Inflammatory Resolution and Vascular Barrier Restoration after Retinal Ischemia Reperfusion Injury

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Inflammatory Resolution and Vascular Barrier Restoration after Retinal Ischemia Reperfusion Injury

Running title: Inflammation and iBRB repair after IR injury

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Abstract:

Background: Several retinal pathologies exhibit both inflammation and breakdown of the inner blood-retinal barrier (iBRB) resulting in vascular permeability, suggesting that treatments that trigger resolution of inflammation may also promote iBRB restoration.

Methods: Using the mouse retinal ischemia-reperfusion (IR) injury model we followed the time course of neurodegeneration, inflammation and iBRB disruption and repair to examine the relationship between resolution of inflammation and iBRB restoration and to determine if minocycline, a tetracycline derivative shown to reverse microglial activation, can hasten these processes.

Results: A 90 min ischemic insult followed by reperfusion in the retina induced cell apoptosis and inner retina thinning that progressed for approximately 2 weeks. IR increased vascular permeability within hours, which lasted for at least 2 weeks and resolved between 3 and 4 weeks after injury. Increased vascular permeability coincided with alteration and loss of endothelial cell tight junction (TJ) protein content and disorganization of TJ protein complexes. Shunting of blood flow away from leaky vessels and dropout of leaky capillaries were eliminated as possible mechanisms for restoring the iBRB. Repletion of TJ protein contents occurred within 2 days after injury, long before restoration of the iBRB. In contrast, the eventual re-organization of TJ complexes at the cell border coincided with restoration of the barrier. A robust inflammatory response was evident a 1 day after IR and progressed to resolution over the 4-week time course. The inflammatory response included a rapid and transient infiltration of granulocytes and Ly6C⁺ classical inflammatory monocytes, a slow accumulation of Ly6C^{neg} monocyte/macrophages, and activation, proliferation and mobilization of resident microglia. The presence of monocyte/macrophages and increased numbers of microglia were sustained until the iBRB was eventually restored. Intervention with minocycline to reverse microglial activation at 1 week after injury promoted early restoration of the iBRB.

Conclusions: These results suggest that iBRB restoration occurs as TJ complexes are reorganized and that resolution of inflammation and restoration of the iBRB following retinal IR injury are functionally linked. **(323 words)**

Keywords:

Ischemia-reperfusion injury, retinal vasculature, blood-retina barrier, tight junctions, resolution of inflammation, microglia, leukocytes, granulocytes, monocytes, myeloid-derived macrophages

Background:

A number of ischemic retinal diseases are characterized by neurodegeneration, sterile inflammation and vascular permeability [1-3]. Human disease and animal models of diabetic retinopathy (DR), retinopathy of prematurity (ROP) and retinal vein occlusions (RVO) all involve retinal ischemia and include increased neural cell death, sterile inflammation and vascular permeability. Vision loss in these ischemic diseases is associated with vascular permeability leading to macular edema [4, 5]. Thus, a major treatment goal in DR and other ischemic retinopathies is the reduction of vascular permeability by restoration of the iBRB [1]. The effectiveness of intra-ocular treatments targeting vascular endothelial growth factor (VEGF) in many DR patients with diabetic macular edema and RVO patients suggests that vascular permeability in ischemic retinal diseases is often driven by VEGF accumulation [6-8]. Clinical studies have also documented increased levels of inflammatory cytokines in the vitreous of DR, ROP and RVO patients [9-11]. In addition, steroids and other anti-inflammatory treatments can reverse retinal edema, even in DR patients that do not respond to anti-VEGF therapy [12, 13]. Together, these studies suggest that inflammation and retinal vascular permeability are intimately related.

Retinal vascular permeability occurs when TJ complexes at endothelial cell borders fail to resist the paracellular transport of ions and large molecules (i.e. plasma proteins) that are normally excluded from the neural retina by the iBRB or increases transcellular flux of molecules through endothelial cells, for example, via plasmalemma vesicle associated protein (PLVAP) vesicles [14]. TJ complexes involve over 40 proteins including members of the claudin family. In particular, the endothelial-restricted member, claudin-5, is required for iBRB formation during retinal development [15]. Members of the membrane-associated zonula occludens family (ZO-1, 2 and 3) organize the junctional complex and are also required for barrier formation [16]. The TJs of the iBRB also include occludin, a transmembrane protein that contributes to barrier regulation. The mechanisms by which the iBRB is dismantled in DR are being elucidated. For example, VEGF causes phosphorylation of occludin on Serine 490 (pS490), leading to its ubiquitination and TJ endocytosis [17, 18]. Conversely, mechanisms signaling the formation of the iBRB during retinal development are also being elucidated (reviewed in [19]). In contrast, the mechanisms controlling iBRB restoration in the adult are relatively unknown.

Likewise, processes that control injury-induced, sterile inflammation and promote resolution are under investigation (reviewed in [20]). In the acute phase of cerebral stroke models microglia quickly react to neuronal damage leading to inflammatory cytokine and chemokine expression. Astrocytes also undergo reactive gliosis and contribute to cytokine expression. Vascular endothelial cells become activated, with increased expression of selectins and cell adhesion molecules, increased permeability and subsequent attraction, adhesion and infiltration of granulocytes, principally neutrophils, and classical pro-inflammatory monocytes (Ly6C^{hi} in mouse), within hours after injury [21]. The microglial population within the infarct also increases via migration from the surrounding tissue and/or proliferation [22]. Together microglia, granulocytes and monocyte-derived macrophages clear dead cells and debris by phagocytosis. Within days, the response transitions to a late phase of

inflammation, with clearance of neutrophils and pro-inflammatory monocytes. Days after injury a relatively small fractions of lymphocytes are included in the infiltrated leukocyte population, including T cells, B cells and natural killer cells. The late phase also coincides with the accumulation of non-classical monocytes and/or macrophages (Ly6C^{neg} in mouse), which originate from either the infiltration of circulating non-classical monocytes or the transformation of Ly6C^{hi} classical monocytes within the tissue [23]. A final reparative phase of the response includes the production of specialized pro-resolving lipid mediators and the expression of anti-inflammatory cytokines by astrocytes and infiltrated lymphocytes, such as gamma-delta T-cells and regulatory T-cells [24].

The rodent retinal IR injury model has often been used to explore mechanisms of ganglion cell death and neuroprotection [25]. This model also induces a rapid increase in vascular permeability together with a robust sterile inflammation [26, 27]. In the present study we examined the temporal effects of retinal IR injury in the mouse on the iBRB integrity, including the modification, expression and organization of TJ proteins that comprise this barrier. We also followed the innate immune responses, including the mobilization and proliferation of microglia and dynamics of various leukocyte populations. The results show that IR injury results in an extended period of inner retinal neurodegeneration that coincides with a progressive innate immune response and sustained vascular permeability that is initiated by TJ protein modification and depletion, as well as disorganization of TJ complexes at the endothelial borders. Eventual resolution of inflammation and restoration of the barrier occurred only after neurodegeneration was complete. Reformation of the iBRB coincided with reorganization of TJ complexes at endothelial borders. Intervention with minocycline hastened restoration of the iBRB, suggesting a link between resolution of inflammation and vascular barrier restoration.

Methods:

Ischemia-reperfusion model

All animals were treated in accordance with the Association for Research in Vision and Ophthalmology. Statement on the Use of Animals in Ophthalmic and Visual Research and the guidelines established by the University of Michigan Institutional Animal Care and Use Committee. The retinal IR model was performed by elevation of intra-ocular pressure (IOP) via saline injection through a 33-gauge needle penetrating the cornea into the anterior chamber and elevation of the saline reservoir by 160 cm to obtain IOP of 85-95 mmHg (measured via a Tonolab rebound tonometer). Ischemia was maintained for 90 min while IOP was periodically monitored. Removal of the needle allowed release of pressure and natural reperfusion. Contralateral eyes with needle injection into the anterior chamber but no increase in IOP served as Sham controls.

Lineage tracing of microglia

Lineage tracing of microglia was performed by Cre-mediated green fluorescent protein (GFP) expression specifically in microglia cells, analogous to that described by Goldman and colleagues [28]. CX3CR1CreERT2 mice [29] (CX3CR1B6J.B6N(Cg)-Cx3cr1^{tm1.1}(cre)Jung>/J, stock No. 025524, Jackson Laboratories, Bar Harbor, ME) were crossed with mT/mG Cre-reporter mice [30] (B6.129(Cg)-Gt(ROSA)26Sor^{tm4}(ACTB-tdTomato,-EGFP)Luo>/J, stock No. 007676, Jackson Laboratories). Induction of tamoxifen (TAM)-inducible Cre-mediated recombination was caused by 3 subcutaneous (s.c.) injections of 160 mg/kg TAM, 48 h apart [31]. A 4-week long chase period following TAM treatment was employed prior to retinal IR injury to allow clearance from circulation of recombined, and therefore mGFP-expressing, CX3CR1⁺/Ly6C^{neg} non-classical monocytes [28].

