

# Nuclear receptor ROR $\alpha$ regulates pathologic retinal angiogenesis by modulating SOCS3-dependent inflammation

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**Pathologic ocular angiogenesis is a leading cause of blindness, influenced by both dysregulated lipid metabolism and inflammation. Retinoic-acid-receptor-related orphan receptor alpha (ROR $\alpha$ ) is a lipid-sensing nuclear receptor with diverse biologic function including regulation of lipid metabolism and inflammation; however, its role in pathologic retinal angiogenesis remains poorly understood. Using a mouse model of oxygen-induced proliferative retinopathy, we showed that ROR $\alpha$  expression was significantly increased and genetic deficiency of ROR $\alpha$  substantially suppressed pathologic retinal neovascularization. Loss of ROR $\alpha$  led to decreased levels of proinflammatory cytokines and increased levels of antiinflammatory cytokines in retinopathy. ROR $\alpha$  directly suppressed the gene transcription of suppressors of cytokine signaling 3 (SOCS3), a critical negative regulator of inflammation. Inhibition of SOCS3 abolished the antiinflammatory and vasoprotective effects of ROR $\alpha$  deficiency in vitro and in vivo. Moreover, treatment with a ROR $\alpha$  inverse agonist SR1001 effectively protected against pathologic neovascularization in both oxygen-induced retinopathy and another angiogenic model of very-low-density lipoprotein receptor (*Vldlr*)-deficient (*Vldlr*<sup>-/-</sup>) mice with spontaneous subretinal neovascularization, whereas a ROR $\alpha$  agonist worsened oxygen-induced retinopathy. Our data demonstrate that ROR $\alpha$  is a novel regulator of pathologic retinal neovascularization, and ROR $\alpha$  inhibition may represent a new way to treat ocular neovascularization.**

ROR $\alpha$  | neovascularization | retinopathy | inflammation | SOCS3

**P**athologic proliferation of blood vessels commonly causes blindness in all age groups, including retinopathy of prematurity in children, proliferative diabetic retinopathy in working-age adults, and neovascular age-related macular degeneration (AMD) in the elderly (1). Development of pathologic ocular angiogenesis is linked with dysregulation of both lipid metabolism (2, 3) and altered inflammation/macrophage function (4). Identification of key controlling mechanisms by which lipids and their metabolites modulate retinal tissue toward (or away from) a proinflammatory, proangiogenic state is critical for developing potential treatments. One potential pivotal regulator of lipid-mediated inflammatory processes is retinoic-acid-receptor-related orphan receptor alpha (ROR $\alpha$ ), a lipid-sensing nuclear receptor that may modify inflammation (5). Genetic variations in ROR $\alpha$  are associated with increased risk of developing neovascular AMD (6–8). However, the functional role of ROR $\alpha$  in pathologic retinal angiogenesis is poorly understood.

ROR $\alpha$  controls diverse biological processes including immunity, cerebellum development, and circadian rhythm (9). As a ligand-dependent transcription factor, ROR $\alpha$  is a suggested receptor for cholesterol, cholesterol sulfate, and other cholesterol-derived oxysterols (10). ROR $\alpha$  regulates lipid metabolism including cholesterol and lipoprotein levels, and hence is implicated in atherosclerosis and vascular contractility control (11). ROR $\alpha$  is also important for regulating immunity and inflammatory response in allergic inflammation (12) and autoimmune diseases (13), as well

as for production of inflammatory cytokines (9, 14–16). ROR $\alpha$  controls its target gene expression through binding as a monomer to a core DNA consensus sequence termed ROR $\alpha$  responsive element (RORE), comprised of an AGGTC A half-site and a 5' AT-rich extension (17, 18). Binding of ROR $\alpha$  to RORE together with its coactivators and corepressors controls transcription of ROR $\alpha$  target genes.

In this study, we investigated whether ROR $\alpha$  acted at the crossroad of lipid metabolism and inflammation to control pathologic retinal angiogenesis. ROR $\alpha$  deficiency significantly suppressed pathologic retinal angiogenesis in a mouse model of proliferative oxygen-induced retinopathy (OIR) with hypoxia-induced neovascularization (19) that mimics retinopathy of prematurity, and some aspects of proliferative diabetic retinopathy in humans. Loss of ROR $\alpha$  resulted in an antiinflammatory retinal environment, which was mediated through its direct transcriptional control of suppressor of cytokine signaling 3 (SOCS3), a critical regulator of tissue inflammation. Suppressing SOCS3 inhibited ROR $\alpha$  deficiency-induced inflammatory and vascular effects in vitro and in vivo. Treatment with SR1001, a synthetic small molecular inverse agonist of ROR $\alpha$ , effectively inhibited pathologic angiogenesis in OIR and spontaneous subretinal neovascularization in very-low-density lipoprotein receptor (*Vldlr*)-deficient (*Vldlr*<sup>-/-</sup>) mice

