Vascular Associations and Dynamic Process Motility in Perivascular Myeloid Cells of the Mouse Choroid: Implications for Function and Senescent Change

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Submitted: October 28, 2013

Accepted: January 14, 2014

Citation: Kumar A, Zhao L, Fariss RN, McMenamin PG, Wong WT. Vascular associations and dynamic process motility in perivascular myeloid cells of the mouse choroid: implications for function and senescent change. *Invest Ophthalmol Vis Sci.* 2014;55:1787–1796. DOI:10.1167/iovs.13-13522

PURPOSE. Immune and vascular alterations in the choroid are implicated in age-related macular degeneration (AMD). As choroidal immune cells are incompletely understood with regard to their physiology and interactions with choroidal vessels, we examined the associations between myeloid and vascular components of the choroid in young and aged mice.

METHODS. Albino CX3CR1^{GFP/+} transgenic mice, whose choroidal myeloid cells possess green fluorescence, were perfused intraluminally with the vital dye DiI to label choroidal vessels. The distribution, morphology, behavior, and vascular associations of resident myeloid cells were examined using time-lapse live confocal imaging and immunohistochemical analysis.

RESULTS. Dendritiform myeloid cells, comprising most of the resident immune cell population in the choroid, were widely distributed across the choroid and demonstrated close associations with choroidal vessels that varied with their position in the vascular tree. Notably, myeloid cells associated with choroidal arteries and arterioles appeared as elongated cells flanking the long axes of vessels, whereas those associated with the choriocapillaris were distributed as a layer of stellate cells on the scleral but not vitreal choriocapillaris surface. While stationary in position, dendritiform myeloid cells demonstrated the rapid process dynamism well suited to comprehensive immunosurveillance of the perivascular space. Myeloid cells also increased in density as a function of aging, correlating locally with greater choroidal vascular attenuation.

CONCLUSIONS. Resident myeloid cells demonstrated close but dynamic physical interactions with choroidal vessels, indicative of constitutive immune-vascular interactions in the normal choroid. These interactions may alter progressively with aging, providing a basis for understanding age-related choroidal dysfunction underlying AMD.

Keywords: eye, choroid, mouse, myeloid cell, macrophage, dendritic cell, vasculature, live imaging, motility

The choroidal vasculature of the eye acts as the circulatory system for the outer retina in animals with a vascularized (holangiotic) retina and for the full thickness of the retina in animals with an avascular (euangiotic) retina. It consists of a system of vessels located in the outer ocular coats that terminates in the choriocapillaris, a fenestrated wide-bore capillary plexus juxtaposed against the outer-blood-retina barrier.¹ Carrying 80% of ocular blood flow, the choroid in the eye constitutes a major point of interaction between systemic circulation and the neural environment.² In common with other homologous junctions of vascular-central nervous system contact, such as the choroid plexus of the brain³ and the blood-labyrinth barrier in the stria vascularis of the cochlea,⁴ choroidal circulation contains a large number of resident immune cells of myeloid origin^{5–8} whose constitutive functions in the maintenance of healthy ocular physiology have not yet been fully defined.

Alterations in the functions of the choroid have been associated with diseases of the outer retina, including age-related macular degeneration (AMD), a disease in which both

vasculature- and immune system-related mechanisms are implicated.^{9,10} Vascular changes in the choroid feature prominently in the histopathology of AMD^{11,12} and are hypothesized to be a factor driving disease progression.¹³ In addition, immune cells in the choroid have been causally associated with pathological choroidal change in AMD,¹⁴ particularly with the formation of choroidal neovascular vessels in the exudative or “wet” form of advanced disease.^{15,16}

However, understanding of pathologic immune changes in the choroid is currently limited by a relative paucity of information about the physiology and function of resident immune cells in the healthy eye. Although choroidal myeloid cells have been located and immunohistochemically characterized in tissue flat-mounts and sections in rat^{17,18} and mouse eyes,⁶ the morphology, distribution, and behavior of these cells at each level of the vascular tree have not been previously described. In the current study, we used confocal microscopy techniques combined with vital labeling of choroidal vasculature and resident myeloid cells to examine the vascular associations and dynamic behavior of choroidal immune cells

in the adult mouse choroid at high resolution. Our novel observations reveal that myeloid cells are distributed throughout the layers of the choroid and are closely associated with vascular elements. In addition, we used live confocal microscopy to show how these resident myeloid cells interact with the perivascular microenvironment through dynamic cellular processes. These characterizations provide support for the proposal that immune cells constitutively signal to vascular cells in ways that are relevant to surveillance of transported materials in the choroid; they also provide a foundation for future study of pathologic specimens and animal models of AMD in which immune and vascular factors within the choroid feature prominently in the disease phenotype.

