Complement facilitates developmental microglial pruning of astrocyte and vascular networks

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Abstract

Microglia, the resident immune cell of the central nervous system, play a pivotal role in facilitating neurovascular development through mechanisms that are not fully understood. This current work resolves a previously unknown role for microglia in facilitating the developmental pruning of the astrocytic template resulting in a spatially organized retinal vascular bed. Mechanistically, our study identified that local microglial expression of complement (C)3 and C3aR is necessary for the regulation of astrocyte patterning and vascular growth during retinal development. Ablation of retinal microglia, loss of C3 or C3aR reduced developmental pruning and clearance of astrocytic bodies leading to increased astrocyte density leading to altered vascular patterning during retinal vascular development. This data demonstrates that C3/C3aR signaling is an important checkpoint required for the finetuning of vascular density during neuroretinal development.

Introduction

High-energy demanding tissues, including neural tissues, require the development of a complex vascular network for their growth, survival, and function. The process of vascular development in neural tissue is orchestrated through highly regulated crosstalk between cell types such as neurons, glial cells, and vascular cells for the establishment of functional blood vessels that contain blood neural barrier properties (Abbott et al., 2006; Fruttiger, 2007; Langen et al., 2019). We now know that the resident immune cell of the central nervous system (CNS), microglia, is a vital contributor in promoting neural-vascular development (Fantin et al., 2010; Rymo et al., 2011). However; the mechanism(s) through which microglial cells dictate vascular growth in the CNS is not fully understood. To further characterize the role of microglia in vascular development, we utilized the retina as a model system allowing us to explore the cellular and molecular contributions of microglia in forming this complex network.

The development of the vascular system in the retina is regulated by non-resident cell types, microglia that populate the retina during early embryonic stages (Santos et al., 2008) followed by astrocytes that enter the retina through the optic nerve head around birth in mice (Fruttiger et al., 1996; Stone and Dreher, 1987; Watanabe and Raff, 1988). Astrocyte migration and template formation in the retina is an essential step for vascular development and interestingly species that lack astrocytes in the retina remain avascular (Schnitzer, 1988; Stone and Dreher, 1987; Zhang and Stone, 1997). In the vascularized retina, ganglion cell derived Pdgfa acts as a chemoattractant that

stimulates astrocyte entry into the retina at the time of birth (Fruttiger et al., 1996; Fruttiger et al., 2000). In response to Pdgfa, astrocytes proliferate and migrate in a centrifugal fashion to form a spatially organized template (that completes around postnatal day (P)-5 thereby allowing subsequent vascular growth to occur (Fruttiger, 2007; Fruttiger et al., 1996; Fruttiger et al., 2000; Gnanaguru et al., 2013). While the vascular template is forming and rearranging, the leading endothelial tip cells follow the astrocyte laid template (beginning at P0) to form the superficial vascular layer by P7 (Dorrell et al., 2002; Gerhardt et al., 2003), followed by the formation of an interconnected deep and intermediate vascular layers that completes around P15 (Fruttiger, 2007). Intriguingly, disruption of astrocyte template formation perturbs subsequent vascular growth (Edwards et al., 2010; Gnanaguru et al., 2013; Tao and Zhang, 2016). Although the importance of astrocyte migration into the retina for vascular growth is known, it remains unclear what signaling mechanism(s) modulate the spatial organization of astrocyte and vascular networks during retinal development. In cases of perturbed or abnormal vascular growth, permanent vision loss can occur (Hartnett, 2020; Holmstrom et al., 2007; Wong et al., 2016), thus understanding these mechanism(s) of vascular development could lead to new strategies for treating retinal pathologies.

Microglia precede astrocytic entry into the neuroretina (Santos et al., 2008) and are known to contribute to vascular growth by facilitating endothelial tip cell anastomoses (Fantin et al., 2010; Rymo et al., 2011). However, to date it was less clear if microglia also interact with astrocytes to assist astrocytic template formation. During neural

development microglia play an indispensable role in the formation of a defined and functional neural circuitry mediated in part through microglia and the complement signaling system (Anderson et al., 2019; Paolicelli et al., 2011; Schafer et al., 2012). The complement system is an integral component of the innate immune system, initially discovered as a component involved in opsonization and clearance of foreign pathogens (Holers, 2014). Recent studies imply that in addition to its immune response function, the complement system regulates wide-variety of biological functions such as synaptic pruning, regulation of glial-microglial crosstalk, clearance of abnormal neovascular growth, removal of stressed or dying cells and modulation of cell metabolism (Anderson et al., 2019; Baudino et al., 2014; Coulthard et al., 2018; Inafuku et al., 2018; Kim et al., 2016; Lian et al., 2016; Mevorach et al., 1998; Mukai et al., 2018; Roy et al., 2013; Schafer et al., 2012; Sweigard et al., 2015). Depending on the context/stimuli, complement activation is initiated through classical, alternative, or lectin pathways (Pitulescu et al.), all of which lead to cleavage of C3 and C5 fragments, i.e. the large C3b and C5b and the small C3a and C5a fragments (Holers, 2014; Merle et al., 2015). In particular, the release of cleaved C3a binding and activation of its cognate C3aR regulates diverse biological functions such as immune cell recruitment, development of the cerebellum, modulation of embryonic neural progenitor proliferation and cognitive performance, chick eye morphogenesis, and astrocyte-microglial crosstalk to elicit inflammatory response in neurodegenerative conditions (Benard et al., 2008; Coulthard et al., 2018; Grajales-Esquivel et al., 2017; Hartmann et al., 1997; Ingersoll et al., 2010; Lian et al., 2016; Litvinchuk et al., 2018). Potentially, suggesting a more ubiquitous role of C3a/C3aR signaling during retinal vascular development.

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134 Prior to superficial retinal vascularization, the astrocyte template undergoes
135 rearrangement and a significant number of astrocytes are eliminated through an
136 unknown mechanism (Chan-Ling et al., 2009; Punal et al., 2019). Given the role of C3
137 in synaptic pruning and microglial-astrocyte crosstalk (Cong et al., 2020; Lian et al.,
138 2016; Schafer et al., 2012), we hypothesized and tested if C3 facilitates microglial
139 pruning of the astrocytic template required for astrocyte spatial organization and
140 subsequent vascular network development.