In situ measurement of retinal layer thicknesses

Retinal thickness was measured *in situ* in mice under anesthesia using a small animal spectral domain optical coherence tomography (SD-OCT) imaging system (Envisu R2200, Bioptigen) and InVivoVue Diver software (Bioptigen). Measurements were made at 4 compass points 350 μ m from center of the optic nerve head and averaged. The total retina spans from the inner limiting membrane (ILM) to the retinal pigment epithelium (RPE). The inner retina spans from the ILM to the inner limit of the outer plexiform layer (OPL). The outer retina spans from the inner limit of the OPL to the RPE.

Measures of cell death

The DNA fragmentation assay was carried out using Cell Death Detection ELISA^{Plus} (Roche) per manufacturer's instructions and as published previously[32]. Optical densities (O.D.) were detected using a microplate spectrophotometer (FluoStar Omega, BMG Labtech) and normalized to wet retinal weight.

Terminal deoxynucleotidyl transferase dUTP nick end labeling (TUNEL) in whole retinas was performed using the Click-iTTM Plus TUNEL assay kit (Thermo Fisher Scientific). Immediately following euthanasia, eyes were removed and fixed in 4% paraformaldehyde in PBS for 30 min at RT and then washed 2 times in PBS. Retinas were removed, and incubated in TBST (0.3% Triton X-100) for 2 h at room temperature (RT) to permeabilize the tissue. Retinas were then placed in terminal deoxynucleotide transferase (TdT) reaction buffer and allowed to equilibrate for 1 h at RT. The TdT reaction buffer was then replaced with cold complete TdT reaction mixture and the retinas incubated overnight at 4°C to allow complete penetration of enzyme into the tissue. TdT reaction mixture was again replaced and the retinas were incubated for 1 h at 37°C to allow TdT reaction to occur. Retinas were then rinsed 3 times with cold PBS, incubated for 30 min in PBS containing 0.1% Triton X-100 and 3% bovine serum albumin (BSA) at 4°C and rinsed again in cold PBS. Retinas were then placed in Click-iT TUNEL reaction cocktail, incubated for 2 h at 4°C to allow tissue penetration of reactants

and incubated again in fresh TUNEL reaction cocktail for 1 h at 37°C to allow the Click-iT reaction to occur. Retinas were then processed for immunofluorescence (IF) to detect retinal ganglion cells (RGC) with anti-RNA binding protein with multiple slicing (RBPMS) antibody (GeneTex GTX118619) diluted 1:100 in PBST with 10% donkey serum and flat-mounted for microscopy. Immunofluorescence confocal microscopy (IF-CM) was performed using a Leica TSC SP5 confocal microscope.

Measures of vascular permeability

To measure retinal vascular leakage, 200 mg/kg body weight of fluorescein isothiocyanate-labeled bovine serum albumin (FITC-BSA, Sigma) was injected into the femoral vein of anesthetized mice and allowed to circulate for 2 h. After transcardiac perfusion with PBS for 3 min to flush vessels, retinal dye accumulation was quantified and normalized to plasma FITC-BSA and retina dry weight as previously described [26].

To visualize vascular leakage the extravascular accumulation of sulfo-NHS-biotin was imaged as described previously [33]. EZ-link Sulfo-NHS-Biotin (300 mg/kg body weight, Thermo Fisher) was perfused into the femoral vein of anesthetized mice and allowed to circulate for 5 min before transcardiac perfusion with PBS for 3 min to flush vessels and then with 2% paraformaldehyde (PFA) for 3 min to fix the sulfo-NHS-biotin in place. Eyes were harvested and postfixed in 1% PFA in PBS for 6 h at 4°C prior to retinal dissection. Dissected retinas were incubated in PBS containing 0.5% Triton X-100 and 0.1 mM CaCl₂ (PBSTC) overnight at 4°C to permeabilize and then probed with antibodies to endothelial marker CD31/PECAM1 (1:75, clone MEC 13-3, BD Bioscience #553370), the TJ protein ZO-1 (1:75, MABT11, Millipore Sigma) and Texas Red Streptavidin (1:500, Vector Lab # SA-5006) and flat mounted for IF-CM as described above.

Quantification of perfused retinal vascular area and empty vascular sleeves

Mice were perfused with FITC-BSA as described above for measurement of vascular permeability. Retinas were then processed and whole-mounted for IF-CM as described above, but using antibodies to CD31/PECAM1 (1:75, clone MEC 13-3, BD Bioscience #550566) and collagen-IV (ColIV, 1:100, Millipore-Sigma AB756P). Using a 40X objective, Z-stacked images of the superficial vascular plexus or the intermediate and deep plexi combined were obtained at compass points approximately 500 μ m from the optic nerve head for each retina and subjected to image analysis (IMARIS 8.2, Bitplane). ColIV IF-positive area was used to define a vascular surface mask, using the Unify Surfaces option. The Colocalize application was used to calculate percent of the ColIV IF map that co-localized with FITC-BSA IF or CD31 antibody IF. For each retinal plexus the 4 values were averaged.

Western blotting

Western blotting for TJ proteins was performed as described previously for the rat retina [26] using antibodies to claudin-5 (1:1000, clone EPR7583, Abcam Cat#ab131259), occludin pS490 (1:400, in house antibody[17]), total occludin (1:1000, Thermo Fisher Cat#71-1500). ZO-1 (1:1000, clone R40.76, Millipore Cat#MABT11), ZO-2 (1:1000, Cell Signaling Technologies Cat#2847) and β -actin (1:5000, Cell Signaling Technologies Cat#3700).

Scoring of TJ protein organization

To assess TJ organization, occludin and ZO-1 protein continuities at vascular endothelial junctions were examined by IF-CM in whole mounted retinas, as performed previously for the rat retina [26]. Mouse retinas were processed and whole-mounted for IF-CM as described above using antibodies to occludin (1:75, Thermo Fisher Cat#71-1500) and ZO-1 (1:75, clone R40.76, Millipore-Sigma Cat#MABT11). At least 4 eyes were assessed for each time point, with images captured at 4

positions 250 μ m from the optic disk. Three separate evaluators scored each image in a masked fashion and the scores were averaged. For each retina the 4 average scores for each vessel type were again averaged to provide a single score profile.

Immunofluorescence of microglia in retinal sections and flat-mounted retinas

IF of retinal sections and whole retinas from mouse eyes was performed essentially as previously described [34]. Primary antibodies used included rabbit polyclonal to the ionized calcium-binding adapter molecule 1 (Iba-1, 1:250, Wako Pure Chemical Industries, Cat#019-19741), rat monoclonal anti-Ki67 (1:100, Thermo Fisher, Cat#14-5698-82), rat monoclonal anti-CD11b (1:100, Thermo Fisher, Cat#14-0112-82), rabbit anti-GFP (1:500, Thermo Fisher, Cat#A21311), *Griffonia simplicifolia* isolectin B4 (IB4, 1:50, Thermo Fisher, Cat#121411). IF-CM images were obtained with a 63X objective with a fixed detection gain for each comparative section. All samples included negative controls with primary antibody replaced with normal IgG (1 mg/ml).

Flow cytometry analysis of retinal immune cell and endothelial cell populations

Following euthanasia, eyes were removed, retinas were quickly dissected and IR or Sham-treated retinas were pooled (4 or more retinas per group) for analysis. Tissues were processed for flow cytometry as previously described [34] and blocked prior to incubation with labeled antibodies. Antibodies included: PerCP-Cy5.5-conjugated rat anti-mouse CD11b monoclonal antibody (1:100, clone M1/70, Thermo Fisher, Cat#550993), APC-Cy7-conjugated rat anti-mouse CD45 monoclonal antibody (1:100, clone 30-F11, Thermo Fisher Cat#557659), PE-conjugated rat anti-mouse Ly6C monoclonal antibody (1:100, clone, HK1.4, Thermo Fisher Cat#12-5932-80) and FITC-conjugated anti-mouse Ly6G (Gr-1) monoclonal antibody (1:75, Clone RB6-8C5, Thermo Fisher Cat#11-5931). Endothelial cell markers included PE-conjugated anti-mouse CD31/PECAM-1 monoclonal antibody (1:50, clone MEC 13-3, Thermo Fisher #561073) and AF488-conjugated IB4 (1:100, Thermo Fisher Cat#121411). After incubating with antibodies and lectin, cells were rinsed 3 times with cold PBS and

analyzed using an LSRII flow cytometer (BD Biosciences) and FlowJo software (Tree Star Inc.). Events representing debris and clumps of cells were gated out in plots of forward scatter area (FSC-A) and side scatter area (SSC-A) and then in plots of forward scatter width (FSC-W) versus FSC-A, in identical fashions for each group prior to analysis of marker antibody and lectin fluorescence intensities. Immune cell populations were first defined by gating on the common leukocyte marker CD45 and the myeloid lineage marker CD11b. CD11b⁺/CD45^{low} microglia were differentiated from CD11b⁺/CD45^{hi} myeloid leukocytes on the basis of their low expression of CD45 [35, 36]. CD11b^{neg}/CD45^{hi} cells representing lymphocytes were not further characterized. Microglia and myeloid leukocytes were further defined by gating for Ly6C, a marker of inflammatory monocytes, and the granulocyte/neutrophil marker Ly6G. Microglia were defined as CD11b⁺/CD45^{low}/Ly6C^{neg}/Ly6G^{neg} cells. Myeloid leukocytes were subdivided into CD11b⁺/CD45^{hi}/Ly6C^{hi}/Ly6G^{neg} (pro-inflammatory, classical monocytes), CD11b⁺/CD45^{hi}/Ly6C^{neg}/Ly6G^{neg} (non-classical monocytes or macrophages) and CD11b⁺/CD45^{hi}/Ly6C^{low}/Ly6G^{neg} (intermediary monocytes) and CD11b⁺/CD45^{hi}/Ly6C⁺/Ly6G⁺ granulocytes. Endothelial cells were defined as CD31⁺/IB4-binding⁺.

