Fractalkine-induced microglial vasoregulation occurs within the retina and is altered early in diabetic retinopathy

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Local blood flow control within the central nervous system (CNS) is critical to proper function and is dependent on coordination between neurons, glia, and blood vessels. Macroglia, such as astrocytes and Müller cells, contribute to this neurovascular unit within the brain and retina, respectively. This study explored the role of microglia, the innate immune cell of the CNS, in retinal vasoregulation, and highlights changes during early diabetes. Structurally, microglia were found to contact retinal capillaries and neuronal synapses. In the brain and retinal exoplants, the addition of fractalkine, the sole ligand for monocyte receptor Cx3cr1, resulted in capillary constriction at regions of microglial contact. This vascular regulation was dependent on microglial Cx3cr1 involvement, since genetic and pharmacological inhibition of Cx3cr1 abolished fractalkine-induced constriction. Analysis of the microglial transcriptome identified several vasoactive genes, including angiotensinogen, a constituent of the renin-angiotensin system (RAS). Subsequent functional analysis showed that RAS blockade via candesartan abolished microglial-induced capillary constriction. Microglial regulation was explored in a rat streptozotocin (STZ) model of diabetic retinopathy. Retinal blood flow was reduced after 4 wk due to reduced capillary diameter and this was coincident with increased microglial association. Functional assessment showed loss of microglial–capillary response in STZ-treated animals and transcriptome analysis showed evidence of RAS pathway dysregulation in microglia. While candesartan treatment reversed capillary constriction in STZ-treated animals, blood flow remained decreased likely due to dilation of larger vessels. This work shows microglia actively participate in the neurovascular unit, with aberrant microglial–vascular function possibly contributing to the early vascular compromise during diabetic retinopathy.

Significance

This work identifies a role for microglia, the innate immune cells of the CNS, in the local control of the retinal vasculature and identifies deficits early in diabetes. Microglia contact neurons and vasculature and express several vasoactive agents. Activation of microglial fractalkine-Cx3cr1 signaling leads to capillary constriction and blocking the renin-angiotensin system (RAS) with candesartan abolishes microglial-mediated vasocstriction in the retina. In early diabetes, reduced retinal blood flow is coincident with capillary constriction, increased microglial–vessel association, loss of microglial–capillary regulation, and altered microglial expression of the RAS pathway. While candesartan restores retinal capillary diameter early in diabetes, targeting of microglial–vascular regulation is required to prevent coincident dilation of large retinal vessels and reduced retinal blood flow.

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suggest regulation of the inner retinal vasculature is more complex (10). Evidence for this comes from the fact that the same light stimulus can induce either vasoconstriction or vasodilatation, and Müller cell-dependent calcium signaling only controls capillaries within the intermediate vascular plexus (11, 12). This suggests the existence of multiple regulatory pathways within the retina.

Recently, it has been proposed that microglia, the innate immune cells of the retina, may also play a role in the neurovascular unit, although direct functional evidence is lacking (13). The conventional view of microglia is that they contribute to disease via the release of proinflammatory and neurotoxic cytokines (14–16). However, it is now recognized that microglia play several important, inflammation-independent roles in the normal brain and retina, such as dynamic synaptic surveillance and synaptic pruning (17–19). Within the retina, microglia are known to be in close contact with the vasculature and impact on vessel development (20). Despite this, the inflammation-independent response of microglia to neuronal signaling and their role in the regulation of vascular tone has yet to be confirmed.

While regulation of retinal blood flow is critical to function (21), vascular dysfunction is known to occur in several pathologies, including diabetic retinopathy (DR). Early in the progression of DR, vascular pathology such as reduced retinal blood flow, microaneurysms, and areas of vascular nonperfusion occur (22). Reduced retinal blood flow, in particular, presents early in humans with diabetes (23–25) and in animal models of diabetes (25). Altered inner retinal vascular regulation is considered a likely precursor to the development of severe vascular pathology in DR (26).

The present study investigates whether retinal microglia form a functional component of the neurovascular unit, and whether signaling through the fractalkine-Cx3cr1 pathway modulates vascular diameter. In addition, the work explores whether altered microglial involvement with the inner retinal vasculature may help explain the reduced retinal blood flow that occurs early during diabetes. Exploring the mechanisms responsible for the tight regulation between neuronal activity and the local blood supply is critical to understanding retinal function in health and disease and may provide an empirical framework for future therapies targeting vascular pathology.

Results

**Microglia Contact both Retinal Vasculature and Neuronal Synapses.**

Microglia within the central nervous system (CNS) have a close association with the vasculature, particularly during development, injury, and disease (20, 27). However, less is known about microglial–vascular interactions in normal tissue. Within the retina, microglial cell bodies typically reside in the plexiform layers, while their processes extend throughout the retina (SI Appendix, Fig. S1). Inspection of the superficial vascular plexus shows microglia tiling the whole tissue (Fig. 1A, enhanced green fluorescent protein [EGFP], green) and in close association with retinal vasculature (Fig. 1A, Inset; isolectin B4 [IB4], red). When microglial process contact with retinal vessels of different diameters is quantified relative to the respective area of each vessel diameter class, microglia are seen to interact with smaller retinal vessels (≤15 μm), particularly the smallest retinal capillaries (<10 μm), when compared to the larger vessels (Fig. 1B) (one-way ANOVA, P < 0.05, 0.001 for 15 to 20 μm and >20 μm, respectively). At the ultrastructural level (Fig. 1C), a microglial process (stained for EGFP) abuts a pericyte, which lies over an endothelial cell lining the capillary lumen. This microglial–pericyte contact is also investigated immunohistochemically using the NG2-DsRed reporter mouse, which labels pericyte somata and processes (Fig. 1D, red). A microglial cell (Fig. 1D, Iba-1, green) is observed to contact two pericyte somata (Fig. 1D, red), with nuclei immunolabeled with DAPI (Fig. 1D, blue). Orthogonal projections (Fig. 1D, top and right) from the boxed area, show direct contact between the two cell types. The contact (Fig. 1D, asterisk) was further imaged at higher resolution to show direct contact between the microglial process (Fig. 1D, green) and pericyte somata (red; asterisk in Fig. 1E; also see SI Appendix, Fig. S2 and Movie S1).

