Inhibition of the Nuclear Receptor RORγ and Interleukin-17A Suppresses Neovascular Retinopathy

Involvement of Immunocompetent Retinomicroglia

Dean M. Talia, Devy Deliyanti, Alex Agrotis, Jennifer L. Wilkinson-Berka

Objective—Although inhibitors of vascular endothelial growth factor (VEGF) provide benefit for the management of neovascular retinopathies, their use is limited to end-stage disease and some eyes are resistant. We hypothesized that retinoic acid–related orphan nuclear receptor γ (RORγ) and its downstream effector, interleukin (IL)-17A, upregulate VEGF and hence are important treatment targets for neovascular retinopathies.

Approach and Results—Utilizing a model of oxygen-induced retinopathy, confocal microscopy and flow cytometry, we identified that retinal immunocompetent cells, microglia, express IL-17A. This was confirmed in primary cultures of rat retinal microglia, where hypoxia increased IL-17A protein as well as IL-17A, RORγ, and tumor necrosis factor-α mRNA, which were reduced by the RORγ inhibitor, digoxin, and the RORγ/RORγ inverse agonist, SR1001. By contrast, retinal macroglial Müller cells and ganglion cells, key sources of VEGF in oxygen-induced retinopathy, did not produce IL-17A when exposed to hypoxia and IL-1β. However, they expressed IL-17 receptors, and in response to IL-17A, secreted VEGF. This suggested that RORγ and IL-17A inhibition might attenuate neovascular retinopathy. Indeed, digoxin and SR1001 reduced retinal vaso-obliteration, neovascularization, and vascular leakage as well as VEGF and VEGF-related placental growth factor. Digoxin and SR1001 reduced microglial-derived IL-17A and Müller cell and ganglion cell damage. The importance of IL-17A in oxygen-induced retinopathy was confirmed by IL-17A neutralization reducing vasculopathy, placental growth factor, tumor necrosis factor-α, microglial density and Müller cell, and ganglion cell injury.

Conclusions—Our findings indicate that an RORγ/IL-17A axis influences VEGF production and neovascular retinopathy by mechanisms involving neuroglia. Inhibition of RORγ and IL-17A may have potential for the improved treatment of neovascular retinopathies. (Arterioscler Thromb Vasc Biol. 2016;36:1186-1196. DOI: 10.1161/ATVBAHA.115.307080.)

Key Words: ganglion cells ■ interleukin-17 ■ macroglia ■ microglia ■ retina ■ retinoic acid receptor

The discovery that inhibition of vascular endothelial growth factor (VEGF) suppressed neovascularization1 revolutionized the management of neovascular retinopathies, such as age-related macular degeneration and diabetic retinopathy.2,3 Anti-VEGF agents are now used to treat the end stages of these diseases when neovascularization and vascular leakage has developed and vision is threatened.4,5 However, the prevention of retinal vasculopathy remains an unmet and urgent need because disease prevalence continues to be an escalating global health burden.2,4 A compounding factor is that a substantial number of eyes insufficiently respond to anti-VEGF agents.3

The retinoic acid receptor–related orphan nuclear receptors (ROR) are transcriptional regulators comprised of RORα, RORβ, and RORγ.6,7 RORs have important roles in various biological processes, including lipid and steroid metabolism, oxidative stress, and tissue pathologies.6 In terms of vasculopathy, a recent study demonstrated that RORα influences neovascularization,8 but little is known about the contribution of other ROR isoforms. We proposed that RORγ has unrecognized proangiogenic properties in the retina because of its central role in interleukin (IL)-17 production.6,8 IL-17 refers to a family of 6 cytokines (IL-17A to F), with IL-17A (often denoted as IL-17) the most investigated and well characterized.9 There is an increasing evidence that IL-17 has proangiogenic effects in autoimmune diseases and tumors via the upregulation of VEGF.10 Of potential relevance to the treatment of neovascular retinopathies is that tumors are resistant to the antiangiogenic effects of VEGF blockade because of the presence of IL-17.11 Yet, there is limited information about the role of RORγ and IL-17 in neovascular retinopathies, albeit the elevated levels of IL-17 in the plasma of patients with diabetic retinopathy12 and retinopathy of prematurity13 is highly suggestive of a causal role for these factors in disease pathogenesis.

RORγ has a predominant role in the differentiation of T helper 17 (Th17) cells14 and production of IL-17 from this...
cell type and other immune cell populations, including natural killer T cells, γδT cells, lymphoid tissue inducer cells, neutrophils, and macrophages. Microglia are the resident immunocompetent cells of the retina, and when chronically activated in disease, release proinflammatory signals that stimulate the production of trophic factors from neuroglial cells. Hence, it is not surprising that activated microglia have a central role in the pathogenesis of neovascular retinopathies. However, it is unknown if retinal microglia produce IL-17, the predominant ROR isoform involved in disease, and if IL-17 stimulates the release of VEGF from the key neuroglial cell populations involved in retinal vasculopathy, namely macroglial Müller cells and neuronal ganglion cells.

We hypothesized that RORγ and IL-17A promote vascular pathology in the ischemic retina by processes involving neuroglial cells and VEGF. We utilized primary cultures of retinal microglia, macroglial Müller cells, and neurons to determine the cellular source of IL-17A and if RORγ influences its production. Furthermore, we investigated a robust model of retinopathy of prematurity, oxygen-induced retinopathy (OIR), to examine the efficacy of RORγ inhibition and IL-17A neutralization on retinal vasculopathy, the expression of angiogenic factors, gliosis, and ganglion cell loss.