Statistics

Tests of statistical significance between groups were performed using Prism 7 (Graphpad Software), and the specific tests applied are indicated in the figure legends.

Results:

Neurodegeneration following retinal IR injury

The mouse IR model was used to examine the time course of vascular and inflammatory responses to retinal ischemic injury. C57BL/6J mice were subjected to retinal ischemia for 90 min by

elevation of IOP via saline delivery to the anterior chamber, which prevents both retinal and choroidal blood flow. SD-OCT imaging revealed alterations to the retinal thickness (**Fig. 1A**). At 6 h and 24 h after IR injury a significant thickening of the total retina by approximately 10% was observed in the IR-injured group, which was primarily due to inner retinal thickening. This suggests a transient inner retinal edema, which coincided with a rapid increase in vascular permeability (see **Fig. 2**). After initial thickening, there was a progressive thinning of retinas in the IR group, which was primarily due to thinning of the ganglion cell layer (GCL) and inner plexiform layer (IPL). Loss of retinal tissue from the inner retina approached a nadir by 13 day after IR injury, with a 36% reduction in mean thickness. This compares to an average 5% reduction in outer retina thickness.

The retinal thinning after IR injury represents neurodegeneration. Examination of cytoplasmic nucleosome-DNA fragments, a measure of retinal cell death by apoptosis or necroptosis in whole retinas, showed a significant 2-fold increase in the IR versus Sham eyes at 4 h after injury, the earliest time measured (**Fig. 1B**). DNA fragmentation reached a maximum at 2 day, with a significant 3-fold increase, and then decreased thereafter, normalizing at 4 wk. The increase in DNA fragmentation corresponded to the appearance of numerous TUNEL-positive cells in the GCL, of which approximately half expressed the RGC marker RBPMS (**Fig. 1C**). This is in keeping with approximately 50% of cell in the GCL being RGC, and the remainder displaced amacrine cells (dAC) [37]. Surprisingly, a small but significant increase in DNA fragmentation was still apparent in IR-injured retinas at 2 wk and 3 wk after injury, when inner retinal thinning had ceased. Sporadic TUNEL staining in the outer nuclear layer (ONL) was identified at 2 wk after IR (**Supplemental Data Fig. S1**), suggesting that the increased DNA fragmentation at 2 wk and 3 wk was due to a relatively small fraction of photoreceptors undergoing apoptosis. Collectively, these studies demonstrate that the majority of neurodegeneration was initially in the inner retina, peaked by 2 day

and then diminished. Loss of a small fraction of photoreceptors persisted at 2-3 wk and was fully resolved by 4 wk after injury.

Effects of IR injury on iBRB integrity

Retinal IR injury disrupts the iBRB and increases vascular permeability. We have demonstrated in rats that transient ischemia induces a rapid and robust increase in retinal vascular permeability to albumin, with a significant increase as early as 15 min after reperfusion and maintained for at least 2 days [9, 26]. We examined the time course of vascular permeability in the mouse IR model by measuring the retinal accumulation of i.v. injected FITC-labeled BSA (**Fig. 2A**). IR caused a very rapid and dramatic increase in vascular permeability with the greatest increase (6-fold versus Sham) at 4 h after reperfusion. A 3-4-fold increase in vascular permeability was sustained for 2 wk. We used i.v. injection of reactive sulfo-NHS-biotin combined with IF analysis of the TJ protein ZO-1 to determine if vascular leak coincided with loss of TJ organization at endothelial borders at 24 h after IR injury (**Fig. 2B**). This analysis made apparent that leakage occurred in regions where TJ organization was disrupted in both the superficial vasculature and the deeper plexi, suggesting that iBRB breakdown allowed paracellular leakage. Eventually the barrier was restored, with no significant difference in permeability between IR and Sham groups by 3 wk after IR.

Restoration of the iBRB as a whole could theoretically occur by three mechanisms: shunting of perfusion away from leaky vessels, pruning of leaky vessels or reformation of TJ structures. Studies have revealed that retinal IR injury in rodents leads to limited capillary degeneration resembling the capillary dropout phenomenon observed in DR pathology [38, 39]. This phenomenon is presumably caused by death of endothelial and mural cells, leaving the vascular basement membrane as collagen-containing empty vascular sleeves, referred to as acellular capillaries. Notably, the increase in empty vascular sleeves formed after IR injury in the mouse are relatively small (i.e. 7/mm² [39]). To determine if shunting of perfusion away from leaky vessels or pruning of leaky

vessels could explain the decline of vascular leakage observed during barrier restoration, we analyzed the effects of IR injury on the perfused capillary area and endothelial cell coverage of vessels at 2 day, 2 wk and 4 wk after IR (**Fig. 3**). Co-localization of perfused FITC-BSA and of CD31 (PECAM1) with ColIV was used to determine changes in perfused vessel area and endothelial coverage of vessels, respectfully. In addition, ColIV-positive capillaries that lacked FITC-BSA and CD31 were counted to determine the density of empty vascular sleeves. At no time was there a significant change in co-localization of FITC-BSA or CD31 with ColIV in injured retinas (**Fig. 3A-B**), suggesting there was no appreciable change in capillary perfusion or loss of endothelial cells. The density of empty vascular sleeves was only slightly increased in IR retinas, but this did not reach significance (**Fig. 3C**). The typical density of empty vascular sleeves that we observed was approximately 300/mm², which is more than an order of magnitude greater than the density observed by the trypsin digest technique employed in previous studies [38, 39]. Importantly, no evidence of FITC-BSA leaking from empty sleeves was observed, suggesting this was not a major route of permeability. To confirm that there was no appreciable loss of endothelial cells, we also employed flow cytometry and gated endothelial cells by CD31 antibody and IB4 isolectin binding. We found no significant changes in the relative populations of CD31⁺/IB4⁺ cells in IR-injured retinas versus Sham control retinas at 2 day, 2 wk or 4 wk after IR injury (**Supplemental Data Fig S2**). In summary, no evidence for any appreciable change in perfusion of vessels, vessel pruning or endothelial cell loss between 2 day and 4 wk after IR injury was observed (**Fig. 2A**). Thus, shunting of perfusion away from leaky vessels and pruning of leaky vessels were eliminated as mechanisms of restoring the iBRB after IR injury.

In order to determine whether changes in content of TJ proteins contributed to barrier loss and restoration, temporal changes in the junctional protein complex were assessed by western blot. Retinas were harvested at 4 h, 1 day, 2 day, 1 wk, 2 wk, and 4 wk after ischemia and processed for

western blotting of TJ proteins (**Fig. 4A**). Phosphorylation of occludin on Ser490 (pS490), total occludin, claudin-5, ZO-1 and ZO-2 protein contents were determined. This analysis revealed a rapid increase in pS490 at 4 h after IR injury, which was sustained for 2 day, similar to that previously reported in rats [26]. This phosphorylation regulates occludin ubiquitination and endocytosis with other members of the TJ complex, creating gaps in the barrier and leading to eventual proteosomal degradation of occludin [40]. In keeping with this, ZO-1, ZO-2 and claudin-5 protein contents all significantly decreased at 1 day after injury, while total occludin protein content trended down (**Fig. 4B**). The levels of these proteins were normalized at 2 day and 1 wk following IR, and even rebounded to be slightly higher in IR retinas at 4 wk after IR (although this was only significant for claudin-5). Thus, phosphorylation of occludin and loss of TJ proteins were associated with the initial loss of barrier properties, but were reversed by 2 day after injury, long before restoration of the barrier. This data show that repletion of TJ proteins was not sufficient to restore the barrier, as leakage continued for weeks after occludin, claudin-5 and ZO protein levels returned to normal.

In order to determine if restoration of barrier function after IR coincided with reorganization of the TJ complex, the continuity of endothelial junctions was evaluated at 4 h, 2 day, 1wk and 4 wk after IR injury by microscopy. Retinas were probed with antibodies to occludin and ZO-1, flat mounted and imaged by confocal microscopy. Occludin and ZO-1 were chosen because occludin regulates barrier properties while ZO-1 acts as a central organizer of the junctional complex [41]. Images of arteries and veins in the superficial vascular plexus and capillaries in the deep vascular plexi were scored for continuity of occludin and ZO-1 protein fluorescence at the borders between endothelial cells in a masked fashion (**Fig. 4C-D**). This analysis indicated that changes in TJ organization were not detectable at 4 h after IR, a time when both occludin Ser490 phosphorylation and vascular permeability were evident. Both occludin and ZO-1 proteins were significantly disorganized in veins and capillaries by 2 d following IR injury. At 2 wk, occludin disorganization

was significant in arteries and veins, but not capillaries. ZO-1 tended to show disorganization in arteries at this time, but the difference between groups was not significant. However, at 4 wk after injury, when the iBRB was fully restored, no differences in TJ proteins organization were apparent between IR-injured and Sham-treated retinas. Thus, reorganization of TJ complexes coincided with restoration of the barrier between 2 wk and 4 wk after injury.

