup>+/−</sup> (*n* = 11) retinas displayed 13.5% ± 1.3% and 13.15% ± 1.8% vascular obliteration, respectively, and increased neovascularization (arrowhead, inset; scale bar: 100 μm); S1p2<sup>−/−</sup> retinas (n = 9) showed significantly decreased capillary-free area (2.6% ± 2.9%; *P* < 0.0001). Scale bar: 200 μm. (F and G) At P17, PAS and hematoxylin–stained cross section of S1p2<sup>+/−</sup> retinas with evident formation of vascular tufts (VT). (H) At P15, the mean number of neovascular nuclei/section for S1p2<sup>−/−</sup> and S1p2<sup>+/−</sup> was 22.5 ± 3.7 (*n* = 2) and 19.58 ± 2.43 (*n* = 4), respectively. **Figure 2** S1p2<sup>−/−</sup> retinas display increased intraretinal vascularization and decreased intravitreal neovascularization during the course of hypoxia. (A and E) At P12, vascular obliteration was similar in S1p2<sup>−/−</sup> (31.2% ± 0.9%; *n* = 3), and S1p2<sup>−/−</sup> whole mount retinas (31.9% ± 3.6%; *n* = 2, *P* = 0.37) stained for GS-lectin. (B and E) At P14, vascular obliteration was also similar in S1p2<sup>−/−</sup> (31.2% ± 0.9%; *n* = 3), and S1p2<sup>−/−</sup> whole mount retinas (31.9% ± 3.6%; *n* = 2, *P* = 0.37) stained for GS-lectin. (B and E) At P14, vascular obliteration was also similar in S1p2<sup>−/−</sup> (34.19% ± 0.74%; *n* = 3) and S1p2<sup>+/−</sup> (32.49% ± 2.4%; *n* = 6; **P* = 0.1) retinas. (C and E) At P16, S1p2<sup>−/−</sup> retinas developed abnormal buds (arrowhead), and the capillary-free area was 16.6% ± 1.9% (*n* = 3); S1p2<sup>−/−</sup> retinas had improved vascular morphology (arrow) and reduced capillary-free area (8.6% ± 1.7%; *n* = 7; ***P* < 0.001). (D and E) At P17, S1p2<sup>−/−</sup> (*n* = 2) and S1p2<sup>+/−</sup> (*n* = 11) retinas displayed 13.5% ± 1.3% and 13.15% ± 1.8% vascular obliteration, respectively, and increased neovascularization (arrowhead, inset; scale bar: 100 μm); S1p2<sup>−/−</sup> retinas (n = 9) showed significantly decreased capillary-free area (2.6% ± 2.9%; *P* < 0.0001). Scale bar: 200 μm. (F and G) At P17, PAS and hematoxylin–stained cross section of S1p2<sup>+/−</sup> retinas with evident formation of vascular tufts (VT). (H) At P15, the mean number of neovascular nuclei/section for S1p2<sup>−/−</sup> and S1p2<sup>+/−</sup> was 22.5 ± 3.7 (*n* = 2) and 19.58 ± 2.43 (*n* = 4), respectively. S1p2<sup>−/−</sup> retinas showed a decreased number, 11.27 ± 2.16 (*n* = 4; **P* < 0.0025). At P17, the mean number of neovascular nuclei/section for S1p2<sup>−/−</sup> and S1p2<sup>+/−</sup> retinas was 37.6 ± 7.03 (*n* = 3) and 34.52 ± 6.2 (n = 5), respectively. S1p2<sup>−/−</sup> retinas displayed a reduced number, 19.62 ± 2.2 (n = 6; **P* < 0.001). Scale bar: 100 μm (F) and 10 μm (G). Values represent mean ± SD. HT, heterozygous.
Furthermore, detailed examination of frozen retinal cross sections during normal development (normoxia) revealed that developing vessels (GS-lectin–staining) of S1p2−/− retinas (P6) spread finely toward the periphery of the retina and are able to form additional capillary networks in inner plexiform layer (IPL), and outer plexiform layer (OPL), whereas S1p2+/− retinas form an incomplete OPL vascular bed (A and B), containing with GFAP (astrocytes). At P17, in KO retinas (D) astrocytes (GFAP) were in close association with vessels (GS-lectin), whereas in WT animals (C), an increased number of abnormal tufts limited interaction with astrocytic processes. (E and F) WT and KO retinas stained for pericyte marker (NG2). Scale bars: 200 μm (C–F) and 50 μm (A, B, and high-magnification views in C–F).