Materials and Methods
Materials and Methods are available in the online-only Data Supplement.

Results
Retinal Microglia, But Not Müller Cells and Ganglion Cells Produce IL-17A Via RORγ
To investigate the source of IL-17A, we performed immunolabeling on retina. Because IL-17A seemed to be present in microglia of OIR mice, we performed dual immunolabeling with IL-17A and the microglial marker, ionized calcium-binding adaptor protein-1 (Iba1). Confocal microscopy revealed that IL-17A immunolabeling was not present in room air controls, but in OIR mice was present in Iba1-positive microglia associated with blood vessels at the retinal surface (Figure 1A). The positive control for IL-17A immunolabeling was mouse spleen (Figure 1 in the online-only Data Supplement). We confirmed that retinal microglia express IL-17 in OIR using flow cytometry to measure the abundance of CD45+CD11b+IL-17A+ cells (Figure 1B).

We next investigated if the ROR isoforms were expressed in retina. In retina of C57BL/6J mice and Sprague Dawley rats, mRNA for RORγ, the predominant ROR isoform involved in IL-17 production, as well as RORα and RORβ were present (Figure 1C). RORγ, the ROR isoform associated with Th17 cells, was not detected, but expressed in mouse and rat thymus, which served as a positive control (Figure 1C). We next studied primary cultures to determine if microglia expressed ROR isoforms, as well as macroglial Müller cells and ganglion cells, as they are key contributors to VEGF production. In all cell types, RORα, RORβ, and RORγ, but not RORδ were detected (Figure 1D). Because the presence of RORγ mRNA does not necessarily result in IL-17A production in disease, we evaluated the effects of hypoxia, the in vitro counterpart of the retinal ischemia that occurs in OIR. In hypoxia, retinal microglia expressed IL-17A protein in cell lysates, which was reduced by the RORγ inhibitor, digoxin (Figure 1E). However, IL-17A was not expressed in Müller cells and ganglion cells exposed to hypoxia, or in the presence of IL-1β, which influences IL-17A production (Figure 1F–1G). As our results indicated that microglia were the main retinal cell type producing IL-17A, we further evaluated if this was linked to RORγ. In cultured microglia, hypoxia increased the protein levels of IL-17A in cell supernatant, IL-17A and RORγ mRNA and the mRNA levels of tumor necrosis factor (TNFα), a cytokine that potentiates IL-17–mediated events (Figure 1H–1K). Digoxin (1 μmol) and the RORγ/RORγ inverse agonist, SR1001, reduced all tested factors, including RORγ mRNA (10 μmol and also 5 μmol for IL-17A and TNFα mRNA; Figure 1H–1K). Hypoxia increased the mRNA levels of RORα which was unaffected by digoxin and SR1001 treatment (Figure II in the online-only Data Supplement).

Retinal Müller Cells and Ganglion Cells Produce VEGF in Response to IL-17A
To determine if Müller cells and ganglion cells produce VEGF in response to IL-17A, we determined if these cell types expressed the IL-17 receptor A (IL-17RA) and IL-17 receptor C (IL-17RC). Both receptors were expressed in Müller cells and ganglion cells as well as microglia, endothelial cells, and pericytes (Figure 2A–2B). In cultured retinal Müller cells, exposure to IL-17A increased the phosphorylation of extracellular-regulated kinase 1/2 (Figure 2C), one of the key intracellular signaling pathways involved in the actions of IL-17A, and stimulated the secretion of VEGF and TNFα (Figure 2D–2E). Ganglion cells also responded to IL-17A by secreting VEGF into cell supernatant (Figure 2F).

RORγ Inhibition Reduced Vasculopathy and Angiogenic Factors in OIR
Digoxin and SR1001’s reduction of IL-17A in vitro suggested that RORγ inhibition might protect against vasculopathy in OIR. The retinal vasculature seemed normal in room air controls (Figure 3A). By contrast, in OIR mice administered vehicle, vasculopathy developed in the expected manner with vaso-obliterration in the central retina and neovascularization in the midperipheral retina (Figure 3A). In OIR, digoxin and SR1001 reduced retinal vaso-obliterration and neovascularization (Figure 3A–3C) as well as vascular leakage (Figure 3D). Both treatments reduced the OIR-induced increase in mRNA and protein levels of VEGF and the VEGF-related factor, placental growth factor (PIGF), as well as TNFα mRNA (Figure
Figure 1. Retinal microglia produce interleukin (IL)-17A via retinoic acid receptor–related orphan nuclear receptor γ (RORγ). A, Representative confocal microscopy images of retina from C57BL/6J mice. In oxygen-induced retinopathy (OIR), dual immunolabeling for IL-17A (green, arrows) and the microglial marker, ionized calcium-binding adaptor protein-1 (Iba1; red, arrows), revealed IL-17A in microglia (arrowheads) associated with blood vessels at the retinal surface (asterisk). IL-17A immunolabeling was not detected in room air controls. Merged images with DAPI nuclear stain (blue). 5 µm cryosections. Magnification ×400. Scale bar, 60 µm. Inset is a high-power image of IL-17A+ microglia. Scale bar, 50 µm. B, Flow cytometry analysis of retina showing IL-17 expression in CD45+CD11b+ microglia. The percentage of IL-17+ microglia is increased in oxygen-induced retinopathy (OIR) compared with controls. ***P<0.001 to control. n=7 to 8 mice per group. C, RORα, RORβ, and RORγ, but not RORγt mRNA, are present in retina from C57BL/6J mice and Sprague Dawley rats. D, RORα, RORβ, RORγ, but not RORγt mRNA, are present in primary cultures of rat microglia, Müller cells, and ganglion cells. E, In microglial cell lysates, IL-17A protein is increased in response to hypoxia (0.5% O2), and reduced by the RORγt inhibitor, digoxin (DIG). F, In Müller cell lysates, IL-17A protein is not present in response to hypoxia and IL-1β, but detected in lysates of rat lymph node, which served as a positive control. G, In ganglion cell lysates, IL-17A protein is not present in response to hypoxia and IL-1β, but detected in lysates of rat lymph node, which served as a positive control. H–K, Microglia exposed to hypoxia expressed IL-17A protein in supernatant and IL-17A, tumor necrosis factor-α (TNFα), and RORγ mRNA in cell lysates, which are reduced by DIG and the RORα/RORγ inverse agonist, SR1001. **P<0.01, and ***P<0.001 to untreated normoxia. #P<0.05, ##P<0.01, and ###P<0.001 to untreated hypoxia. Means±SEM. n=3 independent experiments with triplicate samples within each experiment. INL, inner nuclear layer; IPL, inner plexiform layer; ONL, outer nuclear layer; and U, untreated.
As expected, mice with OIR gained less weight than age-matched room air control mice. Digoxin and SR1001 did not influence the body weights of mice (Table I in the online-only Data Supplement).