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142 To assess the role of microglia in the regulation of the astrocytic and endothelial
143 networks formation during retinal development we utilized an antibody against the
144 purinergic receptor, P2ry12, which is microglial specific (Butovsky et al., 2014). We then
145 depleted microglia using colony-stimulating factor-1 (Csfr1) antagonist (Hilla et al.,
146 2017; Okunuki et al., 2019; Okunuki et al., 2018; Valdearcos et al., 2014), in order to
147 investigate the significance of microglial loss on astrocyte template formation and
148 subsequent vascular development. Data showed that the depletion of microglia severely
149 disrupted astrocytic template patterning and subsequent vascular growth. To
150 understand the mechanism of microglial regulation of astrocyte template patterning, we
151 examined C3 tdTomato and C3aR tdTomato expressing murine retina, as C3a/C3aR
152 signaling is involved in microglia/astrocyte crosstalk (Lian et al., 2016). Results revealed
153 concomitant expression of C3 and C3aR in microglia that are closely associated with
154 astrocytes in the vascularizing and adjacent avascular area. Finally, deficiency of C3 or

C3aR and functionally blocking C3aR illustrated the importance of complement mediated pruning by microglia for the proper development of the astrocytic template.

Results

Microglia immigrate into the retina during embryonic development and closely interact with astrocytes and endothelial cells during postnatal vascular development.

To examine the distribution of microglia prior to astrocyte and endothelial entry into the retina, we assessed E13.5 Cx3cr1-egfp retinas with a microglial specific P2ry12 marker. Analysis showed that P2ry12 and Cx3cr1-egfp double positive microglia were localized within the developing ganglion cell layer as well as in the neuroblastic layer (**Figure S1A**). Since endothelial tip cell migration and vascularization in the retina is entirely dependent on the prior formation of the astrocytic template (P0-P5) (Edwards et al., 2010; Fruttiger et al., 1996; Gnanaguru et al., 2013; Stone and Dreher, 1987; Tao and Zhang, 2016; Uemura et al., 2006), we examined if microglia closely associate with astrocytes during astrocytic template formation (P0) and rearrangement (P5) allowing for subsequent endothelial tip cell migration and vascular patterning.

Analysis of P0 retinal flatmounts revealed the close association of microglial ramified processes with the developing astrocytic template as well as the entering endothelial tip cells around the optic nerve head (ONH) region (**Figure 1A**). In addition, at P0, we noticed the enveloping of microglial processes over astrocytes that are beginning to form a naïve template in the central and peripheral retinal regions required for

subsequent vascular growth (**Figure 1A**). Further developmental analysis at P5 illustrated the existence of intimate interactions between microglia, astrocytes, and endothelial tips cells in the vascularized regions and microglia-astrocyte interactions in the avascular regions (**Figure 1B**). Of note, P2ry12 labeled retinal microglia exhibit more prominent structures as compared to staining with Iba1 or isolectin B4 (**Figure 1C, 1D, and Figure S1B**), which allowed us to assess a clearer role for these cells than previously known (i.e., astrocytes and endothelial cells) during vascular development.

Depletion of microglia is associated with increased astrocytic density and spatial patterning

To further investigate the functional relevance of microglial interactions with astrocytes and endothelial cells during the critical steps of astrocytic template assembly and subsequent vascular development, we designed a strategy to deplete microglia from P0. Colony-stimulating factor 1 receptor (Csf1r) signaling is required for microglial survival (Elmore et al., 2014; Okunuki et al., 2019; Okunuki et al., 2018), and prior work found that Csf1r deletion during early development is embryonic lethal (Chitu and Stanley, 2017), thus we utilized a pharmacologic approach whereby Csf1r is inhibited to deplete microglia (Okunuki et al., 2019; Okunuki et al., 2018). To overcome the technical challenges to deplete microglia from birth, timed-pregnant C57BL6J females were fed a diet incorporated with Csf1r specific antagonist (PLX5622) from E13.5/E14.5. We have recently reported that mice fed a diet containing Csf1r specific antagonist (PLX5622) more effectively depletes microglia in the retina, similar to genetic

approaches, but with minimal off-target effects (Okunuki et al., 2019; Okunuki et al., 2018).

P2ry12 immunostaining of P1 and P5 retinal flatmounts of litters from control diet fed group or PLX5622 diet fed group show that the PLX5622 antagonist successfully depleted microglia in comparison to controls (**Figure S2A and S2C**). Analysis of RNA extracted from P1 and P5 retinas of PLX5622 treated animals also showed significant decrease in Csf1r, Tmem119, and P2ry12 gene expression levels in comparison to the P1 control treated group (**Figure S2B and S2D**). Flowcytometric analysis further confirmed significant microglial depletion (**Figure S3C and S3D**). Following microglial depletion during early retinal vascular development, we examined P1 (active astrocyte template assembly phase) and P5 (active astrocyte template rearrangement and vascular growth phase) retinas of litters from control mice and in mice lacking microglia.

Although microglial depletion did not prevent astrocyte migration into the retina, analysis of P1 retinal flatmounts from pups lacking microglia showed a moderate reduction in the astrocytes migrated area compared to control retinas (**Figure S2E**). While at P5, control retinal flatmounts displayed typical astrocyte honeycomb patterning in the central vascular and peripheral avascular retina (**Figure 2A**). In contrast, examination of litters from mice lacking microglia displayed abnormal clustering of astrocytes in the central vascular region (**Figure 2A**). In the peripheral avascular region, spatial distribution of astrocytes was dramatically increased in pups lacking retinal microglia compared to controls (**Figure 2A and 2C**). Furthermore, quantitative analysis by flowcytometry

showed that the percentage of Pdgfra⁺ (a marker for astrocytes) astrocyte population was significantly increased in pups lacking microglia in comparison to the control group (**Figure 2D, 2E, and S3B**) with no aberrant changes in expression of the retinal astrocytes growth factor Pdgfa (Fruttiger et al., 1996; Fruttiger et al., 2000) (**Figure 2B**). Intriguingly, the abnormal astrocytic density and clustering phenotype persisted even following the completion of vascular development at P15 (**Figure S4**). Astrocyte distribution at P15 retinal flatmounts with and without microglia were assessed using the Gfap marker as it is strongly expressed in mature astrocytes (Chan-Ling et al., 2009). Gfap immunostained P15 retinas of pups lacking microglia showed significant increase in astrocyte distribution along with increased astrocytic template branching in the central and peripheral retinas compared to controls (**Figure S4A and S4B**). P15 retinal flatmounts that lacked microglia also contained abnormal clustering of astrocytes compared to control retinas (**Figure S4C**). Taken together, these findings strongly implicate microglia as playing a significant role in supporting the spatial establishment of the astrocytic template required for retinal angiogenesis.