**Figure 3**
Ischemic S1p2−/− mouse retinas display normal vascular morphology. At P17, hypoxic S1p2−/− retinas formed all 3 vascular networks in the nerve fiber layer (NFL), inner plexiform layer (IPL), and outer plexiform layer (OPL), whereas S1p2+/− retinas form an incomplete OPL vascular bed (A and B), containing with GFAP (astrocytes). At P17, in KO retinas (D) astrocytes (GFAP) were in close association with vessels (GS-lectin), whereas in WT animals (C), an increased number of abnormal tufts limited interaction with astrocytic processes. (E and F) WT and KO retinas stained for pericyte marker (NG2). Scale bars: 200 μm (C–F) and 50 μm (A, B, and high-magnification views in C–F).

Abnormal angiogenesis is attenuated in the intravitreal region of S1p2−/− mice. Intravitreal angiogenesis was determined by counting the nuclei of growing vessels that extend beyond the interface between the retina and vitreous (inner limiting membrane) of PAS and hematoxylin–stained serial cross sections (Figure 2, F and G). S1p2+/− and S1p2−/− mice maintained in normoxia did not show intravitreal angiogenesis (data not shown). At P15, when pathological tufts start developing, the mean number of nuclei counted for S1p2+/− and S1p2−/− retinas was 22.5 ± 3.7 and 19.5 ± 2.43, respectively. The mean number of neovascular nuclei for S1p2−/− retinas was 11.27 ± 2.16 (P < 0.0025; Figure 2H). At P17, when intravitreal neovascularization reached a maximum, the mean number of nuclei counted for S1p2+/− and S1p2−/− retinas that form vascular tufts (VT) was 37.6 ± 7.03 and 34.5 ± 6.2, respectively (Figure 2H). In sharp contrast, the mean number of neovascular nuclei of S1p2−/− retinas was reduced by approximately 50% (19.6 ± 2.2; P < 0.001; Figure 2H). These data suggest that animals that lack S1p2 receptor display greatly reduced pathological intravitreal neovascularization starting at the early stages of the disease.

Ischemic S1p2−/− mouse retinas display normal vascular morphology. In order to characterize the enhanced intraretinal revascularization in more detail, we imaged GS-lectin–stained S1p2+/− and S1p2−/− whole mount retinas at P17 in the mid-peripheral region. Ischemic S1p2−/− retinas exhibited nearly complete and well-defined

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**Ischemic S1p2−/− mouse retinas display normal vascular morphology.** At P17, hypoxic S1p2−/− retinas formed all 3 vascular networks in the nerve fiber layer (NFL), inner plexiform layer (IPL), and outer plexiform layer (OPL), whereas S1p2+/− retinas form an incomplete OPL vascular bed (A and B), containing with GFAP (astrocytes). At P17, in KO retinas (D) astrocytes (GFAP) were in close association with vessels (GS-lectin), whereas in WT animals (C), an increased number of abnormal tufts limited interaction with astrocytic processes. (E and F) WT and KO retinas stained for pericyte marker (NG2). Scale bars: 200 μm (C–F) and 50 μm (A, B, and high-magnification views in C–F).
architecture of the 2 additional capillary networks in IPL and OPL besides the primary vasculature of nerve fiber layer (NFL), whereas $S1p2^{-/-}$ retinas formed poorly organized capillary network in the OPL (Figure 3, B and A, respectively). In addition, $S1p2^{-/-}$ mice (Supplemental Figure 3D), similar to mice maintained in normoxia, displayed normal, almost fully recovered intraretinal vasculature in close association with surrounding long astrocytic processes (Figure 3D). In $S1p2^{-/-}$ retinas, astrocytes (glial fibrillary acidic protein–positive [GFAP-positive] cells) covered the retinal surface, but they were not closely associated with abnormally shaped vessels in the vascular tuft areas (Figure 3C).