**RORγ Inhibition Reduced IL-17A-Positive Microglia and Müller Cell Gliosis in OIR**

To determine if the protective effects of RORγ inhibition in OIR involved microglial-derived IL-17A, we performed dual immunolabeling with Iba1 and IL-17A. The OIR-induced increase in IL-17A immunolabeling in microglia was reduced with both digoxin and SR1001 (Figure 4A). To further evaluate the effect of RORγ inhibition on microglia, we quantitated Iba1-positive microglia. In room air controls, microglia were sparse (Figure 4B), but in OIR control mice administered vehicle, were increased and often associated with blood vessels (Figure 4B). In OIR mice, Iba1-positive microglia were reduced with both digoxin and SR1001 treatment (Figure 4B and 4D). In OIR, the increased production of VEGF by Müller cells is accompanied by their cellular damage, which is readily demonstrated by the increased expression of glial fibrillary acid protein (GFAP). To determine if RORγ inhibition influenced Müller cell gliosis in OIR, we evaluated GFAP immunolabeling. As expected, GFAP immunolabeling was restricted to the retinal surface in room air controls and in OIR control mice was present in the processes of Müller cells (Figure 4C). Both digoxin and SR1001 reduced the OIR-mediated increase in GFAP immunolabeling (Figure 4C and 4E), as well as the mRNA levels of GFAP (Figure 4F).

**IL-17A Neutralization Reduced Vasculopathy and Angiogenic Factors in OIR**

To determine if the direct inhibition of IL-17A protected against retinal vasculopathy, we evaluated the effects of
IL-17A neutralization. The retinal vasculature seemed normal in all room air control groups (Figure 5A). In OIR, IL-17A neutralization reduced retinal vaso-obliteration by 75% and neovascularization by 41% (Figure 5A–5C). Furthermore, in OIR mice, IL-17A neutralization attenuated retinal vascular leakage (Figure 5D) and the increased mRNA and protein levels of VEGF and PlGF, as well as TNFα mRNA (Figure 5E–5I). The IL-17A neutralizing antibody had no effect on the body weight of mice (Table I in the online-only Data Supplement).

**IL-17A Neutralization Reduced Iba1-Positive Microglia and Müller Cell Gliosis in OIR**

We evaluated if IL-17A neutralization protected against the increased microglial cell density and Müller cell gliosis that occurs in OIR. In OIR, IL-17A neutralization reduced Iba1-positive microglia (Figure 6A and 6C) and reduced GFAP immunolabeling (Figure 6B and 6D) and GFAP mRNA (Figure 6E) in retina.

**RORγ Inhibition and IL-17A Neutralization Prevented Ganglion Cell Loss in OIR**

OIR is associated with the death of retinal ganglion cells. We studied the transcription factor Brn3a, a product of the Pou4f1 gene and a robust marker of ganglion cell nuclei, to determine if RORγ inhibition and IL-17A neutralization influenced the loss of ganglion cells. In OIR, fewer Brn3a-positive ganglion cells were detected compared with room air controls (Figure 7). In OIR, digoxin, SR1001, and IL-17 neutralization prevented the reduction in Brn3a-positive ganglion cell nuclei (Figure 7).
Discussion

Recent research has focused on the development of treatment strategies that effectively suppress the excess VEGF and proangiogenic factors produced in neovascular retinopathies. The major finding of this study is that RORγ and IL-17A are previously unrecognized mediators of VEGF production in neuroglial cells of the retina in OIR. Our data indicate that microglia produce IL-17A via RORγ, and IL-17A induces the secretion of VEGF from Müller and ganglion cells. The ability of RORγ blockade and IL-17A neutralization to suppress in OIR the elevated levels of VEGF, associated angiogenic factors and IL-17A, highlights the potential therapeutic use of these treatment approaches. This is supported by these interventions conferring considerable retinoprotection in OIR, including a reduction in capillary degeneration, neovascularization, vascular leakage as well as glial cell dysfunction and ganglion cell loss.