## Significance

**Pathologic retinal neovascularization commonly causes blindness. Retinoic-acid-receptor-related orphan receptor alpha (ROR $\alpha$ ), a lipid-sensing nuclear receptor, is genetically associated with the risk of developing neovascular eye disease in humans. We demonstrate that ROR $\alpha$  expression was highly increased in a mouse model of oxygen-induced proliferative retinopathy with pathologic neovessels. Both genetic deficiency and pharmacologic inhibition of ROR $\alpha$  suppressed pathologic retinal neovascularization in mice with dampened inflammation. ROR $\alpha$  transcriptionally regulated suppressors of cytokine signaling 3 (SOCS3), a negative mediator of macrophage function and inflammation. Suppression of SOCS3 attenuated the protective effects of ROR $\alpha$  inhibition in retinopathy. Our data demonstrate an important role of ROR $\alpha$  in controlling pathologic retinal neovascularization and suggest that ROR $\alpha$  may represent a druggable target for treating ocular neovascularization.**

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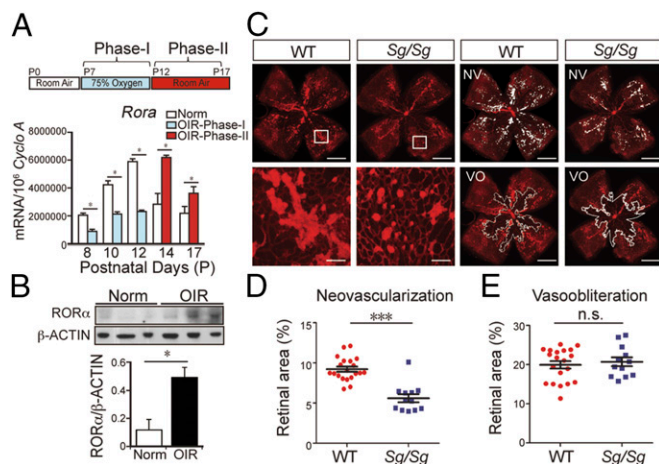
modeling neovascular AMD. Thus, inhibition of ROR $\alpha$ , a novel regulator of pathologic ocular angiogenesis, may be a new approach to potentially treat or prevent pathologic vascular growth in eye diseases.

## Results

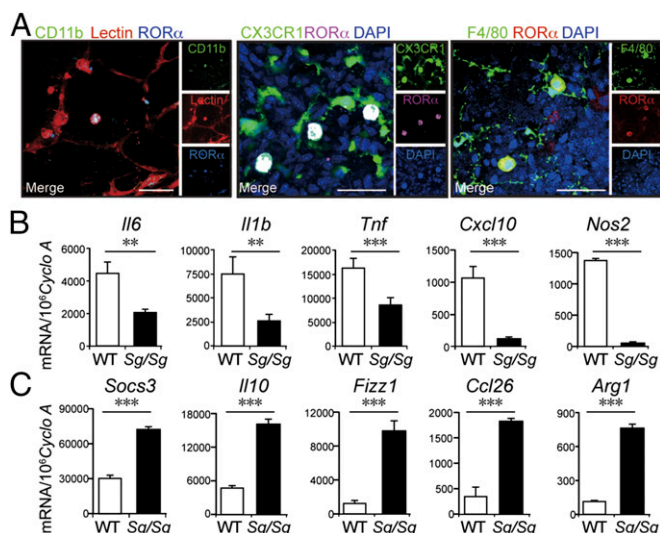
**ROR $\alpha$  Deficiency Significantly Attenuated Pathologic Retinal Neovascularization in OIR.** To induce retinopathy, mice were exposed to 75% oxygen from postnatal day (P)7 to P12. Compared with age-matched room air controls, *Rora* mRNA expression was significantly down-regulated at P8, P10, and P12, during the initial oxygen-induced vessel loss phase (phase I) of OIR, and then up-regulated at P14 and P17 in the second hypoxic and proliferative phase (phase II) (Fig. 1A). Protein levels of ROR $\alpha$  were also significantly up-regulated (approximately fivefold) in P17 OIR retinas (Fig. 1B).

ROR $\alpha$ -deficient *Sg/Sg* mice have a spontaneous deletion in the *Rora* gene with loss of ROR $\alpha$  activity (20). In OIR, *Sg/Sg* mice showed markedly decreased levels of pathologic retinal neovascularization at P17 compared with littermate wild-type (WT) controls (WT:  $9.22 \pm 0.32\%$ ; *Sg/Sg*:  $5.61 \pm 0.50\%$ ;  $n = 12-20$  per group,  $P < 0.001$ ; Fig. 1C and D), with comparable vasoobliteration ( $P = 0.60$ , Fig. 1C and E). ROR $\alpha$  deficiency did not impact normal retinal vasculature as adult *Sg/Sg* retinas showed normal vascular structure and morphology as age-matched WT (*SI Appendix*, Fig. S1). Aortic ring explants isolated from *Sg/Sg* and WT mice showed similar levels of vascular sprouting (*SI Appendix*, Fig. S2), suggesting likely marginal effects of endothelium ROR $\alpha$  on angiogenesis. This notion is consistent with minimal ROR $\alpha$  staining in lectin positive retinal blood vessels (Fig. 2 and *SI Appendix*, Fig. S3), and strong ROR $\alpha$  staining in some lectin positive cells surrounding blood vessels resembling microglia/macrophages morphologically (*SI Appendix*, Fig. S3).