METHODS

Experimental Animals

Because the relative opacity of the choroid in pigmented mice obscures immunofluorescence imaging in choroidal flat-mounts, we crossed CX3CR1^{GFP/GFP} mice¹⁹ on a C57BL/6J background with B6-albino (B6(Cg)-Tyr^{c-2J}/J) mice (Jackson Laboratory, Bar Harbor, ME). Animals in the F1 generation were bred with each other and albino heterozygous CX3CR1^{+/GFP} mice in the F2 generation, identified by genotyping and used in experiments. Wild-type BALB/c mice of young (3–4 months old) and aged (19–21 months old) groups were obtained from Aged Rodent Colonies (National Institutes on Aging, Bethesda, MD). All mice were genotyped and confirmed not to carry the *Crb1*^{rd8} mutation.²⁰ All animals were housed and bred in National Institutes of Health (NIH) animal facilities. Experiments were conducted according to protocols approved by a local Institutional Animal Care and Use committee. All animals were treated in accordance with the Association for Research in Vision and Ophthalmology statement for the use of animals in ophthalmic and vision research.

Labeling and Visualization of Choroidal Vasculature in Choroidoscleral Explants

Choroidal vasculature was labeled by cardiac perfusion with an aqueous solution containing 1,19-dioctadecyl-3,3,39,39-tetramethylindocarbocyanine perchlorate (DiI; product no. D-282; Invitrogen/Molecular Probes, Carlsbad, CA), a lipophilic dye that incorporates into endothelial cell membranes as previously described.^{21,22} Briefly, a DiI perfusate was prepared by diluting a stock solution (6 mg/mL in 100% ethanol) in a diluent of PBS containing 5% glucose in a 1:250 ratio. Experimental animals were euthanized by carbon dioxide inhalation or an overdose of solution containing ketamine (300 mg/kg) and xylazine (30 mg/kg) and perfused intracardially with the DiI perfusate at a rate of 1 to 2 mL/min. For live imaging experiments, DiI perfusion was followed by perfusion with 5 mL of PBS. After being perfused, animals were promptly enucleated, and eye globes were dissected free of ocular muscles and external connective tissue, and anterior segments were removed. The resulting eye cups were immersed in ice-cold oxygenated Ringer solution (125 mM NaCl, 5 mM KCl, 1.5 mM CaCl₂, 0.75 mM MgCl₂/6 H₂O, 1.25 mM NaH₂PO₄, 10 mM D-glucose, 20 mM HEPES, pH 7.35–7.45), and the retinal pigment epithelium (RPE)-sclera-choroid complex was dissected free and flat mounted onto filter paper (product no. HABP045; Millipore, Billerica, MA) with the RPE layer uppermost. For analyses of fixed tissue, intracardiac DiI perfusion was followed by the sequential perfusion of 5 to 6 mL of PBS and 5 to 6 mL of 4% paraformaldehyde. The choroidoscleral explants were prepared by dissection and processed for immunohistochemistry.

Immunohistochemistry

For immunohistochemical analyses, choroidoscleral explants were washed with PBS for 1 hour, incubated in 20 mM of ethylenediaminetetraacetic acid (EDTA) tetrasodium salt hydrate at 37°C for 30 min, and permeabilized with a solution of PBS containing 0.5% Triton X-100 (PBST) for 1 hour (all reagents from Sigma-Aldrich, St. Louis, MO). Explants were transferred to a blocking solution (PBST containing 2% goat serum) for 30 minutes and incubated overnight with the following primary antibodies: anti-major histocompatibility complex class II (MHCII, 1:1000 dilution; BD Pharmingen, San Diego, CA), anti-ionized calcium binding adaptor molecule-1 (Iba1, 1:500 dilution; Wako, Richmond, VA), anti CD-163 (1:500; Santa Cruz Biotechnology, Inc., Santa Cruz, CA), and anti-CD169 (1:500 dilution; AbD Serotec, Raleigh, NC). After being washed with PBS, explants were incubated with secondary antibodies conjugated to either Alexa-405 or Alexa-568 (Invitrogen/Molecular Probes) at a 1:1000 dilution for 1 to 2 hours. Explants were then flat mounted onto glass slides, and images were obtained by confocal microscopy.