Furthermore, in $S1p2^{-/-}$ retinas, pericyte (NG2-positive) staining was seen in all vasculature, in both normal and vascular tuft areas (Figure 3E) (24). In $S1p2^{-/-}$ retinas, pericyte staining was also seen in all vessels (Figure 3F and Supplemental Figure 3C), even though vascular tufts were reduced. These observations suggest that at P17 (peak of neovascularization), $S1p2^{-/-}$ retinas display normal formation of the primary as well as the deeper capillary retinal networks with proper maturation (as indicated by pericyte coverage) of the vasculature.

$S1P2$R modulates vascular patterning but not proliferation in ischemic mouse retina. We next focused on cellular events regulated by the $S1P2$R. To study the proliferation of retinal vessels, we performed a BrdU incorporation assay. BrdU positivity was most pronounced in ECs (GS-lectin–positive cells; Figure 4, E and F); however, a minor fraction of GFAP-positive cells (astrocytes) also incorporated BrdU (Supplemental Figure 3, A and B) (25). At P14 (very early stage of pathological neovascularization), $S1p2^{-/-}$ retinas exhibited similar distribution of BrdU-positive ECs in the primary vasculature (Figure 4, A and C). BrdU-positive cell numbers...
The proinflammatory enzyme COX-2 is induced by S1P-R. To elucidate the molecular mechanisms involved in the regulation of retinal vascularization and intravitreal angiogenesis by S1P-R, we measured mRNA levels of proangiogenic and proinflammatory factors such as VEGF, Ang-2, and iNOS before the initiation of pathologic neovascularization. Expression of angiogenic factors such as VEGF, Ang-2, and iNOS driven retinopathy is known to be regulated by hypoxia-mediated expression of angiogenic factors such as VEGF, Ang-2, and iNOS.

We first focused on vascular patterning as a possible mechanism by which S1P-2 regulates the ROP phenotype. At P15, S1P-2−/− retinas contained new vascular sprouts directed into the central avascular region of the retina, whereas S1P-2+/+ retinas displayed intravitreal neovascular tufts (Figure 4B). In contrast, mitogenic ECs of S1P-2−/− retinas were distributed evenly in the well-formed vascular network of the central retina (Figure 4D). These data suggest that intrinsic proliferation of ECs is not modulated by S1P-2 signaling.

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sion of VEGF, Ang-2, and Flt1 (proangiogenic mediators) as well as TNF-α and iNOS (proinflammatory mediators) was similar in S1p2+/− and S1p2−/− littermates (Figure 6A). However, COX-2 expression was significantly decreased, by approximately 64%, in the S1p2−/− retinas compared with S1p2+/− counterparts. In addition, in WT retinas COX-2 mRNA expression was highly induced during the course of hypoxia, peaking at P16 (4 days of hypoxia) (Figure 6B). These experiments suggest that the proinflammatory mediator COX-2 could be a target of S1P2R pathway, during the hypoxia-driven inflammatory response in the mouse retina (33).

To gain further insights into the cellular mechanism of COX-2 regulation by S1P2R in hypoxic retina, we performed immunohistochemistry on retina cross sections to localize COX-2. Interestingly, S1p2−/− retinas displayed strong COX-2 expression in the retinal nerve cells of the INL and GCL. We also observed enhanced COX-2 staining in the ECs of the INL, while COX-2 was predominantly expressed in the growing vessels of the GCL, which was similar to the tissue expression pattern of S1P2R (Figure 6C and Figure 1D, respectively). In S1p2−/− retinas, COX-2 was still detected in nerve cells of the INL and GCL, but its expression was reduced in vessels of the INL and GCL (Figure 6D). These in vivo experiments suggest that in mouse ischemic retina, S1P2R function induces COX-2 expression in vascular ECs.