**ROR $\alpha$  Was Localized in Retinal Macrophages/Microglia and Mediated Expression of Inflammatory Cytokines.** Retinal microglia/macrophages are important inflammatory regulators of retinal



**Fig. 1.** ROR $\alpha$  deficiency significantly attenuated pathologic neovascularization in OIR. (A) *Rora* mRNA expression in OIR retinas compared with normoxic retinas ( $n = 6$  per group). (B) Quantification of Western blot with ROR $\alpha$  and  $\beta$ -ACTIN antibodies in P17 OIR retinas compared with normoxic retinas (Norm). ( $n = 3$ ). (C) Representative retinal whole mounts from OIR *Sg/Sg* and WT retinas stained with isolectin IB<sub>4</sub> (red) with areas of vasoobliteration (VO) and neovascularization (NV) highlighted (white). Two selected retinal areas (white box) were enlarged to show pathologic neovessels. Quantification of pathologic NV (D) and VO (E) in OIR *Sg/Sg* and WT retinas was expressed as percentage of total retinal areas.  $n = 12-20$  per group. (Scale bar, 1,000  $\mu\text{m}$ ).  $*P < 0.05$ ;  $***P < 0.001$ ; n.s., no significance.



**Fig. 2.** Deficiency of ROR $\alpha$  influenced retinal inflammatory cytokine expression and macrophage markers in OIR. (A) Immunohistochemical localization of ROR $\alpha$  in macrophages/microglia in P17 WT OIR retina flat mounts, costained with macrophage/microglia markers CD11b, CX3CR1, and F4/80. Isolectin IB<sub>4</sub> (red) was used to stain both blood vessels and macrophages/microglia. (Scale bar, 50  $\mu\text{m}$ .) (B) Expression of inflammatory cytokines and macrophage markers in OIR *Sg/Sg* and WT retinas at P17 ( $n = 5$  per group). *Sg/Sg* retinas showed decreased expression levels of *Tnf*, *Il6*, *Il1b*, *Cxcl10*, and *Nos2*. (C) Expression of *Il10*, *Fizz1*, *Ccl26*, *Arg1*, and *Socs3* were increased in OIR *Sg/Sg* retinas at P17 compared with WT.  $**P < 0.01$ ;  $***P < 0.001$ .

angiogenesis (21). Increased levels of proinflammatory cytokines were found in vitreous fluid of patients with proliferative retinopathy (22). We next evaluated whether ROR $\alpha$  controls pathologic retinal neovascularization through modulation of retinal inflammation. In WT OIR retinas, ROR $\alpha$  antibody strongly colocalized with selected population of retinal macrophages/microglia positive for CD11b, CX3CR1, or F4/80 (Fig. 2A). Moreover, ROR $\alpha$  deficiency in *Sg/Sg* retinas significantly suppressed expression of proinflammatory cytokines: interleukin-6 (*Il6*), interleukin-1 beta (*Il1b*), and tumor necrosis factor- $\alpha$  (*Tnf*) ranging from 2- to 3-fold (Fig. 2B) and increased expression of antiinflammatory cytokines *Il10* (Fig. 2C). In addition, *Sg/Sg* retinas demonstrated significant decrease of proinflammatory macrophage markers *iNOS* (encoded by *Nos2*) and *Cxcl10* by 10- to 15-fold compared with WT (Fig. 2B), and reciprocally increased levels of antiinflammatory macrophage markers *Fizz1*, *Ccl26*, and *Arg1* (~2- to 10-fold up-regulation) (Fig. 2C), reflecting an overall shift toward antiinflammatory state. *Socs3*, an inducible negative regulator of inflammation (23, 24), was also significantly increased in *Sg/Sg* OIR retinas (Fig. 2C), yet vascular endothelial growth factor A (*Vegfa*) was not significantly changed (*SI Appendix*, Fig. S4). Together these results indicate that ROR $\alpha$  deficiency promotes retinal inflammation state toward an antiinflammatory environment in OIR.

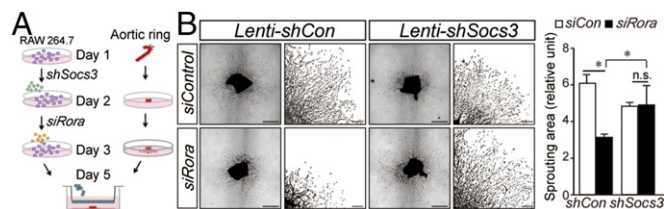
**ROR $\alpha$  Suppressed *Socs3* Transcription Through Binding to Its RORE Site.** ROR $\alpha$  controls target gene transcription by binding to RORE sites (9). The proximal promoter sequences of those genes significantly regulated in *Sg/Sg* OIR retinas all contain at least one potential RORE binding site. Chromatin immunoprecipitation (ChIP) assay was performed with ROR $\alpha$  antibody followed by qPCR to quantitate ROR $\alpha$  binding to candidate DNA regions in P17 OIR retinas. Among the 10 analyzed genes, binding to *Socs3* showed the most significant enrichment compared with IgG control (Fig. 3A). The *Socs3* promoter contains four potential RORE sites (*SI Appendix*, Fig. S5). Direct binding of ROR $\alpha$  to the third site, conserved between human and mouse, was the most strongly



