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Early retinal inflammatory biomarkers in the middle cerebral artery occlusion model of ischemic stroke

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Purpose: The transient middle cerebral artery occlusion (MCAO) model of stroke is one of the most commonly used models to study focal cerebral ischemia. This procedure also results in the simultaneous occlusion of the ophthalmic artery that supplies the retina. Retinal cell death is seen days after reperfusion and leads to functional deficits; however, the mechanism responsible for this injury has not been investigated. Given that the eye may have a unique ocular immune response to an ischemic challenge, this study examined the inflammatory response to retinal ischemia in the MCAO model.

Methods: Young male C57B/6 mice were subjected to 90-min transient MCAO and were euthanized at several time points up to 7 days. Transcription of inflammatory cytokines was measured with quantitative real-time PCR, and immune cell activation (e.g., phagocytosis) and migration were assessed with ophthalmoscopy and flow cytometry.

Results: Observation of the affected eye revealed symptoms consistent with Horner's syndrome. Light ophthalmoscopy confirmed the reduced blood flow of the retinal arteries during occlusion. CX3CR1-GFP reporter mice were then employed to evaluate the extent of the ocular microglia and monocyte activation. A significant increase in green fluorescent protein (GFP)-positive macrophages was seen throughout the ischemic area compared to the sham and contralateral control eyes. RT–PCR revealed enhanced expression of the monocyte chemotactic molecule CCL2 early after reperfusion followed by a delayed increase in the proinflammatory cytokine TNF-α. Further analysis of peripheral leukocyte recruitment by flow cytometry determined that monocytes and neutrophils were the predominant immune cells to infiltrate at 72 h. A transient reduction in retinal microglia numbers was also observed, demonstrating the ischemic sensitivity of these cells. Blood–eye barrier permeability to small and large tracer molecules was increased by 72 h. Retinal microglia exhibited enhanced phagocytic activity following MCAO; however, infiltrating myeloid cells were significantly more efficient at phagocytizing material at all time points. Immune homeostasis in the affected eye was largely restored by 7 days.

Conclusions: This work demonstrates that there is a robust inflammatory response in the eye following MCAO, which may contribute to a worsening of retinal injury and visual impairment. These results mirror what has been observed in the brain after MCAO, suggesting a conserved inflammatory signaling response to ischemia in the central nervous system. Imaging of the eye may therefore serve as a useful non-invasive prognostic indicator of brain injury after MCAO. Future studies are needed to determine whether this inflammatory response is a potential target for therapeutic manipulation in retinal ischemia.

Ischemic injury in the central nervous system (CNS) is a result of a restriction of the blood supply that prevents tissue from fulfilling its metabolic demands. Neurons are especially vulnerable to ischemic events, and given the lack of regeneration in the adult CNS, any sustained injury may result in long-term functional impairment. In rodents, one of the most commonly used experimental models of stroke is the transient middle cerebral artery occlusion (MCAO) model [1]. MCAO is the most common type of infarct and one of the most clinically severe types of stroke [2,3]. In rodents, this experimental procedure involves a transient unilateral occlusion of the MCA with either a filament or clot. This results in a focal infarct of the ipsilateral hemisphere and subsequent reperfusion injury. Despite the translational utility of this model, its reproducibility, and widespread use, several limitations exist. Emerging data suggest that ischemic injury in this model is not confined to the ipsilateral hemisphere but can also involve the ipsilateral retina [4]. This is due to the proximal origin of the internal carotid artery (ICA) to the MCA. The ICA also contributes to the arterial supply of the ophthalmic artery that supplies the inner retina. Thus, unilateral occlusion of the MCA will often result in restricted blood

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flow to the ipsilateral retina in rodents and humans. Although the vascular supply to the eye is dependent on the MCA, the devastating effects of ocular ischemia are less frequently observed in patients with MCA stroke due to the variable status of collateral vessels and retrograde blood flow, which can attenuate the effects of arterial occlusion via alternative routes of blood flow [5]. To date, the inflammatory events that develop in the eye after MCAO, which may have different mechanisms and chronology than those in the brain, have not been examined. As MCA occlusion has the potential to adversely affect ocular function in rodents and humans and can be evaluated non-invasively, the rodent MCAO model is a suitable model for studying the inflammatory response over time in an in vivo model.

In humans, ophthalmic artery occlusions or emboli lead to severe, sudden painless loss of vision due to retinal ischemia. Several experimental studies have reported neuronal apoptosis, retinal thinning, astrocyte activation, and diminished physiologic responses to light stimuli following MCAO in rodents [4,6,7]. As the MCAO model is widely used, the presence of retinal ischemia may potentially confound the interpretation of behavioral outcomes and assessment of long-term recovery. This underreported phenomenon, which occurs more frequently with longer occlusion times, may also provide researchers with the unique opportunity to study the mechanisms that underlie cell death in the brain and the eye. Numerous studies by our laboratory and others have described a stereotypical brain response to ischemic stress, one that begins with excitotoxicity and subsequently gives rise to a robust inflammatory response that can last for days or weeks, influencing overall recovery [8,9]. The eye is considered an immune privileged site; and similar to the brain, the eye has developed unique constraints on ocular immune responses to prevent chronic inflammation [10,11]. Thus, understanding the complex role of inflammatory processes in the eye may yield better insights into the pathogenesis of diseases such as retinal ischemia, including strategies for intervention.

Although neuronal apoptosis has been observed in the retina after MCAO, the contribution of inflammatory signaling to ocular injury has been less well studied. Brain ischemia drives the convergence of several known inflammatory signaling pathways to elicit a sterile inflammatory response; these processes are less understood in ocular ischemia. Sequential recruitment and activation of bone marrow–derived leukocytes to the ischemic brain occurs after stroke, but the degree to which this occurs in the eye after MCAO has not been studied. In this study, the inflammatory response in the eye after MCAO was evaluated. Our findings indicate that the brain and the eye share a conserved inflammatory response to ischemic stress.