When S1P2R was expressed in HUVECs by adenoviral transduction, COX-2 protein expression was significantly increased relatively to that of control cells transduced with adenovirus expressing GFP (AdGFP) (Figure 6E). In addition, we tested whether S1P2R can induce the transcription of the COX-2 gene. For this we measured human COX-2 promoter–driven (phPES2-driven) reporter activity in transfected ECs (34). As expected, the promoter activity of phPES2 that contains the 5′-flanking region (−1432/+59) of the human PTGS2 (COX-2) gene was induced 1.83-fold by PMA in mouse hemangioendothelioma (EOMA) cells. The promoter activity was induced 1.81-fold when S1p2 receptor was transfected, while...
Discussion

The present study investigates the role of S1P signaling in retinal vascular development and abnormal angiogenesis in the ROP model. Although ceramide metabolism has been implicated in retinal photoreceptor function and endocytosis, as well as in diabetic retinopathy, the functional role of S1P in retinal development and pathology has not been addressed (38, 39). This is particularly important as S1P is now accepted as an angiogenic factor and an inducer of vascular maturation (6). A recent report suggests that inhibitors of sphingosine kinase, an enzyme that generates S1P ligand, reduced retinal vascular leakage in the rat diabetic retinopathy model (40). The role of S1P receptors in retinal vasculature has not to our knowledge been addressed previously. We report here that S1P1 receptor is induced during ischemia-driven retinopathy, peaking at the growth phase of pathologic neovascularization. Immunohistochemistry experiments demonstrated that it is expressed in the growing vessels of the INL and GCL, highlighting structures of vascular tufts. It is likely that either hypoxia per se or hypoxia-responsive regulators such as VEGF and Ang-2 could modulate S1P1 receptor expression.

Another key finding of this study is that S1P1 receptor is essential for the pathologic angiogenesis of the retina. At P15 and P17, pathological intravitreal neovascularization was decreased by approximately 50% in S1p1−/− retinas. Importantly, S1p1−/− retinas display enhanced intraretinal revascularization. Enhanced physiological revascularization in the S1p1−/− retina was further confirmed by staining whole mount retinas for astrocytes (astroglia) and pericytes (mural cells), which were associated closely with ECs. Thus, lack of S1P1 shifted the phenotype of the ischemic retinal vasculature from pathologic to normal.

To further probe cellular mechanisms, we quantified EC proliferation; however, the same number of BrdU-positive ECs was observed in both WT and KO retinas at the beginning of pathogenesis (P14), suggesting that regulation of cell proliferation by S1P1 is unlikely to be the primary mechanism involved. Interestingly, detailed examination of the growing vessels at the beginning of neovascularization suggested that S1p2−/− mice display an increased number of ECs with elongated processes (tip cells) that are oriented toward the avascular regions compared with S1p2−/− mice. This alteration in normal vascular patterning may be disrupted by increased expression and signaling of S1P1 in the ECs, thus allowing misdirected angiogenesis in the vireous chamber and concomitant reduced normal retina revascularization. It is possible that exaggerated S1P1 signaling in ECs could contribute to patterning defects. We speculate that retinal endothelial tip cell directionality, which is regulated by signaling pathways such as VEGF (23) and Notch (37, 43), may be disrupted by aberrant S1P1 signaling in the context of ROP. As discussed below, such processes may also be influenced by inflammation.
permeability in the ischemic retinal vasculature is likely the key initiator of the inflammatory events. Indeed, F4/80-positive myeloid cells was observed in the vascular tufts in WT animals, whereas fewer inflammatory cells were associated with the retinal tissue of KO animals even at the very beginning of the pathogenesis.

Inflammatory mechanisms are thought to contribute to pathologic intravitreal angiogenesis (45, 46). iNOS inhibits angiogenesis in the avascular retina through the VEGF/VEGFR2 axis, thus leading to increased intravitreal angiogenesis (29). Ritter et al. showed that activated microglia contributes to enhanced revascularization by restoring appropriate gradient of angiogenic factors (31). Our data suggest that S1P/R-dependent inflammatory response may be important in the initiation and progression of abnormal ocular angiogenesis. Furthermore, abnormal intravitreal angiogenesis and normal retinal vasculature vessel may be interdependent. Our data suggest that S1P signaling via S1P2R may alter the balance between these processes.