Depletion of microglia reduced subsequent astrocyte dependent vascular growth and density

Proper formation of the astrocytic template and microglia derived signaling are necessary for endothelial tip cell migration and subsequent formation of the superficial retinal vascular network (Edwards et al., 2010; Gerhardt et al., 2003; Gnanaguru et al., 2013; Outtz et al., 2011; Rymo et al., 2011; Tao and Zhang, 2016). As microglial depletion resulted in defective assembly of the astrocytic template, we next examined

the effects on subsequent vascular growth (**Figure S5**). Analysis of retinal flatmounts in litters from mice lacking microglia showed that the vascularized area at P1 and P5 were significantly reduced in comparison to litters from control group (**Figure S5A and S5B**). We further confirmed that the PLX5622 treatment did not alter gene expression levels of vegf isoforms, key for retinal vascular growth (**Figure S3E**). Consistent with the previous findings (Fantin et al., 2010; Rymo et al., 2011), depletion of microglia also significantly reduced vascular density and branching points, while dramatically increasing lacunarity in P5 litters of mice lacking microglia compared to litters of control animals (**Figure S5C and S5D**). Examination of P15 retinal flatmounts of litters from mice lacking microglia revealed that the vascular phenotype observed (reduced density, branching points, and increased lacunarity) persisted even after the completion of vascular development (**Figure S6**).

Microglia utilize complement to facilitate spatial astrocytic template for subsequent vascular development

During retinal vascular development, large numbers of astrocytes are eliminated through an uncharacterized mechanism likely facilitated by microglia peaking at P5 (Chan-Ling et al., 2009; Punal et al., 2019), to help spatially organized astrocytic template establishment. In agreement with the previous findings (Chan-Ling et al., 2009; Punal et al., 2019), analysis of P5 retinal flatmounts showed engulfed Gfap positive astrocytic cellular debris localized within the microglial endosomal/lysosomal membrane protein CD68 (**Figures 3A, 3A' and Figure S7A**).

C3 derived signaling is well known to facilitate the refinement of neural architecture by pruning and eliminating dying neurons (Anderson et al., 2019). Therefore, we investigated if C3 regulates microglial pruning of the astrocytic template for the establishment of a defined spatially organized vascular network. Examination of C3 transcript levels during the active astrocytic template rearrangement phase (P5) showed significant increase relative to P0 (early astrocytic template assembly phase) and P15 (vascular development completion phase) (**Figure 3B**). Moreover, assessment of P5 retinal flatmounts using C3 tdTomato reporter mice showed robust localization of C3 in microglia that are closely associated with astrocytes in the vascular growth and adjacent avascular zones at active astrocyte template rearrangement and subsequent vascular developmental phase (**Figure 3C and 3D**).

We next assessed P5 retinal flatmounts of C3 deficient mice along with their WT littermate controls to define how the loss of C3 affected astrocytic template rearrangement. Analysis of C3 deficient P5 retinas showed a significant reduction in the localization of Gfap positive astrocytic cell debris within microglial CD68 compared to WT littermate control retinas (**Figure 3E and Figure S7B**). Notably, in comparison to the WT P5 littermate retinal flatmounts, analysis of Pdgfra immunostained P5 C3 deficient retinal flatmounts displayed a significant increase in astrocyte template density, branching index, and decreased lacunarity, indicative of reduced spatial gap in astrocyte template (**Figure 3F**).

Given that the loss of C3 dramatically increased astrocytes density and spatial patterning (**Figure 3F**), we next analyzed the effect of C3 loss on subsequent vascular growth at P5. Data show that in comparison to WT littermates, P5 C3 deficient retinal flatmounts showed significant increase in the vascularized area (**Figure S8A**). In addition to increased vascular growth, vessel density was significantly increased in the P5 retinal flatmounts of C3 deficient pups compared to P5 WT littermate retinal flatmounts (**Figure S8B**), while the spatial gap was significantly reduced in P5 C3 KO compared to WT littermate controls (**Figure S8B**). These results suggest that C3 plays an important role in regulating spatial astrocytic template assembly for subsequent superficial retinal vascular development.

C3aR activation controls microglia-mediated phagocytosis of astrocytes

C3a/C3aR signaling has been shown to regulate microglia-astrocyte crosstalk (Lian et al., 2016; Litvinchuk et al., 2018). Therefore, we investigated if microglia use a C3a/C3aR dependent signaling mechanism to phagocytose and eliminate astrocytic bodies leading to a spatially organized astrocyte network during vascular development. During the astrocytic template rearrangement phase, expression analysis of C3aR revealed a significant increase at P5 in comparison to P0 and P15 retinas (**Figure 4C**). Further examination of P5 retinal flatmounts of C3aR-tdTomato reporter mice (Quell et al., 2017) showed strong expression of C3aR in microglia within vascular growth and avascular zones (**Figures 4A and 4B**). In order to test if C3aR is involved in the phagocytosis and elimination of astrocyte bodies, we isolated and cultured retinal microglia and astrocytes from P5 pups. After labeling cultured astrocytes with Dil to

track and identify this population, astrocytic cell death was induced by staurosporine treatment. Retinal microglia were treated with Dil-labeled dead astrocytic bodies in the presence or absence of C3aR neutralizing antibodies or control IgG to determine if C3aR facilitates microglial phagocytosis of astrocytic dead cell bodies. Blocking of C3aR significantly reduced microglial engulfment of Dil-labeled dead astrocyte bodies when compared to IgG control (**Figure 4D**). Examination of P5 C3aR KO retinal flatmounts showed a significant reduction in the localization of Gfap positive astrocytic cell debris within microglial CD68 compared to WT littermate control retinas (**Figure 4E**). Analysis of Pdgfra⁺ astrocytic template formation at P5 also showed increased astrocytic density and branching, and reduced spatial distribution in C3aR KO retinas compared to WT littermate controls (**Figure 4E**). Astrocytic template dependent subsequent superficial vascular growth and vascular density were increased with reduced spatial vascular patterning in P5 C3aR KO retinal flatmounts in comparison to its WT littermate controls (**Figures S8C and S8D**). These results illustrate that microglial C3aR receptor facilitates the developmental elimination of astrocytes for spatial patterning and for organized succeeding vascular network formation.

