Microglia and Interleukin-1β in Ischemic Retinopathy Elicit Microvascular Degeneration Through Neuronal Semaphorin-3A

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Objective—Proinflammatory cytokines contribute to the development of retinal vasculopathies. However, the role of these factors and the mechanisms by which they elicit their effects in retina are not known. We investigated whether activated microglia during early stages of ischemic retinopathy produces excessive interleukin-1β (IL-1β), which elicits retinal microvascular degeneration not directly but rather by triggering the release of the proapoptotic/repulsive factor semaphorin-3A (Sema3A) from neurons.

Approach and Results—Sprague Dawley rats subjected to retinopathy induced by hyperoxia (80% O2; O2-induced retinopathy) exhibited retinal vaso-obliteration associated with microglial activation, NLRP3 upregulation, and IL-1β and Sema3A release; IL-1β was mostly generated by microglia. Intraperitoneal administration of IL-1 receptor antagonists (Kineret, or rytvela [101.10]) decreased these effects and enhanced retinal revascularization; knockdown of Sema3A resulted in microvessel preservation and, conversely, administration of IL-1β caused vaso-obliteration. In vitro, IL-1β derived from activated primary microglial cells, cultured under hyperoxia, stimulated the release of Sema3A in retinal ganglion cells-5, which in turn induced apoptosis of microvascular endothelium; antagonism of IL-1 receptor decreased microglial activation and on retinal ganglion cells-5 abolished the release of Sema3A inhibiting ensuing endothelial cell apoptosis. IL-1β was not directly cytotoxic to endothelial cells.

Conclusions—Our findings suggest that in the early stages of O2-induced retinopathy, retinal microglia are activated to produce IL-1β, which sustains the activation of microglia and induces microvascular injury through the release of Sema3A from adjacent neurons. Interference with IL-1 receptor or Sema3A actions preserves the microvascular bed in ischemic retinopathies and, consequently, decreases ensuing pathological preretinal neovascularization. (Arterioscler Thromb Vasc Biol. 2013;33:00-00.)

Key Words: interleukin-1β ■ diabetic retinopathy ■ microglia ■ semaphorin-3A

Ischemic vasoproliferative retinopathies characterized by a retinal microvascular degeneration followed by an abnormal intravitreal neovascularization have recently been associated with neuroinflammatory responses.1–3 Interleukin-1β (IL-1β), a major mediator of inflammation,4–6 has generally been implicated in the development of vasoproliferative retinopathies, such as in diabetes mellitus7 and choroidal neovascularization.8,9 Yet, IL-1β exerts neurotoxicity,2,10–11 vascular repulsion,12 capillary degeneration,13 induction of neovascular tumor growth,14–15 and seems to modulate angiogenesis by directly interacting with vascular endothelial cells or by enhancing the production of proangiogenic factors in a paracrine manner.5,16–18 In the eye, under ischemic conditions, IL-1β is markedly increased in neutrophils recruited into the retina, endothelial, and retinal glial cells.19 Microglia are phagocytic sentinel of the central nervous system, including the retina, and are also needed for neuronal homeostasis and innate immune defense.20,21 However, under severe insults, including hypoxic/ischemic injury, microglia become overactivated and function as a prominent source of cytotoxic oxidant stress and proinflammatory factors.22–24 In this regard, microglial overactivation can contribute to the production of IL-1β, tumor necrosis factor-α, and IL-6 found in high concentrations in tissues of patients and animals with vasoproliferative retinopathy,25–27 and have been linked to the development of ischemic retinopathies in humans28 and animal models.27,29,30

IL-1β exerts its biological effects by interacting with the IL-1 receptor type I (IL-1RI), which is composed of a
ligand-binding unit and a signaling unit named the accessory protein (IL-1RacP). This activity is modulated endogenously by the natural IL-1 receptor antagonist (IL-1Ra), a 17.5-kDa protein that competes with IL-1 for its binding site on IL-1RI. IL-1Ra is considered an important endogenous anti-inflammatory cytokine that blocks all known actions of IL-1β. Previous studies have shown that recombinant IL-1Ra (Kinetics) protects against IL-1β and excitotoxin-induced neurotoxicity. In vitro, the presence of IL-1Ra has been shown to transform amoeboid microglia into ramified cells, and inhibit endothelial cell death induced by IL-1β by decreasing nuclear factor-kB and caspase-3 activation. IL-1Ra-deficient mice are susceptible to neuronal injury after cerebral ischemia, and, conversely, recombinant IL-1Ra attenuates the neurotoxicity. Intravitreal IL-1Ra also markedly reduces laser-induced choroidal neovascularization.

Our laboratory recently highlighted the influence of neuron-derived signaling molecules on endothelial cell function in the retina. These signals include classic neuronal guidance cues, particularly the class III semaphorins, implicated among its functions in inducing apoptosis. However, the specific interplay of microglia and neurons in the pathogenesis of ischemic retinopathies (such as of prematurity and diabetes mellitus), particularly as it applies to IL-1β, on the critical vascular degenerative component, which precedes the aberrant vaso proliferative phase, is not known.

In this work, we found in the oxygen-induced model of retinopathy of prematurity associated with prominent retinal vaso-obstruction (a sine qua non to subsequent preretinal vasoproliferation) that hyperoxia activates microglia in the retina to produce IL-1β, which sustains the activation of microglia and induces microvascular injury not directly, but through the release of proapoptotic/repressive factor semaphorin-3A (Sema3A) from adjacent neurons. Administration of 2 distinct inhibitors of IL-1R (IL-1Ra, and an all-d [protease resistant] oligopeptidyl tyrosine labeled 101.10) abrogated microglial activation, IL-1β release, and Sema3A expression in the retina and resulted in a significant decrease in vaso-obstruction and acceleration of revascularization of the avascular retina in O2-induced retinopathy (OIR), as seen after knockdown of Sema3A.

## Results

### OIR Triggers Early Production of IL-1β and Other Inflammatory Mediators in the Retina; Inhibition of IL-1β Activity Decreases Inflammation and Retinal Vaso-Obliteration

Animals were subjected to OIR as described. At postnatal day 6 (P6) after exposure to hyperoxia (80% O2) for 20 hours, there was an increase in the retinal mRNA expression of the proinflammatory cytokine, IL-1β (4.1-fold; \( P<0.001 \)), the microglial marker, Iba-1 (2.6-fold; \( P<0.01 \)), and the cytotoxic factor, Sema3A (2.1-fold; \( P<0.05 \)), as well as other inflammatory mediators, such as tumor necrosis factor-α (2.1-fold; \( P<0.01 \)) and intercellular adhesion molecule-1 (1.5-fold; \( P<0.05 \)), compared with the normoxic retinas (Figure 1). Interestingly, the major inflammasome protein NOD-like receptor family pyrin domain containing-3 (NLRP3) was also increased in retina of P6 and P8 hyperoxia-exposed pups (Figure IA in the online-only Data Supplement). mRNA expression of endogenous IL-1Ra did not significantly increase, and that of Caspase-1 and vascular endothelial growth factor (VEGF) also remained unchanged (Figure 1). IL-1Ra and 101.10 (started at P5) significantly attenuated hyperoxia-induced changes in IL-1β, tumor necrosis factor-α, intercellular adhesion molecule-1, Iba-1, and Sema3A.

At P8, 72 hours after exposure to high oxygen, IL-1β and Iba-1 mRNA expression was greater than values in normoxia, albeit lower than those at P6 (Figure 1); IL-1Ra decreased further to below normoxia values. In contrast, Caspase-1, intercellular adhesion molecule-1, and VEGF mRNA increased relative to values at P5, although tumor necrosis factor-α and Sema3A remained steady. Western blot analysis confirmed the hyperoxia-induced increase in IL-1β and Sema3A, whereas VEGF expression decreased (Figure IB in the online-only Data Supplement). IL-R antagonists generally decreased values for all parameters except IL-1Ra and VEGF (Figure 1; Figure IB in the online-only Data Supplement).