Activated microglia are an important contributor to neovascular retinopathies because of their release of injurious growth factors, reactive oxygen species, and cytokines.
Here, we provide the first report that retinal microglia express IL-17A in OIR and produce this cytokine in response to hypoxia. Typically, Th17 cells are viewed to be the major source of IL-17 because of the activity of ROR$\gamma$.\textsuperscript{7,9} Although increasing evidence indicates that other immune cell populations, such as macrophages, $\gamma$δT cells, lymphoid tissue inducer cells, and natural killer cells, express ROR$\gamma$ and produce IL-17 when exposed to various pathological stimuli.\textsuperscript{9} We utilized a combination of confocal microscopy, flow cytometry, and primary cultures to demonstrate that retinal microglia are the predominant source of IL-17 in OIR retina. These findings do not exclude possible contributions from other immune cell types expressing IL-17, albeit these populations when present in low abundance in small tissues such as neonatal retina are difficult to detect with techniques, such as flow cytometry. We verified that microglia produce IL-17 via ROR$\gamma$ as digoxin, identified as a ROR$\gamma$ inhibitor that does not influence other ROR isoforms,\textsuperscript{20,31} attenuated the hypoxia-induced increase in IL-17A protein and mRNA levels. To be considered when interpreting these findings is that the actions of digoxin may not entirely be because of ROR$\gamma$, as digoxin acts as a cardiac glycoside to inhibit Na$^+$/K$^+$ ATPase activity\textsuperscript{32} and also suppresses the activation of hypoxia-inducible factor-$\alpha$ (HIF-$\alpha$).\textsuperscript{33} However, our findings were validated with SR1001, a first-in-class highly selective inverse agonist of ROR$\alpha$ and ROR$\gamma$ that effectively suppresses Th17 cell differentiation and IL-17 production.\textsuperscript{23,34} Notably, in microglia, both digoxin and SR1001 reduced the hypoxia-induced upregulation of ROR$\gamma$ but not ROR$\alpha$, indicating that the production of IL-17A is linked to ROR$\gamma$.

**Figure 5.** Interleukin (IL)-17A neutralization reduced vasculopathy in oxygen-induced retinopathy (OIR). A, Representative retinal whole mounts labeled with isolectin to demonstrate the vasculature. Scale bar, 0.25 mm. Room air controls have normal blood vessels. In OIR mice administered the control IgG1 antibody, vaso-obliteration (VO) and neovascularization (arrows) developed. In OIR, IL-17A neutralization reduced vasculopathy. B, Percentage of VO per retina in OIR. C, Percentage of neovascularization per retina in OIR. n=7 to 8 mice per group. ***P<0.001 to OIR+IgG1 control. D, In OIR, vascular leakage was reduced by IL-17A neutralization. In OIR, mRNA and protein for vascular endothelial growth factor (VEGF, E and F), mRNA and protein for placental growth factor (PIGF, G, H), and tumor necrosis factor-$\alpha$ (TNF-$\alpha$) mRNA (I) in retina were reduced by IL-17A neutralization. n=5 to 9 mice per group. **P<0.01, ***P<0.001 to room air controls. #P<0.05, ##P<0.01 to OIR+IgG1 antibody. Mean±SEM.
IL-17 has a key role in host pathogen defense, whereas its overproduction promotes inflammation and damage to tissues in various autoimmune diseases. Additional roles for IL-17 have emerged including the stimulation of angiogenesis via the induction of VEGF and angiogenic and inflammatory cytokines, such as TNFα. IL-17–mediated events occur after its engagement with the IL-17 receptors (RA and RC), and subsequent activation of intracellular signaling pathways, including extracellular-regulated kinase 1/2. In retinopathy of prematurity and diabetic retinopathy, damage to macroglial Müller cells results in this cell type becoming a major producer of VEGF to result in neovascularization and vascular leakage. We uncovered that Müller cells express IL-17RA and IL-17RC and are key responders to IL-17A by stimulating the phosphorylation of extracellular-regulated kinase 1/2 and production of VEGF and TNFα. Ganglion cells also have the capacity to synthesize VEGF in situations of retinal disease. Hence, our finding that IL-17A induces the secretion of VEGF from ganglion and Müller cells highlights the potency of IL-17A as a proangiogenic mediator in the retina. Moreover, our results suggest that microglial-derived IL-17A promotes VEGF production from retinal neuroglial cells, albeit a limitation of this study was that we did not directly establish this relationship. Previous studies indicate that cytokines such as IL-6 may work cooperatively with IL-17 to promote injury in the eye. The elevated secretion of IL-6 from retinal microglia exposed to hypoxia as well as increased IL-6 expression in retinal tissue in the early neovascular phase of OIR suggest that IL-6 and possibly other cytokines may potentiate the actions of IL-17 in OIR.

In 2011, Huh et al performed a chemical screen of 4812 compounds and identified digoxin as a specific inhibitor of RORγ transcriptional activity, findings that have been confirmed by other groups. The specificity of digoxin was demonstrated by its inhibition of Th17 cell differentiation and amelioration of Th17-mediated autoimmune disease in mice. A previous study reported that digoxin reduced retinal neovascularization by a mechanism related to the suppression of hypoxia-inducible factor-1α protein levels and target genes, including VEGF. In this study, we confirmed digoxin’s ability...
to attenuate retinal neovascularization. However, our findings that digoxin reduced IL-17A and RORγ in microglia exposed to hypoxia and also IL-17A–immunolabeled microglia in OIR are consistent with digoxin’s retinoprotective effects being due, at least in part, to antagonism of RORγ. Importantly, we extended the protective actions of digoxin in OIR to a reduction in vision-threatening capillary degeneration and vascular leakage, as well as Iba1-positive microglia, ganglion cell loss and Müller cell gliosis, which contribute to breakdown of the blood retinal barrier. Moreover, in OIR mice, digoxin reduced the elevated levels of powerful proangiogenic factors in the retina, including VEGF, PlGF, and TNFα. PlGF is part of the VEGF family, and both synergizes and forms heterodimers with VEGF to stimulate blood vessel growth. Indeed, both VEGF and PlGF are targeted in antiangiogenic treatments for retinopathy, such as aflibercept, and genetic deletion of PlGF in mice protects the retina against diabetic-induced vascular damage.