Imaging and Analysis of Fixed Choroidoscleral Explants

Choroidoscleral explants labeled with intravascular DiI and/or processed for immunohistochemistry were imaged using epifluorescence and confocal microscopy (LSM 700; Carl Zeiss Microscopy, Thornwood, NY). Epifluorescence images of entire choroidoscleral flat-mounts captured with an ×20 objective were compiled in montage by using image analysis software (AxioVision LE software; Carl Zeiss Microscopy). Confocal multiplane z-series stacks were collected using either an ×20 objective (18–20 μm in total z-depth, with 10–15 z-planes per series) or an ×40 oil-immersion objective (30–40 μm in total z-depth, with 60–80 images per series). Orthographic projections of confocal image stacks were generated from the z-series stacks (ZEN 2011 software; Carl Zeiss Microscopy). Three-dimensional (3D) rendering of confocal image stacks was performed using Volocity 3D image analysis software (Perkin Elmer, Waltham, MA). For analyses, choroidoscleral flat-mount explants were segmented into central and peripheral areas by using a circle centered on the optic nerve, whose radius was half of the distance between the optic nerve and the peripheral edge of the explant. Choroidal myeloid cells were counted manually using epifluorescence and confocal images.

Ex Vivo Live Time-Lapse Imaging of Resident Myeloid Cells in the Choroid

Following DiI perfusion, live choroidoscleral explants from CX3CR1^{+/GFP} samples that had been flat mounted onto filter paper were transferred to a stage-mounted, temperature-controlled (32°C) chamber (Biopetechs, Butler, PA) through which oxygenated Ringer solution was continuously superfused (30–60 mL/h). Tissue preparations were imaged using a confocal microscope (SP2; Leica, Exton, PA) with ×20 (0.50 numerical aperture) and ×40 (0.80 numerical aperture) water-immersion objectives. Multiplane z-series time-lapse images were collected at a resolution of 1024 × 1024 or 512 × 512 pixels. Image stacks traversing volumes of interest were captured every 10 to 60 seconds. Image processing was performed using ImageJ software (National Institutes of Health, Bethesda, MD). Two-dimensional representations of 3D perivascular macrophage structures were created from maximum intensity projections in the z dimension and recursively aligned

in the time dimension to generate time-lapse movies of dynamic choroidal myeloid cells.

Statistical Analysis

Data are means $\pm$ SEM. Statistical comparisons were performed using one-way analysis of variance (ANOVA) and unpaired *t*-tests by using statistical software (GraphPad Prism; GraphPad Software, La Jolla, CA).

RESULTS

General Distribution and Morphology of Resident Myeloid Cells in the Mouse Choroid

Choroidoscleral flat mounts prepared from albino CX3CR1^{GFP/+} (C57BL/6J) background with B6-albino (B6(Cg)-Tyr^{c-2/J}) mice previously perfused with the lipophilic dye DiI were mounted on glass slides with the RPE cell layer uppermost and imaged with confocal microscopy. Resident myeloid cells in the choroid which express green fluorescent protein (GFP) under the control of the CX3CR1 promoter^{19,23} were found throughout the choroid from central to peripheral areas (Fig. 1A). Vascular perfusion of DiI that labeled choroidal vessels intraluminally^{21,22} enabled concurrent visualization of all levels of the choroidal vascular tree from the primary choroidal arteries to the choriocapillaris (Fig. 1A). Under higher magnification, resident CX3CR1-positive (CX3CR1⁺) myeloid cells were observed to have a general perivascular distribution at all levels of the vasculature (Fig. 1B). GFP⁺ myeloid cells demonstrated a broad pattern of immunopositivity to myeloid cell markers including MHCII, Iba1 (Figs. 1C–F), CD11b, and CD68 as previously reported,²³ and CD163 (ED2; Fig. 1G) and CD169 (ED3; Fig. 1H). They were negative for markers of endothelial cells (CD31) and perivascular smooth muscle cells and pericytes (α -SMA and NG2; data not shown). Although most GFP⁺ cells demonstrated dendritiform morphologies with elongated processes, a smaller class of myeloid cells with rounded morphologies and few or no processes was also observed scattered in perivascular spaces at all levels of the choroidal vascular tree (Fig. 1I). Although a predominant proportion ($\approx$ 80%) of GFP⁺ dendritiform cells were also immunopositive for MHCII and Iba1 (Figs. 1J–L, top panels, 1M), rounded cells were significantly more varied in their expression of myeloid markers (Figs. 1J–L, bottom panels, 1N), suggesting a more mixed population. In the absence of definitive markers that can immunophenotypically distinguish dendritic cells (DCs) from macrophages,²⁴ it is likely that the population of CX3CR1⁺ myeloid cells in the normal choroid consists of both resident DCs and MHC class II⁺ macrophages.