At P10, 120 hours after exposure to hyperoxia, the mRNA expression of all inflammatory and apoptotic factors subsided, whereas that of the anti-inflammatory IL-1Ra and of the cytoprotective VEGF increased relative to changes at P8 (during this vaso-obliterative phase).

Both antagonists of IL-1R prevented retinal vaso-obliteration and tended to renormalize vascular density of animals subjected to hyperoxia (Figure 2). Moreover, pups treated with both IL-1R antagonist starting at P8 (3 days after exposure to hyperoxia, which leads to vaso-obliteration) till P10 exhibited a significant increment in normal revascularization (compared with the vehicle-treated rats; Figure 2G), as noted by a gradual decrease in vaso-obliteration; this was further substantiated by a lack of increase in VEGF by P10 (Figure 1). In control normoxic pups, IL-1Ra and 101.10 did not affect developmental retinal vascular density (Figure II in the online-only Data Supplement), consistent with absence of inflammation under normal conditions. Hence, the overall decreased vaso-obliteration by IL-1R inhibitors tended to abolish the hyperoxia-driven surge in VEGF (Figure 1H). Thus, as anticipated, treatments with inhibitors of the IL-1 receptor consequently diminished ensued aberrant preretinal neovascularization (Figure IIIA in the online-only Data Supplement). Additional evidence to substantiate cytotoxic effects of IL-1β on retinal microvasculature was demonstrated by intravitreal injection of IL-1β in normoxia-raised pups (P5), which resulted 24 hours later in a loss of microvasculature (Figure IIIB in the online-only Data Supplement).

To ascertain actions of the all-d peptide 101.10 in retina, its local distribution was studied. 101.10 was conjugated to fluorescein isothiocyanate (FITC), and fluorescence was detected by microscopy in retinas of injected animals. 101.10 localized principally in microglial and endothelial cells in normoxia-raised rats (Figure IV in the online-only Data Supplement). Animals exposed to hyperoxic conditions exhibited a significant increase in vascular and perivascular fluorescence; FITC alone (not conjugated to 101.10) was negligibly detected in retina. Hence, 101.10...
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IL-1β distributes in retina, more so during hyperoxia when IL-1R (to which 101.10 binds) expression increases (Figure 1D in the online-only Data Supplement). To determine the need for IL-1R expression for uptake of 101.10, HEK-Blue cells (that express constitutively IL-1R) and HEK293 cells devoid of IL-1R were stimulated with IL-1β (0–9 hours) in presence of 101.10-FITC; 101.10-FITC internalization was only observed in IL-1R–expressing cells, indicative of the need for IL-1R for cell uptake (Figure V in the online-only Data Supplement).

Together, these results reveal that hyperoxia induces an early increase in the expression of inflammatory mediators in the retina, and show that exogenous IL-1Ra and 101.10 comparably reduce the expression of these inflammatory components, and in turn protect the retina from retinal vascular degeneration, allowing for an accelerated normal vascular regrowth and preventing against ensued preretinal neovascularization.

Figure 1. Hyperoxia triggers the production of inflammatory mediators and other factors in the retina. Quantitative reverse transcriptase polymerase chain reaction analysis on whole retinas from animals exposed to hyperoxia (80% O2) for 20 hours (HypP6) and from P5 to P8 (HypP8) or P5 to P10 (HypP10) treated intraperitoneally with vehicle, interleukin-1 receptor antagonists (IL-1Ra) or 101.10; normoxic control values were set at 1. An increment in the retinal mRNA expression of IL-1β, Iba-1, semaphorin-3A (Sema3A), tumor necrosis factor (TNF)-α, and intercellular adhesion molecule-1 (ICAM-1) was seen from 20 hours after exposure to hyperoxia (80%; at P6) compared with the normoxic retinas. By P8 and P10, the mRNA expression of IL-1β, Iba-1, and Sema3A gradually decreased compared with values at P6. IL-1R inhibitors decreased IL-1β, Iba-1, and Sema3A values at P6 and P8. Values are mean±SEM of 3 to 4 experiments. The fold changes were normalized to 18S as internal control. Significant differences (P value) in the mRNA levels between vehicle and IL-1Ra or 101.10 treatments are shown in the graphs. VEGF indicates vascular endothelial growth factor.

Hyperoxia Leads to Activation of Microglia, Which in Turn Is the Main Generator of IL-1β in Retina

Increased Iba-1 mRNA expression in hyperoxic rats (Figure 1B) was suggestive of microglial activation. We elaborated on this observation by examining microglial Iba-1 immunoreactivity and morphology in situ. IL-1β was mostly found in the inner retina of hyperoxic rats (at P8), largely confined to microglia (Iba-1 immunopositive; a calcium-binding protein expressed by activated microglial cells; Figure 3); baseline immunoreactivity (under normoxic conditions) was very weak (Figure 3). Both exogenous IL-1Ra and 101.10 reduced intensity of IL-1β immunoreactivity in hyperoxic rats (Figure 3). A slight localization of IL-1β was found on endothelium (CD31 immunopositive; Figure VI in the online-only Data Supplement), but hardly on retinal ganglion cells (RGCs; neuronal nuclei immunopositive) and astrocytes (glial fibrillary acidic protein immunopositive; Figure VI in the online-only Data Supplement). IL-1RI expression was increased...
by hyperoxia (at P8), and found throughout all layers of the retina, consistent with its ubiquitous distribution (Figure VII in the online-only Data Supplement); no immunoreactivity to isotype control antibody was detected.

On closer examination, one noted overall increased Iba-1 immunoreactive cells in retinas of hyperoxic rats compared with normoxic ones (Figure 3). Retinal microglia from normoxic rats possessed thin processes that branched as they extended from the cell soma (Figure 3), characteristic of resting microglia. Microglia in retinas of hyperoxic rats were hypertrophied often with amoeboid shape, had fewer processes, which were thicker and shorter, consistent with differentiation into an activated phagocyte (Figure 3). Treatment with inhibitors of IL-1R, IL-1Ra, or 101.10 renormalized the number of activated microglia as well as their morphology, which was more branched out similar to that in normoxic animals (Figure 3); these observations along with those showing decreased Iba-1 mRNA in IL-1R inhibitor–treated animals (Figure 1) suggested that IL-1β participates in the activation of microglia.

**IL-1β Is Released From Microglial Cells Exposed to Hyperoxia In Vitro**

Microglia is activated and IL-1β is released during the hyperoxia-induced vaso-obliteration in OIR (Figures 1 and 3). To confirm microglia as a prominent source of IL-1β under hyperoxic conditions, primary retinal microglial cells from pup rats at P6 were isolated and cultured (Figure 4). Nearly all (>98%) of the isolated DAPI-positive cells from retinas of rats were immunopositive for the microglial specific marker Iba-1 (Figure 4A) as well as lectin Bandeiraea simplicifolia (Figure 4B); cells were immunonegative for macroglial marker glial fibrillary acidic protein (Figure 4C) and for neural marker neuronal nuclei (Figure 4D). Exposure of microglial cells to hyperoxia (80% O₂) reduced significantly the central capillary dropout area to ≈13% (E; #P<0.05 vs hyperoxia [vehicle]), and increased vascular density to ≈78% (F), and promoted revascularization as noted by decrease in vaso-obliteration between P8 and P10 (G; &P<0.001 vs normoxia, #P<0.01 and *P<0.001 vs hyperoxia [vehicle]).

