ANIMAL MODELS

Choroidal Involution Is Associated with a Progressive Degeneration of the Outer Retinal Function in a Model of Retinopathy of Prematurity

Early Role for IL-1 β

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Retinopathy of prematurity (ROP), the most common cause of blindness in premature infants, has long been associated with inner retinal alterations. However, recent studies reveal outer retinal dysfunctions in patients formerly afflicted with ROP. We have recently demonstrated that choroidal involution occurs early in retinopathy. Herein, we investigated the mechanisms underlying the choroidal involution and its long-term impact on retinal function. An oxygen-induced retinopathy (OIR) model was used. *In vitro* and *ex vivo* assays were applied to evaluate cytotoxic effects of IL-1 β on choroidal endothelium. Electroretinogram was used to evaluate visual function. We found that proinflammatory IL-1 β was markedly increased in retinal pigment epithelium (RPE)/choroid and positively correlated with choroidal degeneration in the early stages of retinopathy. IL-1 β was found to be cytotoxic to choroid *in vitro*, *ex vivo*, and *in vivo*. Long-term effects on choroidal involution included a hypoxic outer neuroretina, associated with a progressive loss of RPE and photoreceptors, and visual deterioration. Early inhibition of IL-1 β receptor preserved choroid, decreased subretinal hypoxia, and prevented RPE/photoreceptor death, resulting in life-long improved visual function in IL-1 receptor antagonist-treated OIR animals. Together, these findings suggest a critical role for IL-1 β -induced choroidal degeneration in outer retinal dysfunction. Neonatal therapy using IL-1 receptor antagonist preserves choroid and prevents protracted outer neuroretinal anomalies in OIR, suggesting IL-1 β as a potential therapeutic target in ROP. (*Am J Pathol* 2016, 186: 3100–3116; <http://dx.doi.org/10.1016/j.ajpath.2016.08.004>)

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Retinopathy of prematurity (ROP) is the leading cause of severe visual impairment and blindness in neonates and young children in North America and Europe.¹ ROP has long been considered to be an inner retinal vasculopathy culminating in intravitreal neovascularization, leading to retinal detachment in its most severe form.² The outer retinal dysfunctions, such as defective dark adaptation³ or anomalies in color discrimination,⁴ are detected in many older children formerly afflicted with ROP. In addition, abiotrophic foveal dysplasia is routinely observed by optical coherence tomography,⁵ and loss of retinal pigmentation because of degeneration of retinal pigment epithelium (RPE) has also been reported.⁶ Hence, with increasing survival of extremely premature neonates (at risk of ROP),⁷ an insidious recognition of progressive outer retinal dysfunctions is being observed in former ROP patients, requiring follow-up beyond childhood.⁸

The choroid (a predominantly vascularized tissue) is considered the exclusive source of oxygen and nutrients to the subretina and outer retina.⁹ Recent studies have demonstrated that choroidal integrity is compromised in ROP. Choroidal thinning was reported in several studies in older children and young adults using optical coherence tomography,^{10–12} wherein the choroid was at least 15% thinner in former ROP patients, even in those whose condition had regressed.¹¹ Hence, ROP can no longer be considered only a vasculopathy of the inner retina, but also a disease that affects the largely overlooked choroid, which is indispensable for RPE and photoreceptor integrity. In support of this last claim, we recently showed that oxygen-induced retinopathy (OIR)—evoked choroidal thinning precedes photoreceptor degeneration¹³; however, the cellular and molecular mechanisms implicated in the process, and longer-term outcome, remain unknown.

Intravitreal anti-vascular endothelial growth factor (VEGF) treatment for severe ROP has attracted attention in recent years. Although bevacizumab monotherapy has been shown to be effective in treating zone I stage 3+ ROP,¹⁴ numerous researchers and clinicians have raised concerns regarding its long-term systemic safety,¹⁵ overall effectiveness, and differences in the patient demographics.¹⁶ Intravitreal bevacizumab has been shown to escape into systemic circulation; accordingly, serum VEGF levels were suppressed for up to 8 weeks in treated premature newborns,¹⁷ potentially impeding the critical role of VEGF on development of vital organs. Therefore, there is a need for a novel therapeutic approach for ROP.

A contribution of oxidant stress in the pathogenesis of ROP has long been recognized in human and animal studies.¹⁸ Oxidant stress is intimately intertwined with inflammation.¹⁹ Accordingly, several proinflammatory factors, such as IL-6, IL-18, cyclooxygenase-2, and IL-1 β , have been shown to be elevated in the retina of ROP and OIR subjects.^{20,21} Interestingly, IL-1 β is one of the most abundant cytokines generated in the retina during OIR²² and strongly implicated in neuronal and microvascular degeneration in several ischemic retinopathies, and in turn prevents preretinal neovascularization.^{22,23} IL-1 β

exerts its effects by interacting with the IL-1 receptor type 1. Previous studies have shown that recombinant IL-1 receptor antagonist (IL-1ra; Kineret), a competitive inhibitor of IL-1R, that blocks biological actions of IL-1 β , is able to protect against laser-induced choroidal neovascularization,²⁴ neuronal toxicity,²⁵ and OIR-elicited microvascular degeneration.²² However, the specific role of IL-1 β in choroidal involution is unknown. Hence, we proceeded to elucidate mechanisms of choroidal involution, and systematically explored how preservation of the latter affected long-term changes of the subretina and outer retina of newborns subjected to OIR.

Our findings reveal a prominent role for IL-1 β in choroidal involution associated with a subsequent progression of outer retinal dysfunction. Accordingly, IL-1 β inhibition may serve as a new therapeutic potential to prevent choroidal involution and its long-term impact on retinal degeneration.