The innate immune cell response and resolution following retinal IR injury

Established flow cytometry methods [35, 42, 43] were used to determine the temporal changes in retinal immune cell populations following IR injury (**Fig. 5**). Naive retinas were also examined for comparison. At 1 day following IR there was a substantial accumulation of myeloid leukocytes, primarily granulocytes and pro-inflammatory monocytes expressing high levels of Ly6C (**Fig. 5A-C**). IF detection of leukocytes in flat-mounted retinas clearly showed that at 1 day the CD45⁺ leukocytes were present in the vessel lumen, within the retinal tissue, and in the process of diapedesis (**Fig. 5F-G**). Numbers of granulocytes and inflammatory monocytes rapidly declined between 1 day and 4 day, but were not fully normalized until 4 wk. A lesser population of Ly6C^{neg} monocytes/macrophages accumulated relatively slowly, reaching a maximum at 4 day, and disappearing between 2 wk and 4 wk after IR injury (**Fig. 5D**). At 4 day the microglial population in IR retinas was significantly increased by slightly more than 50% relative to Sham retinas. Microglial numbers in injured retinas declined slowly after 4 day, with no difference between groups by 4 wk after IR (**Fig. 5E**). We also observed a substantial population of CD11b⁺/CD45^{low}/Ly6C⁺/Ly6G⁺ cells of undefined identity that segregated with microglia in the CD11b⁺/CD45^{low} gate (**Supplemental Data Fig. S3**). These cells were greatly increased in IR retinas at 1 day after injury and declined steadily thereafter until becoming insignificantly increased relative to Sham at 4 wk. Thus, full resolution of inflammation coincided with the end of neurodegeneration and restoration of the iBRB.

Because microglia numbers were increased after IR we examined the response of microglial proliferation to IR by probing for the proliferative cell marker antigen Ki-67 in 30 μm sections of IR-injured and Sham retinas (**Fig. 6A**). At 2 day after injury the appearance of Iba-1⁺/Ki-67⁺ cells, suggested microglial proliferation. We then examined Iba-1 and Ki-67 in flat-mounted retinas by confocal microscopy, using the superficial and deep vascular plexi to identify inner and deeper retinal layers, while excluding the outer nuclear layer (**Fig.s 6B-C**). At 2 day after injury, the density of Iba-1⁺ cells near the superficial plexus was increased by 3-fold after IR; the vast majority of these cells were also Ki-67⁺ and there were very few Ki67⁺ cells that were not Iba-1 positive. In contrast, the density of Iba-1⁺ cells in the deeper layers decreased by half after IR, suggesting a migration of microglia from the OPL to the inner retina. There was also a surprising number of Iba-1⁺/Ki-67⁺ cells in Sham-treated retinas relative to naive retinas, which may reflect a response to injury in the contralateral eye. Most of the Iba-1⁺ cells exhibited ramified morphologies indicative of microglia, although their ramification appeared diminished in IR compared to Sham retinas, as well as compared to naive retinas. Indeed, examination of soma size, a sensitive indicator of activation [44], demonstrated significant increase in the IR-injured group relative to Sham (**Fig. 6D**). However, Iba-1 is a general marker of phagocytes and cannot fully distinguish between microglia and monocytes/macrophages [45], and therefore the soma sizes of invading phagocytes could be influencing the apparent soma size changes.

In fact, in IR-injured retinas a minority of Iba-1⁺ cells exhibited an amoeboid morphology suggesting either highly transformed microglia or Iba-1 expressing monocytes that have invaded the retinal tissue. In order to differentiate between microglia and Iba-1⁺ myeloid leukocytes, we utilized lineage tracing of microglia by cross breeding CX3CR1-CreERT2 mice with mT/mG Cre-reporter mice. Recombination-mediated switching from membrane-targeted tdTomato to membrane-targeted GFP expression in microglia and circulating CX3CR1-expressing non-classical monocytes was

induced by repeated treatments with TAM. To insure that microglia were the only cells expressing GFP during the experiment, a 4-wk chase period between TAM treatment and IR injury was used to clear recombined CX3CR1⁺ monocytes from the circulation [28]. At 4 day after IR injury, retinas were probed with antibodies to CD11b, Iba-1 and GFP, and then flat-mounted. In Sham retinas the vast majority (average 95%) of CD11b⁺/Iba-1⁺ were GFP-positive, showing highly efficient Cre-mediated recombination in microglia (**Fig. 7A**). In IR retinas, the GFP⁺ microglia population had expanded and congregated in the GCL, with a 1.8-fold total increase of CD11b⁺/Iba-1⁺/GFP⁺ cells in all retinal layers (**Fig. 7D**) and a 2.5-fold increase in the inner retina (GCL + IPL) (**Fig. 7B**). In contrast, the number of CD11b⁺/Iba-1⁺/GFP⁺ cells in deeper retinal layers (INL + OPL) was unchanged at this time (**Fig. 7C**). These finding confirmed that the retinal microglial population expands and accumulate in the IPL and GCL in response to IR injury. While the populations of infiltrating myeloid leukocytes (CD11b⁺/GFP^{neg}) make up a very small fraction of the total CD11b⁺ cells in Sham treated retinas, this population increased to account for about 30% of the total CD11b⁺ cells after IR. Further, at 4 day after IR the vast majority of CD11b⁺/GFP^{neg} myeloid leukocytes were Iba-1 positive phagocytes.

In order to examine the phagocytic function of Iba-1⁺ cells following IR injury, we probed 30 μ m thick retinal sections with antibodies to Iba-1, CD68 (lysosomal-associated membrane protein 4 or macrosialin), and brain-specific homeobox/POU domain protein 3A (Brn3a). CD68 is constitutively expressed by monocytes/macrophages, but its expression is often used as an indicator of increased phagocytic capacity in microglia [46-48]. Brn3a is a nuclear protein marker expressed by a major fraction of RGC [49]. In IR-injured retinas all Iba-1⁺ cells exhibited dramatically increased levels of CD68 expression relative to Sham retinas (**Supplemental Data Fig. S4**). Iba-1⁺/CD68⁺ cells were often closely associated with nuclei in the GCL, and were apparently engulfing cells, including Brn3a⁺ RGC cells. The results suggest that in response to retinal IR injury, both microglia and

monocyte-derived cells take on a phagocytic phenotype, accumulate in the inner retina and phagocytose dying cells, including RGC.

Intervention treatment with minocycline speeds restoration of the vascular barrier

Because resolution of inflammation and restoration of the iBRB coincided in the IR model, we hypothesized that an intervention treatment targeting the microglial inflammatory phenotype may also trigger restoration of the barrier. Minocycline is a tetracycline derivative that has been shown to have potent neuroprotective and anti-inflammatory properties. In models of stroke, the neuroprotective mechanism of minocycline is attributed to inhibition of microglial activation [50]. To determine if minocycline treatment can promote restoration of the iBRB, we initiated daily systemic treatments at 7 days after IR injury and evaluated vascular permeability at 14 days, which is at least a week prior to spontaneous iBRB restoration. As expected at this time, injured retinas of saline-treated mice still exhibited significantly increased leakage of FITC-BSA relative to Sham retinas. In contrast, minocycline treated mice exhibited retinal vascular leakage that was significantly lower than the saline-treated IR group, and which was not significant higher than either the saline-treated Sham group or the minocycline-treated Sham group. Thus, an interventional treatment targeting the microglia inflammatory phenotype was able to promote restoration of the vascular barrier, suggesting that resolution of their inflammatory response promotes barrier restoration. However, minocycline has pleotropic effects [51] and care is warranted in assuming a direct causal role of microglia in controlling vascular permeability [52].

Discussion:

We show that IR injury of the retina results in significant neuronal cell apoptosis with inner retinal thinning, a progressive and resolving innate immune response, and a sustained loss of the

iBRB followed by spontaneous barrier restoration with minimal vascular restructuring. This model thus presents a unique opportunity to examine interactions between neurodegeneration, neuroinflammation and vascular barrier integrity, as well as signaling mechanisms governing resolution of neuroinflammation and blood-barrier restoration. The temporal coincidence of cessation of neuron death, resolution of inflammation and restoration of the iBRB in this model suggests that these processes may be linked.

The initial increase in DNA fragmentation and vascular permeability response to IR injury were both rapid, being measurable within 4 h after injury (**Fig. 1**). The initial cell death was localized to the GCL, involving both RGC and dAC. A previous time course study [53] of a rat retinal IR model with relatively dramatic inner retinal layer thinning showed very rapid significant loss of Brn3a⁺ RGC and thinning of the GCL, which were detectable by 2 h and 6 h, respectively. This was followed by a much lesser, but significant thinning of the ONL that was detectable by 3 d and accompanied by a loss of cone photoreceptors but not rods. In the mouse model we also found that TUNEL progressed to the ONL with time (**Fig. S1**). However, the fractional photoreceptor loss was quite small. The reason for this temporal shift is not known.