**Figure 2.** Inhibition of interleukin (IL-1) β activity decreases retinal vaso-obliteration and promotes revascularization. Representative images of flat-mounted retinas labeled with *Griffonia simplicifolia* lectin to examine vascularization at P8 (A) and at P10 (D); the central outline delimits the vaso-oblit-erated (VO) region. In the top panels (A–C), rat pups (n=5–6 per group) were treated with vehicle or IL-1R antagonists (IL-1Ra), and maintained in hyperoxia (80% oxygen) from P5 to P8 resulted; one notes a 26% central capillary dropout area (white areas) and 44% decrease in vascular density in controls (compared with pups exposed to 21% oxygen). Whereas IL-1Ra and 101.10 treatments significantly reduced the capillary dropout areas to ≈10% (B; #P<0.05 vs hyperoxia [vehicle treated]), and increased vascular density to ≈85% (C; &P=0.001 vs normoxia, #P<0.05 vs hyperoxia [vehicle]). In lower panels (D–G), rat pups (n=5–6) were maintained in hyperoxia (80% oxygen) from P5 to P10 (D) and treated with vehicle or IL-1R antagonists from P8 (when vaso-obliteration was already present) until P10. In vehicle-treated animals, there was a 29% central capillary dropout area and 48% reduction in vascular density compared with the control. Treatment with both antagonists of IL-1R (starting at P8 and ending at P10; see arrows in G) reduced significantly the central capillary dropout area to ≈13% (E; #P<0.05 vs hyperoxia [vehicle]), and increased vascular density to ≈78% (F), and promoted revascularization as noted by decrease in vaso-obliteration between P8 and P10 (G; &P<0.001 vs normoxia, #P<0.01 and *P<0.001 vs hyperoxia [vehicle]).
Because hyperoxia results in oxidant stress in retina of young developing subjects, we determined its role in inducing IL-1β. The antioxidant glutathione precursor N-acetyl cysteine markedly prevented hyperoxia-induced microglial activation as attested by diminished Iba-1, NLRP3, and IL-1β expression (Figure VIIIB–VIIIE in the online-only Data Supplement).

We also verified in isolated microglia whether IL-1β controls activation of microglia and stimulates further generation of IL-1β, as inferred in vivo using the inhibitors of IL-1R (Figure 1). Indeed, stimulation of microglial cells with IL-1β induced Iba-1 and IL-1β (mRNA) expression, and this effect was abrogated by inhibitors of IL-1R (Figure 4G). This autoamplification of IL-1β expression by exogenous IL-1β was reproduced in IL-1RI–expressing HEK cells as well as in raw-blue mouse macrophages (Figure IXA and IXB in the online-only Data Supplement). Essentially, stimulation with exogenous IL-1β induced an increase in IL-1β mRNA in IL-1RI–expressing HEK cells; this effect was abrogated by IL-1R antagonists; no induction of IL-1β was observed in IL-1RI–devoid HEK293 cells (Figure IXA in the online-only Data Supplement). A similar pattern of response was observed in raw-blue mouse macrophages (Figure IXB in the online-only Data Supplement).

**Figure 3.** Hyperoxia-induced generation of interleukin (IL)-1β is largely produced by activated microglia in the retina. **Left,** Representative confocal images showing immunoreactivity of IL-1β (green) and Iba-1 (red) merged with DAPI (blue and yellow) in retinal cryosections from 8-day-old rats (n=3 per group) after 3 days in normoxia (21% O2) or hyperoxia (80% O2). Co-staining of IL-1β with Iba-1 was detected mainly on activated microglial cells localized in the nerve fiber layer (NFL) or the ganglion cell layer (GCL) in retinas from hyperoxic pups treated with vehicle. Retinas from hyperoxic rat pups treated with IL-1 receptor antagonists (IL-1Ra) or 101.10 reveal a decrement in IL-1β staining and a resting microglia phenotype (Iba-1 labeling) similar to the normoxic animals in the outer nuclear layer (ONL) and GCL. Scale bar, 50 μm. **Right,** Quantitative analysis of the number of Iba-1 immunopositive cells in retinal flat-mounts (n=3–4 per group) from pups exposed to normoxia (21% O2) or hyperoxia (80% O2) from P5 until P8 and treated with vehicle, IL-1Ra or 101.10; &P<0.01 vs normoxia, #P<0.01 vs hyperoxia. Representative photomicrographs from retinal flat-mounts stained with Iba-1 (white) showed a predominant labeling of ramified, nonactivated microglia in animals exposed to normoxic conditions, whereas a large number of amoeboid/activated cells were present in retinas under hyperoxic conditions. Hyperoxic animals receiving IL-1Ra or 101.10 administrations displayed a reverse phenotype of microglia with long protrusions typical of resting microglia. Scale bar, 50 μm.

**Activation of Microglia Is Cytotoxic to Endothelial Cells Through IL-1β–Dependent Induction of Semaphorin-3A in RGCs.** Because microglial activation and increased IL-1β are associated with vaso-oblation during hyperoxia in vivo (Figures 2–4), we next investigated in vitro whether conditioned media from activated microglia in culture (which contains IL-1β; Figure 4F) or whether IL-1β is directly cytotoxic to retinal microvascular endothelial cells. Neither conditioned media from hyperoxia-exposed microglia nor exogenous IL-1β were directly cytotoxic to retinal microvascular endothelial cells. Neither conditioned media from hyperoxia-exposed microglia nor exogenous IL-1β were directly cytotoxic to retinal microvascular endothelial cells. Interestingly, IL-1β has been reported to induce the release of the endothelial proapoptotic and repulsive cue Sem3A from RGCs. First, we confirmed the contribution of Sema3A in vaso-obliteration as seen on exposure to hyperoxia (when IL-1β levels are high; Figure 1). We started by showing that exposure to hyperoxia induced an increase in Sem3A expression in RGCs, and that this increase was abolished by IL-1R antagonists (Figure X in the online-only Data Supplement). Then, we determined the role of Sema3A on vaso-obliteration using shRNA encoded in lentiviral vectors (Lv) as we previously reported. Rat pups were injected at P1 with Lv.shSema3A and the contralateral
A (control) eye with Lv.shGFP; the shSem3A- but not the shGFP-encoded lentivirus knocked down Sem3A expression (Figure 5). As anticipated, Lv.shSem3A, but not Lv.shGFP (control), attenuated the OIR-associated vaso-obliteration at P8 (Figure 5).

We further explored the link between IL-1β and Sem3A. IL-1β induced a time-dependent increase in Sem3A mRNA expression in cultured RGCs, which was significantly attenuated by inhibitors of IL-1R (Figure 6A); similar changes were seen on the protein by Western blot (Figure IE in the online-only Data Supplement). In addition, RGCs stimulated with conditioned media from hyperoxia-exposed (24 hours) microglia (containing IL-1β; Figure 4F) triggered endothelial cell apoptosis as attested by activated caspase-3 (Figure XI in the online-only Data Supplement) and TUNEL positivity (Figure 6; Figure XIIA in the online-only Data Supplement); apoptotic effects of Sem3A were unrelated to VEGF expression (Figure XIIIB in the online-only Data Supplement) as reported. In contrast, hyperoxia-exposed endothelial cells did not exhibit augmented IL-1β formation, and treatment of RGCs with conditioned media from hyperoxia-exposed endothelium did not cause endothelial cytotoxicity (Figure XIC and XIID in the online-only Data Supplement). Together with in vivo evidence that IL-1R inhibitors prevent the early hyperoxia-induced surge in Sem3A (Figure 1C; and Figure X in the online-only Data Supplement), the present findings strongly support an IL-1β–induced Sem3A expression and in turn endothelial cell death, consistent with Sem3A-dependent impaired vascular sprouting. Our results provide evidence for a role for IL-1β, (largely) from sustained activated microglia (Figures 3 and 4), in contributing significantly to hyperoxia-induced vaso-obliteration through a mechanism involving the proapoptotic factor, Sem3A.

**Discussion**

A number of studies have proposed a role for inflammatory mediators in ischemic retinopathies. In this context, the major proinflammatory cytokine IL-1β has been suggested
to contribute to the development of proliferative diabetic retinopathy in humans as well as in animal models, 7,12,25,42 choroidal neovascularization and retinal degeneration 8,9; its role in retinopathy of prematurity has never been specifically evaluated. On one hand, IL-1β is primarily associated with angiogenesis.8,9,18,43 Whereas, on the other hand, IL-1β has also been linked to retinal vascular degeneration 44; this latter aspect remains unexplained. Although microglia are thought to contribute significantly to the generation of cytokines in neural tissue,2,45 their involvement in retinovascular degeneration is elusive; along these lines, microglia (nonactivated) are reported to participate in normal developmental angiogenesis.46 In this study, we show that retinal microglia are overactivated during the vaso-obliteration phase of ischemic retinopathies, and serve as an important source of IL-1β, which induces vascular degeneration not directly but rather by stimulating the generation of proapoptotic/repulsive cue Sema3A from adjacent neurons. Inhibitors of the IL-R, notably exogenous recombinant IL-1Ra and the IL-1R modulator 101.10,39 decreased microglial activation and IL-1β release, and in turn Sema3A expression, consequently diminishing vaso-obliteration, enhancing revascularization and, thus, attenuating resultant pathological preretinal neovascularization in the established model of OIR.