In light of the potential role of DCs in local antigen presentation under conditions of uveitis⁸ and the newly appreciated putative role for choroidal macrophages in regulation of debris clearance in the choriocapillaris/Bruch's membrane complex,²⁵ we undertook the characterization of the detailed morphology of choroidal myeloid cells and their physical association with choroidal vessels at different levels of the vascular tree (primary choroidal arteries, primary choroidal arterioles, terminal choroidal arterioles, and choriocapillaris).

Anatomical Associations of Resident Myeloid Cells With Primary Choroidal Arteries and Arterioles

The main choroidal arteries, which possess wide lumina that are relatively flattened in the anteroposterior direction (Fig. 2A), were flanked laterally by resident dendritiform myeloid cells. These cells were elongated and spindle-shaped, and

FIGURE 1. Distribution and morphologies of GFP-positive resident myeloid cells are shown in the mouse choroid. (A) Choroidoscleral flat-mount from a 3-month-old adult CX3CR1^{+/GFP} mouse that had been perfused intravascularly with lipophilic dye, DiI (red) is shown. Choroidal myeloid cells (green) were found distributed throughout the choroidal layer from central to peripheral areas. Scale bar: 500 μ m. (B) Higher magnification view of the inset in (A) demonstrates that resident myeloid cells showed a generally perivascular distribution. (C–H) Resident myeloid cells were visualized by immunohistochemical staining for myeloid markers in CX3CR1^{+/GFP} mice. Green fluorescent protein-expressing myeloid cells ([C], green) stained positively for MHCII ([D], red) and Iba1 ([E], blue). A merged image of all three markers (F) revealed that choroidal myeloid cells were composed of cells with dendritiform morphologies (upper inset), with a minority of rounded cells with minimal or no processes (lower inset). Most GFP⁺ cells were also immunopositive for CD163 (ED2; [G]) and CD169 (ED3; [H]). (I) Green fluorescent protein⁺ cells with dendritiform morphologies were significantly more prevalent than those with rounded morphologies. (J–N) Myeloid cells with dendritiform morphologies were typically concurrently positive for CX3CR1-GFP (J), MHCII (K), and Iba1 (L) markers (upper panels), as quantified in cell counts (M). Rounded myeloid cells (J–L, lower panels), however, were significantly more varied in their immunopositivity for the three myeloid markers, indicating a more diverse composition (N). Cells were counted from 27 high-magnification fields in nine choroidal flat mounts (*n* = 5 animals). Scale bar: 50 μ m.

their long axes were aligned parallel to artery walls (Figs. 2B, 2C). Orthographic projections (Fig. 2D) and 3D reconstructions (Fig. 2E; Supplementary Movie S1) of confocal images demonstrated that the axial processes of

FIGURE 2. Morphology and distribution of perivascular dendritiform myeloid cells associated with primary choroidal arteries are shown. Choroidal arteries whose lumina were labeled by intravascular Dil perfusion ([A], arterial lumens, *white arrowheads*) were flanked laterally by dendritiform myeloid cells ([B], *asterisk*). These cells were elongated and spindle-shaped and were aligned with the long axis of the artery. Their cellular processes consisted of axial processes (*yellow arrow*) that were parallel and closely associated with the artery wall and projecting processes (*white arrow*) that were directed toward the extravascular space. (C) Merged image demonstrates the close juxtaposition between perivascular myeloid cells and the artery. (D) Orthographic projections demonstrate the lateral perivascular positioning of the myeloid cell (*asterisk* and *insets*) with respect to the vascular wall. (E) Three-dimensional rendering of the same artery shows the lateral flanking position of the perivascular, spindle-shaped myeloid cell (*asterisk*) with the axial (*yellow arrow*) and projecting (*white arrow*) processes. Scale bar: 25 μ m.

myeloid cells were closely juxtaposed with the external vessel wall. In addition, these cells also possessed outward-directed processes that projected into perivascular space (Figs. 2B, 2D, *white arrows*).

Choroidal arterioles, which arise from the primary and secondary branches of the main choroidal arteries, were closely associated with dendritiform myeloid cells (Figs. 3A–C). These cells demonstrated morphologies that were less elongated than those associated with choroidal arteries (Fig. 3B) but had similar perivascular positioning with processes that were oriented circumferentially around the external vessel walls (Figs. 3C, 3D), as well as being directed outward toward the perivascular space.