In summary, these results identify microglia as a key regulator of astrocyte spatial patterning and subsequent vascular development through the C3a/C3aR derived signaling axis.

Discussion

Microglia are essential for neural development (Anderson et al., 2019; Santos et al., 2008; Schafer et al., 2012), and in particular microglia play an important role during neurovascular growth (Fantin et al., 2010; Rymo et al., 2011). In this study, we uncovered a mechanism through which microglia direct neurovascular development in the retina. Results from this study show that the depletion of microglia early during astrocytic patterning and subsequent vascular growth phases increased astrocyte density and caused abnormal astrocyte template formation and reduced vascular growth (**Figure 2, Figures S3, S4, S5, and S6**). Mechanistically, our study identified that microglia derived C3a/C3aR signaling regulates spatial patterning of astrocyte and vascular networks (**Figures 3, 4, and figure S8**). Specifically, deficiency of C3 resulted in dramatic reduction in microglial phagocytosis of astrocyte debris leading to increased astrocyte density and reduced spatial gap (**Figure 3**). In addition, loss of C3aR diminished microglial phagocytosis of dying astrocyte bodies (**Figure 4**), implicating a role for the C3a/C3aR signaling axis in eliminating astrocytes during retinal development. Taken together, these findings uncovered a new functional role for microglia derived C3 signaling in regulating retinal vascular development.

Recent advancements in the discovery of microglial specific markers, such as P2ry12, which are highly expressed during development (Butovsky et al., 2014), allowed us to further define the extensively ramified morphology of microglia during development along with their close interaction with astrocytes during development. The finding of elaborate ramified morphology of microglial associations with astrocytes and endothelial cells during development is particularly intriguing, as prior use of generic myeloid cell

markers such as Iba1 did not reveal the complex morphology or the extent to which microglia associate with astrocytes during development (Fantin et al., 2010; Outtz et al., 2011; Rymo et al., 2011). Given the fact that formation of the astrocyte template is the most critical step for retinal vascular development (Fruttiger, 2007; Fruttiger et al., 1996; Uemura et al., 2006), previous studies provided insights into the microglial regulation of endothelial tip cell fusion during retinal vascular development (Checchin et al., 2006; Fantin et al., 2010; Rymo et al., 2011), however, the role for microglia in astrocyte template assembly or pruning was not clearly known. In this study, the identification of close microglial interactions with astrocytes in the avascular central and peripheral retina at P0 suggested microglia have a role in facilitating astrocyte template assembly. It is known that astrocytes use laminin basement membrane molecules present in the inner limiting membrane (ILM) as a substrate for migration and template formation (Edwards et al., 2010; Gnanaguru et al., 2013; Tao and Zhang, 2016). Deletion of laminin isoforms or the laminin-binding proteoglycans severely disrupt ILM formation, astrocyte template assembly, and succeeding vascularization (Edwards et al., 2010; Gnanaguru et al., 2013; Tao and Zhang, 2016). In addition to known extracellular matrix cues, our study discovered that the depletion of microglia during the early stages of the astrocyte template assembly phase (from birth), results in abnormal clustering of astrocytes leading to defective template formation. This finding strongly suggests that the spaced honeycomb shaped astrocyte patterning is regulated at multiple cellular and extracellular levels (Fruttiger et al., 1996; Gnanaguru et al., 2013; Tao and Zhang, 2016; Uemura et al., 2006), allowing for the formation of an organized vascular network in the retina during development.

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384 Spatial restructuring during development is an important process that helps retinal
385 maturation and function (Anderson et al., 2019; Chan-Ling et al., 2009; Fuerst et al.,
386 2008; Hughes and Chang-Ling, 2000; Li et al., 2015). It is well documented that during
387 the developmental restructuring process, large numbers of retinal ganglion cells are
388 eliminated (Anderson et al., 2019; Li et al., 2015) and microglia, via C3 dependent
389 signaling mechanisms, phagocytose dying ganglion cells to aid spatial organization
390 (Anderson et al., 2019). Similar to ganglion cells (Anderson et al., 2019; Li et al., 2015),
391 large numbers of astrocytes also undergo cell death peaking at P5 and are removed by
392 microglia through unknown mechanisms (Chan-Ling et al., 2009; Punal et al., 2019).
393 Interestingly, the C3b binding receptor CR3 (also known as CD11b or integrin alpha M)
394 involved in neural pruning, was not associated with astrocyte elimination during retinal
395 vascular development (Punal et al., 2019; Schafer et al., 2012).

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397 Several studies have indicated that the C3a anaphylatoxin, and its cognate receptor
398 C3aR involved in immune cell recruitment, mediate astrocyte/microglial crosstalk in the
399 context of neuroinflammation (Lian et al., 2016; Lian et al., 2015; Litvinchuk et al., 2018;
400 Surugiu et al., 2019), thus, in this study we investigated whether C3a/C3aR axis
401 activation also assists in the developmental removal of dying astrocyte bodies/debris by
402 microglia. In contrast to the astrocytic production of C3 in diseased states (Lian et al.,
403 2016; Litvinchuk et al., 2018), our study identified that local expression of C3 and its
404 receptor C3aR were predominantly expressed by microglia during vascular
405 development. It is likely that microglia that are closely interacting with astrocytes deposit

C3 over the template for pruning. Supporting our notion, loss of C3 or C3aR dramatically reduced microglial phagocytic ability to eliminate astrocytes during retinal development. Although we cannot rule out the possible involvement of other complement cleavage fragment receptors, our data provides a compelling role for the C3a/C3aR signaling axis in astrocyte template pruning.