METHODS

Mice: Young adult C57BL/6J and CX3CR1$^{gfp/+}$ male mice (10–14 weeks) of age were pair-housed in barrier cages in a specific pathogen-free facility (12 h:12 h light-dark cycle) [12]. All animals had access to mouse chow and water ad libitum. All procedures were performed in accordance with the U.S. National Institutes of Health Guidelines for the Care and Use of Laboratory Animals and were approved by the Institutional Animal Care and Use Committee of the University of Connecticut Health Center. All analyses were performed blinded to the surgical conditions. All experimental procedures performed in this study complied with the ARVO statement for the use of Animals in Ophthalmic and Vision Research.

Ischemic stroke model: Cerebral ischemia was induced with 90 min of reversible MCAO (20–25 g mice) under isoflurane anesthesia as previously described [13]. To perform surgery, mice were rapidly sedated with 4% isoflurane inhalation anesthesia, and the level of sedation was confirmed by the lack of response to tail pinch. Surgery was performed under 1% continuous isoflurane anesthesia. At the end of ischemia, the animal was briefly re-anesthetized and reperfusion was initiated by filament withdrawal. Rectal temperatures were maintained at approximately 37 °C during surgery and ischemia with an automated temperature control feedback system. A midline ventral neck incision was made, and unilateral MCAO was performed by inserting a 6.0 Doccol monofilament (Doccol Corp, Redland, CA) into the right internal carotid artery 6 mm from the internal carotid and pterygopalatine artery bifurcation via an external carotid artery stump. Following reperfusion, the mice were euthanized at 8 h, 24 h, 72 h, and 7 days. Sham-operated animals underwent the same surgical procedure, but the suture was not advanced into the internal carotid artery.

Fundus image acquisition: Each eye was given a topical anesthetic using one drop of 1% atropine sulfate (Bausch and Lomb, Tampa, FL). After 2 min, a drop of phenylephrine 2.5% solution (Akorn Inc., Lake Forest, IL) was applied topically to each eye to dilate the pupils, followed by a second drop within 10 min. Ketamine (100 mg/kg) and xylazine (10 mg/kg) were injected subsequently into the peritoneum for sedation. The cornea was kept moist with a wetting agent (GenTeal lubricant eye gel, Alcon Laboratories, Fort Worth, TX). Body temperature was maintained at 37 °C at all times on a warming pad. Imaging was performed immediately after this step using a Micron III Imaging system (Phoenix

Research Labs, Inc., Pleasanton, CA). Mice were imaged with the application of GenTeal to the anterior segment of the mouse eye, and the camera lens was placed in contact with the cornea. When the mice had recovered from the anesthesia, they were returned to their cages and housed in their barrier cages.

Tissue harvesting and flow cytometry: Mice were euthanized after stroke or sham surgery with Avertin (2, 2, 2-tribromoethanol, 250 mg/kg i.p, Sigma) and transcardially perfused with 60 ml cold, sterile PBS saline (Lonza, #17-516, Walkersville, MD; KH_2PO_4 144 mg/l, NaCl 9,000 mg/l, Na_2HPO_4 795 mg/l; pH 7.4), and the eyes were harvested. The samples were then processed in a blinded fashion. The eyes were pooled (two per sample) for increased yield and placed in complete RPMI 1640 (Lonza) medium and mechanically and enzymatically digested in collagenase/dispase (1 mg/ml) and DNase (10 mg/ml; both Roche Diagnostics, Indianapolis, IN) for 1 h at 37 °C. The cell suspension was filtered through a 70 µm filter. Leukocytes were harvested from the interphase of a 70%/30% Percoll gradient. Cells were washed and blocked with mouse Fc Block (eBioscience, San Diego, CA) before staining with primary antibody-conjugated fluorophores: CD45-eF450, CD11b-APCeF780, Ly6C-PerCP-Cy5.5, and Ly6G-PE. All primary-conjugated antibodies were purchased from eBioscience. For live/dead discrimination, a fixable viability dye, carboxylic acid succinimidyl ester (CASE-AF350, Invitrogen), was diluted at 1:300 from a working stock of 0.3 mg/ml. Cells were briefly fixed in 2% paraformaldehyde (PFA). Data were acquired on a LSRII using FACsDiva 6.0 (BD Biosciences, Franklin Lakes, NJ) and analyzed using FlowJo (Treestar Inc., Ashland, OR). Fluorescence minus one (FMO) controls were used to determine the positivity of each antibody. The microglia were identified as $CD45^{int}CD11b^{+}Ly6C^{-}Ly6G^{-}$ whereas the infiltrating myeloid cell populations were identified as $CD45^{hi}CD11b^{+}$.

Measurement of phagocytic activity: The eyes were harvested and processed as above, and fluorescent latex beads (Fluoresbrite Yellow Green (YG) carboxylate microspheres; 1 µm diameter; Polysciences) were immediately added to the isolated leukocytes in a final dilution of 1:100 as described [14]. After 1 h incubation at 37 °C with periodic agitation, the cells were washed three times with FACS buffer, resuspended in FACS buffer, stained for surface markers, and fixed in PFA.

Assessment of blood–eye barrier permeability: Vascular integrity was measured as previously described [15]. Briefly, 2 h before the animals were euthanized, Evans Blue (Sigma, St. Louis, MO, 2% in isotonic saline, 4 ml/kg) was injected intraperitoneally. The animals were anesthetized and perfused with 60 ml of cold, sterile PBS. The eyes were harvested, rinsed with PBS, and weighed. The samples were homogenized in 1 ml of PBS using a tissue homogenizer. An equal volume of 100% trichloroacetic acid (TCA) solution was added to each sample and incubated at 37 °C for 1 h. The samples were then centrifuged at 24,000 ×*g* for 20 min at 4 °C. The supernatant was plated 100 µl per well in triplicate, and colorimetric absorbance was measured at 608 nm (EnSpire 2300 Multilabel Reader, Perkin Elmer, Waltham, MA). For quantitative measurement of Evans Blue leakage, the data were normalized to the tissue weight and measured as a concentration (µg/mg tissue weight) of Evans Blue using a standard curve (Evans Blue in 50% TCA).

Thirty minutes before the animals were euthanized, 2 ml/kg of a 10% sodium fluorescein (NaF) solution (Sigma) was injected and processed as above. For each sample, 100 µl of supernatant was measured in triplicate at 400 nm (excitation) and 516 nm (emission). Values obtained from 30% TCA were subtracted from each sample to remove the background.