Confirmation of a central role for RORγ in OIR was achieved with SR1001, a compound that suppresses the actions of RORα and RORγ. SR1001 has been reported to reduce neovascularization in OIR, which was attributed to its effects on RORα. Here, we demonstrated that in OIR, SR1001 has similar effects to digoxin by reducing vasculopathy and proangiogenic factors as well as both IL-17A and RORγ expression. Together, our findings indicate that the beneficial effects of digoxin and SR1001 in OIR involve the RORγ/IL-17A pathway.

A major outcome of this study was that IL-17A neutralization attenuated retinal vasculopathy as well as microglial cell density, Müller cell gliosis, and ganglion cell loss, findings not previously reported in OIR. These results are consistent with evidence that IL-17A promoted blood vessel growth in Matrigel plugs and increased vascular density in tumors and neovascularization in cornea. Of particular interest was the ability of IL-17A neutralization to reduce the elevated levels of VEGF, PlGF, and TNFα in the retina of OIR mice. Currently, anti-VEGF agents such as bevacizumab and aflibercept are used to treat patients with diabetic retinopathy and age-related macular degeneration, although seldom used for retinopathy of prematurity because of VEGF’s key role in neuroprotection in the developing retina. IL-17A blockade may have potential as a new therapeutic avenue for retinopathy of prematurity as well as complement current anti-VEGF approaches for some neovascular retinopathies and have use for eyes resistant to anti-VEGF agents. Supporting this idea is an elegant study by Chung et al showing that IL-17A is responsible for mediating resistance to the antiangiogenic effects of VEGF blockade in the tumor microenvironment.
In conclusion, increasing evidence indicates that reducing IL-17A by RORγ inhibition has beneficial effects in various disease contexts, including diabetes mellitus and arthritis.34,51,52 The ability of RORγ inhibition and IL-17A neutralization to attenuate elevated levels of angiogenic factors and vasculopathy in OIR highlights these approaches as potential new strategies for the treatment of neovascular retinal disease.

Disclosures
None.

References


Inhibition of the Nuclear Receptor RORγ and Interleukin-17A Suppresses Neovascular Retinopathy: Involvement of Immunocompetent Microglia
Dean M. Talia, Devy Deliayanti, Alex Agrotis and Jennifer L. Wilkinson-Berka

Arterioscler Thromb Vasc Biol. 2016;36:1186-1196; originally published online April 7, 2016; doi: 10.1161/ATVBAHA.115.307080

Arteriosclerosis, Thrombosis, and Vascular Biology is published by the American Heart Association, 7272 Greenville Avenue, Dallas, TX 75231
Copyright © 2016 American Heart Association, Inc. All rights reserved.
Print ISSN: 1079-5642. Online ISSN: 1524-4636

The online version of this article, along with updated information and services, is located on the World Wide Web at:
http://atvb.ahajournals.org/content/36/6/1186

Data Supplement (unedited) at:
http://atvb.ahajournals.org/content/suppl/2016/04/06/ATVBAHA.115.307080.DC1
http://atvb.ahajournals.org/content/suppl/2016/04/06/ATVBAHA.115.307080.DC2

Permissions: Requests for permissions to reproduce figures, tables, or portions of articles originally published in Arteriosclerosis, Thrombosis, and Vascular Biology can be obtained via RightsLink, a service of the Copyright Clearance Center, not the Editorial Office. Once the online version of the published article for which permission is being requested is located, click Request Permissions in the middle column of the Web page under Services. Further information about this process is available in the Permissions and Rights Question and Answer document.

Reprints: Information about reprints can be found online at:
http://www.lww.com/reprints

Subscriptions: Information about subscribing to Arteriosclerosis, Thrombosis, and Vascular Biology is online at:
http://atvb.ahajournals.org//subscriptions/
MATERIALS AND METHODS

Inhibition of the nuclear receptor RORγ and interleukin-17A suppresses neovascular retinopathy: involvement of immunocompetent microglia

Dean M. Talia, Devy Deliayanti, Alex Agrotis and Jennifer L. Wilkinson-Berka
Department of Immunology and Pathology, Monash University, Melbourne, Victoria, Australia, 3004

Animals
The Alfred Medical Research and Education Precinct Animal Ethics Committee approved all procedures. C57BL/6J mice and Sprague Dawley rats were purchased from the Animal Resources Centre (Perth, Western Australia).

Primary cultures of retinal cells
The methods to establish and characterize the primary culture of rat retinal microglia, Müller cells and ganglion cells as well as bovine retinal endothelial cells and pericytes are described previously.1, 2 Separate dishes containing purified retinal microglia, Müller cells and ganglion cells were placed in a Modular Incubator Chamber (MIC101; QNA International Pty Ltd, Glenferrie South, VIC, Australia) and exposed to normoxia (21% O2) or hypoxia (0.5% O2) for 4 to 24 hours (microglia), 48 hours (Müller cells) and 8 hours (ganglion cells). The hypoxia conditions were established by pumping the chamber with 0.5% O2, 94.5% N2 and 5% CO2 which was then sealed and placed in a 37°C incubator. Cultures of microglia were administered the retinoic acid orphan nuclear receptors (ROR)γ inhibitor, digoxin3 (0.1, 1 µM; Sigma, St Louis, MI, USA) and the RORα/RORγ inverse agonist, SR10014 (5, 10 µM; Cayman Chemical, Ann Arbor, MI, USA). Müller cell cultures were administered recombinant interleukin (IL)-1β (0, 10, 100 ng/ml, R&D systems, MN, USA) and recombinant IL-17A (0 to 200 ng/ml; Kingfisher Biotech, St. Paul, MN, USA). Ganglion cell cultures were administered IL-1β (0, 1, 10 pg/ml) and IL-17A (0, 1 pg/ml). IL-17A was administered at lower concentrations to ganglion cells than Müller cells, due to the lower survivability of primary cultured neurons5, 6 and their higher sensitivity to stimulatory factors.1 The concentrations of the agents tested were based on previous studies.3, 4, 7, 8 Experiments were repeated 3 times with 3 replicates in each experiment.