Breakdown of the iBRB was already maximal at approximately 4 h after IR and coincided with rapid phosphorylation of occludin Ser490 (**Figs 2 and 4**). Occludin phosphorylation was followed by subsequent loss of occludin, ZO-1, ZO-2 and claudin-5 protein contents at 1 day. Previously, we demonstrated that retinal IR injury in rat induced a rapid and robust increase in retinal vascular permeability to albumin, with a 3- to 4-fold increase when leak was evaluated starting at 15 min after reperfusion [26, 27, 32]. IR-induced occludin Ser490 phosphorylation coincided with VEGF receptor 2 (VEGFR2) activation, as indicated by VEGFR2 Tyr1175 phosphorylation [26]. Treating rats with the anti-VEGF therapeutic, Avastin (bevacizumab), prevented the early increase in permeability, VEGFR2 activation and occludin phosphorylation [26, 32]. These results suggest that

IR induces rapid barrier disruption in a VEGF-driven manner. However, the mechanism by which the barrier is held open for extended periods after IR injury may involve the inflammatory response. For example, we found that preventative minocycline treatment inhibited neuroinflammation following IR and was able to prevent retinal leak at 2 day after injury but not immediately after injury [27]. It is noteworthy that changes in junctional protein content and location were not observed at 4 h when permeability increased (**Fig. 4**). This may be due to changes in the junctional complex that cannot be observed by IF microscopy but coincide with occludin phosphorylation, or may be due to changes in transcellular flux. Studies modeling stroke with transient middle cerebral artery occlusion (tMCAO) revealed that permeability occurs in a stepwise manner, with increased transcytosis through caveolin-1-mediated flux in the first 2 days, followed by increased paracellular flux thereafter [54]. Other studies reveal that the first hours of rat stroke models increase edema dependent on Na/H and Na-K-Cl co-transporter [55].

Organization of endothelial TJ complexes exhibited significant disruption by 2 day after IR injury and was restored to normal between 3 wk and 4 wk after injury (**Fig. 4D**). In contrast, occludin Ser490 phosphorylation returned to normal and ZO protein contents were replenished by 2 day after IR injury (**Fig. 4B**). This suggests that barrier restoration occurred not by simple TJ protein replenishment, but rather through reorganization of the TJ complexes. Alternative hypothetical mechanisms for correcting leakiness include shunting blood flow away from leaky vessels or pruning leaky damaged vessels. However, evaluation of the perfused vessel areas, endothelial coverage of vessels and the density of empty vascular sleeves all failed to provide any evidence of those potential mechanisms (**Fig.s 3**). Thus, the present findings suggest that spontaneous restoration of the iBRB via TJ reorganization occurs after retinal IR injury.

Previous studies using trypsin digests of the retinal vasculature demonstrated that retinal IR injury causes vascular dropout resembling DR pathology [38, 39]. A previous study employing the

same mouse retinal IR model used here and examining trypsin digests found that the total density of acellular capillaries doubled from 7/mm² to 14/mm² [39]. In contrast, utilizing IF probing of flat mounted retinas we found that the number of unperfused ColIV⁺/CD31^{neg} empty vascular sleeves was not significantly increased after IR (**Fig. 3b-3d**). It is also clear that the trypsin digestion method does not detect the high density of ColIV⁺/CD31^{neg} empty sleeves we observed by the IF method (300-400/mm²). These structures were recently identified as pericyte bridges that are formed by pericyte detachment and basement matrix bridge formation, rather than by pericyte and endothelial cell loss [56].

Furthermore, we also used flow cytometry with IB4 and CD31 as markers to quantify retinal endothelial cell numbers after IR and found no significant losses at 2 day, 1 wk or 4 wk after IR injury (**Fig. S2**). In the normal mouse retina, CD31 is specifically and highly expressed by endothelial cells [57]; but it is also moderately expressed on hematopoietic cells, including platelets, monocytes/macrophages, granulocytes and some lymphocytes [58, 59]. Similarly, IB4 binds to α -D-galactosyl end residues on surface glycoproteins of endothelial cells, but also binds to the surface of some leukocytes, including neutrophils [60, 61]. We did not exclude CD45⁺ cells in the analysis, so consequently the CD31⁺/IB4⁺ populations could have included some leukocytes. However, the CD31⁺/IB4⁺ population was not increased in the IR group at 2 day after injury, when the leukocyte population was appreciable. In addition, the numbers of leukocytes in the IR retinas were negligible at 4 wk after injury compared to the numbers of CD31⁺/IB4⁺ cells observed (compare **Fig.s 3B-3D and S2B**). Thus, we found no evidence for appreciable loss of endothelial cells in the mouse retinal IR injury model. In contrast, a previous study [62] used IF methods to detect vascular endothelial cells after retinal IR injury in the rat and observed approximately 30% and 60% decreases in endothelial cell densities in the superficial and deep vascular plexus, respectively. This study also found that IR injury caused approximately 15% and 30% decreases in perfused vessel densities in the

superficial and deep vascular plexus, respectively. However, it should be noted that, in contrast to the present mouse IR model, this rat model exhibited relatively dramatic neurodegeneration with nearly complete thinning of inner retinal layers and total loss of cells in the GCL.

We observed that vascular leak coincided with TJ disorganization and the normalization of vascular permeability coincided with reestablishment of a uniform occludin distribution at the borders of endothelial cells in veins and arteries in the superficial vascular plexus (**Fig. 4D**). Vascular permeability can occur by both paracellular leak and receptor-mediated transcytosis, and because we used FITC-albumin to quantify vascular permeability, transcytosis could contribute to the increased vascular permeability that we observed after IR injury. Our observations co-localizing leakage of sulfo-NHS-biotin with TJ disorganization (**Fig. 2B**) suggest that most early leakage was paracellular, which is consistent with prior findings that inhibition of cytoskeletal and TJ rearrangement prevented BBB permeability in a rodent stroke model [63]. Our results also suggest that the limiting step in iBRB reformation was the reorganization of TJ complexes. In the developing mouse retinal vasculature appreciable paracellular permeability persists until P18 [64]. Analogous to our result in the adult, van der Wijk and co-workers [65] observed that during mouse retinal development TJ proteins were expressed in immature vessels at least 5 days prior to formation of a fully functional iBRB. On the other hand, Chow and Gu [66] attributed the final step of iBRB formation during mouse development to cessation of transcytosis. Although we have not ruled out a role for transcytosis, the observation that TJ reorganization coincided with the eventual normalization of vascular leakage suggests that the majority of the persistent leakage was by the paracellular route. The signals and mechanisms governing TJ reformation leading to iBRB restoration are under investigation.

Following IR injury, the damage-and-repair process begins with a robust inflammatory phase, with granulocyte (neutrophil) and Ly6C^{hi} classical pro-inflammatory monocyte leukostasis and

infiltration over the first 1-2 days, and with microglial activation and proliferation between 1 and 4 days (**Fig.s 5 and 6**). This neuroinflammation evolved with a rapid decline of neutrophil and classical monocyte populations, giving way to a sustained presence of Ly6C^{neg} monocyte/macrophage population that remained until barrier properties were restored (**Fig. 5D**). This is directly analogous to the progression of leukocyte populations that were observed following cerebral IR injury models [21]. The source of Ly6C^{neg} monocytes/macrophages in the IR-injured retina is under investigation. These cells could represent invading Ly6C^{neg} circulation monocytes or could be derived from invading Ly6C^{hi} monocytes that down-regulated Ly6C expression as they differentiated into macrophages. We also observed a population with intermediate Ly6C antigen expression (**Fig. 5A**), which could represent cells in transition. Such a transformation has been well documented in stroke models, with the Ly6C^{neg} cells termed monocyte-derived macrophages (MDM) [67, 68].

We also observed the appearance of a minority population of cells in IR retinas that were initially gated with microglia because, like microglia, they expressed a CD45⁺/CD11b^{low} phenotype but were excluded from the microglia population because they also were positive for Ly6C and Ly6G (**Supplemental Data Fig. S3**). Because the dynamics of this CD45⁺/CD11b^{low}/Ly6C⁺/Ly6G⁺ population did not parallel those of other invading myeloid leukocytes, they are unlikely to be monocytes or granulocytes that were erroneously captured in the initial microglial gate. The identity of these cells is thus unknown and is being examined further.

The retinal IR model should prove useful for determining the mechanisms governing microglial migration and population expansion and contraction following injury. Resident microglia expanded their numbers by day 4, congregating mainly in the inner retina, and then slowly returned to a near normal population size by 4 wk after injury (**Fig.s 5, 6 and 7**). Similarly, microglial proliferation was observed in other models of retinal, cerebral and spinal injury, often using Iba-1 as the sole marker of microglial identity [69-71]. However, the often-used microglial marker Iba-1 does

not differentiate between microglia and invading monocytes. In the present study, both Ki67 positivity of Iba-1⁺ cells (**Fig. 6**) and lineage tracing (**Fig. 7**) strongly suggest that microglial proliferation occurs after retinal IR injury and that the majority of cells in the expanded Iba-1⁺ population originate as resident microglia. However, after IR we observed the appearance of a considerable number of CD11b⁺/Iba-1⁺ cells that were not lineage traced as microglia. Most of these were located in the OPL (**Fig. 7C**). The origin and phenotype of these cells are under investigation.