RNA isolation and qPCR: Eyes were harvested, and RNA was extracted using TRIzol Reagent (Invitrogen, Carlsbad, CA). cDNA was synthesized from 1 µG RNA using an Applied Biosystems High Capacity cDNA Reverse Transcription kit (4,368,813, Applied Biosystems, Foster City, CA) according to the manufacturer's instructions. Quantitative real-time PCR (qPCR) was performed using an Applied Biosystems 7500 machine using SYBR Green (Applied Biosystems) for visualization and quantification according to the manufacturer's instructions. Gene expression was normalized relative to *glyceraldehyde-3-phosphate dehydrogenase* (*GAPDH*) expression. Primer sequences were as follows: GAPDH, forward: 5′-ACC ACC ATG GAG AAG GC-3′, reverse: 5′-GGC ATG GAC TGT GGT CAT GA-3′; *myeloperoxidase* (*MPO*), forward: 5′-AGT TGT GCT GAG CTG TAT GGA-3′, reverse: 5′-CGG CTG CTT GAA GTA AAA CAG G-3′; *tumor necrosis factor-alpha* (*TNF-α*), forward: 5′-GAC CCT CAC ACT CAG ATC ATC TTC T-3′, reverse: 5′-CCT CCA CTT GGT GGT TTG CT-3′; *interleukin 1-beta* (*IL-1β*), forward: 5′- CTG CAG GCT TCG AGA TGA ACA A-3′, reverse: 5′-TGT CCA TTG AGG TGG AGA GCT T-3′; and *monocyte chemoattractant protein-1* (*MCP-1/CCL2*), forward: 5′-TTA AAA ACC TGG ATC GGA ACC AA, reverse: 5′-GCA TTA GCT TCA GAT TTA CGG GT-3′. PCR conditions were as follows: 95 °C for 10 min, followed by 40 cycles of 95 °C for 15 s and 60 °C for 60 s. Amplification reactions were performed in triplicate; relative expression levels were calculated from the average threshold cycle number using the delta-delta Ct method. The GAPDH Ct values did not

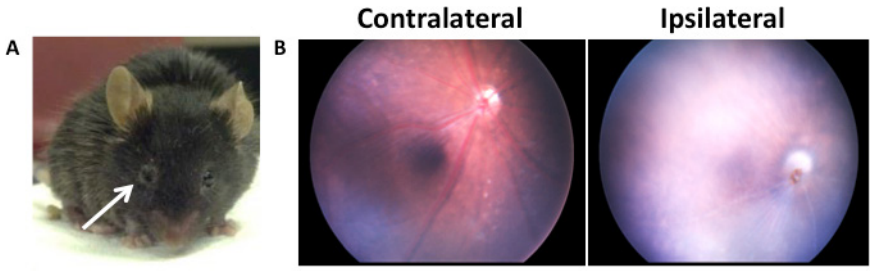

Figure 1. Blood flow reduction through the ophthalmic artery via middle cerebral artery occlusion. The middle cerebral artery (MCA) and the ophthalmic artery in the mouse and occlusion following suture insertion. **A**: A representative image showing the gross observation of acute sensitivity in the ipsilateral eye during MCA occlusion. **B**: Ophthalmoscopic imaging depicts blood flow to the contralateral (left) and ipsilateral (right) retina during MCA occlusion.

significantly differ between the sham and stroke (or ipsilateral and contralateral) groups as previously shown [16-18].

Statistical analyses: The mice were randomly assigned to the sham and stroke surgery groups. Analysis was performed by a blinded investigator. Data from individual experiments are presented as mean ± standard error of the mean (SEM) and assessed with the Student *t* test (GraphPad Prism Software Inc., San Diego, CA). Leukocyte counts were analyzed with one-way ANOVA followed by Tukey's test. Two-way ANOVA with Sidak's method for multiple comparisons was applied to all phagocytosis data. Significance was set at $p<0.05$.

RESULTS

Middle cerebral artery occlusion leads to the early expression of ocular proinflammatory mediators: The ophthalmic artery branches off the internal carotid artery and is simultaneously occluded in the MCAO model of experimental stroke. During and after ischemia, the mice exhibited observable differences in ipsilateral eye sensitivity as evidenced by wincing and closure of the eyelid of the affected ipsilateral eye. By 72 h after stroke, visible white discoloration and inflammatory discharge around the eyelid of the ipsilateral eye were also observed (Figure 1A). A representative image depicts the blockage of blood flow during ischemia in the ipsilateral eye compared to the contralateral eye (Figure 1B). Early gene expression of the proinflammatory cytokines *TNF-α* and *IL-1β* was significantly increased (Figure 2A, $p = 0.04$ and Figure 2B, $p = 0.01$, respectively) in the ipsilateral eye of the mice 8 h after stroke compared to the sham group. A nearly sixfold increase in gene expression of the myeloid cell recruitment chemokine *CCL2* relative to the sham group was also observed 8 h after stroke (Figure 2C, $p = 0.03$). Transcription of MPO, a lysosomal protein released into the extracellular space after myeloid cell degranulation, was modestly but significantly increased (Figure 2D, $p = 0.05$). These data suggest that occlusion of MCA results in ophthalmic artery ischemia and that the local glial populations in the eye are responsive to the acute effects of ischemia. In response, these cells become activated and proinflammatory, and signal recruitment of peripheral myeloid cells into the eye.

Elevated CCL2 production is associated with loss of vascular integrity: The ocular inflammatory response 72 h after reperfusion was also assessed. Interestingly, gene expression of *TNF-α* and *IL-1β* had significantly decreased from their initial elevation at 8 h back to sham levels (Figure 3A, $p = 0.07$ and Figure 3B, $p = 0.27$). However, CCL2 expression remained elevated compared to the sham levels (Figure 3C, $p = 0.01$), and myeloperoxidase expression was further increased to nearly threefold of the baseline at 72 h compared to the sham group (Figure 3D, $p = 0.03$). This suggests that there is augmented recruitment of monocytes and neutrophils and transmigration across the endothelium into the ischemic eye. To see whether the vascular integrity of the blood–eye barrier was compromised after MCAO, high molecular weight (70 kDa, Evans Blue dye) and low molecular weight (376 Da, sodium fluorescein) tracers were separately injected before the mice were euthanized at 72 h. Figures 3E,F show significant leakage of the Evans Blue and sodium fluorescein tracers into the ischemic eye compared to the sham surgery eye ($p = 0.04$ and $p = 0.002$, respectively). These data suggest that although proinflammatory cytokine production by local glial populations may be suppressed after acute injury, ongoing ocular leukocyte infiltration occurs as a result of sustained chemokine expression and increased vascular permeability.