The extent of microglial contact with pericyte somata, processes (NG2-labeled) and capillary areas devoid of pericyte contact (NG2-/IB4+ regions, likely endothelial cells) was quantified in rat retina, with no preference observed for microglial–pericyte or microglial–vascular contact (Fig. 1F).

In addition to contacting retinal vessels (IB4, magenta, asterisk in Fig. 1G), microglia (EGFP, green) are also observed to extend processes into the inner plexiform layer, where neuronal synapses reside (Fig. 1G, VGLUT1 red, arrowheads; DAPI blue). The Fig. 1G, Inset shows a rendering of microglial–neuronal interactions at higher magnification. This is also observed in the human retina (Fig. 1H, DAPI, blue) with microglia (Iba-1, green) contacting both retinal vessels (Fig. 1H, vitronectin, magenta, asterisk) and neuronal synapses (Fig. 1H, VGLUT1, red, arrowheads). When quantified in the Cx3cr1GFP/+ mouse retinae, the majority of microglia (Fig. 1I, EGFP, green) in the inner retina contact both neuronal synapses (Fig. 1I, VGLUT1, blue and retinal vessels (Iba-1, red; Fig. 1I, Inset) (73 ± 13%, rat retina). Individual channels for immunolocalization are shown in SI Appendix, Fig. S3.

**Microglia Modulate Vessel Diameter and Express Vasoactive Genes.**

Within the brain and retina, macroglial (astrocyte and Müller cell) contact with neuronal synapses and vasculature is critical for local control of blood supply in response to neuronal activity (7, 8). To determine whether microglia play a similar role, Cx3cr1GFP/+ retinai were isolated and maintained ex vivo. Microglia were visualized via their expression of EGFP (Fig. 2A, green) and vessels were labeled with rhodamine B (Fig. 2A, red). As the fractalkine-Cx3cr1 axis is thought to mediate neuronal–microglial communication, blood vessels and microglia were imaged while fractalkine (200 ng/mL) or PBS was perfused into the chamber (Movie S2). Vessel diameter change was monitored and expressed relative to the baseline value for the same region of vessel.

In response to fractalkine, blood vessel regions that were associated with microglial processes (m+) constricted (Fig. 2B, m+) (two-way ANOVA; PBS vs. fractalkine, P < 0.001), while those regions that were further away from microglial processes (m−) exhibited no significant alteration in capillary diameter (Fig. 2B, m−) (two-way ANOVA; PBS vs. fractalkine, P = 0.26). These ex vivo preparations showed minimal microglial process movement at the vascular level throughout the imaging, including during fractalkine exposure (SI Appendix, Fig. S4 and Movie S2). When explants lacking Cx3cr1 (Cx3cr1CreERGFP mouse) were exposed to fractalkine, no alteration in vessel diameter was observed compared to PBS controls at regions with (m+: 105.7 ± 2.7% vs. 94.7 ± 2.3%, two-way ANOVA P = 0.52) or without (m−: 98.8 ± 1.2% vs. 97.1 ± 1.3%, two-way ANOVA P = 0.999) microglial contact (Fig. 2B). Further supporting a Cx3cr1-dependent mechanism, preincubation with the Cx3cr1 inhibitor, AZD8797 (28), inhibited fractalkine (FKN)-induced constriction (Fig. 2B, Inset) (FKN 82 ± 2%, FKN+AezD8797 96 ± 2%, t test P = 0.015). Finally, to explore whether this vasoactive role, fragment of fractalkine was retinal-specific, superficial vessels within the rat brain were imaged using a thin skull preparation. These preliminary data showed that while vehicle delivery resulted in no alteration in vessel diameter, the subdural addition of fractalkine lead to a significant constriction of the smaller vessels (Fig. 2C) (repeated-measures two-way ANOVA P < 0.001).
ANOVA, vessels ≤ 15 μm, P < 0.05). While both tissues show a fractalkine-induced constriction, the difference in vessel kinetic response likely reflects the different systems used to explore microglial vasoregulation (ex vivo and in vivo, respectively).

Since the Cx3cr1<sup>GFP/GFP</sup> retina showed no fractalkine-induced vessel constriction, microglial contact with retinal vessels and neurons was explored. High-resolution immunocytochemical analysis of microglia (Fig. 2D, EGFP, green) contact with neuronal synapses (Fig. 2D, VGLUT1, red) and vessels (Fig. 2D, IB4, light blue) allowed specific areas of contact to be quantified. When the volume of contact per individual microglial process making contact with pericyte somata, processes (arrowheads) was calculated, Cx3cr1<sup>GFP/GFP</sup> animals had fewer vessel contacts than animals with one functional copy of Cx3cr1 (Fig. 2E) (Cx3cr1<sup>GFP/GFP</sup> = 7.5 ± 0.4% vs. Cx3cr1<sup>GFP/GFP</sup> = 5.5 ± 0.3%, t test P = 0.004). While there was no difference in neuronal contacts between the two genotypes, Cx3cr1<sup>GFP/GFP</sup> animals showed less microglial process branching (Fig. 2E) (Cx3cr1<sup>GFP/GFP</sup> = 111.5 ± 7.2 vs. Cx3cr1<sup>GFP/GFP</sup> = 92.2 ± 2.1, t test P = 0.03), reflecting the literature showing Cx3cr1<sup>GFP/GFP</sup> to have a more activated inflammatory profile (29). When retinal capillary diameters were compared to C57bl6 control animals, Cx3cr1<sup>GFP/GFP</sup> capillaries were similar to controls (Fig. 2F) (C57bl6 11.3 ± 0.3 μm vs. Cx3cr1<sup>GFP/GFP</sup> 10.9 ± 0.2 μm, one-way ANOVA P = 0.66), while Cx3cr1<sup>GFP/GFP</sup> showed increased capillary diameters (Fig. 2F) (Cx3cr1<sup>GFP/GFP</sup> = 10.9 ± 0.2 μm vs. Cx3cr1<sup>GFP/GFP</sup> = 12 ± 0.4 μm, one-way ANOVA P = 0.047).