Proper spatial assembly of the astrocytic template is a prerequisite for endothelial tip cell migration for the development of retinal vasculature (Edwards et al., 2010; Gnanaguru et al., 2013; Tao and Zhang, 2016). In light of the previous findings (Hellstrom et al., 2007; Pitulescu et al., 2017), our investigation reveals a plausible role for complement signaling creating a spatially organized astrocytic network to fine tune endothelial tip cell morphogenesis during development.

In conclusion, our study identified a novel functional role for the microglia derived C3 signaling axis in defining astrocyte spatial patterning and subsequent vascular growth during development.

Materials and Methods

Mice

All animal procedures were performed in accordance with the Massachusetts Eye and Ear Animal Care Committee. Mice were maintained in a room with a 12 hours light/12

hours dark cycle. C57BL6/J (stock # 00664), C3 KO (stock # 029661), and C3aR KO (stock # 033904) breeding pairs were purchased from Jackson Laboratories. C3 KO and C3aR KO mice were crossed to C57BL6/J mice to create a heterozygous line. Pups generated from the heterozygous lines carrying C3^{+/+} (wild type (WT)), C3^{-/-} (C3 KO), C3aR^{+/+}, and C3aR^{-/-} genotypes were used for the studies. Floxed C3 IRES-tdTomato reporter mice (Kolev et al., 2020) obtained from Dr. Kemper were bred to C57BL6/J mice and maintained on C57BL6/J background. Floxed C3aR-tdTomato reporter knock-in mice (Quell et al., 2017) obtained from Dr. Köhl were maintained on C57BL6/J background.

Microglial depletion during development

Timed-pregnant mice were maintained on control chow diet or chow diet containing Csfr1 inhibitor (PLX5622, Plexxikon Inc. Berkeley, CA) from gestational day 13.5/14.5. Retinas were collected from pups at postnatal days (P) 1, P5, and P15 for downstream analysis.

Isolation and culture of retinal microglia and astrocytes

Astrocytes and microglial cells were isolated by immunopanning methodology (Collins and Bohlen, 2018; Zhang et al., 2016). In brief, six-well culture plates were coated with respective secondary antibodies (10µg/ml diluted in sterile 50mM Tris-HCl, pH 9.5) by incubating for 1 hour at 37°C. After washes, culture plates were coated with 2.5 µg/ml of anti-CD11b or anti-integrin beta5 for overnight in the cell culture hood and washed in 1X PBS. P5 retinas (n=8) were isolated in ice-cold sterile 1X-DPBS (Thermo Fischer

Scientific, Foster City, CA) and incubated in DMEM/F12 containing 5% fetal bovine serum (FBS), collagenase D (1mg/ml,) and DNase (0.1mg/ml) for 30 min at 37°C. The retinas were then gently triturated in DMEM/F12 supplemented with 10% FBS and filtered through a 40µm cell strainer to remove cell clumps and debris. The resultant single cell suspension was incubated in secondary antibody only coated well for 10 min at 37°C to deplete non-specific cell binding. The unbound cell suspension was then transferred to anti-CD11b coated wells and incubated for 20 min 37°C to capture microglia (culture plate was shaken every 5 min to dislodge non-specific cell binding). After depleting microglia, the cell suspension was transferred to integrin beta 5 coated wells to capture astrocytes and incubated for 45 min at 37°C (culture plates were shaken every 10 min to dislodge non-specific cell binding). After removing the unbound cells, microglia and astrocyte cell culture plates were thoroughly washed in serum free DMEM/F12. Microglial cells were grown and maintained in DMEM/F12 supplemented with 5% FBS and 10ng/ml of recombinant mouse csf1, and astrocytes were grown and maintained in DMEM/F12 supplemented with 10% FBS and N-2.

In vitro phagocytosis assay

To examine microglial phagocytosis of dead astrocyte bodies, ~2,500 microglial cells were plated on a 12mm glass coverslip and maintained for 48 hours. Astrocyte cell suspensions were labeled with Vybrant™ Dil cell labeling solution as suggested by the manufacturer (Thermo Fischer Scientific, Foster City, CA) and cell death was induced by incubating the labeled astrocyte cell suspension with 1µM staurosporine (Cayman Chemical Company, Ann Arbor, MI). Microglial cells were then treated with Dil-labeled

dead astrocyte bodies in 1:1 ratio and incubated for 2 hours. For receptor neutralization assays, microglial cells were preincubated with 20µg/ml of anti-C3aR antibody (Catalog # MAB10417) or control IgG (R&D Systems, Inc. Minneapolis, MN) suspended in DMEM/F12 supplemented with 5% heat-inactivated FBS, followed by treatment with Dil-labeled astrocyte dead bodies in the presence of antibodies for 2 hr at 37°C. After thorough washes, cells were fixed in 4% PFA, blocked for 1hr at room temperature (in 10% fetal bovine serum, 0.05%triton-X100, and 0.01% sodium azide in 1X PBS), followed by incubation with P2ry12 antibody (Catalog# AS-55043A, AnaSpec, Fremont, CA) overnight at 4°C and then incubated with goat anti-rabbit Alexa Fluor 488 or 647 (Thermo Fischer Scientific, Foster City, CA), images were acquired using epifluorescent microscope (Axio Observer Zeiss).

Flatmount preparation and immunostaining

Retinal flatmounts were prepared as described previously (Gnanaguru et al., 2013). In brief, eyes were fixed in 2% paraformaldehyde (PFA) for 10-15 min and then dissected in 1X PBS. After discarding the anterior chamber and lens, the retina was separated from the sclera. Retinal flatmounts were prepared with four radial incisions and the flatmounts were then stored in methanol at -20°C until immunostaining was performed. For immunohistochemistry, retinas were rehydrated in 1X PBS and washed at 3X and blocked in blocking buffer (10% fetal bovine serum, 0.05%triton-X100, and 0.01% sodium azide in 1X PBS) for 2hr at room temperature. Following blocking, retinas were then incubated with primary antibodies for 24 hr at 4°C. After washes in 1X PBS, retinal flatmounts were incubated with respective secondary antibodies for 4hr at room

temperature or overnight at 4°C. The flatmounts were then washed in 1X PBS and mounted onto a slide using anti-fade medium (Permaflour, Thermo Fisher Scientific, Waltham, MA).