Anatomical Associations of Resident Myeloid Cells with Terminal Choroidal Arterioles and the Choriocapillaris

Terminal arterioles, which represent the most distal vascular branches prior to the choriocapillaris (Fig. 4A), were densely surrounded with perivascular dendritiform GFP⁺ cells. These cells were only slightly elongated and were primarily stellate in their shape (Fig. 4B). They were located within intervascular spaces at the junctions of vascular branch points (Fig. 4D); 3D rendering of their shape revealed they were “straddling” the spaces between terminal vessel branches, with the ends of their processes in close contact with choroidal vessel walls (Fig. 4E).

At the level of the choriocapillaris, associated GFP⁺ myeloid cells possessed a primarily stellate shape with fine, elongated, radially projecting processes (Figs. 4F, 4G). These cells demonstrated a flattened shape and were laid out in a horizontal network-like arrangement and closely adherent to the scleral surface of the choriocapillaris (Fig. 4H). Their long processes were observed to extend along choriocapillaris vessel surfaces but occasionally projected across and into the gaps between vessels to contact Bruch’s membrane. Interestingly, the choriocapillaris-associated GFP⁺ cells were located only on the scleral

surface of the choriocapillaris. The opposite or vitreal choriocapillaris surface, which is in contact with Bruch’s membrane, was devoid of coverage by resident myeloid cells (Figs. 4I, 4J; Supplementary Movie S2).

FIGURE 3. Morphology and distribution of perivascular dendritiform myeloid cells associated with primary choroidal arterioles are shown. (A) Primary arteriole is outlined by intravascular Dil perfusion (lumen of arteriole, *white arrowheads*). (B) Resident myeloid cell (*asterisk*) shows a ramified morphology with multiple branched processes directed circumferentially around the arteriole and outward toward the extravascular space. These cells have a less elongated and spindle-shape morphology relative to those associated with choroidal arteries. (C) Merged image shows the positioning of resident myeloid cells around arterioles. (D) Orthographic projections show the close juxtavascular position of resident myeloid cells. Scale bar: 25 μ m.

FIGURE 4. Morphology and distribution of perivascular dendritiform myeloid cells associated with terminal choroidal arterioles and the choriocapillaris are shown. (A) Tertiary end-arterioles are outlined by intravascular DiI perfusion and labeling. (B) Resident myeloid cells at this vascular level demonstrated ramified stellate morphologies that were less elongated. (C) Merged image shows these cells were located within intervascular spaces (*asterisk*), with processes that terminate in close proximity to vascular branch points. (D) Orthographic projections show the location of choroidal myeloid cells within intervascular spaces at the junctions of vascular branch points. (E) Three-dimensional rendering of confocal image stacks demonstrate that resident myeloid cells (D, *inset*) were positioned in the “grooves” between terminal arterioles, with their processes spanning the available intervascular spaces. Scale bar: 25 μ m. (F) Choriocapillaris, outlined by intravascular DiI perfusion and labeling, was associated with myeloid cells that possessed flattened stellate morphologies and fine symmetrical tapering processes (G). (H) Merged image shows the positioning of myeloid cells on the surface of the choriocapillaris, forming a loose network. Cellular processes were observed to occasionally extend into intervascular spaces (*white box*). (I) Orthographic projections (*insets*) show the associated myeloid cells were distributed as a flat, two-dimensional layer on the sclera surface (*white arrowhead*) of the choriocapillaris. The vitreal surface of the choriocapillaris (*yellow arrowhead*) lacked myeloid cell coverage. (J) Three-dimensional rendering of the polarized distribution of myeloid cells on the scleral choriocapillaris surface (scleral surface, *white arrowhead*; vitreal surface, *yellow arrowhead*). Scale bar: 25 μ m.

GFP⁺ Resident Myeloid Cells in the Choroid Demonstrate Rapid Dynamic Process Motility

Although resident myeloid cell populations have been described as exhibiting dynamic process motility and “patrolling” behavior in peripheral organs,^{26,27} vessel lumina,²⁸ brain,²⁹ and the retina,³⁰ the behavior of resident myeloid cells in the choroid has not been previously described. Using an ex vivo time-lapse imaging method in living choroidoscleral explants,²¹ we monitored and characterized dynamic behavior of resident

choroidal myeloid cells. In imaging experiments, which extended up to 100 minutes (Supplementary Movie S3), we observed these cells to have cell bodies that were stationary, with little evidence of migration or patrolling behavior observed over this time frame (Fig. 5A). Under high-magnification time-lapse imaging, however, we observed resident myeloid cells demonstrate marked motility of their ramified processes, with rapid and repeated extension and retraction movements. Despite this dynamism, myeloid cells maintained their general cellular morphology and their close associations with the