There was no difference in larger vessel diameter for any genotype (Fig. 2F, inset) (P = 0.87 and 0.94 for Cx3cr1<sup>GFP/GFP</sup> and Cx3cr1<sup>GFP/GFP</sup>, respectively).

RNA sequencing (RNA-seq) was performed on FACS-isolated microglia collected from 12-wk-old dark agouti rats to determine whether microglia expressed vasomodulatory factors. To confirm the purity of sample, the mapped genes were compared to a published list of microglial markers (30), with 23 of 29 markers identified in our gene population, including the microglial-specific marker Tmem119 (SI Appendix, Table S1) (31). The negative microglial fraction (CD11b<sup>+</sup>) was also interrogated for microglial signature genes, with key genes, such as Cx3cr1, Tmem119, and Slc2a5 hardly expressed (<3 copies in >1 x 10<sup>3</sup> transcripts) (SI Appendix, Table S1). The microglial transcriptome was also compared to microglial-enriched genes reported in several studies, with significant overlap observed, while there was little contamination from known neuronal genes (SI Appendix, Fig. S5). The gene population was compared against genes known to be involved in angiogenesis (gene ontology [GO]:0001525, 407 genes) and regulation of blood
vessel diameter (GO:0097746, 310 genes). In total, 268 genes expressed in the microglial population were identified to have roles in angiogenic pathways (Fig. 2G and SI Appendix, Table S2), such as hypoxia inducible factor 1α (Hif1α) and vascular endothelial growth factor A and B (Vegf A/B). When vessel diameter regulation was explored, 41 genes were found to have a role in vasodilation, such as phospholipase A2 (Pla2g6) and sirtuin 1 (Sirt1), while 39 genes were identified with vasoconstriction, including endothelin 1,-3 (Edn1, -3) and arachidonate 5-lipoxygenase (Alox5) and angiotensinogen (Agt) (Fig. 2G, and SI Appendix, Tables S3 and S4, respectively).

As angiotensinogen is a constituent of the renin-angiotensin system (RAS), which is involved in retinal vessel regulation via the angiotensin II receptor type 1 (AT1R) (32, 33), ex vivo experiments were performed using the AT1R antagonist, candesartan. Baseline capillary diameter was averaged over 10 min in rat retinal explants exposed to Ames (black trace) and Ames + candesartan (230 nM) (Fig. 2H, red trace) and after which fractalkine was added (shaded area in Fig. 2H). Similar to that observed in the Cx3cr1GFP−/− mouse (Fig. 2A and B), exposure of the rat retina to fractalkine induced capillary constriction, while candesartan blocked any fractalkine-induced constriction (Fig. 2H). When grouped data were analyzed, candesartan abolished the fractalkine-induced vasoconstriction (Fig. 2I) (80.4 ± 2.0% vs. 97.5 ± 2.3%, t test, P < 0.01).

To further support the role of RAS in microglial-mediated vessel regulation, control C57Bl6 and Cx3cr1GFP/GFP were exposed ex vivo to fractalkine for 2 h, microglia isolated, and the expression of Agt quantified (Fig. 2J,Inset). While exposure to fractalkine increased Agt expression in control retinae, Cx3cr1GFP/GFP retinae that previously exhibited no microglial-mediated constriction (Fig. 2B) showed no expression change (Fig. 2J,Inset) (+FKN, C57Bl6 21.8 ± 3.5 copies per 1,000 copies Hprt vs. Cx3cr1GFP/GFP 7.7 ± 0.6 copies per 1,000 copies Hprt, two-way ANOVA P = 0.017). The present data show that microglia are capable of modulating vascular constriction within the retina and broader regions of the CNS via the fractalkine-Cx3cr1 pathway. While microglia express several gene transcripts for known vasoactive agents, fractalkine-fractalkine-Cx3cr1 pathway. While microglia are capable of modulating vascular constriction within the retina and broader regions of the CNS via the fractalkine-Cx3cr1 pathway. While microglia are capable of modulating vascular constriction within the retina and broader regions of the CNS via the fractalkine-Cx3cr1 pathway.
coupling (23, 25, 34). To explore whether microglial vasoregulation was altered during early diabetes, adult dark agouti rats were rendered diabetic via streptozotocin (STZ) with significant hyperglycemia evident throughout the 4-wk experimental period (SI Appendix, Table S5).

As reduced retinal blood flow is a consistent and early alteration in patients with diabetes and animal models (24, 25), quantitative vessel-dependent kinetic analysis using sodium fluorescein (35) was used to confirm vascular dysfunction. Average normalized fluorescence intensity was calculated over time for every pixel within the fundus image (Fig. 3 A–C, Insets), grouped on vessel type, and en face heat-maps produced (Fig. 3 A–C, fill times), with warmer colors indicating greater time taken to fill (slower blood flow). Vessel-dependent kinetic analysis revealed arterioles in STZ-treated animals took longer to fill (Fig. 3D) (median regression analysis, P < 0.05), reflecting reduced blood flow. Due to the serial nature of the retinal vasculature, this increase in fill time was also observed in retinal capillaries and venules (Fig. 3D) (median regression analysis, P < 0.05), with no vessel-specific deficit identified (median regression analysis, P > 0.05). Drain times were also longer in all retinal vessels (Fig. 3E) (median regression analysis, P < 0.05), with the effect significantly greater than that observed for fill times (median regression analysis, P < 0.05). The reduced arteriolar and venular blood flow in STZ-treated animals revealed using velocimetry (SI Appendix, Fig. S6) and the clinically relevant arterio-venous transit time was also exhibited reduced blood flow (increased transit time) (SI Appendix, Fig. S6D). The decrease in retinal blood flow was independent of systemic change, with systolic blood pressure, blood hematocrit, and intraocular pressure unaltered (SI Appendix, Fig. S7).