Materials and Methods

OIR Model

The OIR model serves as a proxy to the human ocular neovascular disease, retinopathy of prematurity, which is characterized by a late phase of destructive pathological angiogenesis.^{26,27} Newborn Sprague-Dawley rats (Charles River, St. Contant, QC, Canada) were used according to Hôpital Sainte-Justine Animal Care Committee—approved protocols' adherence to the *Association for Research in Vision and Ophthalmology Statement of Use of Animals in Ophthalmic and Vision Research*.²⁸ According to Penn et al,²⁹ newborn rats were placed under oxygen concentration, which cycles at 50% $\pm$ 1% or 10% $\pm$ 1% every 24 hours from postnatal day (P) 0 to P14 (OIR/ROP). On P14, all rats were returned to room air. This model is characterized by retinal avascularity predominantly in the periphery,²⁹ followed by hypoxia-driven inner retinal neovascularization development from P14 until P18. Controls were maintained in room air (21% O₂). In some cases, pups were selected to receive twice-daily i.p. injections of the IL-1R antagonist recombinant protein (Kineret, 20 mg/kg; Swedish Orphan Biovitrum AB, Stockholm, Sweden) from P0 to P14, a regimen successfully tested in previous publications.^{22,23,30} Animals were sacrificed at different ages (P3, P14, P60, P150, and P210), and eyes were collected.

RNA Isolation and Quantitative Real-Time PCR

Eyes were rapidly enucleated and placed into a sterile petri dish resting on ice. The RPE/choroid layer was promptly separated from the neuroretinal layer. Both layers were processed for RNA using TRIzol (Invitrogen, Carlsbad, CA), followed by treatment with DNase I (Qiagen, Hilden, Germany) to remove any contaminating genomic DNA. The DNase-treated RNA was then converted into cDNA using M-MLV Reverse Transcriptase (Invitrogen). PCR primers

targeting rat IL-1 β , tumor necrosis factor- α , IL-6, IL-17, NLRP-3 (NACHT, LRR and PYD domains-containing protein 3), VEGF, pigment epithelium-derived factor, fibroblast growth factor, and platelet-derived growth factor were designed using National Center for Biotechnology Information Primer Blast (Table 1). Quantitative analysis of gene expression was generated using an ABI Prism 7700 sequence detection system and the SYBR Green Master Mix Kit (BioRad, Hercules, CA). Gene expression was calculated relative to 18S universal primer pair (Ambion, Waltham, MA) expression using the Δ Ct method.

MTT Assay

MTT assays were performed to assess endothelial cell viability. Briefly, rat brain microvascular endothelial cells (1.8 $\times$ 10⁵ cells; Cell Applications, Inc., San Diego, CA) were seeded in 48-well plates, cultured with Endothelial Cell Growth Medium-2 microvascular media (Lonza, Basel, Switzerland), and treated for 24 hours with recombinant rat IL-1 β (400-01B, 0 to 5000 pg/mL; PeproTech, Rocky Hill, NJ). After the treatment, the medium was removed and MTT (5 mg/mL, 30 μ L/well) was added, followed by incubation at 37°C for 4 hours. Afterward, supernatants were carefully removed, and dimethyl sulfoxide (30 μ L/well; Sigma-Aldrich, St. Louis, MO) was added to the cells. After insoluble crystals were completely dissolved, absorbance at 590 nm was measured using a microplate reader (Awareness Technology, Inc., Palm City, FL). Cell viability was expressed as a percentage of optical density relative to control.

Microvascular Sprouting from Choroid Explants

Choroids from juvenile rats were prepared as previously described.³¹ Briefly, choroids were cut into approximately

2 $\times$ 1-mm pieces and placed in Matrigel (BD Biosciences, Mississauga, ON) with 2% fetal bovine serum and incubated in 37°C, 5% CO₂ for 48 hours before treatment. Images of individual explants were taken before and 48 hours after treatment (with 5 nmol/L IL-1 β or saline). The choroids were stained or not (bright-field) for endothelium marker with fluorescent-conjugated lectin (FL-1101; Vector Labs, Burlington, ON, Canada) and for fibroblast marker with tetramethylrhodamine isothiocyanate (TRITC)-labeled phalloidin (00027; Biotium, Fremont, CA); the sprouting covered areas were quantified with ImageJ software version 1.46r (NIH, Bethesda, MD; <http://imagej.nih.gov/ij>).

Hypoxyprobe (Pimonidazole Hydrochloride) Staining

Hypoxyprobe (Hypoxyprobe, Inc., Burlington, MA) binds to proteins only under hypoxic conditions (hypoxia pO₂ <10 mmHg). Hypoxyprobe is dissolved in saline to obtain a stock solution of 100 g/L. Rats received Hypoxyprobe (60 mg/kg) via i.p. injection. Hypoxyprobe is distributed to all tissues but binds only to cells that have oxygen concentrations less than a pO₂ of 10 mm Hg at 37°C. Sixty minutes later, rats were sacrificed; their eyes were enucleated and further processed into cryosections. To detect Hypoxyprobe-protein adducts, a primary fluorescein (fluorescein isothiocyanate)-conjugated mouse monoclonal antibody (included in the kit) was used.

MitoTracker Orange Staining

MitoTracker Orange CMTMRos (ThermoFisher Scientific, Waltham, MA) is an orange-fluorescent dye that stains mitochondria in live cells, and its accumulation is dependent on membrane potential. The dye is well retained after aldehyde fixation. Rat eyes were enucleated and immediately dissected into eyecups. Eyecups were then incubated in mammalian Ringer solution containing 500 nmol/L MitoTracker Orange for 1 hour at 37°C. Stained eyecups were further processed into cryosections. MitoTracker Orange can be excited at 554 nm and observed at 576 nm (orange-red color).