A major feature in this study applies to the pivotal role of microglia not only in generating IL-1β, but also through this cytokine in orchestrating neurovascular degeneration through the release of Sema3A. Although microglia are known to be in intimate contact with neovascular tufts29,30 because they guide the proliferating vascular bed,68 mechanisms for their role in vascular degeneration is vague. We found that in response to hyperoxic stress, microglia are activated and release IL-1β (in vivo and in vitro), which in turn induces its endothelial cytotoxic effects via RGC-derived Sema3A; our findings do not fully exclude a contribution of cytokines by other retinal cells.19 Consistent with these findings, hyperoxic stress is tightly intertwined with inflammation 47 through receptors activated by products of peroxidation, such as CD36,48 TLR4, and stimulation of the inflammasome 49; this oxidant stress41 seems to explain the generation of IL-1β during hyperoxia as observed here and by others.50 Conversely, we found that interfering with IL-1R actions preserved retinal vasculature, enhanced revascularization, and, as a result, prevented preretinal neovascularization. In this context, we observed that it is not so much the number of microglia that changes with the stress stimulus (hyperoxia) but rather their activation. These observations are consistent with microglial activation in human diabetic retinopathy28 as well as in experimental ischemic retinopathies.39 Interestingly, we also found that IL-1β maintains activation of microglia, and inhibition (or downregulation) of IL-1R prevents increased Iba-1 in microglia and IL1β expression in different cell types. Along these lines, inhibition of microglial activation (using minocycline) has been found to diminish cytokine release and reduce...
progression of ischemic retinopathy; whereas exogenous IL-1Ra reduces intercellular adhesion molecule-1 expression and reinstates microglial ramification (resting condition); microglial activation is also reduced in IL-1R1 null mice subjected to hypoxic/ischemic insults. Together our observations, supported by other reports, suggest an autoactivation of microglia through IL-1β.

In the present work, we uncover a new mechanism for IL-1β in inducing retinal microvascular degeneration, specifically by stimulating RGCs to produce the proapoptotic/repressive factor Sema3A. By 20 hours of hyperoxia, microglia were activated through an IL-1β–dependent process, resulting in release of IL-1β (along with other inflammatory markers) and (consequently) in Sema3A; this in vivo induction could be reproduced and better characterized in vitro on cultured microglia exposed to hyperoxia; in turn, IL-1β was also shown to induce Sema3A in isolated RGCs. Consequently, Sema3A was responsible for microvascular endothelial cell death (in vitro and in vivo); hence, inhibition of IL-1R or of Sema3A (anti-Sema3A, or through silencing shRNA-Sema3A) exerted a relative preservation of retinal endothelium. By 120 hours of hyperoxia (at P10), retinal vascular degeneration was found to be pronounced, resulting in a relatively hypoxic inner retina, which triggers an increase in VEGF expression in an attempt to reestablish O2 supply; this does not exclude a direct role for IL-1β in inducing VEGF expression; however, there was a general suppression of inflammatory markers at P10 (compared with P8), which may be attributable to the relative increase in IL-1Ra. Interestingly, one also notes at P10 unaltered levels of Sema3A (compared with normoxia), consistent with a rate of vaso-obliteration, which decreases from P8-P10 compared with P5-P8. Inhibition of IL-1R prevents the early (P8) rise in Sema3A, which contributes to vaso-obliteration, and facilitates revascularization; re-establishment of the O2 supply to the retina suppresses a hypoxia-driven surge in VEGF at P10. A schematic model depicting our overall observations is presented in Figure 8.

A new aspect conveyed in this study applies to the efficacy of the IL-1R modulator 101.10 in ischemic retinopathies. This small stable peptide was as effective as recombinant IL-1Ra. Recombinant IL-1Ra is approved for rheumatoid arthritis, but exhibits certain drawbacks, such as pain at the site of administration, need for injection (rather
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than oral administration), and increased risk for infections. The allosteric modulator of IL-1R 101.10 may offer certain advantages, including possibly through its pharmacological selectivity diminish certain adverse effects such as the risk for infections.39 101.10 may represent a prototype for a new class of small (molecule) inhibitors of IL-1R, which so far are all large molecules requiring parenteral administration.55

In summary, we hereby present a critical role for IL-1β in contributing to the early (and consequently as well to the late) phase of OIR, by activating microglia that are major contributors in IL-1β generation; on one hand, IL-1β sustains microglial activation, and on the other induces adjacent RGCs to produce Sema3A. The latter causes microvascular degeneration contributing to the early vaso-obliteration detected in ischemic retinopathies, which predisposes to subsequent preretinal neovascularization. Distinct inhibitors of IL-1R, notably recombinant IL-1Ra and the modulator 101.10, prevent hyperoxia-induced microglial activation, the surge in IL-1β formation, retinal vaso-obliteration and, consequently, preretinal neovascularization.

Disclosures

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Figure 7. Endothelial apoptotic effects of retinal ganglion cell (RGC)-conditioned media containing semaphorin-3A (Sema3A) obtained by stimulation of RGC-5 with interleukin (IL)-1β are abolished by the immunoneutralization of Sema3A or by blocking the activation of the IL-1 receptor. Representative micrographs of apoptotic neurovascular endothelial cells (RBMVECs) detected by TUNEL (green nuclei) 24 hours after treatment of RGC-5 stimulated or not with IL-1β (RGC-CM), recombinant Sema3A (Sema3A) or conditioned media from RGC-5 cells previously stimulated with IL-1β (0.5 ng/mL) in absence (RGC-CM+IL-1β) or presence of IL-1R antagonists (IL-1Ra; RGC-CM+IL-1β+IL-1Ra and RGC-CM+IL-1β+101.10), or RGC-CM+IL-1β immuno-neutralized with a polyclonal anti-Sema3A antibody (2 μg/mL; RGC-CM+anti-Sema3A). Cells were counterstained with DAPI (blue) to detect nuclei. Scale bar, 100 μm. Histogram compiles the percentage of TUNEL-positive cells determined in 3 different fields for each group (n=3) and normalized to the control (RGC-CM). $P=0.05 vs Norm-RGC Medium, ***P<0.05 vs RGC-CM+IL-1β.
Figure 8. Microglia activation contributes to vascular regression in ROP through interleukin (IL)-1β-dependent stimulation of semaphorin-3A (Sema3A) in retinal ganglion cells: Inhibition of IL-1β activity decreases retinal vaso-obliteration and promotes revascularization. Microglial cells maintain the neuronal homeostasis and immunologic integrity of the healthy retina. Hyperoxia as seen in O$_2$-induced retinopathy and retinopathy of prematurity activates microglia, which become a prominent source of proinflammatory autoactivating IL-1β. IL-1β then stimulates in a paracrine manner the production of Sema3A from adjacent retinal ganglion cells, and in turn induces endothelial cell death. Pretreatment with IL-1R blockers prevents this cascade, retinal vaso-obliteration, and subsequent pre-retinal neovascularization.

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Microglia and Interleukin-1β in Ischemic Retinopathy Elicit Microvascular Degeneration Through Neuronal Semaphorin-3A
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MICROGLIA AND IL-1β IN ISCHEMIC RETINOPATHY ELICIT MICROVASCULAR DEGENERATION THROUGH NEURONAL SEMAPHORIN 3A

MATERIALS AND METHODS

Animal Care

All experimental procedures were approved by the Animal Care Committee of the Hôpital Ste-Justine in accordance with guidelines established by the Canadian Council on Animal Care.