As vessel change affects blood flow in DR (36, 37), the morphology of large-diameter vessels was assessed from fluorescein images at peak fluorescent intensity. No change in large vessel pathology of large-diameter vessels was assessed from fluorescein angiography (P < 0.05). To determine whether the retinal capillary constrictions in diabetes were accompanied by altered microglial association. While microglia exhibited a similar association with large-diameter arterioles and venules (Fig. 4A) (two-way ANOVA, P = 0.99 and P = 0.66, respectively), microglial-capillary association was reduced in STZ-treated animals (Fig. 4D) (two-way ANOVA, P < 0.05). In addition, microglial-pericyte association (Fig. 4B, Inset, microglia green, Iba-1; pericytes light blue, NG2, vessels red, IB4) was increased within the central retina of STZ-treated animals (Fig. 4B) (two-way ANOVA, P < 0.05). There was no vessel dropout (Fig. 3G), nor loss of retinal pericytes (SI Appendix, Fig. S8) at this early stage of diabetes.

The association of microglia with pericytes and capillary areas lacking pericyte contact was further explored in control and STZ-treated animals using quantitative image analysis (Fig. 4 C, Inset, rendered image showing pericyte somata red; pericyte processes green; pericyte-free vessel blue and skeletonised microglia). While there was no specific preference for microglia to contact pericyte somata, processes or capillary areas lacking pericytes (Fig. 4C) (two-way ANOVA, P = 0.16), there was increased microglial association with all three at 4 wk of diabetes (Fig. 4C) (two-way ANOVA, P < 0.01). To determine whether this microglial effect was specific, or a result of a more generalized macroglial response as has been shown in later stages of diabetes (38, 39), astrocyte density and Müller cell giosis were quantified. Vessel-specific astrocyte coverage (Fig. 4D) and Müller cell giosis (Fig. 4E) were unaltered after 4-wk STZ treatment (two-way ANOVA, P > 0.92 and 0.99, respectively).

Previous work has shown blood-retinal barrier (BRB) integrity is compromised early in diabetes (40). Using vessel-dependent blood flow analysis (Fig. 3 A–E), we used the return to baseline after fluorescein peak (fluorescein offset) as a measure of BRB integrity. While no alteration in offset was observed for larger vessels, retinal capillaries showed a significant increase, indicative of fluorescein leakage/reduced BRB integrity (Fig. 4F) (median regression analysis, P < 0.05). A breakdown in BRB can lead to immune cell infiltration and microglial activation, with microglial migration and morphological change indicative of classic activation observed 1 mo post-STZ (41). To assess whether altered microglial–vessel association occurred in the context of monocyte involvement/microglial activation, whole-mounts were colabeled with IB4 and Iba-1 and the number and morphology of microglia quantified in central and peripheral retina. Despite the increase in capillary fluorescein offset, there was no difference in the number of monocytes-retinal microglia (Fig. 4G) (two-way ANOVA, central P = 0.4, peripheral P = 0.9), or microglial morphology after 4 wk of hyperglycemia (Fig. 4H) (two-way ANOVA, cell body area P > 0.99, process length/cell P = 0.15, branch points/cell P > 0.99). Despite this, Cx3cr1 and fractalkine expression was increased in the diabetic retina (SI Appendix, Fig. S9). RNA-seq analysis of microglial isolates from 4-wk control and STZ-treated animals showed that of the 254 differentially expressed genes, 22 inflammatory response genes were identified, 15 of which were positive regulators (GO:0050729), while 12 were negative regulators of inflammation (GO:0050728) (Fig. 4F and SI Appendix, Tables S6 and S7). Importantly, chemokine and cytokines normally associated with microglial activation—including Tlr2, Il-1β, Cxcl10, Tnf-a, Il-1α, C1q—were not altered and there was no expression of the infiltrating monocyte marker marker, Ccr2, in our RNA-seq dataset (31, 42). Thus, at this early stage of diabetes (4 wk) when retinal capillaries are constricted, there is increased microglial–capillary interaction, which is independent of monocyte recruitment, classic microglial activation, and a more generalized macroglial response.

**Retinal Microglia Contact with Capillaries and Pericytes Is Increased in Early Diabetes, Independent of Activation.** The extent of microglial (Fig. 4 A, Inset, green, Iba-1) contact with arterioles, capillaries, and venules (Fig. 4 A, Inset, red, IB4) was quantified for control and STZ-treated animals to determine whether the retinal capillary constriction in diabetes was accompanied by altered microglial association. While microglia exhibited a similar association with large-diameter arterioles and venules (Fig. 4A) (two-way ANOVA, P = 0.99 and P = 0.66, respectively), microglial-capillary association was increased in STZ-treated animals (Fig. 4D) (two-way ANOVA, P < 0.05). In addition, microglial-pericyte association (Fig. 4B, Inset, microglia green, Iba-1; pericytes light blue, NG2, vessels red, IB4) was increased within the central retina of STZ-treated animals (Fig. 4B) (two-way ANOVA, P < 0.05). There was no vessel dropout (Fig. 3G), nor loss of retinal pericytes (SI Appendix, Fig. S8) at this early stage of diabetes.

**Microglial Expression of Vasoactive Genes and Control of Capillary Constriction Are Altered in Early Diabetes.** To determine whether there was a loss of retinal vasomotor control during early diabetes and is altered early in diabetic retinopathy
diabetes, breathable oxygen was used to induce hyperoxic challenge and capillary diameter within the superficial vascular plexus was quantified using OCTA (Fig. 5A, Upper). While control animals showed a distinct vasoconstriction in response to 100% oxygen, no constriction was observed in STZ-treated animals (Fig. 5B) (two-way ANOVA, \( P < 0.05 \)). To explore whether this dysfunction was also evident in the diabetic retina, angiography was performed in vivo to measure capillary diameter in control and STZ-treated animals, with the vessels measured shown in green. (Scale bar, 50 \( \mu \)m.) (H) OCTA was performed in vivo to measure capillary diameter in control and STZ-treated (Inset) animals, with the vessels measured shown in green. (Scale bar, 50 \( \mu \)m.) (I) Decreased capillary diameter was observed in STZ-treated animals (\( n = 12 \)) compared to control (\( n = 10 \)) within the superficial vascular plexus. No alteration was observed in the intermediate/deep vascular plexi. Group data expressed as mean \( \pm \) SEM, \(^*\) \( P < 0.05 \).