Oxygen-induced retinopathy (OIR)
OIR was induced in C57BL/6J mice according to our previous protocol.9 OIR develops over two phases featuring vaso-obliteration followed by neovascularization. Phase I occurred when neonatal mice at postnatal day age (P) 7 were exposed to 75% O2 cycled with 20% O2 for 2 hours per day until P11. Phase II of OIR occurred when mice were exposed to room air from P12 to P18. Comparisons were made to control mice exposed to only room air. Mice were administered by intraperitoneal injection, digoxin (0.25mg/kg/day, Sigma), SR1001 (25mg/kg/day, Cayman Chemical) or vehicle (100% dimethyl sulfoxide). Separate groups of mice received by intraperitoneal injection, mouse anti-IL-17A neutralizing monoclonal antibody (clone 17F3, 100 µg/day, BioXCell, Branford, CT, USA) or mouse IgG1 isotype control monoclonal antibody (100µg/day, BioXCell). Treatments were between P9 to P18. Doses were based on previous studies10, 11 and administered in a 20µl volume. Mice were euthanazed at P18 with sodium pentobarbitone (170mg/ml, Virbac, Peakhurst, NSW, Australia).

Confocal microscopy for IL-17A and microglia
Eyes were embedded in optimal cutting temperature compound (Sakura-Finetek, Zoeterwoude, NL) and frozen in isopentane on dry ice. Five µm cryosections were transferred to slides (SuperFrost; Menzel-Gläser, Braunschweig, Germany), treated with 100% methanol for 1 minute and rehydrated with 0.1M phosphate buffered saline (pH 7.4).
Sections were incubated in 5% BSA for 30 minutes at room temperature and incubated simultaneously with antibodies against anti-mouse IL-17A (1:100, BioXCell) and the microglial marker, anti-ionized calcium binding adaptor protein-1 (Iba1, 1:1000, Wako, Tokyo, Japan) at 4°C overnight. Sections were incubated with goat anti-mouse Alexa Fluor 488 (1:500, Molecular Probes, Eugene, OR, USA) and goat anti-rabbit Alexa Fluor 568 (1:500, Molecular Probes) for 1 hour at room temperature and stained with 4',6-diamidino-2-phenylindole (DAPI, 300 nM, Molecular Probes). Each experiment contained a negative control without the primary antibody and an isotype IgG control. The positive control for IL-17A immunolabelling was mouse spleen (Figure I in the online-only Data Supplement). Sections were visualised with a Nikon A1 laser confocal microscope and images analysed with Nikon image software (NIS-Elements AR 3.0). Four mice per group were evaluated.

**Immunohistochemistry for microglia and Müller cell gliosis**
Using an established method,3 3µm paraffin sections were incubated overnight at 4°C with anti-Iba1 to detect microglia (1:1000, Wako) and anti-mouse glial fibrillary acidic protein (GFAP) to detect Müller cell gliosis (1:500, DakoCytomation, Glostrup, Denmark). A negative control and isotype IgG control were included in each experiment. Iba1 immunolabeling was visualized with a Vectastain ABC standard kit (Vector Laboratories Inc., CA, USA) and DAB substrate chromagen system (Dakoctomation). GFAP immunolabeling was visualized with Alexa Fluor 488-conjugated goat anti-rabbit IgG (1:200, Life Technologies, VIC, Australia). For quantitation, 8 sections at least 60µm apart were randomly selected from each eye. In each section, 4 non-overlapping fields spanning the entire retina were captured at x400 magnification using a Spot digital camera (SciTech, VIC, Australia). Image J (v3.1, National Institutes of Health, Bethesda, Washington, USA) was used to set a threshold for immunolabelling which was applied to all fields. Five to 7 mice per group were evaluated.

**Flow cytometry of retina**
Both retina from each mouse were pooled and digested in collagenase (1mg/ml) and DNase (15U/ml) in RPMI 1640 for 15 minutes at 37°C. The homogenised tissue was filtered through a 40µm strainer (BD Biosciences, San Jose, CA) and enzyme activity quenched with fluorescent activated cell sorting (FACS) buffer (phosphate buffered saline with 2% fetal calf serum and 0.1% sodium azide). The cells were then exposed to Fc Block (BD Biosciences) and resuspended in FACS buffer containing rat anti-mouse CD45, CD11b and IL-17 (BD Biosciences), for 30 minutes at 4°C. This was followed by fixation and permeabilization using the Cytofix/Cytoperm kit (BD Biosciences) and IL-17 staining. FACS analysis was performed on a BD LSR II flow cytometer (BD Biosciences). Dead cells were excluded through staining with either propidium iodide or Invitrogen LIVE/DEAD Fixable Aqua (Life Technologies, VIC, Australia). A minimum of 1 million events were collected per sample and analysed with FlowJo software (Tree Star, Ashland, Oregon, USA). Results are expressed as the percentage of IL-17+CD45+CD11b+ gated cells. Seven to 8 mice per group were evaluated.