Intravitreal Injection of IL-1 β

On P7 and P10, Sprague-Dawley rats were kept under deep ketamine-induced anesthesia and injected with rat IL-1 β (400-01B, 1 μ L at 5 ng/ μ L; PeproTech) intravitreally in the left eye (*n* = 10). Half of the IL-1 β -injected rats (*n* = 5) received twice-daily i.p. injections of Kineret (20 mg/kg) from P7 to P14. For the controls, sterile phosphate-buffered saline was injected into the right eye of the same rat. On P14, the animals were euthanized and the eyes were removed and processed for cryosection analysis.

mERG

Detailed electroretinogram (ERG) recording and analysis methods on rats were described before.³¹ Given the central-

Table 1 Primers Used in This Study

cDNA	Primer sequences
Il1b	Fwd: 5'-CATCTTTGAAGAAGAGCCCG-3' Rev: 3'-GGGATTTTGTCTGTGCTTGT-5'
Il6	Fwd: 5'-GACTGATGTTGTTGACAGCCACTGC-3' Rev: 3'-TAGCCACTCCTTCTGTGACTCTAACT-5'
Tnf	Fwd: 5'-CTATGTGCTCCTCACCCACA-3' Rev: 3'-TGGAAGACTCCTCCCAGGTA-5'
Vegfa	Fwd: 5'-CAATGATGAAGCCCTGGAGT-3' Rev: 3'-AATGCTTTCTCCGCTCTGAA-5'
Il17a	Fwd: 5'-ACTTTCCGGGTGGAGAAGAT-3' Rev: 3'-TGGCGGACAATAGAGGAAAC-5'
Nlrp3	Fwd: 5'-CCAGGAGTTCTTTGCGGCTA-3' Rev: 3'-GCCTTTTTCGAACTTGCCGT-5'
Serpinf1	Fwd: 5'-GGCAGTGGGCAACCAAGTTTG-3' Rev: 3'-GCAGCTGGGCAATCTTGCA-5'
Pdgfb	Fwd: 5'-CTGCCTCTCTGCTGCTACCT-3' Rev: 3'-TCTGTACGCGTCTTGCACTC-5'
Fgf2	Fwd: 5'-CCGTTTGGATCCGAGTTTA-3' Rev: 3'-CCCACACGTCAAACCTACAGC-5'

Fwd, forward; Rev, reverse.

temporal predominance of cones in rodents, the centers of multifocal ERG (mfERG) stimuli were aligned accordingly. Before ERG recordings, the rats were anesthetized with a mixture of ketamine hydrochloride (85 mg/kg) and xylazine (5 mg/kg). Pupils were dilated with drops of 1% cyclopentolate hydrochloride. Recordings were obtained using a tungsten loop electrode (active electrode) carefully placed over the corneal surface of the left eye, and was kept in place with 2% methylcellulose (Gonioscopic solution; Alcon Laboratories, Fort Worth, TX), which also helped prevent corneal desiccation. Grass disk (model E6GH; Grass Instruments, Quincy, MA) and subdermal needle electrodes (model E2; Grass Instruments) were placed in the mouth as the reference while inserted in the tail as the ground. The signals were amplified 100,000 times within a 10- to 100-Hz bandwidth. mfERG responses were evoked using the Veris 5.1b program (Electro-Diagnostic Imaging, Inc., Redwood City, CA) connected to a video camera display positioned at the center of the eye as viewed on the monitor. The stimulus matrix consisted of 37 hexagons presented in a pseudorandom (m-sequence) manner at a frame rate of 75 Hz for a total recording time of 4 minutes. The luminance of the white and black hexagons was 200 and 0 cd/m², respectively, and the background luminance was 100 cd/m².

Scotopic ERG

ERGs were recorded with a data acquisition system (Acknowledge; Biopac MP 100WS; BioPac Systems, Inc., Goleta, CA), according to techniques as previously reported.³² After 12 hours of dark adaptation, rats were anesthetized as above mentioned under dim red light. After dilating the pupils with 1% cyclopentolate hydrochloride solution (Mydracyl; Alcon Laboratories, Fort Worth, TX), a DTL silver-coated conductive nylon yarn electrode (27/7 X-Static; Sauquoit Industries, Scranton, PA) was placed on the cornea as active electrode with 2% methylcellulose (Gonioscopic solution) for prevention of corneal desiccation. Similar to mfERG, reference (model E5 disk electrode; Grass Technologies, Quincy, MA) and ground (model E2 subdermal needle electrode; Grass Technologies) needles were placed in the mouths and tails of rats, respectively. The rats were then placed in a custom-made dark chamber with a light stimulus for the recording of full-field ERGs (10,000 $\times$, 1- to 1000-Hz bandwidth, 6 dB of attenuation) using analog preamplifiers (model P511; Grass Technologies). Scotopic responses were evoked by flashes of white light spanning a 6.0-log-unit range in 0.3-log increments.

RPE Flat Mounts

Rat eyes were enucleated, fixed in 4% paraformaldehyde for 15 minutes at room temperature, and sectioned at the limbus. Retinas and choroids were fixed separately for additional 15 minutes in acetone at -20°C. Specimens were incubated overnight at 4°C with TRITC-labeled phalloidin

(Biotium, 00027) diluted in phosphate-buffered saline supplemented with 10% fetal calf serum and 0.1% Triton X-100. RPE morphological analysis to detect abnormal (large distorted) RPE was performed manually, and was similar to that reported by others,^{33,34} and used in automated software systems.³⁵

Ocular Tissue Preparation

Animals were perfused by phosphate-buffered saline and 4% paraformaldehyde. For histology sections, eyes were immediately collected, dehydrated by alcohol, and embedded in paraffin. Sagittal sections (5 μ m thick) were cut by microtome (Leica, RM 2145). Eyes for cryopreparation were further fixed in 4% paraformaldehyde overnight. Posterior eyecups were frozen in OCT medium. Samples were then cut into sagittal sections (10 μ m thick) and processed for immunohistochemistry.