In order to gain insight into the molecular mechanisms by which S1P2Rs facilitate intravitreal neovascularization, we profiled the expression of proangiogenic and proinflammatory molecules. We found that expression of the proinflammatory molecule COX-2 is significantly reduced in KO retinas. This observation is in agreement with previous reports that identify COX-2 as a promoter of retinal and corneal neovascularization, as well as tumor angiogenesis (33, 47, 48). Indeed, COX-2–specific inhibitors significantly reduced vascular tufts in the ROP model (33, 49). We propose that in the ischemic retina, S1P2R induces COX-2, leading to increased inflammatory response and enhanced intravitreal neovascularization most likely through proangiogenic PGE2 (30–53).

However, COX-2 inhibitor treatment did not stimulate normal retinal vascularization, suggesting that additional targets regulated by S1P2 are involved. We propose that eNOS may be one such molecule. We observed that at the early stage of pathologic angiogenesis (P14), S1P2+/− retinas have increased expression of eNOS protein in comparison with S1P2+/- retinas. By performing in vitro experiments in ECs, we show that S1P2R can directly downregulate eNOS protein expression. eNOS is a major source of NO, a potent vasodilator that facilitates proper blood flow and inhibits microvascular congestion (54, 55). Interestingly, our observations are somewhat discordant with a previous study in which eNOS-deficient mice developed reduced intravitreal neovascularization in oxygen-induced retinopathy (56). This may be due to the fact that eNOS-deficient mice are less susceptible to hyperoxia-induced pruning of the vessels from P7 to P12, whereas we observed increased eNOS expression in S1P2+/- hypoxic retinas at P14. In addition, the pharmacologic NOS inhibitor (L-NNa) that was used for these studies appears to be a competitive nonselective inhibitor of all 3 NOS isoforms (eNOS, neuronal NOS [nNOS], and iNOS). It has been reported that eNOS mRNA stability is reduced under hypoxic conditions or upon thrombin stimulation and inflammation (57–59). Interestingly, Rho/ROCK-associated kinase activation that is downstream of the S1P2/G12/13 Receptor pathway is known to mediate hypoxia-dependent inhibition of eNOS expression in ECs (60, 61). Thus, we speculate that in ischemic retinas, S1P2R negatively regulates eNOS expression, possibly through the Rho/Rho kinase pathway, which may contribute to retinal vascular congestion and pathological angiogenesis.

In conclusion, we show for the first time to our knowledge that the S1P2R pathway is an essential inducer of pathological neovascularization and inhibits hypoxia-triggered revascularization in the retina. In this regard we suggest that therapeutic compounds that specifically inhibit S1P2 G protein–coupled receptor would inhibit pathologic angiogenesis while promoting physiological revascularization of the ischemic retina. Regulation of the plasticity of vascular phenotype by S1P2 may also be useful in other ischemia-driven vascular diseases.

**Methods**

*Animals.* C57BL/6 x 129Sv mice with targeted disruption of the S1p2 gene were generated as previously reported (13). Mice were maintained on a mixed C57BL/6 x 129Sv genetic background, and experiments on KO mice were performed with appropriate littermate controls. All procedures involving mice were approved by the University of Connecticut Health Center Animal Care Committee. ROP was induced according to a protocol established by Smith et al. (20). Briefly, pups (P7) with nursing mother were transferred into an air-tight incubator and were exposed to an atmosphere of 74% ± 1% oxygen for 5 days. At P12, pups were returned to room air.

*RNA isolation and RT-PCR analysis.* RNA was extracted (RNasy kit; QIA-GEN) from mouse retinas. First-strand cDNA was synthesized using random hexamers, murine leukemia virus reverse transcriptase, and accompanying reagents (Invitrogen) for 1 hour at 37°C. Mouse RT-PCR primers shown in Supplemental Table 1 were designed with Primer Express software (version 2.0; Applied Biosystems). Amplification and data analysis were performed with an ABI Prism 7900HT Sequence Detection System (Applied Biosystems). mRNA levels were quantified and corrected for cyclophilin A and expressed as fold induction over the corresponding control.