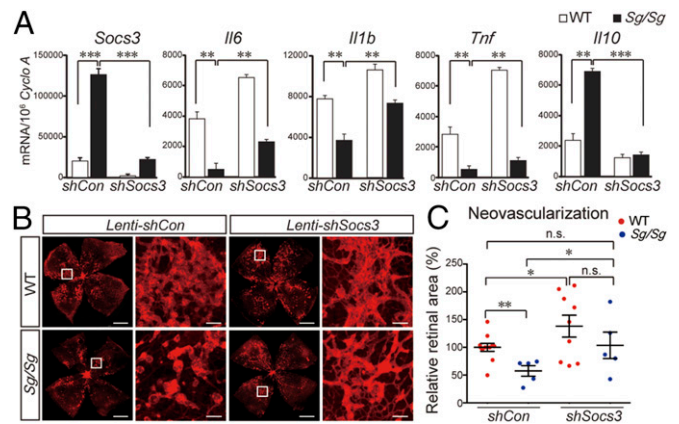
we cocultured WT aortic rings with macrophages to evaluate the effects of modulating macrophage ROR $\alpha$  on aortic ring sprouting. RAW 264.7 cells were pretreated with lenti-*shSocs3* and *siRora* with respective controls, then cocultured with WT aortic rings in Transwells (Fig. 5A). ROR $\alpha$ -deficient (*siRora*) RAW 264.7 cells significantly suppressed aortic ring sprouting compared with control (*siCon*)-treated RAW cells by ~50% ( $n = 8$ ,  $P < 0.05$ ; Fig. 5B). Suppression of *Socs3* in ROR $\alpha$ -deficient (*siRora/shSocs3*) macrophages largely reversed the effects of macrophage ROR $\alpha$  on aortic ring sprouting (Fig. 5B), indicating that *Socs3* mediates the angiogenic effect of macrophage ROR $\alpha$  on vascular growth.

**SOCS3 Mediated the Inflammatory and Vascular Effects of ROR $\alpha$  in OIR.** To determine whether SOCS3 is functionally important for ROR $\alpha$ -mediated retinal neovascularization, we silenced *Socs3* expression with intravitreal injection of lenti-*shSocs3* before exposing WT and *Sg/Sg* mice to OIR. Lenti-*shSocs3* effectively suppressed ~85% of *Socs3* expression in both WT and *Sg/Sg* OIR retinas compared with their respective lenti-controls (Fig. 6A). Lenti-control-treated *Sg/Sg* retinas exhibited decreased levels of *Il6*, *Il1b*, and *Tnf*, increased levels of *Il10* (Fig. 6A), and significantly reduced pathologic neovascularization compared with littermate WT retinas in OIR ( $P < 0.01$ ,  $n = 5-9$  per group, Fig. 6B and C), similar to the dampened inflammation and reduced pathologic neovessels observed in nontreated *Sg/Sg* vs. WT OIR retinas (Fig. 1). Importantly, lenti-*shSocs3* injection in *Sg/Sg* OIR retinas significantly abolished ROR $\alpha$ -deficiency-induced dampening of inflammatory cytokines (Fig. 6A) and ROR $\alpha$ -deficiency-induced protection from pathologic neovascularization, compared with lenti-control injected *Sg/Sg* eyes ( $P < 0.05$ ,  $n = 5$  per group, Fig. 6B and C), back to the levels comparable to lenti-*shSocs3*-treated WT retinas (no significance,  $n = 5-9$  per group, Fig. 6B and C). Together these results support the idea that SOCS3 mechanistically mediates the inflammatory and vascular effects of ROR $\alpha$  in OIR.

**A Synthetic Inverse Agonist of ROR $\alpha$  Suppressed Pathologic Neovascularization in OIR and *Vldlr*<sup>-/-</sup> Mice.** SR1001, a synthetic inverse agonist of ROR $\alpha$  (13), dose dependently increased *Socs3* expression in macrophage RAW 264.7 cells (Fig. 7A). Moreover, SR1001 treatment from P12 to P17 in WT OIR mice significantly reduced pathologic neovascularization at P17 by ~30% ( $P < 0.01$ ,  $n = 14-20$  per group) compared with littermate vehicle controls, without affecting vasoobliteration (Fig. 7B and C), suggesting that pharmacologic inhibition of ROR $\alpha$  was effective in suppressing retinopathy. On the other hand, SR1078, a ROR $\alpha$  agonist validated in the liver (26), dose-dependently suppressed *Socs3* expression level in RAW 264.7 cells and significantly increased the levels of pathologic neovascularization ( $P < 0.01$ ,  $n = 14-16$  per



**Fig. 5.** Macrophage ROR $\alpha$ -regulated aortic ring vascular growth through modulation of *Socs3*. (A) Illustration of coculture showing RAW 264.7 cells were pretreated with lenti-*shSocs3* and *siRora*, or respective controls, before being cocultured with normal aortic rings in Transwells. (B) Images of aortic ring sprouts ( $n = 8$  per group) with selected areas enlarged. Sprouting areas were quantified. (Scale bars, 1,000  $\mu\text{m}$  for original images and 150  $\mu\text{m}$  for enlarged images.) \* $P < 0.05$ ; n.s., no significance.



**Fig. 6.** Inhibition of *Socs3* abolished the inflammatory and neovascular effects of ROR $\alpha$  deficiency in OIR. (A) Expression of *Il6*, *Il1b*, *Tnf*, and *Il10* in P17 *Sg/Sg* and WT OIR retinas intravitreally injected with lenti-*shControl* or lenti-*shSocs3* at P5 ( $n = 6$  per group). (B) Representative retinal whole mounts from P17 OIR WT or *Sg/Sg* mice intravitreally injected with lenti-*shControl* or lenti-*shSocs3*, with retinal vessels stained by isolectin IB<sub>4</sub> (red) and selected areas enlarged (white box). (C) Quantification of pathologic neovascular areas normalized to levels in control-treated WT retinas.  $n = 5-9$  per group. (Scale bars, 1,000  $\mu\text{m}$  for original images and 100  $\mu\text{m}$  for enlarged images.) \* $P < 0.05$ ; \*\* $P < 0.01$ ; \*\*\* $P < 0.001$ ; n.s., no significance.