FIGURE 5. Dendritiform resident myeloid cells are nonmigratory but demonstrate rapid dynamic process motility. **(A)** GFP⁺ resident myeloid cells in *ex vivo* choroidoscleral explants from a CX3CR1^{+/GFP} mouse were monitored using time-lapse confocal live imaging. Images of choriocapillaris myeloid cells taken at time 0 (green, top) and 100 minutes (red, middle) were highly overlapping when superimposed (bottom), demonstrating an absence of significant cellular migration or soma translocation within this time interval. **(B)** Resident myeloid cells demonstrated dynamic motility in their dendritiform processes. Comparison of a confocal image of a choroidal artery-associated myeloid cell (L, arterial lumen), captured at a single time point (top), to that of a summed image of 100 individual images (captured every 10 seconds over 1000 seconds) (bottom) demonstrates the coverage of perivascular space by motile processes. Note that the elongated morphology and parallel alignment to the vessel wall are maintained despite marked process movement. **(C)** Comparison of a confocal image of a choriocapillaris-associated myeloid cell captured at a single time point (top) to that of a summed image of 30 images (captured at 42-second intervals over 1000 seconds) (bottom) illustrates the dynamism of the processes and their ability to occupy the extravascular space on the surface of the choriocapillaris. **(D)** Time-lapse images (sequential images taken 2 minutes apart) from a recording of a spindle-shaped myeloid cell associated with a primary arteriole demonstrates varying motility in its processes; processes that were directed outward into the extravascular space (process 1) demonstrated more prominent dynamism than juxtavascular axial processes closely associated with the vessel wall (processes 2 and 3) that were relatively more stable. **(E)** Time-lapse images (sequential images taken 2 minutes apart) from a recording of a stellate-shaped myeloid cell associated with the choriocapillaris (C) demonstrate dynamism in all processes (1–4).

vascular walls (Supplementary Movie S4). Superimposing time-lapse images captured over a period of 17 minutes demonstrated that these process movements provided efficient coverage of the perivascular space along the wall of the artery (Fig. 5B).

Stellate-shaped GFP⁺ cells on the scleral surface of the choriocapillaris also demonstrated rapid and repeated exten-

sions and retractions of their radially directed processes (Supplementary Movies S5, S6) that effectively covered the scleral surface of the choriocapillaris (Fig. 5C). Process movements occurred in all directions in the horizontal plane without any obvious polarization (Fig. 5E), in contrast to the spindle-shaped cells around choroidal arteries that were closely

aligned with the vessel wall (Fig. 5D). In the minority population of myeloid cells with rounded morphology, time-lapse imaging revealed them to be relatively stationary and lacking any extension of transient cellular processes (Supplementary Movie S7).

Aging in the Choroid Involve Concurrent Vascular and Myeloid Cell Alterations

Because the choroidal vasculature has been described as undergoing age-related alterations in structure,^{31,32} we aimed to correlate changes in GFP⁺ resident myeloid cells with changes in choroidal vascular structure. We performed anatomical studies in young (3–4 months old) and aged (19–21 months old) wild-type BALB/c mice, labeling resident myeloid cells with MHCII immunohistochemistry and choroidal vessels with DiI perfusion. We observed that the general distributions of resident myeloid cells in both young and aged choroid tissues under low magnification were quite similar; myeloid cells in both age groups were distributed across both central and peripheral areas in a perivascular manner (Figs. 6A, 6B). However, choroidal arteries and arterioles in the aged choroid demonstrated greater tortuosity (Figs. 6C, 6D, left panels) and vascular attenuation (Figs. 6C, 6D, right panels) than those in young choroid. The density of dendritiform myeloid cells was significantly higher in the aged than in the young choroid in both the central (Fig. 6E, top left) and peripheral (Fig. 6E, top right) portions of the choroid, whereas the densities of the minority population of rounded myeloid cells were slightly but not significantly higher in the aged choroid (Fig. 6E, bottom). In the choriocapillaris, associated dendritiform myeloid cells were increased in density in the aged compared to those in the young choroid (Figs. 6F–H); in the corresponding areas, the vascular density of the choriocapillaris (assessed as the fraction of area occupied by choriocapillaris vessels in the same horizontal plane) was also significantly lower in the aged than in the young choroid (Fig. 6H, right).