**Vaso-oblation and neovascularization**
Retinal wholemounts were prepared as described previously.9 Briefly, each retina was stained with fluorescein isothiocyanate conjugated *Griffonia* (Bandeiraea) simplicifolia BS-I lectin (1:100, Sigma). Individual retinal images were taken at 100x magnification using a digital microscope camera (AxioCam MRc 6.1.0.0., Carl Zeiss, Gottingen, Germany) attached to a Zeiss Axio X1 microscope (Carl Zeiss). Entire retinal montages were achieved using the tiling tool in the AxioObserver software (v5.3, Carl Zeiss). Image J was used to quantitate vaso-oblation using the freehand tool, and neovascularization by pseudocolourizing aggregates of blood vessels. Seven to eight mice per group from 3 different litters of mice were evaluated.
Vascular leakage
Albumin levels in retina were measured using a mouse albumin ELISA kit (Bethyl Laboratories, TX, USA) and normalized to dry retinal weight. Five to 9 mice per group were evaluated.

Quantitative real-time PCR (qPCR)
qPCR was performed as described previously. The primer sequences are found below in Table I. Total RNA was isolated from single retina of 5 to 9 mice per group using the RNeasy mini kit (Qiagen, Doncaster, VIC, Australia), and then 1µg of RNA was subjected to DNase treatment (DNA-free kit, Ambion, Carlsbad, CA, USA) and reverse transcription (First Strand cDNA synthesis kit, Roche, Switzerland). For cultured cells, RNA was extracted using the RNeasy mini kit (Qiagen) and 500ng of RNA was used. The average CT values for ROR isoforms are 20 (rat RORα), 20 (rat RORβ), 29 (rat RORγ), 21 (mouse RORα), 20 (mouse RORβ) and 27 (mouse RORγ). mRNA expression was normalized to 18s rRNA endogenous control and the relative fold difference in expression was calculated using the comparative 2\(^{-\Delta\Delta Ct}\) method.

ELISA
Using an established protocol, retina were homogenized on ice in 0.01M sodium phosphate buffer (pH 9.5) containing protease/phosphatase inhibitor cocktail (1:100, Sigma). The total protein concentration was quantitated using a colormetric assay (Biorad, CA, USA). Undiluted retinal lysates were assayed in duplicate using ELISA kits for rat vascular endothelial growth factor (VEGF) (#DY493, R&D systems). In cultured cells, protein levels of rat IL-17A (eBioscience, San Diego, CA, USA), VEGF (Duoset, R&D systems) and tumor necrosis factor-α (TNFα) (Duoset, R&D systems) were evaluated in cell supernatant. Supernatants for IL-17A levels were concentrated 5X using 10K MW cut-off centrifugal filters (Millipore, Cork, Ireland). Three independent experiments with 3 replicates were used.

Western blot
Methods are described previously. Cultured cells were homogenized in RIPA buffer containing 1:100 protease and phosphatase inhibitor cocktail (Sigma). Total protein levels were quantitated using the Biorad assay. Fifty µg of protein was fractionated by SDS gel electrophoresis and transferred to PVDF membranes (Biorad). Membranes were incubated overnight at 4°C with anti-extracellular-signal-regulated kinase (ERK)1/2 (1:1000, Cell Signaling, MA, USA), as well as anti-placental growth factor (PIGF) (1:200, Cell Signaling), and then washed and stained with goat anti rabbit IgG conjugated with HRP (1:4000) for 1 hour at room temperature. Membranes were developed using the ECL chemiluminescent kit (Thermo, IL, USA) and a film developer and then washed in TBST overnight and incubated with an anti-β-actin antibody (1:1000, Cell Signaling) as loading controls. Quantitation was performed using the v22 Biorad Quantity One 1-D analysis software and optical densities expressed as the ratio between corresponding protein and β-actin.