Microglia and macrophage populations are highly activated in the fundus after tMCAO: To examine microglia activation and monocyte recruitment in the ischemic eye, CX3CR1-GFP-expressing mice were assessed using an ophthalmoscope with fluorescence capability. The number and size of CX3CR1-GFP-positive cells were increased in the ipsilateral eye compared to the contralateral eye (Figure 4A). The measured fluorescence intensity was threefold higher after

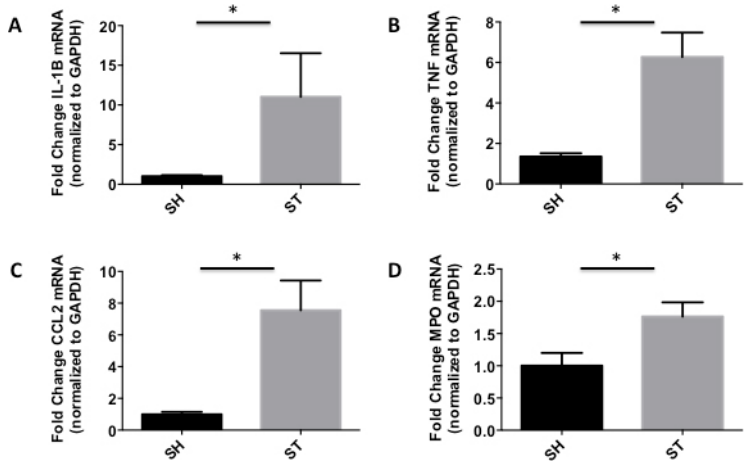

Figure 2. Acute expression of inflammatory mediators in the ipsilateral eye after MCAO. Quantitative real-time PCR analysis shows a significant relative fold increase in interleukin-1 beta (IL-1β) (**A**) and tumor necrosis factor (TNF) (**B**) mRNA expression in the ipsilateral eye 8 h after stroke compared to sham surgery. At 24 h, expression of the inflammatory signaling genes *monocyte chemoattractant protein-1* (*CCL2*) (**C**) and *myeloperoxidase* (*MPO*) (**D**) is increased following ischemia. For all experiments, n = 4/group. Error bars show mean ± standard error of mean (SEM). Abbreviations: GAPDH = glyceraldehyde 3-phosphate dehydrogenase, SH = sham, ST = stroke. *p<0.05; **p<0.01; ***p<0.001.

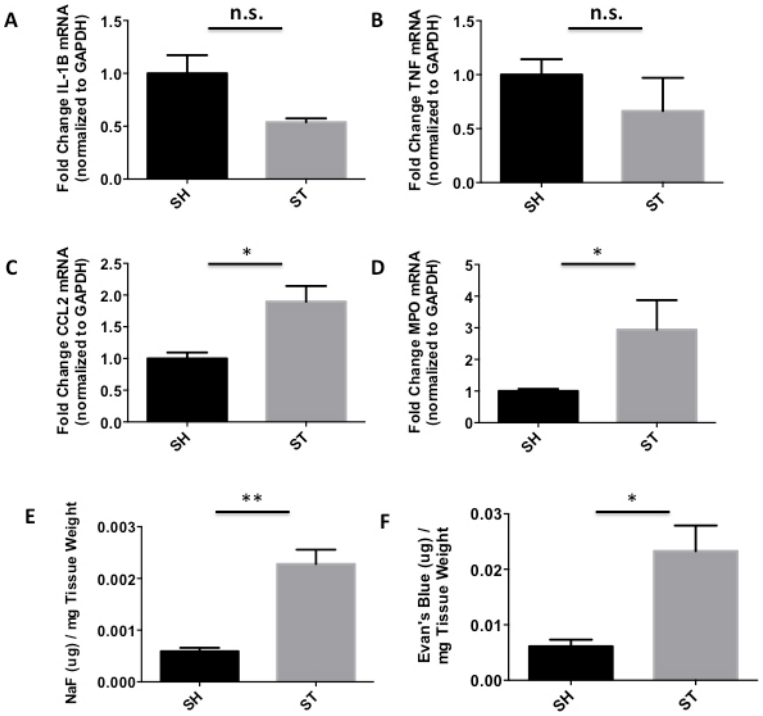

Figure 3. Differential cytokine expression and vascular permeability at 72 h. Quantitative real-time PCR analysis shows no difference in interleukin-1 beta (IL-1β) (**A**) and tumor necrosis factor (TNF) (**B**) mRNA expression in the ipsilateral eye after stroke compared to sham at 72 h. Expression of the inflammatory signaling genes *monocyte chemoattractant protein-1* (*CCL2*) (**C**) and *myeloperoxidase* (*MPO*) (**D**) continued to be increased at 72 h following ischemia. For all quantitative real-time PCR experiments, n = 5/group. Vascular permeability in the eye was measured following injection of low molecular weight (sodium fluorescein (NaF), **E**) and high molecular weight (Evans Blue, **F**) dye tracers at 72 h in the stroke and sham groups (n = 10/group). Error bars show mean ± standard error of mean (SEM). Abbreviation: GAPDH = glyceraldehyde 3-phosphate dehydrogenase, SH = sham, ST = stroke, NaF. *p<0.05; **p<0.01; ***p<0.001.