Based on the loss of vasomotor control in the diabetic retina and the dysregulation of the microglial RAS pathway, animals were rendered diabetic and treated with candesartan (Fig. 5C) or vehicle in their drinking water. At 4 wk post-STZ, capillary diameter and retinal blood flow were quantified. OCTA analysis of superficial retinal capillaries showed a decrease in diameter within the vehicle control group, similar to that observed in Fig. 3I (Fig. 5D) (91.8 \( \pm \) 2\%, two-way ANOVA, \( P < 0.05 \)). This capillary constriction was not evident in STZ-treated animals exposed to candesartan, with diameters returning to control levels (Fig. 5D) (99.9 \( \pm \) 1.8\%, two-way ANOVA, \( P > 0.99 \)). However, despite this, retinal blood flow remained slower, with arterio-venous transit time increased in the vehicle and candesartan STZ-treated animals (Fig. 5E) (median regression analysis \( P < 0.05 \) and \( P < 0.001 \), respectively). Quantification of larger retinal vessels (arterioles and venules) showed systemic delivery of candesartan resulted in an increase arteriovenous ratio in the STZ-treated animals compared to candesartan-treated control (Fig. 5F) (STZ 0.94 \( \pm \) 0.01, control 0.84 \( \pm \) 0.01, two-way ANOVA \( P < 0.05 \)) and vehicle-treated control and STZ animals (Fig. 5F) (control 0.798 \( \pm \) 0.03, STZ 0.86 \( \pm \) 0.02, two-way ANOVA \( P < 0.001 \) and 0.05, respectively).
Fig. 4. Microglia increase their contact with retinal capillaries after 4 wk of STZ-induced diabetes. (A) Whole-mounted retina from control (Inset) and STZ-treated animals were labeled for Iba-1 (microglia, green) and IB4-FITC (blood vessels, red) and the extent of microglial and vessel contact quantified for each vessel type. While no difference in large vessel contacts occurred, microglia–capillary contact increased in the central retina of the STZ-treated animals (filled bars, n = 11). (Scale bar, 50 μm.) (B) Control (Inset) and STZ-treated animals were labeled for Iba-1 (microglia, green), NG2 (pericytes, light blue), and IB4-FITC (blood vessels, red) and the extent of microglia–pericyte contact quantified for each vessel type. Microglial–pericyte association increased within the central retina of STZ-treated animals (filled bars, n = 11). (Scale bar, 50 μm.) (C) Using similar immunolabeling as in B, microglial association with pericyte somata, processes, and capillary areas lacking pericyte contact was quantified. The image analysis render (Inset) highlights pericyte somata (red), pericyte processes (green), and pericyte-free vessels (blue), while microglia touching each of these regions were skeletonized and color-coded for quantification. While there were no preferential association, all contacts were increased in STZ-treated (unfilled bars, n = 5) compared to control (unfilled bars, n = 5) retinae (STZ treatment P = 0.0015). (Scale bar, 50 μm.) (D and E) Macroglial change was assessed in control (unfilled bars, n = 11) and STZ-treated (filled bars, n = 11) retinae, with no alteration in astrocyte coverage (D), nor Muller cell gliosis (E) observed (n = 6). (F) VFA was used to quantify fluorescein offset as a measure of RBB integrity. While arterioles and venules showed no change, capillary offset was increased in STZ-treated animals (unfilled bars, control n = 23; filled bars STZ, n = 21). (G and H) The inflammatory status of microglia was assessed morphologically and no difference was found in the number of monocytes/macrophage in central and peripheral retina (G, n = 11), cell soma size, mean process length, or process branching points (H, n = 5 control, n = 8 STZ). (I) RNA-seq data from retinal microglia taken from control and STZ-treated rats were screened for genes involved in the positive (GO:0050729) and negative (GO:0050728) regulation of inflammation. While some inflammatory genes were altered, key inflammatory genes were unchanged after 4 wk of diabetes. Data represented as mean ± SEM, *P < 0.05.

Overall, these data show that in early diabetes, retinal vasomodulation is aberrant, with a loss of microglial-mediated vasomotor and specific dysregulation of the RAS. However, treatment with the AT1R inhibitor, candesartan, did not restore retinal blood flow, despite dilating the retinal capillaries.

Discussion

The present study examined the role of microglia in local control of inner retinal blood supply. Microglia preferentially contact retinal capillaries that reside in the superficial vascular plexus, as well as contacting neuronal synapses within the inner retina. A role for microglia in vasomodulation within the retina and brain was identified, where addition of fractalkine induced capillary constriction. Subsequent characterization within the retina showed this vasomodulation to be dependent on microglial contact and Cx3cr1 signaling. The microglial transcriptome contained gene transcripts for known vasoactive agents, while the AT1R inhibitor, candesartan, blocked capillary constriction, suggesting microglial vasoregulation likely occurs via modulation of local RAS. This was supported by data showing fractalkine-Cx3cr1–mediated up-regulation of angiotensinogen. Microglial vasoregulation was further explored in the context of vascular dysfunction during early diabetes. After 4 wk of experimental diabetes, retinal blood flow was reduced, coincident with constriction of retinal capillaries within the superficial plexus and increased microglial–capillary association. However, there was no indication of classic microglial activation, nor a more generalized macroglial response. RNA-seq data showed altered microglial expression of components of the RAS and there was a loss of microglial-mediated capillary constriction during diabetes. Finally, treatment with candesartan restored retinal capillary diameter in STZ-treated animals; however, retinal blood flow remained reduced.