Primary antibodies used: Rat anti-Pdgfra (CD140A, clone: APA5, BD Biosciences, San Jose, CA), anti-chicken GFAP (Aves Labs, Inc, Davis, CA), rabbit anti-P2ry12 (a gift from Dr. Butovsky, Brigham and Women's Hospital), rat anti-mouse CD68 (Clone: FA-11, Biolegend, Dedham, MA), anti-rabbit Iba1 (Catalog# 019-19741, FUJIFILM Wako Chemicals, Richmond, VA), and goat anti-CD31 (Catalog # AF3628, R&D Systems, Inc. Minneapolis, MN).

Secondary antibodies used: Donkey anti-chicken 594 (Biotium, Inc. Fremont, CA), anti-rat-594, donkey anti-rabbit-488, and donkey anti-goat-647 (Thermo Fisher Scientific, Waltham, MA)

Cryosectioning

The enucleated eyes were fixed in 4% PFA for 2hr at room temperature and then the dissected posterior eyecups were cryopreserved in 10%, 20%, and 30% sucrose and then frozen in Tissue-Tek® O.C.T compound (Ted Pella, Inc. Redding, CA). The eyecups were then cut at 12µm thickness and used for immunohistochemistry.

Flow cytometry

Singe cell suspensions were prepared from retinas that were dissected in ice-cold HBSS and then incubated with collagenase D (1mg/ml) and DNase (0.1mg/ml) (Millipore Sigma) in DMEM/F12 supplemented with 5% FBS for 30 min at 37°C. After

filtering the cells through a 40µm cell strainer, single cell suspensions were blocked with anti-mouse CD16/32 monoclonal antibody and then stained with APC anti-Pdgfra (CD140a, clone: APA5), and PE anti-p2ry12 (clone: S16007D, Biolegend, Dedham, MA). Dead cells were distinguished with DAPI stain. The data for flow cytometry was acquired by Cytoflex S (Beckman Coulter, Indianapolis, Indiana) and analyzed using FlowJo version 10.1.

Real-time PCR

Retinas were isolated in ice-cold PBS and RNA was extracted using RNA STAT-60™ as recommended by the manufacturer. After determining the RNA concentration using NanoDrop™, 500ng of total RNA was used for cDNA synthesis using SuperScript™ IV VILO™ Master Mix. Real-time PCR reactions were performed in the CFX384™ Real-time PCR platform (Bio-Rad, Hercules, CA) using SYBR Green master mix (Applied biosystems, Thermo Fischer Scientific, Foster City, CA) to determine the relative expression level of *Pdgfa*, *P2ry12*, *Tmem119*, *Csf1r*, *Vegfc*, *Vegf120*, *Vegf164*, and *Vegf188* (Darland et al., 2011). Sequences of primers used: *Ppia* (TTC ACC TTC CCA AAG ACC AC and CAA ACA CAA ACG GTT CCC AG), *Pdgfa* (ATTAACCATGTGCCCCGAGAA and GTATCTCGTAAATGACCGTCCTG), *P2ry12* (CCAGTCTGCAAGTTCCACTAAC and GAGAAGGTGGTATTGGCTGAG), *Tmem119* (GGTCCTTCACCCAGAGC and GGAGTGACACAGAGTAGGC), *Csf1r* (TGT ATGTCTGTCATGTCTCTGC and AGGTGTAGCTATTGCCTTCG), *Vegfc* (GAAGTTCCACCATCAAACATGC and CAGCGGCAT ACTTCTTCACTA), *Vegf120* (GCCAGCACATAGGAGAGATGAGC and CGGCTTGTCACATTTTTCTGG), *Vegf164*

(GCCAGCACATAGGAGAGATGAGC and CAAGGCTCACAGTGATTTTCTGG), and
Vegf188 (GCCAGCACATAGGAGAGATGAGC and AACAAGGCTCACAGTGAACGCT).

Image analysis

Samples were imaged using an epifluorescent microscope (Axio Observer Zeiss) or confocal microscopy (SP8, Leica). For 3D image reconstruction of Z-stack images, Amira® 2019.4 software tool was used.

Analysis of astrocyte and vascular coverage

Tiled images of the entire retinal surface immunostained for Pdgfra or CD31 were acquired. Using NIH-ImageJ (version 2.1.0/1.55c)) software, scale measurements were set and an outline of the astrocyte or vascular growth front was created using manual freehand selection tool total area of the Pdgfra or CD31 immunostained area was measured. Tip cells were manually quantified in all four quadrants using ImageJ multi-point tool.

Microglia immunostained cellular area measurement

Images of retinal flatmounts co-labeled with CD31 and P2ry12 or CD31 and Iba1 were acquired in all the retinal quadrants near the tip cell and avascular peripheral regions by confocal microscopy. Outline of P2ry12 or Iba1 stained cell area was manually drawn using Image J (version 2.1.0/1.53c) freehand selection tool and each immunostained cell area was measured and quantified.

Analysis of astrocyte and vessel density, branching, and lacunarity

Astrocyte and blood vessel covered area were quantified using ImageJ (version 2.1.0/1.53c) Angiogenesis Analyzer plugin tool. In brief, using free hand selection tool

the edges of astrocyte or blood vessel stained region was manually selected and the area was determined. Tip cells were manually quantified in all four quadrants using ImageJ Wand tracing tool. Vascular density, branching index, and lacunacity was analyzed using ImageJ Angiogenesis Analyzer.

Statistical analysis

All the other graphs and statistical analysis were performed using Prism version 9 software. All data were presented as the mean +/- SEM. Statistical differences between the two groups were determined by unpaired t test. The 'n' and the level of statistical significance were noted in the legend of each figure.

Data availability

Images and flow cytometry data used for the analysis are uploaded to Harvard institutional repository. Upon acceptance these data will be publicly available (<https://dataverse.harvard.edu/dataset.xhtml?persistentId=doi:10.7910/DVN/CDJYDT>). All raw data will also be available by reaching out to the Lead Contact, Gopalan Gnanaguru (gopalan_gnanaguru@meei.harvard.edu).