Statistics
All data were analysed using the GraphPad Prism Software (v.5, San Deigo, California, USA). Normality was assessed by the Pearson, Shapiro-Wilk and Kolmogorov-Smirnov normality tests. Analyses were performed using a one-way ANOVA followed by appropriate posthoc analysis correcting for the number of comparisons and unpaired t-tests (parametric), or a Kruskal-Wallis test followed by Mann-Whitney U tests (nonparametric). Investigators were masked to the groups. A value of \(p<0.05\) was considered significant.
<table>
<thead>
<tr>
<th>Gene</th>
<th>Species</th>
<th>Forward primer</th>
<th>Reverse primer</th>
</tr>
</thead>
<tbody>
<tr>
<td>IL-17A</td>
<td>Rat</td>
<td>CAGGACGAGCGACCATGAG</td>
<td>TTCAGTAGCAGCAACAGCATCA</td>
</tr>
<tr>
<td>IL-17RA</td>
<td>Rat</td>
<td>CTGC GCCCTCCTCAGTCT</td>
<td>GTTCAGATGGAGATAGATGTTTTCG</td>
</tr>
<tr>
<td>IL-17RA</td>
<td>Bovine</td>
<td>TCTGGTGTCAGGTTTGAAGT</td>
<td>AAGTTGGCTGAAAGCATACTC</td>
</tr>
<tr>
<td>IL-17RC</td>
<td>Rat</td>
<td>GACCTCAAACATATTCTTTA</td>
<td>GCCAGAAGCTGTCTCCTACAGA</td>
</tr>
<tr>
<td>IL-17RC</td>
<td>Bovine</td>
<td>GGACCACAGACCATACCTTG</td>
<td>CAGATGCTGTCTCAGACAGAT</td>
</tr>
<tr>
<td>GFAP</td>
<td>Mouse</td>
<td>CTGGAGGTGAGGGACAA</td>
<td>CAGCCTCGAGTTGTTTCAATC</td>
</tr>
<tr>
<td>PIGF</td>
<td>Mouse</td>
<td>CTGCTGGGAAACACTACAG</td>
<td>GCTGCAGACCCACACTTCTC</td>
</tr>
<tr>
<td>TNFα</td>
<td>Mouse</td>
<td>GCCTATGCTCTACCTTCTTC</td>
<td>CACTTTGCTGTGTTTGGCTACGA</td>
</tr>
<tr>
<td>RORα</td>
<td>Rat</td>
<td>TCCTCACAAGGAGAGCTT</td>
<td>AGCACAACACTGCACACTCAC</td>
</tr>
<tr>
<td>RORα</td>
<td>Mouse</td>
<td>GAGACAAATCGTCAGGATCC</td>
<td>GTCGCAACTCAACAGCTCTTCT</td>
</tr>
<tr>
<td>RORβ</td>
<td>Rat</td>
<td>CCGGGATAAACATGTCTGAGA</td>
<td>CTGCCATGCGACGCTGATG</td>
</tr>
<tr>
<td>RORβ</td>
<td>Mouse</td>
<td>CGAGGAATCAAGGCCTATCA</td>
<td>GCCGAACTCACCACCTGACT</td>
</tr>
<tr>
<td>RORγ</td>
<td>Rat</td>
<td>TCTGTGCAAGACTCATCGACA</td>
<td>GTCTGTGAGCTTTCCACATG</td>
</tr>
<tr>
<td>RORγ</td>
<td>Mouse</td>
<td>GGACAGGGCCACACAGA</td>
<td>ACAGATCTTGCAGGGATCAC TTC</td>
</tr>
<tr>
<td>RORγt</td>
<td>Rat</td>
<td>TGAGGCACCCGCTGAGA</td>
<td>TGCAAGGGATCACTTCAATTGTG</td>
</tr>
<tr>
<td>RORγt</td>
<td>Mouse</td>
<td>GGGAGCGAATCTCAGTCTAG</td>
<td>TGTCACACAGATCTTGGCAA</td>
</tr>
<tr>
<td>VEGF</td>
<td>Mouse</td>
<td>AGCAGAATGTCATGAGTA</td>
<td>TCAATCGGACCGCAGTACG</td>
</tr>
</tbody>
</table>

References


SUPPLEMENTAL MATERIAL

Inhibition of the nuclear receptor RORγ and interleukin-17A suppresses neovascular retinopathy: involvement of immunocompetent microglia

Dean M. Talia, Devy Deliyanti, Alex Agrotis and Jennifer L. Wilkinson-Berka
Department of Immunology and Pathology, Monash University, Melbourne, Victoria, Australia, 3004

Supplemental Figures and Figure Legends

Supplemental Figure I. Immunolabeling for IL-17A in C57BL/6J mouse spleen. Three μm paraffin section of spleen showing IL-17A-positive cells (green). DAPI (blue), nuclear stain. Magnification=x400. Scale bar=60μm. In each experiment, mouse spleen served as a positive control for IL-17A immunolabeling.

Supplemental Figure II. mRNA levels of RORα in primary cultures of rat retinal microglia. Microglia were exposed to normoxia or hypoxia (0.5% O₂) for 4 hours. U, untreated. RORα mRNA levels were increased in untreated microglia exposed to hypoxia compared to normoxia controls. In microglia exposed to hypoxia, RORα mRNA levels were unchanged by the RORγ inhibitor digoxin (DIG) and the RORα/ RORγ inverse agonist, SR1001. *p<0.05, **p<0.01, ***p<0.001 to untreated normoxia. Data are Mean±SEM. n=3 independent experiments with triplicate samples within each experiment.
**Supplemental Tables**

<table>
<thead>
<tr>
<th>Groups</th>
<th>Treatment</th>
<th>Body Weight (g)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Room air control</td>
<td>IgG1</td>
<td>8.08±0.11</td>
</tr>
<tr>
<td>Room air control</td>
<td>Vehicle</td>
<td>8.36±0.26</td>
</tr>
<tr>
<td>Room air control</td>
<td>IL-17A neutralization</td>
<td>7.42±0.15</td>
</tr>
<tr>
<td>OIR</td>
<td>IgG1</td>
<td>5.88±0.11**</td>
</tr>
<tr>
<td>OIR</td>
<td>Vehicle</td>
<td>6.98±0.17**</td>
</tr>
<tr>
<td>OIR</td>
<td>IL-17A neutralization</td>
<td>6.09±0.14**</td>
</tr>
<tr>
<td>OIR</td>
<td>Digoxin</td>
<td>6.56±0.21**</td>
</tr>
<tr>
<td>OIR</td>
<td>SR1001</td>
<td>6.49±0.28**</td>
</tr>
</tbody>
</table>

**Supplemental Table I**: Body weights of control and OIR mice at postnatal day 18. Body weights of OIR pups are lower than body weights of room air control pups. **p<0.01 to room air controls. n=6 to 8 mice per group. Values are mean±SEM.
RORγ and IL-17A inhibition

Retinal ischemia

Activated microglia
RORγ

IL-17A secreted

Retinal Müller cells and ganglion cells injured

Angiogenic factors produced (e.g. VEGF, PIGF)

Retinal microvascular injury