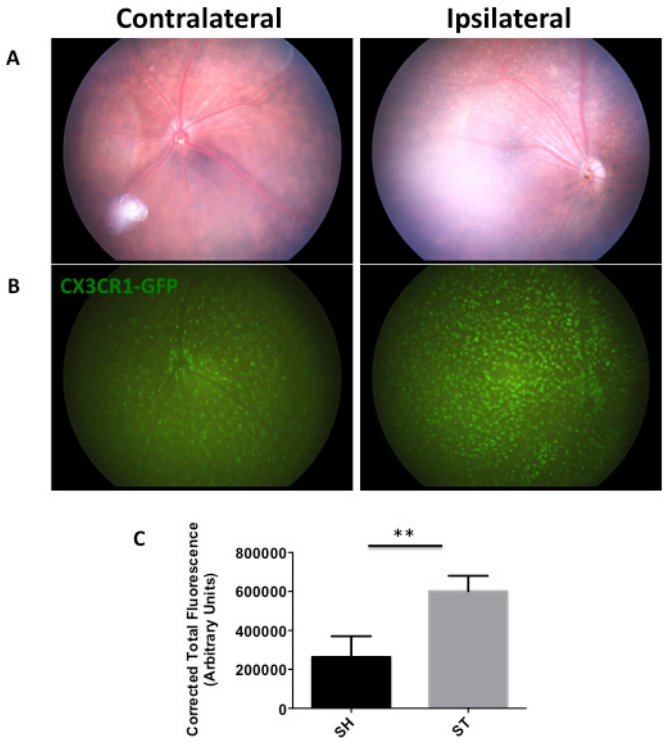

Figure 4. Increased number of microglia/macrophages in the retina at 72 h. **A**: Representative light depicting a significant increase in CX3CR1-GFP-positive microglia and macrophages in the ischemic ipsilateral eye at 72 h compared to the contralateral side. **B**: Fluorescence ophthalmoscopic images depicting a significant increase in CX3CR1-GFP-positive microglia and macrophages in the ischemic ipsilateral eye at 72 h compared to the contralateral side. The green fluorescence signal intensity was measured in the sham and stroke groups at 72 h after reperfusion (n = 4/group, **C**). Error bars show mean ± standard error of mean (SEM). Abbreviations: SH = sham, ST = stroke. *p<0.05; **p<0.01; ***p<0.001.

MCAO (Figure 4B, p = 0.001). Because the green fluorescent protein (GFP) signal is present on all macrophage subsets [19], these data suggest that there is either extensive microglia ($CX3CR1^{hi}$) proliferation or robust infiltration of monocytes ($CX3CR1^{lo}$) after ischemic ocular injury at 72 h.

MCAO results in the acute infiltration of debris-clearing bone marrow–derived myeloid cells and a transient reduction in retinal microglia numbers: To determine the cellular dynamics of inflammatory leukocytes in the ischemic eye over the course of 7 days, flow cytometry was used to distinguish the resident microglia ($CD45^{int}CD11b^{+}$) populations and the recruited bone marrow–derived myeloid populations ($CD45^{hi}CD11b^{+}$; Figure 5A) as previously described [20,21]. Infiltrating myeloid cells were further categorized as $Ly6C^{+}Ly6G^{-}$ monocytes (and their derivatives) or $Ly6C^{+}Ly6G^{+}$ neutrophils according to the gating strategy shown in Figure 5B. This method of identification has been previously validated and applied to retinal injury models [22-24]. The absolute number of infiltrating leukocytes is significantly increased at 72 h after MCAO (Figure 5C, p<0.05) but subsequently returns to baseline numbers by 1 week. Our data show that the majority of the cellular infiltrate is composed of myeloid cells (Figure 5A). Neutrophils make up the largest percentage of this infiltrate at 72 h after MCAO; however, the relative composition of myeloid cells in the ischemic eye is restored to baseline by 7 days (Figure 5E).

Microglia cell counts were significantly decreased (Figure 5C, p<0.05), albeit transiently at 72 h but returned to baseline by 1 week. Consistent with an activated and severely compromised microglia population, these cells showed significant increases in the cellular granularity (Figure 6A, p = 0.02) and the relative surface expression of the activation marker CD11b (Figure 6B, p = 0.002) compared to the sham group. Retinal microglia exhibited an increased potential to phagocytize beads at 72 h after MCAO compared to the sham group (Figures 6C–D, p<0.05), but this capacity returned to baseline levels by 7 days. Overall, the bone marrow–derived myeloid cells displayed a significantly enhanced ability to phagocytize material compared to the resident microglia (group effect with two-way ANOVA, p<0.001). Perivascular and/or choroid macrophages in the healthy eye were more efficient at phagocytosis than the resident microglia. The percentage of phagocytic myeloid cells in the ischemic eye was further increased at 72 h after MCAO when neutrophils and monocytes were the predominant leukocytes (Figure 6D, p<0.05) but fell significantly below the baseline potential by 1 week (p<0.05). These data suggest an inverse relationship between the number of microglia and infiltrating myeloid cells in the eye early after ischemic injury. Although these leukocyte dynamics and inflammatory processes were largely resolved by 7 days, the ischemic sensitivity of microglia, as well as their role in recruiting bone marrow–derived phagocytes to