Microglial Vasomodulation within the Retina. The present data show that microglia are intimately associated with retinal
vasculature, directly apposing pericytes and capillary areas free from pericytes, yet showing no particular preference for direct contact. Highlighting the functional significance of this interaction, stimulation of the microglial-specific receptor Cx3cr1 via its sole ligand fractalkine induced vasoconstriction, not only within the mouse and rat retina, but also in the brain. While the role of fractalkine-induced vessel constriction in the brain requires significantly more work to confirm microglial/Cx3cr1 involvement in areas exhibiting constriction, within the retina this effect was spatially discrete, occurring only in areas associated with microglial processes and was dependent on Cx3cr1 signaling, with Cx3cr1GFP/GFP retinae exhibiting no constriction, altered microglia-vessel contact and capillary diameter. While the previously reported alterations in the retina of Cx3cr1GFP/GFP mice may impact on these vascular outcomes (44), the Cx3cr1 inhibitor AZD8797 data showing a lack of fractalkine-induced constriction, is in agreement with the Cx3cr1GFP/GFP results, directly implicating microglia in the capillary response to fractalkine. While previous work has identified microglia as a component of the blood–brain barrier (45), and involved in retinal and brain vascular development (20, 46), this report of microglial-mediated vasomodulation is unique. Furthermore, our data and those of others show microglia also monitor and modulate neuronal synapses during development, throughout adulthood, and in response to activity (17, 47, 48), raising the possibility that microglia may contribute to neurovascular coupling, the process through which local blood flow is regulated by neuronal activity. As previous work in the retina suggests the existence of Müller cell-independent vasoregulatory mechanisms (11, 12), microglial vasoregulation may constitute one such alternative pathway, particularly within the superficial plexus. Further work exploring the structure of microglial–neuronal contact, its temporal response to altered neuronal activity, and its fractalkine dose–response profile will be required to properly characterize the role of microglia in the neurovascular unit.

Microglial RAS Involvement in Capillary Constriction. In order for microglia to directly mediate vessel constriction, they must express vasoactive factors. The RNA-seq data from isolated retinal microglia highlighted several genes for vasoactive agents, including endothelin (Edn1, 3), angiotensinogen (Agt), and arachidonate 5-lipoxygenase (Alox5), all of which are known to induce microglial-neuronal contact, its temporal response to altered neuronal activity, and its fractalkine dose–response profile will be required to properly characterize the role of microglia in the neurovascular unit.
Fractalkine-induced microglial vasoregulation occurs within the retina and is altered early in diabetic retinopathy

Microglial Involvement in Capillary Constriction during Early Diabetes and Its Effect on Retinal Blood Flow.

Microglia contact pericyte somata and processes, it is tempting to speculate that microglial control of these vessels lies the pathophysiology of early and later-stage DR (25, 61, 62), in contrast to larger retinal vessels that showed no alteration, there was a significant reduction in capillary diameter (∼9%) within the superficial plexus. To our knowledge this is a unique finding and while the change in capillary diameter is small, it would lead to large effect on blood flow, since capillaries constitute the majority of retinal vasculature (58). One estimate indicated a 6% dilation in capillary diameter (∼0.32 μm) generated the majority of blood flow increase evoked by neuronal activity (5). In addition to static vessel change, retinal capillaries from STZ-treated animals failed to constrict after hyperoxic challenge. This report of in vivo retinal capillary diameter measurement during vascular challenge is unique; however, previous human studies have reported altered hyperoxic retinal vessel responses (blood flow) in patients with type 1 (59) and type 2 (60) diabetes.

As changes in capillary network have been suggested to underlie the pathophysiology of early and later-stage DR (25, 61, 62), it is tempting to speculate that microglial control of these vessels contribute to the vascular dysfunction in early diabetes. The data showing an increase in the number of microglial processes associated with capillaries, increased microglial Agt expression, and the restoration of capillary diameter after candesartan cilexetil treatment all support this hypothesis. Even the increased microglial expression of aryl hydrocarbon receptor (Ahr), a negative regulator of vasoregulatory substances (43), may be incorporated into this theory,
since recent work shows it contributes to vessel stiffness (65). Therefore, the increased Air and Agt expression may contribute to the phenotype of smaller and less-responsive retinal blood vessels in early diabetes. Additional support for a microglial-specific effect on the retinal vasculature during diabetes comes from work in STZ-treated Cx3cr1GFP/GFP animals, which showed increased acellular capillaries after 4 mo of hyperglycemia (64). Further work using the STZ-treated Cx3cr1GFP/GFP model is required to specifically explore the capillary constriction evidenced early in diabetes.

The microglial dysregulation of the RAS suggests this pathway is altered in diabetes. These data are supported by our supplementary data (SI Appendix, Fig. S9) and previous studies showing increased angiotensinogen within the vitreous of individuals with proliferative DR (65) and increased vitreal AngII concentrations and elevated retinal AngII, ATII, and AT2R levels in rodent models of diabetes (66, 67). As well as causing vasoconstriction, AngII is also known to uncouple pericytes from the endothelium, thereby altering vessel permeability and contributing to the development of microaneurysms, a key clinical determinant of DR thereby altering vessel permeability and contributing to the development of microaneurysms, a key clinical determinant of DR

AngII is also known to uncouple pericytes from the endothelium, thereby altering vessel permeability and contributing to the development of microaneurysms, a key clinical determinant of DR thereby altering vessel permeability and contributing to the development of microaneurysms, a key clinical determinant of DR

while candesartan cilexetil is a prodrug that is generally activated during gas-...
animal to 100% oxygen via a nose cone (3 L/min). After a baseline image was taken, follow-up mode was used to acquire a second capillary image in the same retinal location, after 2 min of oxygen breathing.

Immunocytochemistry. Rat, mouse, and human retinae were processed for indirect immunofluorescence in whole-mount or cross-section, as previously described (80, 81). Retinal microglia were labeled with rabbit anti-ionized calcium-binding adapter molecule 1 (Iba-1, 1:1,000; Wako) or expressed EFR (Cx3cr1GFP, Cx3cr1GFP/Griffonia simplicifolia II 485 (FITC 1:75; Sigma-Aldrich; 647 fluorophore 1:100; Thermo Fisher Scientific). While IBA4 has shown cross-reactivity with brain microglia and activated retinal microglia (82, 83), we observe no cross-reactivity in fixed retinal tissues. We also show better vessel coverage using IBA4 compared to the endothelial marker CD-31 (80, 81). Retinal microglia were labeled with rabbit anti-ionized calpain (80, 81). Retinal microglia were labeled with rabbit anti-ionized calpain (80, 81). Retinal microglia were labeled with rabbit anti-ionized calpain (80, 81).