Retinal vaso-obliteration model

Since we focused on retinal vaso-obliteration we utilized a well-established model of retinopathy of prematurity – the oxygen-induced retinopathy model (OIR), characterized by exposing the animals to 80% O₂ during the first days of birth, when vasculature is still immature, and susceptible to damage caused by hyperoxia leading to retinal vaso-oblitration, a hallmark of the initial phase of ischemic retinopathies in humans¹,²; relevantly, this model mimicks the relative hyperoxia premature infants are exposed to relative to levels in utero. Hence, Sprague-Dawley albino rat pups were placed with their mothers in an 80% oxygen environment from P5 to P10, when normal retinal vasculature reaches the periphery. The ability of IL-1Ra or 101.10 to curb vaso-obliteration was tested in two different animal groups exposed to O₂ from P5-P8 (first group) and P5-P10 (second group). Briefly, in each group the pups were randomly selected to receive twice daily 20 µl saline (vehicle), IL-1Ra (10 mg/kg) or 101.10 (1.5 mg/kg) intraperitoneally from P5 to P8 in the first group and from P8 to P10 in the second group; pups were euthanized respectively on P8 and P10. Eyes were enucleated and retinas dissected. Vaso-obliteration was evaluated in retinal flat-mounts. Control animals were maintained in room
air (21% O₂). All other conditions (e.g., light exposure, temperature, feeding, etc.) were similar for both treatment groups.

**Retinal neovascularization model**

Within 4 hours after birth, litters of Sprague-Dawley albino rats (Charles River, St. Constant, Québec, Canada) were placed with their mothers in an oxygen regulated environment (OxyCycler A820CV; BioSpherix Ltd., Redfield, NY) adjusted to alternate between 50% and 10% of oxygen every 24 hours for 14 days. At postnatal day 14 (P14), pups were transferred to room air (21% O₂) for 4 days. Pups were randomly selected to receive twice daily 20 µl saline (vehicle), commercial interleukin-1 receptor antagonist Kineret® [(Amgen, Canada, Inc) by intraperitoneal injections (10 mg/kg) or twice daily the recently reported allosteric modulator of IL-1 receptor, labelled 101.10 [(peptide sequence: rytvela; synthesized by Elim Biopharmaceuticals, Hayward, CA) by intraperitoneal injections (1.5 mg/kg) from birth to sacrifice; the efficacy of doses used has previously been demonstrated. This treatment protocol aimed at preventing vaso-obliteration (induced by high O₂) and consequently neovascularisation. On day P18, rats were anaesthetized with isoflurane (2%) and sacrificed by decapitation. Eyes were enucleated and retinas dissected. Neovascularization was evaluated in retinal flat-mounts stained with lectins. Control animals were maintained in room air (21% O₂) throughout the 18 days. All other conditions (e.g., light exposure, temperature, feeding, etc.) were similar for both treatment groups.

**Retinal Flat-Mounts**

In all cases the eyes were enucleated and fixed in 4% paraformaldehyde for one hour at room temperature and then stored in PBS at 4°C until used. The cornea and lens were removed and the retina was gently separated from the underlying choroid and sclera under a dissecting microscope. Then, the retinas were subjected to 100% cold methanol (-20°C) for 10 minutes,
and incubated overnight at 4°C in 1% Triton X-100-1 mM CaCl$_2$/phosphate-buffered saline (PBS) with the TRITC-conjugated lectin endothelial cell marker *Bandeiraea simplicifolia* (1:100; Sigma-Aldrich, St. Louis, MO). Retinas were washed in PBS and mounted on microscope slides (Bio Nuclear Diagnostics Inc, Toronto, ON) under cover slips with Fluoro-Gel® (Electron Microscopy Sciences, Hatfield, PA) as the mounting media.

Retinas were photographed under an epifluorescence microscope (Nikon, Eclipse E800, Rockland, MA) using a digital camera (Nikon, DXM1200, Rockland, MA) and vascular density was calculated for the full retina surface by using the software AngioTool recently described. $^6$ AngioTool computes several morphological and spatial parameters including vascular density by assessing the variation in foreground and background pixel mass densities across an image. $^6$ Vascular density in study groups was normalized with that of untreated groups raised in 21% O$_2$. Vaso-obliterated areas were assessed as the retinal area devoid of vasculature over the total retinal area. Neovascularization was analyzed using the SWIFT-NV method, $^7$ that consists of a set of macros that was developed to quantify all the pixels represented by neovascular tufts and clusters, but not normal vessels in lectin-stained retinal whole mounts.

**Retinal uptake of 101.10 in vivo.**

Pups were randomly selected to receive 40 µl of the peptide 101.10 labelled with fluorescein isothiocyanate (101.10-FITC), or FITC (alone; Sigma-Aldrich) as control by intraperitoneal injections (3 mg/kg) or enteral administration (5 mg/kg) in rats exposed to normoxia (21% O$_2$) or hyperoxia (80% O$_2$). Animals were euthanized 3 hours after drug administration, and retinas were evaluated in retinal flat-mounts co-stained with lectins and 4,6-diamidino-2- phenylindole, dihydrochloride (DAPI; 0.1 µg/mL; Molecular Probes).

**Intravitreal injections of IL-1β**

On day P5, pup rats were anaesthetized with isoflurane (2%) and intravitreally injected
into the left eye with 5ng/1µl of IL-1β, and sterile PBS was injected into the contralateral eye as control. Twenty-four hours after the administration of IL-1β, the animals were euthanized, the eyes were removed, and retinal flat-mounts stained by the lectin technique and vascular density evaluated using image J.

**Immunofluorescence in retinal cryosections**

Seventy two hours after exposure to hyperoxia, eyes of rats treated or not with IL-1Ra or 101.10 and their corresponding controls (n=3 in each group) were enucleated and fixed in 4% paraformaldehyde at room temperature for 4 hours, incubated in 30% sucrose overnight, and then frozen and cut. Sagittal cryosections were blocked in PBS containing 1% bovine serum albumin, 1% normal goat serum, 0.1% Triton X- 100 and 0.05% Tween-20 for 1 h and double labeled overnight at 4°C with a 1:300 dilution of anti-IL-1β polyclonal (R&D Systems), 1:300 dilution of anti-IL-1RI polyclonal (Santa Cruz Biotechnology, Inc), 1:200 of anti-Sema3A polyclonal (Abcam, Inc) or a 1:100 dilution of the anti-IL-1β monoclonal antibodies and antibodies for cell-specific proteins: microglia [Iba-1, 1:500; Wako Chemicals USA, Inc], astrocytes [GFAP, 1:500; Dako, Carpinteria, CA], neurons [NeuN, 1:100, Chemicon] and endothelium [anti-CD31, 1:100 Immunologicals Direct). The primary antibodies were labeled for 2 h with Alexa-594-conjugated goat anti-rabbit or anti-mouse IgG, or Alexa-488 goat anti-rabbit or anti-mouse IgG obtained from Molecular Probes (Eugene, OR) and used at dilutions of 1:1000 (anti-rabbit) or 1:500 (anti-mouse), respectively. Incubation with isotype antibody was used as control. Labeled retinas were examined with a laser scanning confocal microscope (Zeiss LSM 510).

**Primary cultures of retinal microglia cells**

Microglial cells were isolated from retinas of rats (6 days old) according to the method
described previously. Briefly, the eyes were enucleated, and the retinas were collected in ice-cold 0.01 M PBS. They were soaked in Dulbecco’s modified Eagle’s medium/F12 (1:1) (DMEM; Invitrogen, CA, USA) supplemented with 100 U/ml penicillin and 100 µg/ml streptomycin, and then washed in Hank’s balanced salt solution, cut into small pieces, and digested with 0.5% trypsin for 30 min at 37°C. Then DMEM/F12 (1:1) with 10% fetal bovine serum (FBS; Hyclone, Logan, UT, USA) was added to terminate trypsinization, the retinal pieces were manually dissociated by trituration and centrifuged. The supernatant was eliminated and the cell pellet was resuspended in DMEM/F12 (1:1) + 10% FBS supplemented with 10 ng/ml recombinant murine granulocyte–macrophage colony-stimulating factor (rmGM-CSF, PreproTech, Rocky Hill, NJ, USA) and allowed to grow at 37°C in 5% CO₂ in 75-cm² flasks at a density of 1X10⁶ cells/mm². All cultures were maintained in a humidified CO₂ incubator (Sanyo Biomedical) and fed every 2 days. After 2 weeks, microglia were harvested by shaking the flasks at 200 rpm for 4 h in an orbital shaker at 37°C. The cell suspension was centrifuged at 1000 rpm for 5 min, and the cell pellet was resuspended in DMEM/F12 (1:1) + 10% FBS. The purity of the microglia in the cultures was determined by immunostaining the cells with Ionized Calcium Binding Adaptor Molecule-1 (Iba-1), Glial Fibrillar Acidic Protein (GFAP), or Neuronal Nuclei (NeuN), as specific markers for microglia, astrocytes and neurons, respectively. The percentage of microglial cells was about 97%, which was established after 2 weeks.