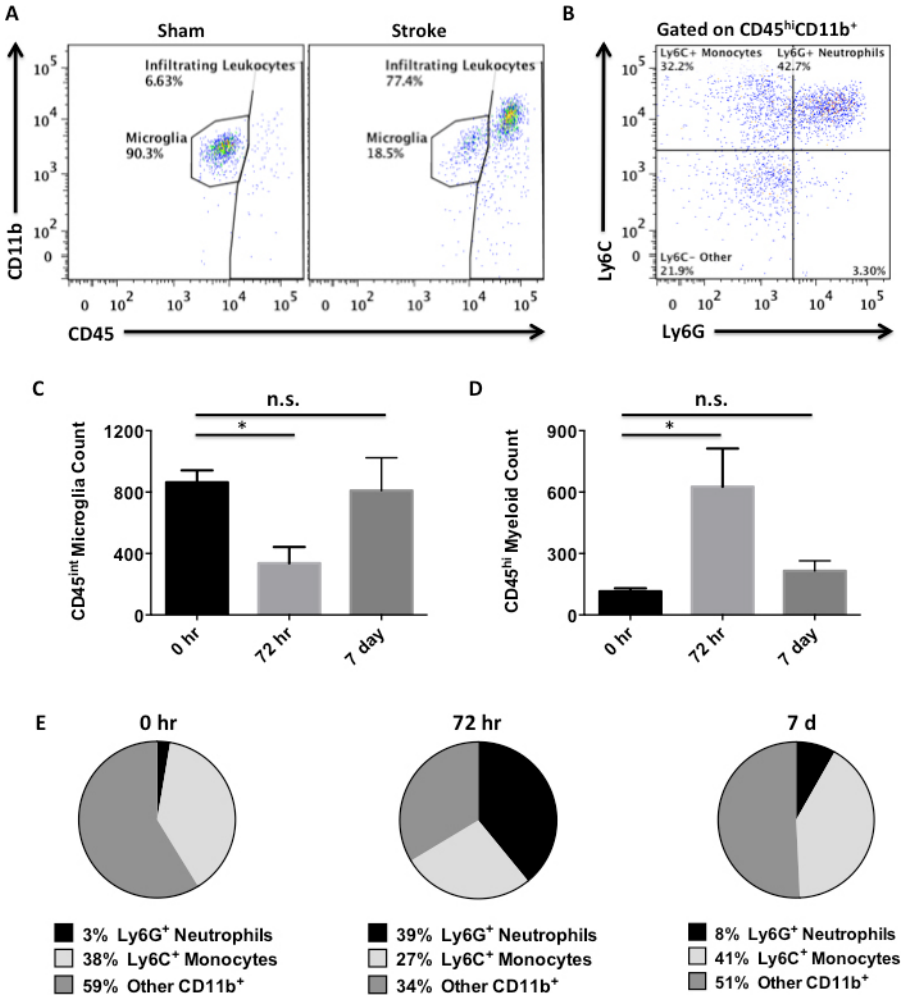

Figure 5. Changes in the number of resident retinal microglia and infiltrating bone marrow–derived myeloid cells over 7 days after MCAO. Mice were subject to 90 min of occlusion followed by 72 h or 7 days of reperfusion and analyzed with flow cytometry (n = 5/group). **A**: A representative dot plot shows resident microglia ($CD45^{int}CD11b^{+}$) and infiltrating leukocyte ($CD45^{hi}$) populations in the ipsilateral sham and stroke eyes at 72 h. **B**: A representative dot plot shows the identification of monocyte ($Ly6C^{+}Ly6G^{-}$) and neutrophil ($Ly6C^{+}Ly6G^{+}$) subsets of infiltrating myeloid ($CD45^{hi}CD11b^{+}$) cells. **C**: The absolute number of resident microglia was quantified in the sham and stroke groups. **D**: The absolute number of infiltrating leukocytes was quantified in the sham and stroke groups. **E**: Pie charts depict the cellular composition of the infiltrating myeloid ($CD45^{hi}CD11b^{+}$) cell fraction in sham (0 h) and at 72 h and 7 days. Error bars show mean ± standard error of mean (SEM). *p<0.05; **p<0.01; ***p<0.001.

the injured eye, implies that microglia play an important role in mediating the inflammatory response after ocular stroke.

DISCUSSION

This work demonstrates that transient MCAO induces a robust inflammatory response in the eye due to simultaneous occlusion of the ophthalmic artery. This induces a sterile inflammatory response characterized by early induction of proinflammatory mediators and chemoattractant signals that recruit leukocytes to the injured eye. As the injury evolves, these proinflammatory signals that likely arise from local glial populations are significantly attenuated by 1 week. In the acute period following MCAO, the vascular integrity of the blood–eye barrier becomes severely compromised, coinciding with a reduction in microglia and a significant influx of transmigrating monocytes and neutrophils. Although infiltrating myeloid cells are major producers of proinflammatory cytokines, we have demonstrated that these cells are also highly efficient phagocytes responsible for most of the debris clearance after ischemia. These findings highlight the complex nature of the sterile inflammatory response in the ischemic eye, not unlike the brain, in which the acute response is detrimental to tissue integrity but seemingly

necessary to facilitate subsequent recovery processes that restore homeostasis and visual restoration.

In humans, atherosclerosis is the major cause of ocular ischemia due to stenosis and hypoperfusion or embolic occlusion of the internal carotid artery [25]. Retinal artery occlusion is a medical emergency and the ocular equivalent of an ischemic stroke in the brain. In both cases, the duration of ischemia is the determining factor in outcome, and ocular ischemia may be manageable with early delivery of intravenous tissue plasminogen activator [26]. Visual impairments persist in approximately 20% of stroke survivors in the first 3 months, although for reasons that are not always understood [27,28]. Retinal damage has been observed in several models of experimental ischemic stroke, including after MCAO [4,29,30]. Recent work has shown that this ocular ischemia can have functional consequences. Within the first 4 to 24 h after branch retinal vein occlusion, necrosis can be seen in the affected areas of tissue. In the weeks following injury, a cascade of events occurs that includes apoptosis and inflammation. These contribute to inner retinal cell layer atrophy, optic nerve degeneration, and vision impairment [31,32]. Understanding the sequence of inflammatory events that occurs after ocular ischemia may help ophthalmologists develop therapies and prognostic markers of outcome. Microvascular abnormalities in the retina have been found to predict the risk of cerebral infarcts, clinical stroke events, and stroke mortality [33-35].

It has been reported that stroke is often accompanied by temporary (amaurosis fugax) or permanent vision loss [36]. The ophthalmic artery is the source of the blood supply to the inner retina and originates proximal to the origin of the MCA. Occlusion of the MCA therefore hinders blood to the ipsilateral retina, making it a purely non-invasive vascular model of retinal ischemia [4,29] as there is no mechanical damage or disruption of the blood–retina barrier [36]. In animals, the severity (i.e., duration) of MCAO determines the degree of retinal functional deficits [6]. Selective retinal cell death has been reported in the inner and outer nuclear layers after MCAO, and colocalization of glial fibrillary acidic protein (GFAP) and glutamine synthetase was increased at 72 h after ischemia, suggesting that activated Müller glia enhance glutamate excitotoxicity. Interestingly, in that study the