Immunohistochemistry

Cryosection samples were stained with TRITC-conjugated (GSA) lectin endothelial cell marker (1:100; Sigma-Aldrich, L5264) or by using a mouse anti-RPE65 monoclonal antibody (NB100-355, 1:1000; Novus Biologicals, Littleton, CO) or a rabbit anti-NALP3 polyclonal antibody (bs-10021R, 1:1000; Bioss, Woburn, MA). Cryosections were then assessed by epifluorescent microscopy (E800; Nikon Eclipse, Melville, NY) or confocal microscope (Olympus, Richmond Hill, ON, Canada).

Quantification of Immunofluorescence Tissue Staining

Immunofluorescence quantification is performed using ImageJ 1.42q, following a protocol previously described.³⁶ Briefly, regions of interest were defined using the Polygon tool. The image was split into the two or three color channels (RGB Merge/split function) to obtain one image per channel. Regions of interest and an area next to them (as background) were measured. The corrected total cell fluorescence is calculated as follows: Integrated Density – (Area of Selected Cell $\times$ Mean Fluorescence of Background Readings). Five frames per images were measured and averaged to obtain the final reading.

Quantification of Choroidal Thickness

As mentioned in *Immunohistochemistry*, cryosection samples were stained with TRITC-conjugated lectin endothelial cell marker (1:100; Sigma-Aldrich, L5264) to indicate choroidal endothelium. The thickness of the choroid was measured on digital images taken under $\times 20$ amplification using computer software ImageJ 1.42q. Because both control and treated eyes were embedded in the same mode and stained with the same procedure, the differences induced by artifacts are minimized.

Western Blot

Protein extracts (40 μg) were loaded, separated by voltage potential, and transferred to polyscreen polyvinylidene difluoride transfer membrane (PerkinElmer, Waltham, MA), as previously reported.²³ Respective proteins were revealed by goat anti-IL-1β polyclonal antibody (AF-501-NA, 1:500; R&D Systems, Minneapolis, MN) or mouse anti-β-actin monoclonal antibody (NB600-501, 1:4000; Novus Biologicals). Antibodies and markers used in this study are listed in Table 2.

Electron Microscopy

Eyes were fixed for 1 hour in 2.5% glutaraldehyde in cacodylate buffer (0.1 mol/L, pH 7.4). Then, eyes were dissected, fixed for another 3 hours, post-fixed in 1% osmium tetroxide in cacodylate buffer, and dehydrated in graduated ethanol solutions. The samples were included in epoxy resin. Ultra-thin sections (80 nm) were contrasted by uranyl acetate and lead citrate and then observed with an electron microscope JEOL 100-CXII (JEOL, Peabody, MA) with 80 kV.

Statistical Analysis

One- or two-way analysis of variance was used for processing data. Bonferroni post hoc analysis was used for calculating significance between groups. Two-tailed *t*-tests were used to test for significance between two means. Dose-response curves were fitted using nonlinear regression dose-response (with variable slope) using GraphPad Prism (GraphPad Software Inc., La Jolla, CA). Results are presented as means ± SEM for all studies; statistical significance was set at *P* = 0.05.

Results

IL-1β Is Produced in Abundance in the Subretina by RPE Cells during Choroidal Involution

We have previously described that choroidal vascular involution begins early during the development of OIR.¹³ We assayed several major proinflammatory cytokines and growth

factors in the RPE/choroid complex of rat pups subjected to OIR or raised in normoxia at different time points. Tumor necrosis factor-α, IL-6, and particularly IL-1β mRNAs were substantially higher than controls on P10 and P14 (Figure 1A). IL-1β protein was also augmented in RPE/choroid complex from OIR animals at P14, as noted by immunoblot (Figure 1B) and immunohistochemistry (Figure 1C), compared to controls; IL-1β immunoreactivity also seemed to localize on RPE cells and diffuse to the adjacent choroid (Figure 1C). Concordant with these observations, immunoreactivity of NLRP3 and caspase-1 of inflammasomes, which produce mature IL-1β from pro-IL-1β, was highly increased and largely confined to RPE cells (Figure 1, D and E). However, activated (Iba-1 positive) microglia/macrophage (difficult to distinguish in subretina), the predominant source of IL-1β in the inner retina during OIR,²² were modestly detected and were clearly not augmented in the subretina (Supplemental Figure S1). In contrast to cytokines, concomitant measurements of major growth factors, such as pigment epithelium-derived factor, fibroblast growth factor, platelet-derived growth factor, and VEGF-A, were not significantly altered in the RPE/choroid complex from OIR pups (Supplemental Figure S2). Hence, in OIR, IL-1β increases in the subretina, to a significant extent, in RPE cells.

IL-1β Induced Choroidal Endothelium Cytotoxicity

We evaluated the effects of IL-1β on endothelial cell viability in *in vitro*, *ex vivo*, and *in vivo* assays. IL-1β elicited a direct concentration-dependent cytotoxicity to cultured endothelial cells treated for 24 hours (Figure 2A). This effect was corroborated in an *ex vivo* choroidal microvascular angiogenesis assay, where IL-1β also exerted a dose-dependent inhibition of choroidal microvasculature sprouting (Figure 2B). This effect of IL-1β was abolished by coadministration of the selective IL-1ra (Kineret), a competitive inhibitor of IL-1R (Figure 2B); the result profile was equivalent on bright-field and on lectin-stained (endothelial marker) analyses (Figure 2, B–D). Choroidal microvasculature sprouting can be distinguished from fibroblasts based on their morphological differences (Supplemental Figure S3). Furthermore, intraretinal IL-1β administration caused a significant decrease in choroid thickness in newborn rats, which was abrogated by coadministration of IL-1ra (Figure 2, E and F). Interestingly, IL-1β administration *in vivo* did not affect photoreceptor integrity, as neither caspase 3-immunoreactivity nor photoreceptor density was affected (Supplemental Figure S4).