**Microglia activation in vitro and quantification in vivo**

To study whether IL-1β regulates the activation of microglia, isolated retinal microglial cells (150,000 cells/well) were treated with IL-1β (0.5 ng/ml) in presence or absence of IL-1Ra or 101.10. After 24 hours, the mRNA expression of IL-1β and Iba-1 was analyzed by qPCR. For microglia quantification, retinal flat-mounts stained with an anti-Iba-1 polyclonal antibody were used. Four representative fields in the central (vasoobliterated) zone were randomly selected in each retina. Activated microglial cells were recognized by their short and thick processes and
their increased immunoreactive staining of Iba-1 (as well as IL-1β).\textsuperscript{10,11} The average number of Iba-1 positive cells from the four fields for each retina was calculated by using Image J. The mean and standard error of these averages from at least 4 retinas in each group was calculated.

**Preparation of microglia-conditioned media (MGCM)**

To study the effects of hyperoxia on expression of IL-1β, microglial cells (250, 000 cells) were cultured in plates of 25 cm\(^2\) (Sarstedt, Inc, Newton, NC, USA) with DMEM/F12 (1:1) + 10% FBS. After 24 hours the cells were starved with DMEM/F12 (1:1) free of FBS for 24 hours. Then the cells were exposed to hyperoxia (80% oxygen and 20% nitrogen; Hyp-MGCM) in a modular incubator chamber (Billups-Rothenberg, Inc) and maintained in a humidified CO\(_2\) incubator at 37\(^\circ\) C for 4, 12, and 24 h. Microglial cells in matching controls (Nor-MGCM) were incubated at 37\(^\circ\) C in an incubator with 95% air and 5% CO\(_2\) and collected at the same time points.

**Reverse transcription and quantitative real-time PCR analysis.**

Eyes were enucleated and retinas were rapidly dissected and processed for RNA using TRizol (Invitrogen). Total cellular RNA was isolated by acidic phenol/chloroform extraction followed by treatment with DNase I (Roche Diagnostics, Mannheim, Germany) to remove any contaminating genomic DNA; 500 ng of RNA was reverse transcribed into cDNA with Moloney murine leukemia virus reverse transcriptase (Promega, Madison, WI) in 25\(\mu\)L of reaction mixture. PCR primers targeting for IL-1β; (5'-CATCTTTGAGAGAGACCCCG-3' and 5'-GGGATTTTTGTGGTTGCTGT-3'), Sema3A; (5'-GAGTGCTTATTCCACGACCA-3' and 5'-AATGCTTCTCCGCTCTGAA-3'), VEGF; (5'-CAATGATGAAGCCCTGGAGT-3' and 5'-AATGCTTCTCCGCTCTGAA-3'), Iba-1; (5'-AGAGGTGTTCCAGTGCTCCGA-3' and 5'-GTCTCTCGTCCACCGGT-3'), ICAM1; (5'-TGCAAGCCGGAAAGCAGATGT-3' and 5'-CACGATCAGAAGCCCGCAAT-3'), IL1Ra; (5'-TCTGTGTGGCTAACCACAT-3' and 5'-
ACTTGGGGGATTGTCAAGTG-3'), Caspase-1; (5'-AAGGCACGAGACCTGTGCGAT-3' and 5'-ACCACTCGGTCCAGGAAATGCG-3'), TNF-α; (5'-CTATGTGCTCCTCACCACA-3' and 5'-TGGAAGACTCCTCCAGGTA-3'), IL-1R1; (5'-TGAATGTGGGCTGAAGAGCAC-3' and 5'-CGTGACGTGAGATCAGTT-3'), NLRP3; (5'-TGCGATCGGATGGT-3' and 5'-ACGGCGTTAGCAGAAATCCA-3') were designed using Primer Bank and NCBI Primer Blast software. Quantitative analysis of gene expression was generated using an ABI Prism 7700 Sequence Detection System and the SYBR Green Master mix kit (BioRad) and gene expression was calculated relative to 18S universal primer pair (Ambion) expression using the ΔcT method.

**Western Blot**

Retinal samples were obtained as described above. Three retinas from different animals per sample (n=3 samples per group) were collected in tubes (Precellys Lysing Kit, 0.5 ml, MEDICORP, Montreal, CA) containing 100 µl lyses buffer and proteases inhibitors and then homogenized in a tissue homogenizer (Precellys®24 Cat. 03119.200.RD000) at 5000 rpm-3x15seg. The samples were then centrifuged at 10,000 rpm during 10 minutes at 4°C and the supernatant was collected. 50 µg of protein was loaded on an SDS-PAGE gel and electroblotted onto a PVDF membrane. After blocking, the membranes were incubated overnight with 1:200 rabbit antibody to VEGF-A (sc-152; Santa Cruz Biotechnology), 1:1000 rabbit antibody to Sema3A (ab23393; Abcam), 1:200 goat antibody to rat IL-1β (MAB501; R&D), 1:500 rabbit antibody to IL-1R (sc-689; Santa Cruz Biotechnology) and 1:1000 mouse antibody to β-actin (sc-47778; Santa Cruz Biotechnology). After washing, membranes were incubated with 1:5000 horseradish peroxidase conjugated anti-rabbit, anti-goat or anti-mouse secondary antibodies (Amersham) for one hour at room temperature.
MTT assay

MTT [3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide] assays were performed to assess endothelial or microglia cell viability. Briefly, rat brain microvascular endothelial cells (RBMVEC, Cell Applications, Inc, 1.8 × 10^6 cells) were seeded in 48-well plates and treated for 24 h with recombinant rat IL-1β (500 pg/mL; 400-01B; PeproTech), recombinant Sema3A (R&D Systems), normoxic or hyperoxic microglia-conditioned media (Norm-MGCM or Hyp-MGCM), Norm-MG-RGC-CM or Hyp-MG-RGC-CM or with normoxic or hyperoxic endothelial-RGC-conditioned media (Nor-EC-RG-CM or Hyp-EC-CM) in presence or absence of IL-1Ra or 101.10. To analyze microglia cells viability, the cells were cultured in 6-well plates (2.4 x 10^5) and exposed to hyperoxia (80% O_2) or normoxia (21% O_2) in presence or absence of N-acetylcysteine (NAC; 8 mM). After treatment in both types of cultures, the medium was removed and MTT (5 mg/mL, 30 µl/well) was added followed by incubation at 37°C for 4 h. Afterwards, supernatants were carefully removed, and DMSO (30 µl/well, Sigma-Aldrich, St. Louis, MO) was added to the cells. After insoluble crystals were completely dissolved, absorbance at 590 nm was measured using microplate reader (Awareness Technology, Inc). Cell viability was expressed as a percentage of optical density relative to control.