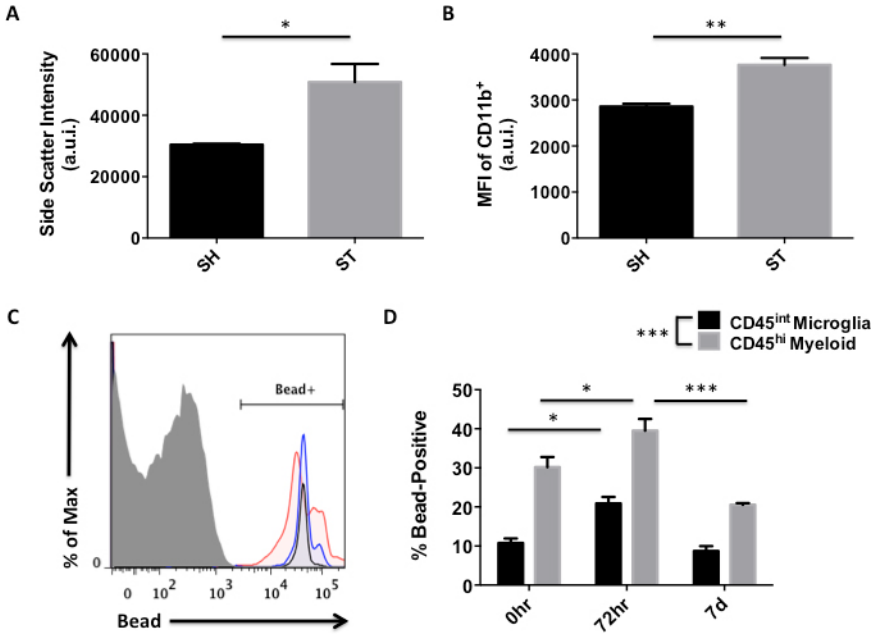

Figure 6. Phagocytic activity of resident microglia and infiltrating myeloid cells in the ischemic retina. **A**: The mean side scatter intensity was quantified at 72 h after sham or stroke. **B**: The mean fluorescence intensity of $CD11b^+$ microglia was quantified at 72 h after sham or stroke. The phagocytic potential of retinal myeloid cells after middle cerebral artery occlusion (MCAO) was assessed using ex vivo fluorescent bead assay, and the percentage of phagocytic myeloid cells was measured using flow cytometry. **C**: A representative histogram shows the relative phagocytic activity of resident microglia after sham (black) and stroke (blue) and infiltrating $CD45^{hi}CD11b^+$ myeloid cells (red). Positive gating was determined using fluorescence minus one (FMO) control (shaded gray). **D**: The percentages of bead-positive $CD45^{int}CD11b^+$ microglia versus $CD45^{hi}CD11b^+$ myeloid cells were quantified at three time points. Error bars show mean ± standard error of mean (SEM). Abbreviations: MFI = mean fluorescence intensity, a.u.i. = arbitrary units of intensity, SH = sham, ST = stroke. *p<0.05; **p<0.01; ***p<0.001.

deficits in retinal function after MCAO were transient with moderate occlusion duration, and thus unlikely to confound behavioral tests that require visual cues beyond 9 days [6]. Another study showed no differences in electroretinogram wave frequency at 4 weeks after stroke (tMCAO) in rats [37], but as in the brain, the duration of ischemia is probably the critical factor in functional outcome [38]. We have shown that the ocular inflammatory response to MCAO is largely resolved by 7 days after reperfusion, mirroring that of the ischemic brain [39,40]. Our study showed, for the first time, that the non-invasive assessment of inflammatory biomarkers in the retina following MCAO might be used to potentially predict and monitor injury in the ischemic brain.

TNF-α is an important biomarker in stroke and has been shown to be largely detrimental in retinal ischemia [41-44]. Treatment with an anti-TNF-α-blocking antibody showed improved function, and transgenic mice that lack TNF-α signaling also had less functional impairment as evidenced by the preservation of retinal thickness and electroretinogram wave amplitude [45]. Mice with retinal ischemia secondary to increased ocular pressure showed elevated TNF-α expression as early as 3 h after ischemia, which peaked between 12 and 24 h, and then dramatically decreased by 72 h. This time course has been observed after hypoxia in other genes involved in oxidative stress and excitotoxicity (i.e., *Nos1 (nNOS)* ID 24598, OMIM 163731; *Nos2 (iNOS)* ID 24599, OMIM 163730; *Nos3 (eNOS)* ID 24600, OMIM 163729, *COX-2*), with an initial increase at 24 h followed by a decrease at 72 h [46,47], consistent with our findings. It is unclear what precipitates the decrease in the expression of TNF-α and other proinjury signals given the simultaneous occurrence of vascular permeability and leukocyte infiltration; however, it is possible that the cells that make these substances are dead or no longer functioning. Interestingly, whereas many inflammatory mediators appear to peak and then return to baseline before 72 h in the retina, cytokine expression levels in the brain peak between 12 and 24 h and remain elevated for days under similar duration of ischemic insult [39,48]. This may point to unique immune suppressive factors present in the eye designed to quench excessive inflammation and spare the retina from irreversible injury. Immunomodulating neuropeptides such as alpha-melanocyte stimulating hormone are constitutively produced by the posterior eye and have anti-inflammatory effects on myeloid cells and can enhance polarization of the microglia and macrophages toward a more alternatively activated state [49]. Recent evidence suggests these neuropeptides have protective effects following retinal ischemia warranting further investigation into their therapeutic potential [50,51].

An intact blood–retinal barrier is essential for the functional and structural integrity of the retina [52]. The inner and outer blood–retinal barriers are formed by tight junctions between adjacent endothelial or RPE cells [53]. We found that small and large molecular weight tracers were increased in the eye after MCAO. The increase in Evans Blue dye, which complexes with serum albumin (70 kDa), indicates that the retinal immunity is altered, at least, temporarily. Importantly, the entry of high molecular weight proteins suggests that the ischemic eye may be accessible to systemically administered drugs and amenable to therapy.