Choroidal Involution Is Sustained into Adulthood and Associated with Local Hypoxia, and Early Neonatal Inhibition of IL-1β Receptor Prevents Choroidal Thinning and Extent of Hypoxia

We evaluated if IL-1ra (administered daily i.p.) during the early phase of retinopathy (from P1 until P14) could prevent

Table 2 Antibodies and Markers Used in This Study

Antibodies/markers	Companies, catalog no.
TRITC-labeled phalloidin	Biotium, 00027
TRITC-conjugated lectin	Sigma, L5264
Fluorescein-conjugated lectin	Vector Labs, FL-1101
Anti-RPE65 monoclonal antibody	Novus Biologicals, NB1000-355
Anti-NALP3 polyclonal antibody	Bioss, Bs-10021R
Anti-rat IL-1β polyclonal antibody	R&D Systems, AF-501-NA
Anti-β-actin monoclonal antibody	Novus Biologicals, NB600-501

NLRP3, NACHT, LRR and PYD domains-containing protein 3; RPE, retinal pigment epithelium; TRITC, tetramethylrhodamine isothiocyanate.

Figure 1 IL-1 β is immediately and markedly elevated in the RPE/choroid (Ch) complex from rats exposed to the oxygen-induced retinopathy (OIR) model. **A:** Expression levels of proinflammatory genes in OIR animals. Graph showing the quantitative PCR analysis on RPE/choroid from animals exposed to cycling oxygen (50% and 10%) or their normoxic controls (NOR; set as 1). During early induction of retinopathy (at P10), proinflammatory IL-1 β , IL-6, and tumor necrosis factor (TNF)- α were up-regulated (3.17-, 2.15-, and 1.80-fold, respectively). Immediately after completing cycling oxygen exposure (at P14), IL-1 β increased to 6.3-fold in the RPE/choroid. In comparison, IL-6 and TNF- α were moderately increased (2.65- and 3.30-fold, respectively). Fold changes were normalized to 18S as internal control. The dotted line indicates the relative level of the control group (set as 1). **B:** Western blot of RPE/choroid complex from normoxic controls, OIR rats, and/or OIR rats treated with IL-1 receptor antagonist (IL-1ra; Kineret). IL-1 β (17 kDa) protein level was markedly increased in OIR rats but significantly reverted with IL-1ra administration. β -Actin protein (42 kDa) was used as internal control. **C:** Immunohistochemistry revealed IL-1 β (green arrows) was colocalized with the RPE layer (red arrow) of OIR animals. **D:** Immunohistochemistry indicated specific colocalization between NLRP3, LRR, and PYD domains-containing protein 3 (NLRP-3) (green arrows), a component of inflammasome that produces IL-1 β , and retinal pigment epithelium-specific 65-kDa protein (RPE65) in P14 OIR animals. **E:** Immunohistochemistry indicated specific localization of caspase 1, the active enzyme that produces mature IL-1 β , in the RPE of P14 OIR animals (red arrows). Data are expressed as means $\pm$ SEM (**A**). $n = 3$ to 4 experiments, and representative images from 4 experiments are shown (**B–E**). * $P < 0.05$, ** $P < 0.01$. Scale bars: 10 μ m (**C**); 50 μ m (**D** and **E**). P, postnatal day.

choroidal involution of these animals. A choroidal thinning sustained into adulthood was observed in vehicle-treated animals subjected to OIR, in contrast to a robust thickening of the choroid in normoxia-raised animals (Figure 3). Although IL-1ra-treated newborn animals subjected to OIR showed a significant preservation of choroidal thickness well into mid-life ages (P210), daily IL-1ra (from P1 until P14) in animals raised in normoxia did not affect choroidal thickness (Figure 3).

Because the choroid is considered the exclusive source of oxygen and nutrients to the subretina and outer retina,⁹ we determined if the robust 50% involution of the choroid in OIR compromised tissue oxygenation, as would be expected³⁷ based on Poiseuille's equation, whereby blood flow is

directly related to the fourth power of vessel radius.³⁸ Hypoxia was detected using the marker Hypoxyprobe [by binding to thiol groups of proteins under these conditions, which spots severe hypoxia ($pO_2 < 10$ mmHg)³⁹]. Consistent with sustained choroidal thinning (Figure 3), robust Hypoxyprobe staining was detected in the rod outer segments and RPE/choroid complex in adolescent and adult rats (respectively at P60 and P210) subjected to OIR (Figure 4, A, C and E). Early neonatal IL-1ra, which preserved choroid thickness (Figure 3), expectedly ameliorated OIR-induced subretinal hypoxia (Figure 4, A, C and E). Consistent with these observations, mitochondrial integrity (using MitoTracker Orange, which first requires oxidation of normally functional mitochondria before fluorescence and mitochondrial sequestration) was disrupted