In models of neurodegeneration microglia play a key role in removal of dead neurons and debris by phagocytosis [47]. Following IR injury, microglia are likely to remove by phagocytosis dying RGC and dAC in the GCL, as well as synapses in the IPL. We were able to identify Iba-1⁺ cells enveloping Brn3a⁺ RGC soon after injury (**Fig. S4**). Although this phagocytic role may be shared by invading monocytes, microglia are the more capable phagocytes and seem responsible for the majority of debris removal in both cerebral IR injury and spinal cord injury models [72, 73]. The current studies suggest the hypothesis that the extent of IR injury and rate of neuronal death induces microglial population expansion to meet the demand for removal of dead cells and their processes.

We also found that intervention treatment with minocycline at 1 wk after IR injury was able to hasten restoration of the vascular barrier (**Fig. 8**). Minocycline is an effective anti-inflammatory agent that is often used to target microglial activation in models of brain injury and stroke [50]. We previously demonstrated that pretreatment with minocycline inhibited both the inflammatory response and vascular leakage after retinal IR injury in rat [27]. Similarly, Ahmed and co-workers showed that minocycline was neuroprotective in the mouse retinal IR model when delivered prior to injury and continued for 5 days following injury [74]. In that study, minocycline treatment did not alter the morphological changes exhibited by microglia following IR, but did increase their expression of the M2 marker arginase-1, as well as retinal expression of the M2-inducing cytokine IL-4.

Conclusions:

The current study reveals the intimate relationship between the time-course of retinal neurodegeneration, resolution of inflammation and reformation of the iBRB after IR injury, and the retinal IR model should prove valuable in examining the causal relationship of these processes. The intimate time-course of inflammatory resolution and barrier restoration coupled with the effect of intervention with minocycline suggest that resolution of inflammation and restoration of the iBRB are associated processes after retinal IR injury. Altering the microglial phenotype could be an effective method to promote iBRB restoration in retinal ischemic diseases.

List of abbreviations:

APC = allophycocyanin

Brn3a = brain-specific homeobox/POU domain protein 3A

BSA = bovine serum albumin

CD11b = cluster of differentiation molecule 11b

CD31 = cluster of differentiation molecule 31

CD45 = cluster of differentiation molecule 45

CD68 = cluster of differentiation molecule 68

ColIV = collagen-IV

Cy5.5 = cyanine5.5

Cy7 = cyanine7

DR = diabetic retinopathy

dAC = displaced amacrine cells

DNA = deoxyribonucleic acid

ELISA = enzyme-linked immunosorbant assay

FITC-BSA = fluorescein isothiocyanate-labeled bovine serum albumin

FSC-A = forward scatter area

FSC-W = forward scatter width

GCL = ganglion cell layer
GFP = green fluorescent protein
IB4 = *Griffonia simplicifolia* isolectin B4
h = hour
Iba-1 = ionized calcium-binding adapter molecule 1
iBRB = inner blood-retinal barrier
IF = immunofluorescence
IF-CM = immunofluorescence confocal microscopy
IgG = immunoglobulin G
ILM = inner limiting membrane
IOP = intra-ocular pressure
IPL = inner plexiform layer
IR = ischemia-reperfusion
i.v. = intravenous
Ly6C = lymphocyte antigen 6C
Ly6G = lymphocyte antigen 6G
MDM = monocyte-derived macrophages
min = minute
mT/mG = membrane-targeted tdTomato/membrane-targeted GFP
OCT = optical coherence tomography
O.D. = optical density
ONL = outer nuclear layer
OPL = outer plexiform layer
PBS = phosphate-buffered saline
PBSTC = PBS containing 0.5% Triton X-100 and 0.1 mM CaCl₂
PE = phycoerythrin
PECAM-1 = platelet endothelial cell adhesion molecule-1
PerCP = peridinin chlorophyll protein
PFA = paraformaldehyde
PLVAP = plasmalemma vesicle associated protein
pS490 = phosphorylated serine 490
RBPMS = RNA binding protein with multiple slicing
RGC = retinal ganglion cell
ROP = retinopathy of prematurity

RPE = retinal pigment epithelium
RT = room temperature
RVO = retinal vein occlusions
SD-OCT = spectral domain optical coherence tomography
Ser490 = serine 490
SSC-A = side scatter area
TAM = tamoxifen
TBST = tris-buffered saline with Triton X-100
TdT = terminal deoxynucleotide transferase
tMCAO = transient middle cerebral artery occlusion
TJ = tight junction
TUNEL = terminal deoxynucleotidyl transferase dUTP nick end labeling
Tyr1175 = tyrosine 1175
VEGF = vascular endothelial growth factor
VEGFR2 = vascular endothelial growth factor receptor 2
wk = week
ZO-1 = zonula occludens 1
ZO-2 = zonula occludens 2

Declarations:*Ethics approval and consent to participate*

Animal protocols used in this study were reviewed and approved by the University of Michigan Institutional Animal Care and Use Committee (IACUC). Consent to participate is not applicable.

Consent for publication

Not applicable.

Availability of data and materials

The data sets generated and analyzed during the current study are available from the corresponding author upon reasonable request. The mouse strains used are commercially

available.

Competing interests

The authors declare that they have no competing interests to report.

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Author contributions

SFA and DAA obtained funding, designed experiments, evaluated results and wrote the manuscript. SS, AM, CML, DK, HH, and XL designed and performed experiments, evaluated and reported results.

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Authors' information

None.

Footnotes

None.

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Figure Legends:

Figure 1: Retinal IR injury induced edema and neurodegeneration. (A-D) At the indicated times following IR injury, total retinal thickness and thickness of the inner and outer retinal layers were measured *in situ* using SD-OCT. Measurements were made at 4 compass points 350 μ m from center of the optic nerve head and averaged. (A and B) Total retina spans from the inner limiting membrane (ILM) to the retinal pigment epithelium (RPE). Note that (B) shows the same data as (A) with an amplified y-axis. (C) Inner retina spans from ILM to the OPL. (D) Outer retina spans from the OPL to the RPE. Note that the y-axis scales are equal in (C) and (D). A total of n=30 eyes/group were initiated at time zero but, due to cataract formation in some injured eyes, IR group size declined to 15 eyes/group at day 28. * $p \leq 0.05$, ** $p \leq 0.01$ and *** $p \leq 0.001$ for IR versus sham by t-test. (E) At the indicated times following IR injury, ongoing cell death was evaluated in Sham and IR-injured retinas using a DNA fragmentation assay with n=6-8/group. * $p \leq 0.05$, ** $p \leq 0.01$ and *** $p \leq 0.001$ for IR versus Sham by t-test. (F) TUNEL staining and IHC of the RGC marker RBPMS was performed on flat-mounted Sham and IR retinas harvested at 1 day following injury.

Figure 2: Retinal IR injury caused breakdown of the iBRB that resolved after 2 weeks. (A) At the indicated times following IR injury, vascular permeability in Sham and IR retinas was evaluating

by measuring the FITC-BSA accumulation into retinal tissue with n=8/group: **p≤0.01. **(B)**

Representative images of permeability to sulfo-NHS-biotin in the superficial retinal vasculature (top panels) and the deep retinal vasculature (lower panels) obtained by confocal microscopy of Sham and IR retinas at 24 h after injury. Images show IF detection of the endothelial marker CD31 (PECAM-1, green) and the TJ protein ZO-1 (magenta), together with intravenously-injected sulfo-NHS-Biotin (red) as an indicator of leakage from the vasculature. Note areas of sulf-NHS-biotin leak corresponding to disruption of ZO-1 localization at endothelial cell borders.

Figure 3: Restoration of the vascular barrier following IR injury did not coincide with diminished perfused capillary volume or loss of vessel endothelialization. **(A)** Mice were perfused with FITC-BSA and immuno-probed for CD31 (PECAM-1) and ColIV followed by flat mounting and confocal microscopy. Arrows indicate empty sleeves that are positive for ColIV but lack FITC-BSA perfusion and CD31 **(B-D)** Percent of FITC-BSA and CD31 co-localized with ColIV was determined at 2 day **(B)**, 2 wk **(C)** and 4 wk **(D)** after IR injury. **(b-d)** Density of ColIV-positive, FITC-negative and CD31-negative empty sleeves were determined at 2 day **(b)**, 2 wk **(c)** and 4 wk **(d)** after IR injury. No significant differences were observed between Sham and IR groups using both parametric (t-test) and non-parametric (u-test) statistics.

Figure 4: Restoration of the vascular barrier coincided with reorganization of TJ complexes.