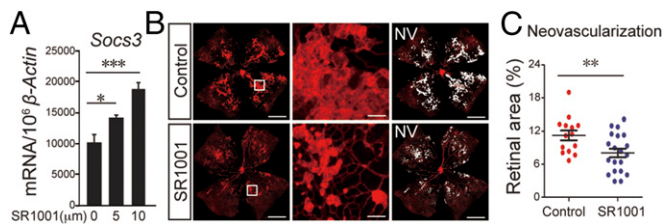
group) in OIR (SI Appendix, Fig. S7), indicating that activation of ROR $\alpha$  worsened retinopathy.

Next SR1001 treatment was evaluated in an additional angiogenic model of *Vldlr*<sup>-/-</sup> mice, which develop spontaneous pathologic subretinal neovascularization, modeling neovascular AMD, retinal angiomatous proliferation, and macular telangiectasia (27-29) (Fig. 8A). The spontaneous subretinal neovascularization in *Vldlr*<sup>-/-</sup> mice invades the normally avascular photoreceptor layer starting at P12 and reaches retinal pigment epithelium (RPE) at P16 (Fig. 8A). *Socs3* expression levels were significantly suppressed in *Vldlr*<sup>-/-</sup> retinas compared with WT retinas (Fig. 8B), whereas *Rora* levels were comparable (SI Appendix, Fig. S8). Daily SR1001 treatment in *Vldlr*<sup>-/-</sup> mice from P5 to P15 led to significant induction of retinal *Socs3* expression at P16 (Fig. 8C). Importantly, SR1001 treatment significantly inhibited both the number and area of subretinal neovascular lesions in *Vldlr*<sup>-/-</sup> mice at P16 by ~50% ( $P < 0.001$ ,  $n = 9-12$  per group, Fig. 8D-F). These data suggest that ROR $\alpha$  inhibition by SR1001 suppresses subretinal neovascularization in *Vldlr*<sup>-/-</sup> retinas, a relevant model for AMD, corroborating the findings in the OIR model.

## Discussion

Our study presents evidence for a critical role of ROR $\alpha$  in two models of pathologic ocular neovascularization, an oxygen-induced ischemic retinopathy and the *Vldlr*<sup>-/-</sup> mice with spontaneous subretinal neovascularization. *Rora* expression was significantly regulated in OIR, consistent with a suggested role of *Rora* as a hypoxia-inducible factor (HIF) target gene (30). ROR $\alpha$  may thus be modulated synergistically by tissue ischemia and hypoxia, in addition to lipid-based ligands, to influence the angiogenic response. Our findings support a proangiogenic role of ROR $\alpha$  as its deficiency suppressed pathologic retinal neovascularization; yet in a hind limb ischemia model, ROR $\alpha$  was reported as a negative regulator of angiogenesis (31), potentially reflecting a functionally plastic role of ROR $\alpha$  in regulating angiogenesis in a disease- and organ-dependent manner.

Suppression of pathologic neovascularization in ROR $\alpha$ -deficient *Sg/Sg* retinas was associated with decreased retinal inflammation. Many inflammatory mediators were increased in vitreous fluid of patients with proliferative diabetic retinopathy



**Fig. 7.** SR1001 suppressed pathologic neovascularization in OIR. (A) *Socs3* mRNA expression in SR1001-treated RAW 264.7 cells. (B) Representative retinal whole mounts from WT OIR littermates treated with SR1001 or vehicle control were stained with isolectin IB<sub>4</sub> (red). Areas of neovascularization (NV) were highlighted (white) and selected retinal areas (white box) enlarged. (C) Quantification of pathologic NV in SR1001 and vehicle control-treated retinas.  $n = 14$ –20 per group. (Scale bars, 1,000 μm for original images and 100 μm for enlarged images.) \* $P < 0.05$ ; \*\* $P < 0.01$ ; \*\*\* $P < 0.001$ .

or retinopathy of prematurity (32) including *TNFα*, *IL-1β* (33), *IL-6* (34), and chemokines such as *CXCL10* (35), suggesting that chemokine-induced recruitment of inflammatory cells and inflammatory cytokines are important for clinical retinopathy. In mice, depletion of *TNFα* resulted in decreased pathologic retinal neovascularization (36), and inhibition of *IL-1β* also suppressed diabetic retinopathy (37) and pathologic ocular angiogenesis (38). These studies suggest that proinflammatory cytokines are potentially detrimental to retinopathy, which may underlie the protective effect of *RORα* deficiency in suppressing inflammation-associated pathologic retinal neovascularization in OIR.

Whereas our study localized *RORα* mainly in macrophages/microglia, these may not be the sole cellular source of retinal *RORα*. Clodronate liposome depletion of macrophage largely suppressed but did not completely abolish *Rora* expression, suggesting other cellular source of *RORα*. Previous studies have reported the presence of *RORα* in retinal neurons including retinal ganglion cells (39) and a subset of cone photoreceptors (25), as well as in human aortic vascular cells (40). Therefore, potential contribution from vascular endothelium or neuronal *RORα* toward the observed vascular effects in the retina is still possible.