A

Figure 2 IL-1β has a negative impact on endothelial cells *in vitro*, *ex vivo*, and *in vivo*. **A:** Graph showing the percentage of survival on rat brain endothelial cells (RBECs) cultured with increased concentrations of rat IL-1β (from 0 to 5000 pmol/L). MTT assay was performed to measure cell viability. IL-1β dose dependently reduced RBEC viability. **B:** Representative images from choroidal explants cultured in absence or presence of IL-1β (from 0.5 to 5000 pmol/L) and IL-1ra (Kineret, 10 mg/mL) for 48 hours; explants were also stained with fluorescein-conjugated lectin (green) for specific detection of endothelium. Bright-field and fluorescent images were taken. Choroidal sprouting was dose dependently inhibited by IL-1β. The increase in sprouting in the control group was diminished by 50 and 5000 pmol/L IL-1β. IL-1ra coinubation effectively restored choroidal sprouting. **C** and **D:** Analyses based on bright-field and fluorescent images revealed equivalent observations. **E** and **F:** Representative P14 images of choroid thicknesses from rats intravitreally treated with vehicle (control), IL-1β (1 μL at 5 ng/μL on P7 and P10), or IL-1β plus IL-1ra (Kineret, 20 mg/kg twice a day from P7 to P14). Compared to the control group ($27.73 \pm 2.39 \mu\text{m}$), rats that received intravitreal injection of IL-1β showed significantly thinner choroid ($14.99 \pm 2.47 \mu\text{m}$), whereas IL-1ra-treated animals showed a preservation of choroidal thickness, as seen in the graph ($25.64 \pm 1.66 \mu\text{m}$). **Brackets** delineate choroidal thickness. Data are expressed as means $\pm$ SEM (**A**, **C**, **D** and **F**). $n = 4$ to 5 experiments (**B** and **E**). * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$, and **** $P < 0.0001$. Scale bar = 20 μm (**E**). P, postnatal day.

B **C** **D** **E** **F**

A **B** **C** **D** **E**

Figure 4 Persistent subretinal hypoxia was detected in oxygen-induced retinopathy (OIR) animals. **A:** Cross sections of OIR retinas were stained with Hypoxyprobe to indicate areas of hypoxia. On P60, control animals had minimal Hypoxyprobe staining in the retina. In contrast, the OIR group showed positive signals throughout the outer segment of photoreceptors, retinal pigment epithelium (RPE), and choroid (Ch; **arrows**). In IL-1ra-treated OIR rats, only sporadic Hypoxyprobe signals were detected. **B:** MitoTracker Orange was used to selectively label functional mitochondria (**arrows**). Positive MitoTracker Orange signals were found throughout the retina and RPE in control groups [normoxia (NOR) and NOR + IL-1ra]. MitoTracker Orange signals markedly reduced in OIR group. IL-1ra-treated OIR rats exhibited preserved MitoTracker Orange signals at outer segments of the photoreceptors and RPE. **C:** On P210, control group exhibited minute Hypoxyprobe in photoreceptor outer segments, whereas age-matched OIR rats exhibited robust signals at RPE and choroid. As expected, IL-1ra-treated OIR group showed diminished staining. **D:** Representative images of three animals are shown. **E:** Representative figures of four animals are shown. Data are expressed as means $\pm$ SEM (**D** and **E**). * $P < 0.05$, ** $P < 0.01$. Scale bar = 30 μ m (**A–C**). INL, inner nuclear layer; ONL, outer nuclear layer; P, postnatal day.

Figure 5 Structural abnormalities in retinal pigment epithelium (RPE) from oxygen-induced retinopathy (OIR) animals. **A–T:** Representative images of RPE flat mounts from animals at different ages (P3, P14, P60, P150, and P210) and previously exposed to cycling oxygen (OIR) or maintained in normoxia (NOR) and treated or not with IL-1ra (from P1 until P14) were labeled with tetramethylrhodamine isothiocyanate–conjugated phalloidin. Normoxic controls (NOR and NOR + IL-1ra) had RPE cells with their typical polygonal cell shape. However, RPE flat mounts in OIR revealed a progressive increasing number of pleomorphic and enlarged cells with age (**arrows**). **U:** Statistical analyses shown in the graph revealed a higher proportion of pleomorphic RPE cells in OIR animals, especially at P150 and P210. Data are expressed as means $\pm$ SEM (**U**). $n = 4$ experiments (**A–T**). * $P < 0.05$, ** $P < 0.01$. Scale bar = 20 μ m (**A–T**). P, postnatal day.

by hypoxia (after OIR-induced choroidal involution) (Figure 4, B and D) and was preserved in IL-1ra–treated animals (Figure 4, B and D).

OIR Elicits RPE and Photoreceptor Degeneration

On the basis of sustained OIR-induced choroidal thinning, subretinal hypoxia, and disrupted mitochondria, we evaluated longitudinally the integrity of the subretinal and outer retinal RPE and photoreceptors. RPE normal hexagonal flat mount structure was maintained into a polygonal shape with age in intact animals (Figure 5, A, B, E, F, I, J, M, N, Q, and R). No changes in RPE morphology were detected at P14 (Figure 5, C, G, D, and H). However, by P60, a gradual age-dependent disruption of RPE morphology was intensified in animals raised in hyperoxia during the newborn period, resulting in loss of their hexagonal shape (Figure 5, K, O, S, and U). Correspondingly, electron microscopy analysis of RPE cells from control and P60 OIR animals showed normal cellular arrangement (Figure 6, A–F, I, and J). RPE cells from OIR animals at older ages (P150 and P210)

revealed cytoplasmic vacuolization (Figure 6, G and K), representing mitochondria with minimal residual cristae, suggestive of ghost mitochondria,⁴⁰ and a 50% reduction in RPE65 mRNA expression by P150 (Figure 6N). Again, IL-1ra–dependent choroidal preservation prevented gradual degeneration of RPE in animals subjected to OIR as newborns (Figure 5, L, P, T, and U, and Figure 6, H and L).