**Histology and immunohistochemistry.** For intravitreal neovascularization, 12 sections (6 μm thick, 30 μm apart) from each eyeball were PAS and hematoxylin stained (PAS kit; Sigma-Aldrich). Vascular cell nuclei growing beyond the INL were counted. For S1P2 staining, heat epitope retrieval of tissue sections was performed in 10 mM citrate buffer (pH 6.0). For F4/80 and COX-2 staining, tissue sections were pretreated with Pronase E (Sigma-Aldrich) for 5 minutes. Sections were stained with primary antibody overnight at 4°C: rabbit polyclonal anti-S1P-R (62, 63) (1:200), mouse anti-COX-2 (1:800; Cayman Chemical). Primary antibody detection was performed with VECTASTAIN ABC kit (Vector Laboratories). Counterstaining was performed with methyl green or Mayer hematoxylin.

*Retina whole mount preparation, quantification of avascular area, and immunofluorescence.* Eyes were enucleated and fixed in 4% PFA for 15 minutes. Retinas were dissected out and postfixed for 15 minutes. To visualize endothelium, retinas were stained with Alexa Fluor 594–conjugated GS-lectin (20 μg/ml; Molecular Probes; Invitrogen). Primary antibodies were: FITC-conjugated mouse anti–œ SMA (1:100; Sigma-Aldrich), rabbit anti–GFAP (1:200; Dako), rabbit anti–NG2 (1:200; Chemicon), mouse anti–BrU (1:200; Chemicon). Secondary antibodies were: Alexa Fluor 488–conjugated goat anti-rabbit antibody (1:200; Molecular Probes; Invitrogen). Retinas were visualized using a Zeiss LSM 510 confocal microscope. Avascular and total retina areas were quantified with ImageJ (http://rsb.info.nih.gov/ij/). For FITC–RCA–1 (50 μl, 2 mg/ml; Vector Laboratories) perfusion, mice were anesthetized with Avertin (Sigma-Aldrich), injected in the left ventricle with RCA 1, which was allowed to circulate for 2 minutes. Unbound lectin was removed with 1% BSA–PBS perfusion for 1 minute followed by 4% PFA–PBS fixation for 5 minutes. Eyes were enucleated, postfixed, and stained as described above. For tip cell quantification, sprouts were counted in 4 different fields of the retinal mid-periphery, and the mean number of tip cells per retina was calculated. For BrDU–positive cell quantification, Image-Pro Plus image analysis software (version 5.1.1.38, Media Cybernetics) was used to count fluorescent pixels per total retinal area.

**Luciferase activity experiments.** EOMA cultures (3 × 104 cells/well) on a 6-well plate were grown 1 day before the transfection. To measure transfection...
10. Sanna, M.G., et al. 2006. Enhancement of capillary rupture vector pHPE2(–1432/+59), 0.3 µg of the gene of interest (pcDNA3.1-S1p2 or pcDNA3.1-S1p1), and 25 ng of pCMV-β-gal mixed with Lipofectamine 2000 (Invitrogen) were introduced into the cells as described by the manufacturer. Twenty-four hours after transfection, cells were treated with PMA (100 nM) for 5 hours, if necessary. Cells were harvested, and Luciferase and β-gal activity were determined with a Luciferase Assay System (Promega) and Western-Light and Western-Star system (Applied Biosystems), respectively. The amount of plasmid DNA was made constant by adding pcDNA 3.1, and Luciferase activity was normalized to micrograms of protein content.

Western blot analysis. Retinas or cells were solubilized in 2x SDS sample buffer (20 mM DTT, 6% SDS, 0.25 M Tris pH 6.8, 10% glycerol, bromophenol blue, protease inhibitors, 1 mM sodium orthovanadate, and 1 mM NaF), sonicated, boiled and separated by SDS-PAGE gel electrophoresis. Membranes were incubated with the following antibodies: anti-actin (Sigma-Aldrich), anti-ENOS (BD Biosciences—Pharmingen), anti-COX-2 (Cayman), and anti-β (Invitrogen). Cells were treated overnight with 10 µm Y-27632 (Calbiochem). Immunoreactive band density was quantified with IQMac version 1.2 software (Molecular Dynamics).

Adenoviral vector construction and production. CDNA encoding S1P-V5 tag was subcloned into the p Shuttle-CMV vector that was used to produce recombinant adenovirus using bacteria-AdEasy vector system (AdEasy kit; Quantum Biotechnologies) as described by the manufacturer.

Statistics. Statistical differences were assessed using the 2-tailed Student’s t test. P values less than 0.05 were considered significant.

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