Mechanistically *RORα*-mediated inflammatory and vascular effects may act in part through SOCS3, by direct repression of its transcription. Interestingly *RORα* is generally considered a constitutively active transcription factor, yet our discovery of transcriptional suppressive activity of *RORα* on *Socs3* may reflect the possibility of a negative response element of the *Socs3* RORE site. Negative response elements mediate direct transcriptional suppression by nuclear receptors such as the glucocorticoid receptor (41), and similar mechanisms may also exist for *RORα* that will require further study. *RORα* suppression of *Socs3* promoted retinal inflammation, consistent with previous reports showing increased inflammation in *Socs3*-deficient macrophages in myeloid-specific *Socs3* knockout mice (28). Depletion of *Socs3* in *Tie2*-expressing cells also promoted pathologic neovascularization in OIR (42), reflecting an endogenous inhibitory role of SOCS3 in blood vessels. Low levels or transient induction of *RORα* in the endothelium may also potentially mediate endothelial SOCS3-dependent inflammation. Inhibiting SOCS3 effectively reversed the inflammatory and vascular effect of *RORα* deficiency, yet potential direct *RORα* transcriptional regulation of other inflammatory mediators, such as *Arg1* and *Il10*, may be additional contributing factors, reflecting a likely multifactorial process regulated by *RORα*, which will be further investigated.

As a receptor for cholesterol derivatives, *RORα* is involved in regulation of cholesterol homeostasis and may influence cellular inflammatory and angiogenic responses to lipid metabolites. However, whether *RORα*-related lipid metabolites may directly influence proliferative retinopathy is still unclear, although

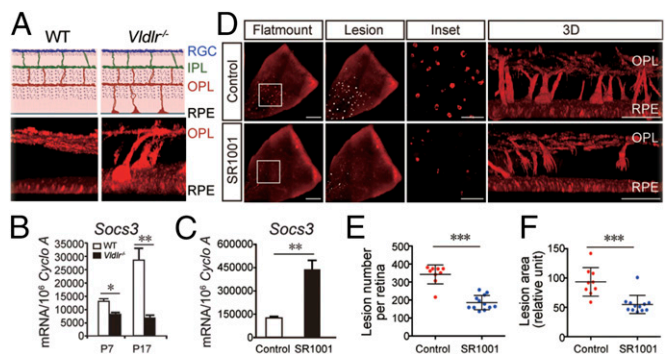
dyslipidemia is closely linked with both clinical diabetic retinopathy and AMD. Whereas no data are available regarding the influence of *RORα* on human proliferative retinopathy, genetic variations of *RORα* were linked with increased risks of developing neovascular AMD (6–8); however, the functional consequence of the AMD-associated *RORα* SNPs awaits further investigation.

Our findings indicate that SR1001, a high-affinity synthetic inverse agonist of *RORα*, effectively suppressed pathologic neovascularization in both OIR and *Vldlr*<sup>-/-</sup> mice, without causing the staggering phenotype or obvious gross toxicity, consistent with lack of adverse events observed in previous studies with prolonged SR1001 treatment in adult or diabetic mice (13, 43). However, further evaluation on retinal neuronal function will be needed to fully characterize the safety profile of SR1001. Additional structural optimization of SR1001 may also allow development of more potent and selective *RORα* inhibitors with even stronger anti-angiogenic effect for translational use. Whereas SR1001 binds specifically to the ligand-binding domains of both *RORα* and *RORγ* (13), *Rorc* expression levels were much lower in the retinas compared with *Rora*, and relatively unchanged in OIR (*SI Appendix, Fig. S9*), suggesting that potential side-effect contribution from *RORγ* to the observed effects of SR1001 is likely marginal.

In summary, this study provides the first direct evidence to our knowledge for a critical role of an immunoregulating nuclear receptor *RORα* in experimental proliferative retinopathy through modulating tissue inflammation via transcriptional regulation of *Socs3*. Modulation of a nuclear receptor such as *RORα* may thus serve as a completely new approach to potentially treat vascular eye diseases without directly impacting angiogenic growth factors, such as VEGF, that are essential for vascular homeostasis, and may have broad therapeutic value for potentially other vascular disorders precipitated by inflammation-mediated angiogenesis.

## Materials and Methods

**Mice.** All animal studies were performed according to protocols reviewed and approved by the Institutional Animal Care and Use Committee at the Boston Children's Hospital. *Rora* heterozygous staggerer mice (*Sg/+*) were obtained



**Fig. 8.** SR1001 treatment suppressed spontaneous subretinal neovascularization in *Vldlr*<sup>-/-</sup> mice. (A) Schematic illustration (Top) and 3D reconstruction (Bottom) of spontaneous subretinal neovascularization in *Vldlr*<sup>-/-</sup> mice at P16, stained with isolectin IB<sub>4</sub> (red). RGC, retinal ganglion cell; IPL, inner plexiform layer; OPL, outer plexiform layer; RPE, retinal pigment epithelium. (B) *Socs3* mRNA expression in WT and *Vldlr*<sup>-/-</sup> retinas at P7 and P17,  $n = 3$  per group. (C) *Socs3* mRNA expression levels in SR1001 (P5–P15) or vehicle control-treated P16 *Vldlr*<sup>-/-</sup> retinas,  $n = 3$  per group. (D) Representative images of a quadrant of *Vldlr*<sup>-/-</sup> retinas with SR1001 treatment (P5–P15) or littermate *Vldlr*<sup>-/-</sup> retinas with vehicle-control treatment. Lesions were highlighted (white) and enlarged in Inset and 3D. (E and F) Quantification of the number and total area of subretinal vascular lesions in SR1001 or control-treated *Vldlr*<sup>-/-</sup> mice.  $n = 9$ –12 per group. (Scale bar, 500 μm for flat mount, 250 μm for Inset, and 100 μm for 3D.) \* $P < 0.05$ ; \*\* $P < 0.01$ ; \*\*\* $P < 0.001$ .