Based on gradual degeneration of RPE, which is critical to photoreceptor homeostasis,⁴¹ we justifiably examined age-dependent changes in photoreceptor density and function after OIR. As seen with RPE integrity, photoreceptor density was not altered early in newborn (P14) exposed to hyperoxia (Figure 7, A–D). By P60 (but not before), photoreceptor cell density in the OIR animals started to decrease gradually with age to reach approximately 50% of values seen in normoxia-raised animals by P210 (Figure 7, E–G, I–K, M–O, and Q). On the other hand, early choroidal involution was associated with diminished photoreceptor function by P30, as evidenced by reduced scotopic a-wave amplitude (photoreceptor generated) of the flash ERG in OIR-subjected animals

Figure 6 Retinal pigment epithelial (RPE) mitochondrial anomaly in the subretina of oxygen-induced retinopathy (OIR) animals. **A–M:** Representative RPE electron micrographs from animals at different ages (P60, P150, and P210) and previously exposed to cycling oxygen (OIR) or maintained in normoxia (NOR) and treated or not with IL-1ra (from P1 until P14). In OIR animals, the number of ghost mitochondria (defined by reduced cristae; **arrows**) was markedly increased on P150 (22.30 per 225 μm^2) and persisted to P210 (27.73 per 225 μm^2). The increment of ghost mitochondria in OIR was abrogated by IL-1ra on P150 (**H**) and P210 (**L**). **N:** Real-time quantitative PCR analyses showed similar Rpe65 expression levels between normoxia and OIR animals at P14 and P60. However, Rpe65 levels were significantly reduced in OIR group on P150 and P210, in line with the timing of ghost mitochondria. Representative images of four samples per group are shown. Data are expressed as means $\pm$ SEM (**M** and **N**). * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$, and **** $P < 0.0001$. Scale bar = 5 μm (**A–L**). P, postnatal day.

(Figure 8, A–C, E–G, I–K, M, and N); similar reductions in mfERG amplitudes were observed, especially for the most vulnerable central zone (Supplemental Figure S5). Early IL-1ra treatment of OIR-exposed rats markedly

attenuated photoreceptor cell loss and corresponding attenuation of the a-wave and mfERG amplitudes (Figure 7, H, L, and P, Figure 8, D, H, L, and P, and Supplemental Figure S5), as anticipated.

Figure 7 Photoreceptor population decline in oxygen-induced retinopathy (OIR) rats. **A–Q:** Representative cross sections stained with hematoxylin and eosin from animals at different ages (P14, P60, P150, and P210) exposed to cycling oxygen (OIR) or maintained in normoxia (NOR) and treated or not with IL-1ra. The number of photoreceptors, whose nuclei resided in the outer nuclear layer (ONL), was measured and represented in the schematic. The number of photoreceptors in the OIR groups (**G**, **K**, and **O**) became significantly reduced compared to normoxia controls (**E**, **I**, and **M**; **F**, **J**, and **N**) on P60; further decrement was observed until P210. IL-1ra treatment (**H**, **L**, and **P**) salvaged photoreceptor population. **Brackets** delineate the thickness of the ONL. Representative images of four to five samples are shown. Data are expressed as means $\pm$ SEM (**Q**). * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$, and **** $P < 0.0001$. Scale bar = 50 μ m (**A–P**). GCL, ganglion cell layer; INL, inner nuclear layer; P, postnatal day.

Q Photoreceptor response (a-wave)

Figure 8 Photoreceptor functional degeneration in oxygen-induced retinopathy (OIR) rats. **A–Q:** Scotopic electroretinogram analysis of animals at different ages (P30, P60, P150, and P210) and exposed to cycling oxygen (OIR) or maintained in normoxia (Nor) and treated or not with IL-1ra (from P1 until P14). The a-wave is generated by the photoreceptors, whereas the b-wave is derived from the inner retina, predominantly Muller and bipolar cells. Across all ages (P30 to P210), a-wave amplitude from OIR animals (**C, G, K, and O**) was significantly lower than normoxia controls (**A, E, I, and M; B, F, J, and N**). The effect of OIR on photoreceptor response became evident early on. A progressive photoreceptor function decline was observed as animals aged. On P210, a-wave response in OIR group was 91.7 μ V—a 72.6% reduction compared to the NOR group (283.2 μ V). IL-1ra treatment (**D, H, L, and P**) rescued photoreceptor functions in OIR animals; its effect became statistically significant on P150 and P210. Data are expressed as means $\pm$ SEM (**Q**). $n = 4$ to 7 animals (**A–P**). * $P < 0.05$, ** $P < 0.01$, and **** $P < 0.0001$. P, postnatal day.

Discussion

The inner retina has long been considered to be the primary region affected in human ROP and animal models.⁴² Lately, dysfunctions of photoreceptors are detected in a significant number of older children formerly afflicted with ROP; these outer retinal dysfunctions are composed of defective dark adaptation because of rod dysfunction,³ abnormal cone function,⁴³ and retinal depigmentation.⁴⁴ Moreover, the

apparent progressive nature of these changes is suggested.⁴⁵ Hence, with increasing survival of extremely premature neonates (at higher risk of ROP), an insidious recognition of late-onset photoreceptor dysfunction is being observed.

Our laboratory first described choroidal involution in the OIR model of ROP as a plausible explanation for outer retinal disorders.¹³ This observation was corroborated by several independent clinical investigations,^{10–12,46} wherein choroidal thinning was reported in children and adolescents

Figure 9 Schematic diagrams of long-term dysfunctionality of the outer retina from animals subjected to oxygen-induced retinopathy (OIR). **A:** During early OIR, the choroid starts to become thinner (involution). Unlike inner retinal vasculature degeneration, where revascularization occurs, choroidal involution persists throughout life. Accordingly, subretinal hypoxia is detected after choroidal involution. Pleomorphic retinal pigment epithelial (RPE) cells begin to appear on P60 (adolescence), and their number continues to augment when OIR animals age. Meanwhile, a large number of mitochondria lacking cristae (termed ghost mitochondria) appear on P150 (adulthood). Starting on P60 (adolescence), fewer photoreceptors are observed in OIR animals. Photoreceptor population continues to decline as the animal ages. Subsequently, photoreceptor function follows a similar trend—OIR animals elicit marked attenuated photoreceptor responses starting on P60 (adolescence) and continue to deteriorate as they age. **B:** A schematic representation of choroidal involution associated with OIR and subsequent long-term degeneration of the outer retina. IL-1 β is produced by RPE cells in response to retinopathy. Acting in a paracrine manner, IL-1 β triggers involution of the choroid (the sole blood supply to RPE and photoreceptors), and subsequently, severe hypoxia in the outer retina. As choroidal involution persists, RPE cells gradually become pleomorphic and ghost mitochondria (within RPE) start to emerge, likely because of metabolic imbalance. With a global reduction in oxygen and nutrients, as well as dysfunctional RPE cells, photoreceptor population and function also take a downturn. Early anti-IL-1 β treatment rescues choroid integrity, thereby preventing outer retinal hypoxia, RPE abnormalities, and photoreceptor population and functional decline. P, postnatal day; ROP, retinopathy of prematurity.