TUNEL assay

Retinal ganglion cell line (RGC-5, 2.5 x 10^4 cells) was kindly provided by Neeraj Agarwal (University of North Texas Health Science Center, Fort Worth, TX). RGC-5 supernatant (RGC-CM) was collected after 24 hours of stimulation with Hyp-MGCM, Norm-MGCM or directly with pure recombinant rat IL-1β (0.5 ng/ml) in presence or absence of IL-1Ra or 101.10. Hyp-MG-RGC-CM, Norm-MG-RGC-CM or RGC-CM+IL-1β was centrifuged to remove debris and filtered with 0.2-µm filters (Millipore). To eliminate the Sema3A present in the RGC-CM, the collected medium was filtered with a Centricon (10000 MWCO, Millipore). The concentrated medium was
discarded and the filtered media was immu-neutralized with a polyclonal anti-Sema3A antibody (2 µg/ml, Abcam) in the RBMVEC assay. RBMVEC (1.8 × 10^5 cells) were seeded in 48-well plates and treated for 24 h with Hyp-MG-RGC-CM, Norm-MG-RGC-CM or IL-1β-RGC-CM. After the treatment, the medium was removed and TUNEL assay was performed with an *in situ* cell-death detection kit (ApopTag® Plus, Chemicon International.) according to the manufacturer’s instructions. After TUNEL staining, the samples were counterstained with 4,6-diamidino-2-phenylindole, dihydrochloride (DAPI; 0.1 µg/mL; Molecular Probes).

**Immunofluorescence in RBMVEC cells**

After the treatment with Norm-MG-RGC-CM or Hyp-MG-RGC-CM for 24 hours, the medium was removed and rat brain microvascular endothelial cells (RBMVEC, Cell Applications, Inc) were washed two times with PBS, fixed with 4% paraformaldehyde for 10 minutes and incubated with a 1:500 dilution of activated caspase-3 polyclonal antibodies (Cell Signaling Technology) or a 1:300 dilution of the anti-β-actin monoclonal antibodies (Santa Cruz Biotechnology, Inc). The primary antibodies were labelled for 2 h with Alexa-488 goat anti-rabbit (1:1000) or Alexa-594-conjugated goat anti-mouse (1:500). RBMVEC cell were co-stained with DAPI (0.1 µg/mL; Molecular Probes) and examined with a laser scanning confocal microscope (Zeiss LSM 510).

**ELISA**

Conditioned media from microglial cells exposed to hypoxic or hyperoxic conditions in presence or absence of N-acetyl cysteine (NAC; Sigma-Aldrich) was collected and concentrated in Ultrafree-4 centrifugal filter unit (10,000 NMWL, Millipore) at 3,500 rpm for 10 minutes at 4°C. The concentrated media (5 times) was collected and analyzed by ELISA according to the
manual instructions (Rat IL-1β/1L-1F2 immunoassay R&D Systems, Inc, Cat. RLB00) to determine the IL-1β concentration in each condition.

**Internalization of 101.10 in HEK cells**

HEK-Blue™ IL-33/IL-1β cells (InvivoGen) designed to detect bioactive IL-1β (generated by stable transfection of the IL1-RL1 gene) and HEK293 cells (ATCC®) that do not express the IL-1RI were used. Both cell types were cultured following the manufacturer's protocols. Briefly, HEK-Blue™IL-1β and HEK-293 cells were cultured in 24 well plates at densities of approximately 50,000 cells/well in 500 µl of complete growth medium consisting of DMEM supplemented with 4.5 g/l glucose, 10% FBS, 50 U/ml penicillin, 50 mg/ml streptomycin, 100 mg/ml Normocin™ and 2 mM L-glutamine for HEK-Blue™ IL-1β cells or ATCC-formulated Eagle's Minimum Essential Medium, supplemented with 10% fetal bovine serum [FBS], 100 U/ml penicillin, and 100 µg/ml streptomycin for HEK 293 cells. All cultures were maintained in a humidified incubator at 5% CO₂ and 37 °C. After overnight incubation, the cells were starved with Eagle's Medium or DMEM free of FBS for 4 hours. Then the cells were stimulated with human recombinant IL-1β (50 ng/ml/well) and incubated with 101.10-FITC or FITC alone (control) for 3, 6 and 9 hours. After incubation the cells were washed three times with PBS, fixed with 4% paraformaldehyde, stained with DAPI. Microphotographs showing the uptakes 101.10-FITC were made by confocal microscopy.

**IL-1β stimulation in RAW-Blue mouse macrophage and HEK blue cells**

Transfection with siRNA macrophages was performed as follows. Twenty micrograms of siRNA complex was mixed with 8 ml of 1mg/ml polyethylenimine solution (Polyscience Inc.) for 30 minutes and added to RAW-Blue mouse macrophages (Invivogen). The following day cells were treated with mIL-1β (50 ng/ml) at 37°C for 4 hours. HEK blue and 293 cells were treated
with human recombinant IL-1β in presence or absence of $1 \times 10^6$ M of IL-1Ra or 101.10. After treatment, the cells were collected in TRizol (Invitrogen) and total RNA was isolated. 500 ng of RNA was combined to qScript cDNA SuperMix (Quanta Bioscience) and cDNA synthesis was performed following the manufacturer’s protocol. Quantitative real-time PCR was performed on MxPro3000 (Stratagene) using iTaq SYBR Green SuperMix with ROX (Bio-Rad). siRNA primers and qPCR primers were synthesized by Alpha DNA and sequences were: siRNA: simIL-1RF$_{557}$AGUAACCGUAACUGUUATT; simIL-1R$_{557}$UAACACAGUUACGGUUACUTT; simIL-1RF$_{1794}$GAAAGACCACAGUCUGCAATT; simIL1RR$_{1794}$UUGCAGACUGUGGUCUUUCTT; mIL1RF: AACCTTTGACCTGGGCTGTC; mIL1RR: CAGAGGATGGGCTCTTCTTCAA; mIL1bF: AGATGAAGGGCTGCTTCCAAA; mIL-1bR: GGAAGGTCCACGGGAAAGAC; mRNA expression levels were normalized against 18S rRNA endogenous control levels in each sample and calculated relative to control vehicle-treated cells.

**Lentivirus production**

Lentiviral vectors (HIV-1 derived) were prepared as we previously reported$^{12}$ by transfecting HEK293T cells with a vector plasmid containing the small hairpin RNA (shRNA) against Sema3A or green fluorescent protein together with the third generation packaging plasmids pV-SVG, pMDL, and pREV (Open Biosystems).

**Intravitreal injections of lentivirus**

P2 rat pups were anesthetized with 3.0% isoflurane and injected intravitreally with 1 µL of lentivirus using a 10-µL Hamilton syringe fitted with a 50-gauge glass capillary tip. Approximately 500 ng/µL of lentivirus shGFP or containing shSema3A was injected. From P5 to P8 the animals were exposed to hyperoxia (80% oxygen). At P8, the animals were sacrificed and vaso-obliteration was assessed in retinal flat-mounts.
Statistical Analysis

Results are expressed as mean ± SEM. Two-tailed independent Student t tests was used to analyze data. Comparisons between groups were made using 1-way ANOVA followed by the post hoc Bonferroni’s multiple comparison test. Statistical significance was set at p<0.05.
REFERENCES