(A) At the indicated times following IR injury, retinas were removed and proteins in whole retinal lysates examined by western blotting with antibodies to occludin phosphorylated on serine 490 (pS490), total occludin protein, ZO-1, ZO-2 and claudin-5. A representative blot is shown. **(B)** Quantification of 4 replicate western blot experiments. Occludin pS490 immunoreactivity was normalized to that of total occludin. ZO-1, ZO-2, and claudin-5 immuno-reactivities were normalized

to that of β -actin. $*p \leq 0.05$, $**p \leq 0.01$, $***p \leq 0.001$ by t-test. **(C)** At various times following IR injury (2 day and 4 wk samples shown) retinas were removed and immuno-probed with antibodies to occludin and ZO-1, followed by flat mounting and confocal microscopy. **(D)** Histograms represent the evaluation of TJ continuity at endothelial cell borders using a blinded rank (1-5) scoring system. In the graph, green indicates completely continuous, yellow indicates 75% to 100% continuous, pink indicates 50% to 75% continuous, orange indicates 25% to 50% continuous and red indicates 0% to 25% continuous border staining. For each retina four images equidistant from the optic disc were averaged with $n=4$ retinas for each condition. $*p \leq 0.05$, $****p \leq 0.0001$ by t-test.

Figure 5: IR injury induced progressive changes in innate immune cell populations within the retina. **(A)** Representative scatter-graphs showing the flow-cytometric analysis used to quantify immune cell populations in the retina. After gating for single cells, events were gated into $CD11b^+/CD45^{low}$ (principally microglia), $CD11b^+/CD45^{hi}$ myeloid leukocytes, and $CD11b^{neg}/CD45^{hi}$ lymphocytes. Myeloid leukocytes were further gated into $CD11b^+/CD45^{hi}/Ly6C^{hi}/Ly6G^{neg}$ classical monocytes, $CD11b^+/CD45^{hi}/Ly6C^{neg}/Ly6G^{neg}$ non-classical monocytes or MDM, and $CD11b^+/CD45^{hi}/Ly6C^+/Ly6G^+$ granulocytes. Microglia-like cells were further gated to quantify $CD11b^+/CD45^{low}/Ly6C^{neg}/Ly6G^{neg}$ microglia. **(B-E)** At the indicated times following IR injury, flow-cytometric analysis was used to quantify microglia and leukocyte populations in Sham and IR-injured retinas, including: granulocytes **(B)**, classical monocytes **(C)**, non-classical monocytes/MDM **(D)** and microglia **(E)**. For each analysis 4 retinas were pooled and analyzed with $n=4$ pools of retinas for each group at 1 day, 4 day, 1 wk and 4 wk following IR injury. $*p \leq 0.05$, $**p \leq 0.01$ and $***p \leq 0.001$ by one-way ANOVA with Bonferroni and Sidak multiple comparison test. **(F)** Representative images obtained by confocal microscopy of the superficial retinal vasculature of at the optic nerve head and periphery of Sham and IR retinas at 24 h after injury. Images show IB4 (green, Note: binds to

endothelium, with arterial>venous, and to some leukocytes), ColIV (red), and CD45 (magenta). Note that CD45⁺ leukocytes appear both within vessel lumen and within the retina. **(D)** Confocal microscopy images showing IB4 (green), ZO-1 (red) and CD45 (magenta) of a superficial vascular region of an IR-injured retina at 24 h after injury (Sham not shown). Note that the vessel is exhibiting disorganization of endothelial TJ complexes coinciding with extravasation of CD45⁺ leukocytes.

Figure 6: Retinal IR injury caused microglia proliferation and mobilization toward the GCL.

(A) Representative images of IF of Iba-1 (microglia/phagocyte marker) and Ki-67 (proliferative cell marker) in retinal sections from Sham and IR retinas at 2 days following IR injury. **(B)** Representative confocal microscopic images of IB4, Iba-1 and Ki-67 IF in flat-mounted retinas from naive (no treatment), Sham-treated and IR-injured retinas. Z-stacks of images from retinal layers containing the superficial vascular plexus (GCL and nerve fiber layer) and the deep vascular plexus (IPL, INL and OPL). **(C)** Quantification of microglia and proliferative cells in retinal layers. Cell counts from confocal microscopic images (as shown in A, 4 images/retina/depth) were used to quantify Iba-1⁺ and Ki-67⁺ cells and the extent of co-localization of these markers with n=2 retinas/group for naive and n=4 retinas/group for Sham and IR and *p≤0.05, **p≤0.01 and ***p≤0.001 for IR versus Sham by one-way ANOVA. **(D)** Effect of IR injury on microglia/phagocyte activation evaluated by increase in soma size, an indication of loss of process ramification and/or an amoeboid morphology. Confocal microscopic images of Iba-1 IF in (B) were used to evaluate soma sizes (areas) of Iba-1⁺ microglia/phagocytes. Note that Iba-1⁺ cells in IR-injured retinas had significantly larger mean soma size than both naive and Sham retinas. *p<0.05, ***p<0.001 by one-way ANOVA.

Figure 7: Lineage tracing of microglia show their increase in the inner retinal and appearance of CD11b⁺/Iba1⁺ myeloid leukocytes after IR injury. CX3CR1-CreERT2 knockin driver mice crossed with mT/mG reporter mice were used to lineage trace microglia as GFP⁺. A 4-wk washout period after TAM treatment was used to clear GFP⁺ Cre-recombined monocytes from circulation prior to IR injury. **(A)** Representative images of GFP, CD11b and Iba-1 expressing cells in inner (GCL+IPL) and deep (INL+OPL) layers of Sham and IR-injured retinas at 4 days after injury. Note that essentially all CD11b⁺ cells in the Sham retina are GFP⁺ microglia expressing Iba-1. Quantification and categorization of CD11b⁺ cells in the inner **(B)**, deep **(C)**, and combined **(D)** layers. Note that the number of CD11b⁺/Iba-1⁺/GFP⁺ microglia nearly doubles in the inner layers, while not changing in the deep layers. An appreciable population of CD11b⁺/Iba-1⁺/GFP^{neg} myeloid leukocytes appears in both layers after IR injury, while there are relatively few myeloid leukocytes that are negative for Iba-1. *p≤0.05, **p≤0.01 and ***p≤0.001 for IR versus Sham by u-test.

Figure 8: Intervention treatment with minocycline promotes restoration of the iBRB. Mice were treated by daily injections of minocycline (50 µg/g-BW i.p.) or saline vehicle beginning 7 days after retinal IR injury. At 14 days following IR injury, vascular permeability in Sham and IR retinas was evaluating by measuring the accumulation of intravenously injected FITC-BSA in retinal tissues. n=13-16/group. ***p≤0.001 by 2-way ANOVA with Tukey post-hoc test.

Supplemental Data Figure S1: Apoptosis is ongoing in the outer nuclear layer at 2 weeks after IR injury. Nuclei with fragmented DNA were detected using the Click-iT™ Plus TUNEL assay kit (Thermo Fisher Scientific) on flat-mounted retinas. Representative images of TUNEL staining (Magenta) in the ONL of Sham and IR-injured retinas obtained by confocal microscopy (63X). Nuclei were counterstained with Hoechst (blue). Hoechst staining was used to determine the ONL. A

Z-stack of confocal microscope images spanning from the OPL up to the outer boarder of the ONL are shown. Scale bars = 10 μ m.

Supplemental Data Figure S2: IR injury did not cause loss of endothelial cells. (A) At the indicated times following IR injury, flow-cytometric analysis was used to quantify CD31⁺/IB4⁺ endothelial cell numbers in retinas. For each analysis 2 retinas were pooled, enzymatically dissociated, probed with antibody to CD31 (PECAM1) and with IB4, and analyzed by flow cytometry. (B) CD31⁺/IB4⁺ cells were quantified as percentage of total events. No significant differences were observed between Sham and IR groups using both parametric t-test and non-parametric u-test statistics.

Supplemental Data Figure S3: IR injury induced the appearance of a CD11b⁺/CD45^{low}/Ly6C^{neg}/Ly6G^{neg} cell populations within the retina. (A) Representative scatter-graphs showing the flow-cytometric analysis used to quantify immune cell populations in the retina. After gating for single cells, events were gated into CD11b⁺/CD45^{low} cells and then further gated to separate CD11b⁺/CD45^{low}/Ly6C⁺/Ly6G⁺ cells from CD11b⁺/CD45^{low}/Ly6C^{neg}/Ly6G^{neg} microglia. (B) At the indicated times following IR injury, flow-cytometric analysis was used to quantify CD11b⁺/CD45^{low}/Ly6C⁺/Ly6G⁺ cell populations in Sham and IR-injured retinas. For each analysis 4 or more retinas were pooled and analyzed with n=4 pools of retinas for each group at 1 day, 4 day, 1 wk and 4 wk following IR injury. *p $\leq$ 0.05 and ***p $\leq$ 0.001 by one-way ANOVA with Bonferroni and Sidak multiple comparison test.