Fractalkine-induced microglial vasoregulation occurs within the retina and is modulated by visual experience. Two days after induction of retinal microglia, we assessed the percentage of vessel area covering the total retinal area. For all subjective measurements, individuals were blinded to the treatment group. Microglial, glial, pericyte histology, and vessel interaction. Microglia, pericytes, and astrocytes from STZ-treated and control tissue were analyzed in the normal and pathologic brain. Axonal transport studies on the structure and function of the retinal microvasculature in diabetes. The clinical implications of recent studies on the structure and function of the retinal microvasculature in diabetes. Diabetologia 58, 871–885 (2015).

Microglial isolation and RNA-seq. Retinae from control and 4-wk STZ-treated rats (n = 5 control, n = 4 STZ, 12-wk-old) were isolated, papain-digested (Worthington Biochemical), and labeled with CD11b–FITC conjugate (Miltenyi Biotech) for microglial isolation (FACSArria III, BD Bioscience). Both CD11b–FITC (microglial-positive) and CD11b–PE (microglial-negative) fractions were collected. RNA was isolated and RNA-seq performed as in SI Appendix. To explore fractalkine regulation of microglial RAS, retinae from C57Bl6 and Cx3cr1GFP animals (n = 6) were incubated as above with fractalkine (200 ng/mL; R&D Systems) or PBS for 2 h at 37 °C. Retinal microglia were isolated via FACS using the CD11b and EGF labels. RNA was isolated and Smart-2q performed with 13 cycles of preamplification followed by quantitative PCR (SI Appendix).

Statistical Analysis. Statistical significance was determined by two-tailed unpaired Student’s t test, two-way ANOVA, or repeated-measures ANOVA depending on the experiment (Prism 6.0, GraphPad). Where required, a Tukey post hoc analysis was performed. Blood flow analysis was undertaken using median regression analysis (STATA, StataCorp). Alpha levels were set at 0.05. Numerical values are expressed as SEM unless otherwise stated.

Data Availability. The data reported in this paper have been deposited in the Gene Expression Omnibus (GEO) database, https://www.ncbi.nlm.nih.gov/geo (accession no. GSE139276) (88).