MICROGLIA AND IL-1β IN ISCHEMIC RETINOPATHY ELICIT MICROVASCULAR DEGENERATION THROUGH NEURONAL SEMAPHORIN 3A

SUPPLEMENTARY FIGURES AND FIGURE LEGENDS

A

NLRP3 expression in the retina

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Supplemental Figure I. NALP3 inflammasome expression in the retina and protein detection of different mediators induced by hyperoxia. Measurement by quantitative PCR of the mRNA expression of NLRP3 inflammasome in retinas (n=3) from rat pups exposed to normoxia (21% O₂) or hyperoxia (80% O₂) during 1, 3 and 5 days (A). *p<0.01 vs 21% O₂. Representative Western blots showing IL-1β (17 kD), Sema3A (~95 kDa) and VEGF (21 kDa) immunoreactive bands in retinal homogenates at P8 (B) or P10 (C) after normoxic (21% O₂; as control) or hyperoxic (80% O₂) exposure treated with vehicle, IL-1Ra or 101.10. Expression of IL-1R (80 kDa) was increased by hyperoxia and suppressed by both IL-1R antagonists (D). RGC-5 cells treated with IL-1β (0.5 ng/ml) for 24 h and evaluated by Western blot in cell lysate showed increased Sema3A expression that was suppressed with IL-1Ra or 101.10 (E). In all the cases β-actin (42 kDa) was used as internal control.
Supplemental Figure II. Administration of IL-1 antagonists does not affect normal retinal vascularization. Representative lectin-stained retinal micrographs showing vascularisation, and vascular density histogram of animals treated with intraperitoneally with vehicle, IL-1Ra or 101.10 from P5 to P14 (sacrifice). Data are the mean ± SEM for n=3 independent experiments.
Supplemental Figure III. IL-1R inhibitors prevent pre-retinal neovascularization, and intravitreal IL-1β increases Sema3A expression and causes retinal microvascular injury. Representative lectin-stained retinal flat mounts (showing vascularity) at P18 (A) and compiled histograms of percent neovascularization. Intraperitoneal administration of IL-1Ra or 101.10 from P0-P14 prevented pre-retinal neovascularization. Data are the mean ± SEM for n=3 independent experiments. *$p<0.01$ vs normoxia, $^\#p<0.01$ vs OIR + vehicle. (B) Lectin-stained retinal photomicrographs in animals treated with intravitreal IL-1β (5ng/1µl) or vehicle (PBS). Below representative micrographs are the compiled histograms of vascular density and Sema3A mRNA expression. $^\ast p<0.01$ vs vehicle. Scale bar = 50 µm.
Supplemental Figure IV. The peptide 101.10 (rytvela) is mostly distributed in the retina in microglia and endothelial cells during hyperoxia. Peptide 101.10 was conjugated to FITC (green) and injected intraperitoneally in animals (at P5) exposed to normoxia (A) or hyperoxia (B); FITC alone was used a negative control (C). Fluorescence was analyzed 3-6 hours after peptide administration by confocal microscopy in retinal flat-mounts stained with lectin (red). Primarily upon exposure to hyperoxia (80% O₂), 101.10-FITC co-localized with lectin (yellow), corresponding to microglial (thick arrows) and endothelial (thin arrows) cells. Nuclei were counter-stained with DAPI (blue). Scale bars = 50µm.
Supplemental Figure V. Cellular internalization of 101.10 is dependent on the presence of the IL-1R. Internalization of 101.10-FITC in HEK blue cells (expressing IL-1R) was increased time-dependently at 3, 6 and 9 hours after stimulation with IL-1β; HEK 293 cells (devoid of IL-1R) do not internalize IL-1R. FITC alone was used as control (n=3-4 per group).
Supplemental Figure VI. IL-1β is minimally produced by astrocytes, neurons and endothelium during hyperoxia. Representative confocal images showing immunoreactivity to IL-1β (green), GFAP, NeuN, CD31 (red), merged with DAPI (blue and yellow) in retinal cryosections (n=3) from 8-day-old rats after 3 days of hyperoxia (80% O₂). A slight co-localization of IL-1β with CD31 (endothelial marker) was detected. Scale bar = 50 µm.
Supplemental Figure VII. Localization of IL-1R in the retina. Representative confocal images from 3 experiments showing the immunoreactivity to IL-1RI (principally the inner plexiform layer [IPL], in green), IgG’s (Control) and DAPI in retinal cryosections from 8-day-old rats exposed for 3 days to normoxia or hyperoxia (80% O₂). IL-1RI immunoreactivity was increased by hyperoxia and mainly detected on neurons (NeuN) in the ganglion cell layer (GCL), and very slightly in microglia (Iba-1) and vessels (CD31), but not in astrocytes (GFAP). Scale bar = 50 µm.
Supplemental Figure VIII. NLRP3 inflammasome, IL-1β and Iba-1 expression in microglia cultures are increased during hyperoxia. Measurement by quantitative PCR of the mRNA expression of NLRP3 (B), IL-1β (C), Iba-1 (F) and IL-1β secretion measured by ELISA (D) or cell viability evaluated by MTT assay (E) in primary microglial cultures exposed to hyperoxia (80% O₂) or normoxia (21% O₂) in presence or absence of N-acetylcysteine (NAC; 8mM). Data are the mean ± SEM for n=4-5 independent experiments. *p<0.01 versus 21% O₂.
RAW BLUE MOUSE MACROPHAGES

A

HEK Blue Cells

IL-1β mRNA expression/18S (relatively change to control)

Control + IL-1β L-1β+IL-1Ra L-1β+10.10 + IL-1β

HEK 293

B

IL-1β mRNA expression/18S (relatively change to control)

Control +IL-1β siRNA IL-1R+IL-1β

* #
Supplemental Figure IX. Autostimulation of IL-1β in HEK blue cells and macrophages. (A) Exogenous IL-1β (50 ng/ml; 4 h) induced IL-1β mRNA expression (qPCR) in HEK blue cells (containing IL-1R) but not in HEK 293 cells devoid of IL-1R; induction was prevented by IL-1Ra and 101.10. Histogram values are mean ± SD of three independent experiments. * p<0.05 vs Control, &## p<0.05 vs IL-1β treatment. (B) Exogenous IL-1β (50 ng/ml; 4 h) induced IL-1β mRNA expression (qPCR) in RAW-Blue mouse macrophages; silencing of IL-1RI (siRNA-IL-1RI [see insert]) prevented this induction. Histogram values are mean ± SD of three independent experiments. # p<0.05 vs Control.
Supplemental Figure X. Semaphorin 3A (Sema3A) is largely produced by retinal ganglion cells (RGC) in the retina under hyperoxia. Representative confocal images showing immunoreactivity of Sema3A (green) and NeuN (red) merged with DAPI (blue) in retinal cryosections (n=3) from 8-day-old rats after 3 days in normoxia (21% O₂) or hyperoxia (80% O₂). Co-staining of Sema3A with NeuN (yellow) was detected mainly on retinal ganglion cells (RGC) from hyperoxic pups treated with vehicle. Retinas from hyperoxic rat pups treated with IL-1Ra or 101.10 reveal a decrement in Sema3A staining similar to the normoxic animals in the ganglion cell layer (GCL). Scale bar = 50 µm.
Supplemental Figure XI. Conditioned media from retinal ganglion cells (RGC-5) stimulated with hyperoxic-microglia conditioned media (Hyp-MG-RGC-CM) induces activation of caspase-3 on endothelial cells (RBMVEC). Representative photomicrographs of endothelial cells (n=3) positively labeled with activated caspase-3 polyclonal antibody (green) or β-actin monoclonal antibody (red) on RBMVEC treated for 48 hours with conditioned medium from RGC-5 cells previously stimulated with normoxia-(21% O_{2}; Norm-MG-RGC-CM) or hyperoxia-exposed (80% O_{2}) microglia media in absence (vehicle; Hyp-MG-RGC-CM) or presence of IL-1Ra or 101.10. Co-localization of activated caspase-3 and β-actin staining was merged with RBMVECs nucleus stained with DAPI (blue). Scale bar = 50µm.
Supplemental Figure XII. IL-1β released from neuro-microvascular endothelial cells (RMBVEC) exposed to hyperoxia does not induced Sema3A release on RGC-5 cells. The treatment with recombinant Sema3A (4.5 nM) for 24 hours decreased significantly (48%) the cell viability (evaluated by MTT assay) on RBMVEC cells compared to the control (A). *p<0.05 vs Vehicle. But, the mRNA VEGF levels determined by qPCR did not change with the Sema3A treatment (B). IL-1β protein was evaluated by ELISA (C) in the media of RBMVEC exposed to 21% or 80% of oxygen levels for 24 hours. IL-1β levels from RBMVEC exposed to hyperoxia does not significantly change compared to control. No changes were observed in cell viability (D) evaluated by MTT assay on RBMVEC cells (n=3) treated with conditioned media from RGC-5 previously treated with hyperoxic endothelial cell conditioned media (Hyp-EC-RGC-CM, containing IL-1β). By contrast, the conditioned media from RGC-5 cells treated with hyperoxic microglial cell conditioned media (Hyp-MG-RGC-CM, containing high levels of IL-1β, used as positive control), increased the expression of the pro-apoptotic Sema3A from cultured RGC and induced a significant decrement (35%; p<0.05) in the endothelial cell viability compared to the control (D). *p<0.05 vs Nor-EC-RGC-CM.