from The Jackson Laboratory (stock no. 000237) and bred together to generate homozygous and wild-type littermates. C57BL/6J mice (stock no. 000664) and *Vldlr*<sup>-/-</sup> mice (stock no. 002529) were both obtained from The Jackson Laboratory.

**Oxygen-Induced Retinopathy.** OIR was carried out as described previously (19), with mouse pups exposed to 75% oxygen at postnatal day (P) P7–P12 followed by room air. At P17, mice were anesthetized, and retinas dissected followed by fluoresceinated isolectin IB<sub>4</sub> (Invitrogen) staining to visualize vessels on whole-mount retinas. Areas of retinal vasoobliteration and pathologic neovascularization were quantified as a percentage of total retinal areas using Adobe Photoshop and Image J.

**Statistics.** Results were presented as mean ± SEM for animal studies and mean ± SD for nonanimal studies. Two-tailed *t* tests (two groups) or ANOVA

(more than two groups) were performed. Differences were considered significant if *P* ≤ 0.05.

Other expanded materials and methods are available in *SI Appendix, SI Methods*.

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- Dorrell M, Uusitalo-Jarvinen H, Aguilar E, Friedlander M (2007) Ocular neovascularization: Basic mechanisms and therapeutic advances. *Surv Ophthalmol* 52(Suppl 1):S3–S19.
- Busik JV, Esselman WJ, Reid GE (2012) Examining the role of lipid mediators in diabetic retinopathy. *Clin Lipidol* 7(6):661–675.
- Curcio CA, Johnson M, Huang JD, Rudolf M (2009) Aging, age-related macular degeneration, and the response-to-retention of apolipoprotein B-containing lipoproteins. *Prog Retin Eye Res* 28(6):393–422.
- Nguyen DV, Shaw LC, Grant MB (2012) Inflammation in the pathogenesis of microvascular complications in diabetes. *Front Endocrinol (Lausanne)* 3:170.
- Bitsch F, et al. (2003) Identification of natural ligands of retinoic acid receptor-related orphan receptor alpha ligand-binding domain expressed in Sf9 cells—a mass spectrometry approach. *Anal Biochem* 323(1):139–149.
- Silveira AC, et al. (2010) Convergence of linkage, gene expression and association data demonstrates the influence of the RAR-related orphan receptor alpha (RORA) gene on neovascular AMD: A systems biology based approach. *Vision Res* 50(7):698–715.
- Schaumberg DA, et al. (2010) Prospective study of common variants in the retinoic acid receptor-related orphan receptor  $\alpha$  gene and risk of neovascular age-related macular degeneration. *Arch Ophthalmol* 128(11):1462–1471.
- Jun G, et al. (2011) Influence of ROBO1 and RORA on risk of age-related macular degeneration reveals genetically distinct phenotypes in disease pathophysiology. *PLoS One* 6(10):e25775.
- Jetten AM (2009) Retinoid-related orphan receptors (RORs): Critical roles in development, immunity, circadian rhythm, and cellular metabolism. *Nucl Recept Signal* 7:e003.
- Kallen J, Schlaeppi JM, Bitsch F, Delhon I, Fournier B (2004) Crystal structure of the human RORalpha ligand binding domain in complex with cholesterol sulfate at 2.2 Å. *J Biol Chem* 279(14):14033–14038.
- Saito T, et al. (2013) Pivotal role of Rho-associated kinase 2 in generating the intrinsic circadian rhythm of vascular contractility. *Circulation* 127(1):104–114.
- Halim TY, et al. (2012) Retinoic-acid-receptor-related orphan nuclear receptor alpha is required for natural helper cell development and allergic inflammation. *Immunity* 37(3):463–474.
- Solt LA, et al. (2011) Suppression of TH17 differentiation and autoimmunity by a synthetic ROR ligand. *Nature* 472(7344):491–494.
- Journiac N, et al. (2009) The nuclear receptor ROR(alpha) exerts a bi-directional regulation of IL-6 in resting and reactive astrocytes. *Proc Natl Acad Sci USA* 106(50):21365–21370.
- Delerive P, et al. (2001) The orphan nuclear receptor ROR alpha is a negative regulator of the inflammatory response. *EMBO Rep* 2(1):42–48.
- Yang XO, et al. (2008) T helper 17 lineage differentiation is programmed by orphan nuclear receptors ROR alpha and ROR gamma. *Immunity* 28(1):29–39.
- Jetten AM, Kurebayashi S, Ueda E (2001) The ROR nuclear orphan receptor subfamily: Critical regulators of multiple biological processes. *Prog Nucleic Acid Res Mol Biol* 69:205–247.
- Lechtken A, Zündorf I, Dingermann T, Firla B, Steinhilber D (2006) Overexpression, refolding, and purification of polyhistidine-tagged human retinoic acid related orphan receptor RORalpha4. *Protein Expr Purif* 49(1):114–120.