Supplemental Data Figure S4: Iba-1⁺ phagocytes express CD68 and engulf cells in the GCL after IR injury. (A) Representative confocal microscope images of retinal sections from a Sham

retina at 4 days after IR injury showing nuclei (Hoechst staining), microglia/phagocytes (Iba-1), Brn3a (a nuclear protein expressed in a major subset of RGC) and CD68 (a marker of phagocytes). Note that in the Sham retina there are few microglia/phagocytes in the GCL and essentially no detectable CD68. **(B)** Representative image of IR-injured retina section at 4 days suggesting mobilization of Iba-1⁺ microglia and close association of Iba-1⁺/CD68⁺ phagocytes with nuclei in the GCL. **(C)** 3-dimensional reconstructions using Imaris software showing a Iba-1⁺ microglia/phagocyte engulfing a Brn3a⁺ RGC in the GCL of an IR retina. The left image shows nuclei (blue), Iba-1 (green) and CD68 (magenta). The right image shows Brn3a (red), Iba-1 (green) and CD68 (magenta).

Fig. 1

Fig 2

Fig 3

Fig 4

Fig 5

Fig. 6

Fig. 7

Fig. 8

Supplemental Data:

Supplemental Data Fig. S1

Supplemental Data Fig. S2

A

SSC-A

FSC-A

FSC-W

FSC-A

Single Cells 95.5

G1 78.8

All Retinal cells

Sham

IR

CD11b

CD45

Microglia 0.20

ML 0.025

Lymphocytes 0.012

Microglia 0.24

ML 0.29

Lymphocytes 0.025

Microglia-like cells

Sham

IR

Ly6C

Ly6G

Microglia 1.02

3.48

94.7

0.80

Microglia 8.47

28.8

61.8

0.98

$CD11b^{+}/CD45^{low}/Ly6C^{+}/Ly6G^{+}$

Supplemental Data Fig. S4

Figures

Figure 1

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Figure 2

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Images show IF detection of the endothelial marker CD31 (PECAM-1, green) and the TJ protein ZO-1 (magenta), together with intravenously-injected sulfo-NHS-Biotin (red) as an indicator of leakage from the vasculature. Note areas of sulf-NHS-biotin leak corresponding to disruption of ZO-1 localization at endothelial cell borders.

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Restoration of the vascular barrier following IR injury did not coincide with diminished perfused capillary volume or loss of vessel endothelialization. (A) Mice were perfused with FITC-BSA and immuno-probed for CD31 (PECAM-1) and ColIV followed by flat mounting and confocal microscopy. Arrows indicate empty sleeves that are positive for ColIV but lack FITC-BSA perfusion and CD31 (B-D) Percent of FITC-BSA and CD31 co-localized with ColIV was determined at 2 day (B), 2 wk (C) and 4 wk (D) after IR injury. (b-d) Density of ColIV-positive, FITC-negative and CD31-negative empty sleeves were determined at 2 day (b), 2 wk (c) and 4 wk (d) after IR injury. No significant differences were observed between Sham and IR groups using both parametric (t-test) and non-parametric (u-test) statistics.

Figure 4

Restoration of the vascular barrier coincided with reorganization of TJ complexes. (A) At the indicated times following IR injury, retinas were removed and proteins in whole retinal lysates examined by western blotting with antibodies to occludin phosphorylated on serine 490 (pS490), total occludin protein, ZO-1, ZO-2 and claudin-5. A representative blot is shown. (B) Quantification of 4 replicate western blot experiments. Occludin pS490 immunoreactivity was normalized to that of total occludin. ZO-1, ZO-2, and

claudin-5 immuno-reactivities were normalized to that of β -actin. $*p \leq 0.05$, $**p \leq 0.01$, $***p \leq 0.001$ by t-test. (C) At various times following IR injury (2 day and 4 wk samples shown) retinas were removed and immuno-probed with antibodies to occludin and ZO-1, followed by flat mounting and confocal microscopy. (D) Histograms represent the evaluation of TJ continuity at endothelial cell borders using a blinded rank (1-5) scoring system. In the graph, green indicates completely continuous, yellow indicates 75% to 100% continuous, pink indicates 50% to 75% continuous, orange indicates 25% to 50% continuous and red indicates 0% to 25% continuous border staining. For each retina four images equidistant from the optic disc were averaged with $n=4$ retinas for each condition. $*p \leq 0.05$, $****p \leq 0.0001$ by t-test.

Figure 5

IR injury induced progressive changes in innate immune cell populations within the retina. (A) Representative scatter-graphs showing the flow-cytometric analysis used to quantify immune cell populations in the retina. After gating for single cells, events were gated into CD11b⁺/CD45^{low} (principally microglia), CD11b⁺/CD45^{hi} myeloid leukocytes, and CD11b^{neg}/CD45^{hi} lymphocytes. Myeloid leukocytes were further gated into CD11b⁺/CD45^{hi}/Ly6C^{hi}/Ly6G^{neg} classical monocytes,

CD11b⁺/CD45^{hi}/Ly6C^{neg}/Ly6G^{neg} non-classical monocytes or MDM, and CD11b⁺/CD45^{hi}/Ly6C⁺/Ly6G⁺ granulocytes. Microglia-like cells were further gated to quantify CD11b⁺/CD45^{low}/Ly6C^{neg}/Ly6G^{neg} microglia. (B-E) At the indicated times following IR injury, flowcytometric analysis was used to quantify microglia and leukocyte populations in Sham and IR-injured retinas, including: granulocytes (B), classical monocytes (C), non-classical monocytes/MDM (D) and microglia (E). For each analysis 4 retinas were pooled and analyzed with n=4 pools of retinas for each group at 1 day, 4 day, 1 wk and 4 wk following IR injury. *p≤0.05, **p≤0.01 and ***p≤0.001 by one-way ANOVA with Bonferroni and Sidak multiple comparison test. (F) Representative images obtained by confocal microscopy of the superficial retinal vasculature of at the optic nerve head and periphery of Sham and IR retinas at 24 h after injury. Images show IB4 (green, Note: binds to endothelium, with arterial>venous, and to some leukocytes), ColIV (red), and CD45 (magenta). Note that CD45⁺ leukocytes appear both within vessel lumen and within the retina. (D) Confocal microscopy images showing IB4 (green), ZO-1 (red) and CD45 (magenta) of a superficial vascular region of an IR-injured retina at 24 h after injury (Sham not shown). Note that the vessel is exhibiting disorganization of endothelial TJ complexes coinciding with extravasation of CD45⁺ leukocytes.

Figure 6

Retinal IR injury caused microglia proliferation and mobilization toward the GCL. (A) Representative images of IF of Iba-1 (microglia/phagocyte marker) and Ki-67 (proliferative cell marker) in retinal sections from Sham and IR retinas at 2 days following IR injury. (B) Representative confocal microscopic images of IB4, Iba-1 and Ki-67 IF in flat-mounted retinas from naive (no treatment), Sham-treated and IR-injured retinas. Z-stacks of images from retinal layers containing the superficial vascular plexus (GCL and nerve

fiber layer) and the deep vascular plexus (IPL, INL and OPL). (C) Quantification of microglia and proliferative cells in retinal layers. Cell counts from confocal microscopic images (as shown in A, 4 images/retina/depth) were used to quantify Iba-1+ and Ki-67+ cells and the extent of co-localization of these markers with n=2 retinas/group for naive and n=4 retinas/group for Sham and IR and *p≤0.05, **p≤0.01 and ***p≤0.001 for IR versus Sham by one-way ANOVA. (D) Effect of IR injury on microglia/phagocyte activation evaluated by increase in soma size, an indication of loss of process ramification and/or an amoeboid morphology. Confocal microscopic images of Iba-1 IF in (B) were used to evaluate soma sizes (areas) of Iba-1+ microglia/phagocytes. Note that Iba-1+ cells in IRinjured retinas had significantly larger mean soma size than both naive and Sham retinas. *p<0.05, ***p<0.001 by one-way ANOVA.

Figure 7

Lineage tracing of microglia show their increase in the inner retinal and appearance of CD11b+/Iba1+ myeloid leukocytes after IR injury. CX3CR1-CreERT2 knockin driver mice crossed with mT/mG reporter mice were used to lineage trace microglia as GFP+. A 4-wk washout period after TAM treatment was used to clear GFP+ Cre-recombined monocytes from circulation prior to IR injury. (A) Representative images of GFP, CD11b and Iba-1 expressing cells in inner (GCL+IPL) and deep (INL+OPL) layers of Sham and IR-injured retinas at 4 days after injury. Note that essentially all CD11b+ cells in the Sham retina are GFP+ microglia expressing Iba-1. Quantification and categorization of CD11b+ cells in the inner (B), deep (C), and combined (D) layers. Note that the number of CD11b+/Iba-1+/GFP+ microglia nearly doubles in the inner layers, while not changing in the deep layers. An appreciable population of CD11b+/Iba-1+/GFPneg myeloid leukocytes appears in both layers after IR injury, while there are relatively few myeloid leukocytes that are negative for Iba-1. * $p \leq 0.05$, ** $p \leq 0.01$ and *** $p \leq 0.001$ for IR versus Sham by u-test.

Figure 8

Intervention treatment with minocycline promotes restoration of the iBRB. Mice were treated by daily injections of minocycline (50 µg/g-BW i.p.) or saline vehicle beginning 7 days after retinal IR injury. At 14 days following IR injury, vascular permeability in Sham and IR retinas was evaluating by measuring the

accumulation of intravenously injected FITC-BSA in retinal tissues. n=13-16/group. *** $p \leq 0.001$ by 2-way ANOVA with Tukey post-hoc test.