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1. A. Ames III, Y. Y. Li, E. C. Heher, C. R. Kimber, Energy metabolism of rabbit retina as related to function: High cost of Na+ transport. J. Neurosci. 12, 840–853 (1992).
2. D. Y. Yu, S. J. Cringle, Oxygen distribution and consumption within the retina in vascularized and avascular retinas and in animal models of retinal disease. Prog. Retin. Eye Res. 28, 175–208 (2011).
3. J. P. Campbell et al., Detailed vascular anatomy of the human retina by projection-resolved optical coherence tomography angiography. Sci. Rep. 7, 42201 (2017).
4. T. E. Kornfield, E. A. Newman, Regulation of blood flow in the retinal trilaminar vascular network. J. Neurosci. 34, 11504–11513 (2014).
5. C. N. Hall et al., Capillary pericytes regulate cerebral blood flow in health and disease. Nature 508, 55–60 (2014).
6. C. E. Riva, E. Logean, B. Falsini, Visually evoked hemodynamic response and assessment of neurovascular coupling in the optic nerve and retina. Prog. Retin. Eye Res. 24, 214–225 (2005).
7. M. R. Metea, E. A. Newman, Signalling within the neurovascular unit in the mammalian retina. Exp. Physiol. 92, 635–640 (2007).
8. T. Takanou et al., Astrocyte-mediated control of cerebral blood flow. Nat. Neurosci. 9, 260–267 (2006).
9. E. A. Newman, Calcium increases in retinal glial cells evoked by light-induced neuronal activity. J. Neurosci. 25, 5502–5510 (2005).
10. E. A. Newman, Functional hyperemia and mechanisms of neurovascular coupling in the retinal vasculature. J. Cereb. Blood Flow Metab. 33, 1685–1693 (2013).
11. K. R. Biesecker et al., Glial cell calcium signaling mediates capillary regulation of blood flow in the retina. J. Neurosci. 36, 9435–9445 (2016).
12. A. Mishra, A. Hamid, E. A. Newman, Oxygen modulation of neurovascular coupling in the retina. Prog. Natl. Acad. Sci. U.S.A. 108, 17827–17831 (2011).
13. J. A. Filosa, H. W. Morrison, J. A. Iddings, W. Du, K. J. Kim, Beyond neurovascular coupling, role of astrocytes in the regulation of vascular tone. Neuroscience 323, 96–109 (2016).
14. J. G. Griswol et al., The role of microglia in diabetic retinopathy. J. Ophthalnol. 2014, 705783 (2014).
15. J. T. Tang, T. S. Kern, Inflammation in diabetic retinopathy. Prog. Retin. Eye Res. 30, 343–358 (2011).
16. U. K. Hanisch, H. Kettenmann, Microglia: Active sensor and versatile effector cells in the normal and pathologic brain. Nat. Neurosci. 19, 1387–1394 (2017).
17. M. E. Tremblay, R. L. Lowery, A. K. Majewska, Microglial interactions with synapses are modulated by visual experience. Proc. Biol. Sci. 8, 1005027 (2010).
18. M. E. Tremblay, A. K. Majewska, A role for microglia in synaptic plasticity? Commun. Integr. Biol. 4, 220–222 (2011).
19. J. E. Lee, K. J. Liang, R. N. Faris, W. T. Wong, Ex vivo dynamic imaging of retinal microglia using time-lapse confocal microscopy. Invest. Ophthalvm. Vis. Sci. 49, 4166–4176 (2008).
20. D. Checchin, F. Sennlaub, E. Levasseur, M. Leduc, S. Chemtob, Potential role of microglia in retinal blood vessel formation. Invest. Ophthalvm. Vis. Sci. 47, 3595–3602 (2006).
21. B. Tan, E. Mason, B. MacLellan, K. K. Bizheva, Correlation of visually evoked functional and blood flow changes in the rat retina measured with a combined OCT+ERG system. Invest. Ophthalvm. Vis. Sci. 50, 1673–1681 (2009).
22. N. Cheung, P. Mitchell, T. Y. Wong, Diabetic retinopathy. Lancet 376, 124–136 (2010).
23. F. Tayyari et al., Retinal blood flow and retinal oxygen saturation in mild to moderate diabetic retinopathy. Invest. Ophthalvm. Vis. Sci. 56, 6796–6800 (2015).
24. S. E. Bursell et al., Retinal blood flow changes in patients with insulin-dependent diabetes mellitus and no diabetic retinopathy. Invest. Ophthalvm. Vis. Sci. 37, 886–897 (1996).
25. A. C. Clermont, S. E. Bursell, Retinal blood flow in diabetes. Microcirculation 14, 49–61 (2007).
26. C. Y. Cheung, M. K. Ikram, R. Klein, T. Y. Wong, The clinical implications of recent studies on the structure and function of the retinal microvasculature in diabetes. Diabetologia 58, 871–885 (2015).
40. A. Carmo, J. G. Cunha-Vaz, A. P. Carvalho, M. C. Lopes, Effect of cyclosporin-A on the
43. A. K. Lund, M. B. Goens, N. L. Kanagy, M. K. Walker, Cardiac hypertrophy in aryl hydro-
44. A. I. Jobling et al.
50. S. J. Liu
30. I. M. Chiu
29. S. Gyoneva et al.
33. H. Kawamura et al.
56. K. Hamilton, L. Dunning, W. R. Ferrell, J. C. Lockhart, A. MacKenzie, Endothelium-
57. L. E. Downie
53. J. Matsumoto
35. F. Hui
17. Y. S. Gao, X. Chen, N. Timothy, L. P. Aiello, E. P. Feener, Characterization of the vitre-
ous proteins in diabetes without diabetic retinopathy and diabetes with prolifera-
tive diabetic retinopathy. J. Proteome Res. 7, 2516–2525 (2008).
1. S. Byon et al., Effect of angiotensin II type 1 receptor blocker and angiotensin con-
verting enzyme inhibitor on the intraocular growth factors and their receptors in streptozotocin-induced diabetic rats. Int. J. Ophthalmol. 10, 896–901 (2017).
1. J. H. Kim, J. H. Kim, Y. S. Yu, C. S. Cho, K. W. Kim, Blockade of angiotensin II attenu-
ates VEGF-mediated blood-retinal barrier breakdown in diabetic retinopathy. J. Cereb. Blood Flow Metab. 29, 621–628 (2009).
6. N. Chaturvedi et al., DIRECT Programme Study Group, Effect of candesartan on preven-
tion (DIRECT-Prevent 1) and progression (DIRECT-Protect 1) of retinopathy in type 1 dia-
betes: Randomized placebo-controlled trials. Lancet 372, 1394–1402 (2008).
2. P. T. Yeh, H. W. Huang, C. M. Yang, W. S. Yang, C. H. Yang, Astasaxanthin inhibits
expression of retinal oxidative stress and inflammatory mediators in streptozotocin-
induced diabetic rats. PLoS One 11, e0146338 (2016).
2. P. T. Yeh et al., Effect of fenofibrate on the expression of inflammatory mediators in a
diabetic rat model. Curr. Eye Res. 44, 1121–1132 (2019).
1. M. E. Lott et al., Impaired retinal vasodilator responses in prediabetes and type 2 dia-
betes. Acta Ophthalmol. 91, e462–e463 (2019).
2. A. Mandecka et al., Influence of flickering light on the retinal vessels in diabetic pa-
tients. Diabetes Care 30, 3048–3052 (2007).
5. G. B. Arden, S. Saviprasad, Hypoxia and oxidative stress in the causation of diabetic
retinopathy. Curr. Diabetes Rev. 7, 291–304 (2011).
6. N. Horio et al., Angiotensin AT1 receptor antagonist normalizes retinal blood flow and
acytcholine-induced vasodilatation in normotensive diabetic rats. Diabetologia
47, 113–123 (2004).
5. P. S. Kulkmari, H. Hamid, M. Barati, D. Butulija, Angiotensin II-induced constric-
tions are masked by bovine retinal vessels. Invest. Ophthalmol. Vis. Sci. 40, 721–728 (1999).
2. D. Guarini, S. Majumdar, J. K. Mitra, Role of metabolism in ocular drug delivery. Curr. Drug Targets
5, 507–515 (2004).
5. F. Y. Tang et al., Determinants of quantitative optical coherence tomography angi-
ography metrics in patients with diabetes. Sci. Rep. 7, 2575 (2017).
6. K. A. Vessey et al., CCI3/CX3CR1 knockout mice have inner retinal dysfunction but are
not an accelerated model of AMD. Invest. Ophthalmol. Vis. Sci. 58, 7833–7846 (2012).
5. A. I. Jobling et al., Nanosecond laser therapy reverses pathologic and molecular changes
in age-related macular degeneration without retinal damage. FASEB J. 29, 696–710 (2015).
3. W. Ma et al., Absence of TGFβ signaling in retinal microglia induces retinal degenera-
tion and exacerbates choroidal neovascularization. eLife 8, e8019 (2019).
3. A. Linenmann et al., Macrophage/microglia activation factor expression is restricted to
lesion-associated microglial cells after brain trauma. Glia 53, 412–419 (2006).
2. P. Bankhead, C. N. Shoffield, J. G. McGeown, T. M. Curtis, Fast retinal vessel detec-
tion and measurement using wavelets and edge location refinement. PLoS One 7, e32435 (2012).
2. E. Zudaire, L. Gambardella, C. Kurcz, S. Vermeren, A computational tool for quantita-
tive analysis of vascular networks. PLoS One 6, e27385 (2011).
3. A. C. Schneider, W. S. Rasband, K. W. Eliceiri, NIH Image to Image: 25 years of image
analysis. Nat. Methods 9, 671–675 (2012).
3. A. M. Fontainhas et al., Microglial morphology and dynamic behavior is regulated by
ionotropic glutamatergic and GABAergic neurotransmission. PLoS One 6, e15973 (2011).
3. S. A. Mills et al., RNAseq analysis of retinal microglia during early diabetes. Gene Expres-
tion Omnibus. https://www.ncbi.nlm.nih.gov/geo/query/acc.cgi?acc=GSE139276. Depos-
it 19 October 2019.