Author contributions

GG and KMC designed research and wrote the manuscript; GG performed research and analyzed data; SJT and KY analyzed data; RM performed embryonic study, CK and JK generated and provided reporter mice.

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Conflict of interest

The authors declare no competing interests

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Figures

Figure 1. Microglia populate the retina early in embryonic development and are recruited to sites of astrocyte template formation and vascularization. **A.** Cross section of an embryonic (E) day 13.5 Cx3cr1-gfp expressing retina immunostained for P2ry12 and DAPI nuclear stain (blue). Overlaid images reveal Cx3cr1-gfp and P2ry12 colocalization within a microglia cell population. **(B and C)** Retinal flatmounts prepared from P0 and P5 pups were immunostained for Pdgfra, P2ry12, and CD31 and Z-stack images were acquired and 3D-reconstructed to examine microglial interaction with astrocytes and endothelial tip cells early during vascular development. **B.** Representative 3D-reconstructed image near the optic nerve head (ONH), central, and peripheral retina revealing microglia (P2ry12-green) have a close association with astrocytes (Pdgfra-grey), and endothelial tip cells (CD31-red) (n=3). **C.** Representative 3D-reconstructed image of P5 C57BL/6J retinal flatmount immunostained for Pdgfra (astrocyte marker), P2ry12 (microglial marker), and CD31 (endothelial cell marker). Confocal z-stack images were taken near the leading tip cell area and avascular peripheral retina to visualize microglial interaction with endothelial cells and astrocytes (n=5). **D.** Representative 3D reconstructed P5 retinal flatmount immunostained for either P2ry12 or Iba1 revealing microglia morphology. Areas with increased intensity are indicated as hotspots (n=4). **E.** Quantification of P2ry12 and Iba1 localized expression around tip cells and avascular regions of the retina (n=4). Scale bars: A is 100 μ m, B, C, and D are 10 μ m. All error bars represent $\pm$ S.E.M. Statistical differences were calculated by unpaired *t* test. **** P < 0.0001

Figure 2. *Csfr1r* specific antagonist effectively depletes retinal microglia early in vascular developmental stage. Time-pregnant C57BL/6J mice from gestational day E13.5/E14.5 were fed a control or *Csfr1r* specific antagonist (PLX5622) diet and littermate controls were analyzed at P1 and P5 developmental time points. (A-F). **A.** Representative P1 retinal flatmounts from control or PLX5622 treated groups showing staining of *P2ry12* (Green). **B.** Real-time PCR analysis of relative gene expression

levels of microglia markers, *Csf1r*, *P2ry12*, and *Tmem119* at P1 (n=3) in the retinas of control or PLX5622 groups. **C.** Representative anti-P2ry12 immunostaining of retinal flatmounts showing microglial distribution at P5. **D.** Real-time PCR analysis of relative gene expression levels of microglia markers, *Csf1r*, *P2ry12*, and *Tmem119* at P5 (n=5) in the retinas of control or PLX5622 groups. **E.** P1 retinal flatmounts isolated from control or PLX5622 groups were immunostained for *Pdgfra*, the percentage of astrocyte coverage (indicated by dotted lines in images) was quantified and shown in the graph (n=4). Scale bar: A, C, and D are 500µm. All error bars represent ± S.E.M. Statistical differences between control and PLX-5622 group was calculated by unpaired *t* test. * P < 0.05, *** P < 0.001, **** P < 0.0001

Figure 3. Microglia depletion in early vascular development increases astrocyte density and causes abnormal template formation. Time-pregnant C57BL/6J mice from gestational day E13.5/E14.5 were fed either a control or *Csfr1* specific antagonist (PLX5622) diet and littermates were analyzed at P5. (A-E). **A.** Representative P5 retinal flatmount from control or PLX5622 groups immunostained for Pdgfra and P2ry12. Cell morphology and patterning of astrocytes (Pdgfra, grey) and microglia (P2ry12, Green)

were observed in avascular central and peripheral retina (n=4). Scale bar: 50 μ m. **B.** Real-time PCR analysis of relative gene expression levels of *Pdgfa* at P5 (n=5) in the retinas of control or PLX5622 groups. **C.** Images of control and PLX5622 P5 retinal flatmounts immunostained for *Pdgfra* in the central region of all four quadrants were acquired. Using 'NIH-ImageJ Angiogenesis Analyzer' software, astrocyte density, branching index, and lacunarity were quantified and shown (n=5). **D.** Astrocyte (*Pdgfra*-APC+) live cell frequencies were assessed in P5 retinas in either control or PLX5622 groups by flow cytometry (n=3). **E.** Quantification of flow cytometry data (**D**) showing the percentage of *Pdgfra*-APC+ live cells in control and PLX5622 groups. All error bars represent $\pm$ S.E.M. Statistical differences between control and PLX5622 group was calculated by unpaired *t* test. * *P* < 0.05, *** *P* < 0.001, **** *P* < 0.0001

Figure 4. Microglial depletion reduces vascular growth and density. Time-pregnant C57BL/6J mice from gestational day E13.5/E14.5 were fed a control diet or diet containing

Csfr1 specific antagonist (PLX5622) and the littermates were analyzed at respective ages (A-E). **A and B.** P1 and P5 retinal flatmounts from control and PLX5622 group immunostained for CD31 (endothelial cell marker) and vascularized area (indicated by dotted lines in the images) were quantified and shown in the bar graphs (n=4) **C.** Representative images of P5 retinal flatmount from control and PLX5622 group immunostained for CD31, Pdgfra, and P2ry12 showing microglial interaction with endothelial cells and astrocytes in the vascularized area. **D.** Images of control and PLX5622 P5 retinal flatmounts immunostained for CD31 in the vascularized central region of all four quadrants were acquired. Using 'NIH-ImageJ Angiogenesis Analyzer' software, vessel density, branching index, and lacunarity were quantified and shown (n=5). Scale bars: A and B are 500 μ m, C is 50 μ m. All error bars represent $\pm$ S.E.M. Statistical differences between control and PLX5622 group was calculated by unpaired *t* test. * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$, **** $P < 0.0001$