- Smith LE, et al. (1994) Oxygen-induced retinopathy in the mouse. *Invest Ophthalmol Vis Sci* 35(1):101–111.
- Hamilton BA, et al. (1996) Disruption of the nuclear hormone receptor RORalpha in staggerer mice. *Nature* 379(6567):736–739.
- Ritter MR, et al. (2006) Myeloid progenitors differentiate into microglia and promote vascular repair in a model of ischemic retinopathy. *J Clin Invest* 116(12):3266–3276.
- Kaštelan S, Tomić M, Gverović Antunica A, Salopek Rabatić J, Ljubić S (2013) Inflammation and pharmacological treatment in diabetic retinopathy. *Mediators Inflamm* 2013:213130.
- Starr R, et al. (1997) A family of cytokine-inducible inhibitors of signalling. *Nature* 387(6636):917–921.
- Qin H, et al. (2012) SOCS3 deficiency promotes M1 macrophage polarization and inflammation. *J Immunol* 189(7):3439–3448.
- Fujieda H, Bremner R, Mears AJ, Sasaki H (2009) Retinoic acid receptor-related orphan receptor alpha regulates a subset of cone genes during mouse retinal development. *J Neurochem* 108(1):91–101.
- Wang Y, et al. (2010) Identification of SR1078, a synthetic agonist for the orphan nuclear receptors ROR $\alpha$  and ROR $\gamma$ . *ACS Chem Biol* 5(11):1029–1034.
- Li C, et al. (2007) Biochemical alterations in the retinas of very low-density lipoprotein receptor knockout mice: An animal model of retinal angiomas proliferation. *Arch Ophthalmol* 125(6):795–803.
- Hua J, et al. (2011) Resveratrol inhibits pathologic retinal neovascularization in *Vldlr*<sup>-/-</sup> mice. *Invest Ophthalmol Vis Sci* 52(5):2809–2816.
- Dorrell MI, et al. (2009) Antioxidant or neurotrophic factor treatment preserves function in a mouse model of neovascularization-associated oxidative stress. *J Clin Invest* 119(3):611–623.
- Chauvet C, Bois-Joyeux B, Berra E, Pouyssegur J, Danan JL (2004) The gene encoding human retinoic acid-receptor-related orphan receptor alpha is a target for hypoxia-inducible factor 1. *Biochem J* 384(Pt 1):79–85.
- Besnard S, et al. (2001) Increased ischemia-induced angiogenesis in the staggerer mouse, a mutant of the nuclear receptor RORalpha. *Circ Res* 89(12):1209–1215.
- Sato T, Kusaka S, Shimojo H, Fujikado T (2009) Simultaneous analyses of vitreous levels of 27 cytokines in eyes with retinopathy of prematurity. *Ophthalmology* 116(11):2165–2169.
- Demircan N, Safran BG, Soyulu M, Ozcan AA, Sizmaz S (2006) Determination of vitreous interleukin-1 (IL-1) and tumour necrosis factor (TNF) levels in proliferative diabetic retinopathy. *Eye (Lond)* 20(12):1366–1369.
- Koskela UE, Kuusisto SM, Nissinen MJ, Savolainen MJ, Liinamaa MJ (2013) High vitreous concentration of IL-6 and IL-8, but not of adhesion molecules in relation to plasma concentrations in proliferative diabetic retinopathy. *Ophthalmic Res* 49(2):108–114.
- Nawaz MI, et al. (2013) Autocrine CCL2, CXCL4, CXCL9 and CXCL10 signal in retinal endothelial cells and are enhanced in diabetic retinopathy. *Exp Eye Res* 109:67–76.
- Gardiner TA, et al. (2005) Inhibition of tumor necrosis factor-alpha improves physiological angiogenesis and reduces pathological neovascularization in ischemic retinopathy. *Am J Pathol* 166(2):637–644.
- Kowluru RA, Odenbach S (2004) Role of interleukin-1beta in the development of retinopathy in rats: Effect of antioxidants. *Invest Ophthalmol Vis Sci* 45(11):4161–4166.
- Lavalette S, et al. (2011) Interleukin-1 $\beta$  inhibition prevents choroidal neovascularization and does not exacerbate photoreceptor degeneration. *Am J Pathol* 178(5):2416–2423.
- Steinmayr M, et al. (1998) staggerer phenotype in retinoid-related orphan receptor alpha-deficient mice. *Proc Natl Acad Sci USA* 95(7):3960–3965.
- Besnard S, et al. (2002) Expression and regulation of the nuclear receptor RORalpha in human vascular cells. *FEBS Lett* 511(1-3):36–40.
- Surjit M, et al. (2011) Widespread negative response elements mediate direct repression by agonist-liganded glucocorticoid receptor. *Cell* 145(2):224–241.
- Stahl A, et al. (2012) SOCS3 is an endogenous inhibitor of pathologic angiogenesis. *Blood* 120(14):2925–2929.
- Solt LA, Banerjee S, Campbell S, Kamenecka TM, Burris TP (2015) ROR inverse agonist suppresses insulinitis and prevents hyperglycemia in a mouse model of type 1 diabetes. *Endocrinology* 156(3):869–881.