DISCUSSION

Composition of Resident Myeloid Cells in the Adult Mouse Choroid

We used transgenic CX3CR1^{+/GFP} mice, which demonstrate GFP labeling in multiple CX3CR1-expressing myeloid-derived cells, to study resident cells within the mouse choroid. These cells likely have prolonged residence times in the normal choroid that are at least on the time scale of weeks, as suggested by previous myeloablation and transplantation studies.³³ The cytoplasmic GFP in CX3CR1-expressing myeloid cells provided a detailed delineation of morphological features in living cells, which, when combined with the labeling of choroidal vasculature with the fluorescent vascular dye DiI via cardiac perfusion, enabled myeloid cell associations with choroidal vessels to be clearly and vividly visualized. As previously noted in rodents and humans, resident myeloid cells in the choroid can be grouped into two general morphological categories: a predominant category consisting of dendritiform cells with ramified processes, and another smaller category of rounded cells with minimal or no processes.^{5–7} Although in the rat choroid, dendritiform myeloid cells have been subcategorized into macrophages and dendritic cells according to marker expression,^{17,18} in the mouse choroid, CX3CR1-expressing cells coexpress MHCII as well as CD169, CD163, and CD68 (markers associated with macrophages).²³ Although it is accepted that mature DCs

constitutively express high levels of MHC class II and costimulatory molecules as part of their functional repertoire as professional antigen presenting cells, other cells, including activated macrophages can also express this molecule. As such, definitive subclassification of these resident myeloid cells into dendritic cells and macrophages in tissue flat-mounts using immunohistochemical markers alone is difficult.²⁴ Indeed, although many studies rely on CD11c expression for confirmatory evidence of DCs, this molecule is also expressed on macrophages.³⁴ In this study, we chose to be cautious and used the term “resident myeloid cell” to refer more generally to the dendritiform GFP⁺ cells we observed in the mouse. Although they likely have heterogeneities as a group, distinct subcategories based on their vascular associations or dynamic behaviors were not evident. The minority population of rounded myeloid cells, however, demonstrated more heterogeneity in the expression of myeloid markers, suggesting diversity in their cellular composition which may include circulating monocytes and myeloid progenitor cells.⁶ These rounded cells are unlikely to include significant numbers of lymphocytes or natural killer cells, by virtue of their expression of MHCII and Iba1.

Functional Implications for Vascular Associations of Dendritiform Myeloid Cells

Dendritiform myeloid cells in the choroid, despite their prominent and constitutive presence, are not well understood with regard to their functions in the healthy choroid or to the interactions they have with other cells in the choroid. The novel data presented here describe how dendritiform myeloid cells are closely juxtaposed with vascular elements and how their morphologies and orientations demonstrate a progressive patterning at different levels of the choroidal vasculature tree, suggesting differential functions at each level.

The close physical associations observed between myeloid cells and vascular cells indicate that myeloid cells may play a role in the maintenance of choroidal vascular structure. At the level of choroidal arteries and arterioles, dendritiform myeloid cells were elongated and closely aligned with the long axes of vessels, with their ramified processes fasciculating along and around vessel walls. This close relationship presents indirect evidence that constitutive signaling by these cells may constitute either a constant trophic effect on vascular cells or a cellular signal that maintains their physiological phenotype. More distally in the terminal arterioles, myeloid cells were less tightly associated with vessel walls and were positioned with their processes spanning intervascular connective tissue spaces and are thus well positioned to play a role in vascular patterning. In the brain and retina, microglia, the resident specialized macrophages or myeloid cells, have been shown to regulate the branching patterns of developing blood vessels³⁵; interventions that perturb microglial numbers^{36–38} or microglial-derived signals³⁹ can produce alterations in retinal vascular architecture. Dendritiform myeloid cells may potentially play an analogous role in vascular development in the choroid.

Another function of resident myeloid cells may be to regulate the overall permeability of choroidal vessels to blood-borne solutes and cells. In the vascular bed of the stria vascularis in the cochlea, a resident population of perivascular macrophages⁴ regulates vascular permeability as a function of macrophage activation state⁴⁰ via macrophage-derived secreted molecules.⁴¹ Although the choriocapillaris of the eye is a fenestrated capillary bed lacking tight junctions, the number and structure of the fenestrations are sensitive to angiogenic-factor signaling,^{42,43} indicating that immune cells, which are known sources of angiogenic factors, can have the ability to