Figure 5. C3 is expressed in microglia and loss of C3 reduces microglial pruning of astrocytes. **A.** Representative z-stack images of P5 retinal flatmount showing GFAP and CD68 immunostaining (n=5). **A'.** Enlarged 3D-reconstructed boxed area in (**A**) revealing microglia internalized GFAP astrocyte debris within CD68+ lysosomes (green). **B.** Real-time PCR analysis of retinas isolated at P0 (n=3), P5 (n=5), and P15 (n=5) showing age-

dependent relative C3 mRNA levels. **C.** C3-tdTomato (C3-tdT) expressing P5 retinal flatmount immunostained for P2ry12 and Pdgfra to label microglia (blue) and astrocytes (green) respectively. **D.** Representative 3D-reconstructed image from C3-tdT expressing P5 retinal flatmount immunostained for P2ry12 and Pdgfra reveal C3 reporter expression within microglia (P2ry12, green) that are closely interacting with astrocytes (Pdgfra, grey) (n=5). **E.** Z-stack images were acquired from P5 WT and C3 KO retinal flatmount immunostained for P2ry12, GFAP, and CD68 and 3D-reconstructed to visualize microglia (P2ry12) engulfed astrocyte (GFAP) bodies within microglial lysosomal/endosomal membrane protein CD68). Bar graph shows the percentage of GFAP debris localized within P2ry12, CD68 co-labelled microglia between WT and C3 KO retinas (n=3). All error bars represent $\pm$ S.E.M. In **B.** statistical differences at P0, P5, and P15 were calculated by one-way analysis of variance followed by a post Dunnett's multiple comparisons. * P = 0.02. Statistical differences between WT and C3 KO in E and F was calculated by unpaired *t* test. * P < 0.05, ** P < 0.01. Scale Bars: A is 15 μ m, C is 75 μ m, D and E are 10 μ m.

Figure 6. C3 deletion increases astrocytic template and vascular densities at P5. **A.**

Representative P5 retinal flatmount from WT and C3 KO immunostained for Pdgfra showing astrocyte template patterning in the avascular region (n=5). Bar graphs represent the quantitative measurements of astrocyte density, branching index, and lacunarity using ImageJ Angiogenesis Analyzer software tool (n=5). **B.** Representative P5 Retinal flatmounts of WT and C3 KO immunostained for CD31 showing vascular outgrowth and quantification of vascularized area in WT and C3 KO (n=5). **C.** P5 retinal flatmounts of WT and C3 KO were immunostained for CD31 and images were taken in

958 the central region to assess vessel density and spatial branching (lacunarity) using
 959 'ImageJ Angiogenesis Analyzer' software tool (n=5). Scale bars: **A** is 500 μm , **B** is 50 μm ,
 960 and **C** is 20 μm . All error bars represent $\pm$ S.E.M. Statistical differences between WT and
 961 C3 KO was calculated by unpaired *t* test. ** $P < 0.01$, *** $P < 0.001$.

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Figure 7. Microglia expresses C3aR and loss of C3aR reduces microglial pruning of astrocytes. **A.** Representative P5 C3aR-tdTomato (C3aR-tdT) expressing retinal flatmount showing Pdgfra and P2ry12 immunolocalization around the vascular growth front (n=3). Arrows indicates the localization of tdTomato in P2ry12 expressing microglia. **B.** Representative 3D-reconstructed image from C3aR-tdTomato expressing

P5 retinal flatmount immunostained for P2ry12 and Pdgfra reveal C3aR reporter expression within microglia (P2ry12, green) that are closely interacting with astrocytes (Pdgfra, grey) (n=3). **C.** Real-time PCR analysis of retinas isolated at P0 (n=3), P5 (n=5), and P15 (n=5) showing C3aR relative mRNA levels. **D.** Primary microglial culture isolated from P5 retina were treated with Dil-labeled retinal astrocyte apoptotic bodies in the presence of control IgG or anti-C3aR IgG for 2 hours and the number of Dil-astrocyte bodies engulfed microglia in the two treatment conditions were quantified and shown in the bar graph (n=3). **E.** Z-stack images were acquired from P5 WT and C3aR KO retinal flatmount immunostained for P2ry12, GFAP, and CD68 and 3D-reconstructed to visualize microglia (P2ry12) engulfed astrocyte (GFAP) bodies within microglial lysosomal/endosomal membrane protein CD68). Bar graph shows the percentage of GFAP debris localized within P2ry12, CD68 co-labelled microglia between WT and C3aR KO retinas (n=3). All error bars represent $\pm$ S.E.M. Statistical differences at P0, P5, and P15 were calculated by one-way analysis of variance followed by a post Dunnett's multiple comparisons. * $P = 0.007$. Statistical differences between IgG control and anti-C3aR IgG was calculated by unpaired t test. Statistical differences between WT and C3aR KO in E and F were calculated by unpaired t test. ** $P < 0.01$, *** $P < 0.001$, and **** $P < 0.001$. Scale Bars: A is 75 μm , B and E is 10 μm , D is 40 μm ,

Figure 8. Loss of C3aR increases astrocytic template and vascular densities at P5. **A.**

Representative P5 retinal flatmount from WT and C3aR KO immunostained for Pdgfra showing astrocyte template patterning in the avascular region (n=5). Bar graphs represent the quantitative measurements of astrocyte density, branching index, and lacunarity using ImageJ Angiogenesis Analyzer software tool (n=5). **B.** Representative P5 Retinal flatmounts of WT and C3aR KO immunostained for CD31 showing vascular outgrowth and quantification of vascularized area in WT and C3aR KO (n=5). **C.** P5 retinal flatmounts of WT and C3aR KO were immunostained for CD31 and images were taken

1024 in the central region (bottom panel) to assess vessel density, and spatial branching
 1025 (lacunarity) differences using 'ImageJ Angiogenesis Analyzer' software tool (n=5). Scale
 1026 bars: **A** is 50 μm , **B** is 500 μm , and **C** is 50 μm . All error bars represent $\pm$ S.E.M. Statistical
 1027 differences between WT and C3 KO was calculated by unpaired *t* test. * $P < 0.05$, **** P
 1028 < 0.0001 .

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