The role of inflammation in neuronal injury and degeneration in the ischemic eye is not entirely understood. A previous study showed that minocycline treatment attenuated retinal vascular permeability, CCL2 expression, and infiltration of $MHCII^+$ myeloid cells in rats subject to pressure-induced retinal ischemia [54]. Interestingly, however, despite these anti-inflammatory actions, minocycline did not prevent retinal cell death, suggesting that retinal cell death occurs in part independently of inflammation. Others have shown that selective COX-2 inhibitors prevented apoptotic death of retinal ganglion cells but not the breakdown of the blood–retinal barrier or microglia activation [46]. Moreover, CX3CR1 immunoinhibitory signaling in microglia was found to be inconsequential after retinal ischemia, implying that neuronal loss in the retina occurs independently of microglia activation [55]. This suggests that the ocular environment evolved unique ways of coping with the deleterious effects of inflammation.

In this study, neutrophils were the most actively recruited myeloid cell after ischemia, which differs from that seen in the brain, in which the bulk of infiltrating cells at 72 h are $Ly6C^+Ly6G^-$ monocytes [39]. Although neutrophils are early responders to ischemic injury in the brain, their removal by microglia and monocytes is important to avoid further bystander damage (reactive oxygen species [ROS], matrix metalloproteinases [MMPs], etc.) [56]. We found that myeloperoxidase levels are increased 24 h after ischemia, the likely source of which is neutrophils, although microglia also upregulate MPO after injury [57]. Whether the blood–eye barrier is more permissive to neutrophil recruitment than the blood–brain barrier after ischemia and what chemotaxic signals underlie these differences remain to be investigated.

The retina is sensitive to ischemia due to the retina's exposure to light, high oxygenation consumption, firing frequency, and continual RPE phagocytic activity [58]. The high-energy requirements are obligatory for constant renewal and turnover of retinal photoreceptors and recycling of photopigments by the RPE. We demonstrated that

$CD45^{hi}CD11b^{+}Ly6C^{+}$ perivascular macrophages exhibited comparably greater phagocytic activity under normal conditions compared to resident retinal microglia. This may be due to the finite lifespan of perivascular macrophages. The precise role of microglia in the healthy retina is not well-known, although they clearly function as phagocytes and antigen presentation cells [59,60]. Our study suggests that retinal microglia behave similarly to that of their counterparts in the brain. Microglia granularity and phagocytic activity substantially increase after stroke, the degree of which is dependent on the severity of the ischemic insult [38,39]. With severe injury, microglia have also been shown to undergo apoptosis, indicating that they, similar to other CNS cells, are susceptible to the deleterious effects of severe ischemia [61]. We have previously shown that microglia exhibit a positive correlation between phagocytic activity and TNF production at 72 h after stroke, when numbers are similarly diminished in the brain. This same ischemia-sensitive phenotype was also seen in the retina, as we observed a reduction in microglia numbers at 3 days after a severe ischemic insult. However, whether cellular activation precedes microglial death in this model is not fully substantiated by our data. Future studies are required to determine whether stroke-induced microglia activation and cell injury and death are two independent processes or the same acting in parallel. Alternatively, it is possible that some populations of microglia become activated while others undergo cell death independent of activation, depending on their proximity to the site of ischemia. How this disturbance in the retinal microglia population impairs debris clearance and influences retinal repair remains unknown. Nevertheless, microglia numbers were found to rebound by 7 days, suggesting that any functional impairment on day 3 is likely compensated by the influx of debris-clearing leukocytes.

Microglia are required for normal retinal growth and blood vessel formation [62-64]. We demonstrated for the first time that retinal microglia are sensitive to the effects of ischemia and are significantly decimated at 72 h during peak inflammation. Given the morphological and phenotypic similarities between microglia and monocytes, this finding could easily go unnoticed or misinterpreted as microglia proliferation given the concurrent increase in the number of transmigrating $CD45^{hi}$ monocyte-derived macrophages. The possibility exists that activated retinal microglia shift from $CD45^{int}$ to $CD45^{hi}$ status after stroke. However, previous work by our laboratory and others using bone marrow chimeric mice suggests that CD45 expression remains stable after stroke [39,65]. Because these two types of cells share similar myeloid origin and overlapping lineage markers, it is difficult to discriminate between them using standard immunohistochemical protocols (i.e., TUNEL assay). In fact, heterogenous populations of microglia and macrophages have previously been observed in the post-ischemic retina [66]. Based on the relative CD45 expression level of resident ($CD45^{int}CD11b^{+}Ly6C^{-}Ly6G^{-}$) and infiltrating myeloid cells ($CD45^{hi}CD11b^{+}$), we were able to demonstrate that there is an inverse relationship between microglial numbers and monocyte infiltration in the ischemic eye. The depletion of the resident retinal microglia niche after ischemia may therefore favor the recruitment of new monocytes that establish residence that become microglia-like in function and sustain the pool. Studies using bone marrow chimeras have found that bone marrow–derived macrophages can replace the endogenous pool of retinal microglia within 6 to 12 months, implying that microglia turnover is slow and via a peripheral source [67,68]. However, microglia turnover as measured using parabiosis implied that replenishment by bone marrow–derived macrophages is unlikely [69]. Moreover, we have shown that myeloid infiltration is highly attenuated by 1 week, although the functional ramifications and lasting presence of the residual immigrant populations remain to be known. Vitreal macrophages have been shown to be important for post-ischemic angiogenesis and recovery after retinal injury, in which depletion of macrophages stunted neovascularization [70]. Further investigation into the activation status and functional role of the infiltrating monocyte population may be fruitful.

In summary, we have found that there is a robust inflammatory response in the eye following MCAO and the leukocyte dynamics after injury mirror and differ from that occurring simultaneously in the brain. Examining the retina after stroke may provide us with an accessible window to visualize the dynamics of inflammation and repair. Ocular manifestations after stroke may predict changes in brain injury in a non-invasive manner [34]. Our findings indicate that the inflammatory response in the eye following MCAO has similar kinetics but an altered leukocyte composition compared to those occurring in the brain despite similar durations of ischemia. This may be due to the ischemic sensitivity of specific cell types, altered reperfusion dynamics, or a unique combination of immunoinhibitory factors present in each CNS tissue. Nevertheless, the inflammatory biomarkers described in our study combined with retinal imaging techniques [71] may serve as a useful prognostic tool in predicting brain injury following MCAO, providing a novel non-invasive longitudinal method for assessment of stroke recovery.

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