with a history of ROP, indicating that choroid involution persists; inner retinal vascular changes resolve. In the present study, we explored mechanisms underlying choroidal involution secondary to OIR, and investigated if targeting these pathological processes would prevent long-term deterioration in subretinal and outer retinal structure and function. We hereby uncovered a significant role for IL-1 β in choroidal involution associated with OIR. IL-1 β was found to be specifically toxic to choroidal endothelium, but not directly to photoreceptors (and RPE); its potency is in line with that reported for other actions of IL-1 β .^{47,48} The ensued choroidal involution curtailed outer and subretinal oxygenation, and resulted in a protracted degeneration of RPE and photoreceptors. Early inhibition of IL-1 β receptor prevented choroidal involution and ensuing local hypoxia,

and in turn preserved RPE and photoreceptors in the long-term in the adult.

A salient feature of the present study lies in the chronological order of outer and subretinal pathologies relative to the choroidal involution. Essentially, the choroid rapidly involutes on exposure to hyperoxia, which is in line with our previous observations.¹³ This OIR-induced choroidal involution at P14 results in a marked outer and subretinal hypoxia, which is associated with a progressive degeneration of RPE, as revealed by cell morphology, electron microscopy, and specific cell marker protein (RPE65); concomitantly, photoreceptor integrity was compromised, as shown on cell counts and function. These observations infer a significant role for prolonged hypoxia in protracted RPE and photoreceptor injury after OIR. Early in the response to hypoxia,

there is rapid vasodilatation and accumulation of (dephosphorylated) adenosine and of Krebs cycle intermediates (mostly succinate),²⁷ followed by stabilization of hypoxia-inducible factor-1 α , redox alteration, and the activation of survival factors—which altogether are intended to attempt to reinstate oxygen supply and protect the tissue. However, as hypoxia is sustained, it leads to a maladaptive response that ultimately results in cell degeneration.⁴⁹

These inferences related to the effect of choroidal involution on outer retina and subretina are supported by the following evidence: i) Diminished choroidal circulation has been suggested using a variety of techniques (angiography, laser Doppler flowmetry, and color Doppler imaging) in subretinal disorders, such as age-related maculopathy.⁵⁰ ii) Hypoxia-inducible factor-1 α , a major inducer of the prominent endothelial growth factor (VEGF), has been detected in subretinal cells of patients with hypoxia-triggered choroidal neovascular membranes.⁵¹ iii) Prolonged choroidal ischemia has been proposed to cause degeneration of the RPE and photoreceptors.⁵² iv) The photoreceptor degeneration, in turn, diminishes visual acuity.⁵³ Our findings are consistent with a sustained outer retinal hypoxia after OIR, resulting in injury to RPE and photoreceptors, as shown in the scheme presented in Figure 9.

Further evidence in support of choroidal involution in delayed outer retinal injury is substantiated by prevention of OIR-induced choroidal involution by IL-1ra, which, in turn, avoids subretinal hypoxia and circumvents in the long run RPE and photoreceptor damage. In line with this claim is the fact that cytotoxicity to IL-1 β was only observed on choroidal endothelium, but not on RPE and photoreceptors, as previously documented,⁵⁴ and by absence of RPE and photoreceptor injury in the newborn (at P14).

Although not all patients with a history of ROP develop outer retinal dysfunction, the stage of ROP serves as a key predicting factor. Children with former severe ROP performed poorer than those with mild cases, which, in turn, exhibited inferior performance compared to children without ROP.⁵⁵ Because the 50%/10% OIR model in this study reliably reproduces a more severe form of retinopathy,²⁹ including tortuous retinal vessels (akin to plus disease), advanced neovascularization, vitreous hemorrhage, and retinal detachment, OIR mimicks severe cases of the disease at greater risk of developing progressive outer retinal abnormalities.

With increasing survival of extremely premature neonates (<28 weeks) and very-low-birth-weight (<1500 g at birth) infants,⁷ long-term changes afflicting the subretina are being detected in older children and young adults with history of ROP and those at risk of ROP.⁴³ This is especially true in emerging economies, such as China and India.⁵⁶ Other ophthalmic conditions associated with inflammation, such as diabetic retinopathy⁵⁷ and geographic atrophy,⁵⁸ also exhibit choroidal atrophy. Kineret (anakinra), which is used to treat severe rheumatoid arthritis in adults and children, may be a safe and effective option to tackle retinal

pathology associated with ROP (and possibly other ischemic retinopathies).

In summary, our observations uncover a critical role for IL-1 β in OIR-evoked choroidal degeneration and subsequent long-term subretinal degeneration. This is the first study to describe progressive injury to photoreceptors in the OIR model of ROP, in an attempt to reproduce clinical observations.⁴⁵ Our findings set forth new concepts and mechanistic notions for ROP and its long-term outcomes, underlining the importance of prolonged follow-up of ROP. Early administration of IL-1 receptor blockers to subjects at risk of ROP may not only benefit the inner retina,²² but also prevent choroidal rarefaction and ensued (RPE and) long-term photoreceptor demise.

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Supplemental Data

Supplemental material for this article can be found at <http://dx.doi.org/10.1016/j.ajpath.2016.08.004>.

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