FIGURE 6. Aging is associated with increased resident myeloid cell number and structural alterations in choroidal vasculature. (A, B) Comparison of the overall distribution of resident myeloid cells (MHCII-labeled, green) in young (3- to 4-month-old; [A]) and aged (19- to 21-month-old; [B]) mice demonstrated a similar general distribution throughout the central and peripheral areas of the choroid. (C, D) Primary choroidal arteries and primary arterioles (DiI perfusion, red) demonstrated changes with aging that were evident as increased vascular tortuosity ([C, D], yellow arrow) and increased vascular attenuation ([D], red arrow). (E) The overall density of dendritiform resident myeloid cells (upper panels) were concurrently increased in the aged choroid relative to the young choroid in both central ($P = 0.0005$) and peripheral ($P = 0.0051$) areas (Mann-Whitney U test, $n = 18$ imaging fields in five animals in each comparison). The density of myeloid cells with rounded morphologies were slightly but not significantly increased in the aged versus the young choroid (lower panels). Myeloid cells located across the full thickness of the choroid were counted. (F, G) At the level of the choriocapillaris, dendritiform myeloid cells (left panels) in aged choroid retained a stellate morphology as in the young choroid but demonstrated increased process numbers and ramification. DiI perfusion revealed that the aged choriocapillaris was significantly more attenuated with enlarged intervascular spaces relative to the young choriocapillaris (middle panels). (H) The density of choriocapillaris-associated myeloid cells were increased significantly with aging ($P = 0.034$), whereas the choriocapillaris vascular density (defined as the percentage of total area covered by choriocapillaris vessels) decreased significantly (Mann-Whitney U test, $P < 0.0001$; $n > 7$ imaging fields from five animals in each comparison).

alter transport of materials and cells to and from the choriocapillaris.

If choroidal myeloid cells perform constitutive cellular signaling in the healthy choroid, what may be the vascular cell types responding to these signals? In our previous work, we have described the structure and distribution of α -SMA-positive mural cells in mouse choroid, which are composed of smooth muscle cells and pericytes.²¹ Pericytes have been found to play

important roles in regulating neutrophil and monocyte egress^{44,45} and in stabilizing capillary bed structure.⁴⁶ Given the distributions and positions of myeloid cells and α -SMA⁺ pericytes in the choroid, it is clear that these two cell types are closely apposed in the choroid and are likely to exchange intercellular signals. For example, both myeloid cells and pericytes show a polarized distribution on the scleral surface of the choriocapillaris and are thus well positioned to overlap and

interact with each other via cell contact-mediated or secreted cues. The nature of these cellular interactions is the current focus of our ongoing work.

Functional Implications for Dynamic Process Motility of Dendritiform Myeloid Cells

We show here for the first time that dendritiform myeloid cells in the choroid, in addition to their intimate vascular associations, demonstrate rapid and repeated dynamism in their processes in situ. There is compelling evidence these cells provide extensive physical coverage of the perivascular space over a short time period. This dynamic behavior is also observed in the processes of CX3CR1-expressing dendritic cells in the lamina propria of the intestine²⁶ and tubules in the kidney,²⁷ which enable the sampling of antigens and the clearance of pathogens in the vicinity. Similarly, resident myeloid cells in the choroid, by virtue of their constitutive MHCII expression, dynamic behavior, and localization at the interface between the circulation and the outer retina, are likely to serve important immunosurveillance functions, including pathogen detection, antigen presentation, autoimmunity, and the regulation of the outer retinal immune environment.⁸ In the choriocapillaris, resident myeloid cells extend fine tapering processes that repeatedly probe vascular surfaces and gaps between vessels and can thus gain access to the basal surface of Bruch's membrane. As suggested in previous ultrastructural studies,¹⁷ this contact may enable choroidal myeloid cells to sample retinal antigens that are exocytosed from RPE cells in order to appropriately regulate autoimmune responses.

Possible Involvement of Resident Myeloid Cells in Senescence-Related Ocular Pathologies

Although resident myeloid cells may contribute positively to the healthy functioning of the young choroid, they may also play causal roles in age-related changes in the choroid. The human choroidal vasculature has been noted to undergo aging changes as shown by age-related decreases in overall choroidal thickness, choriocapillaris density, vessel diameter, and choroidal blood flow.^{31,32,47} These aging choroidal changes have been hypothesized to contribute to the progression in AMD in terms of drusen formation^{48,49} and the evolution of advanced forms of disease.¹³

The factors driving senescent change in the choroid are not well understood but have been related to concurrent aging changes involving the immunologic activity in the RPE/choroid complex. These changes have included increases in chemokine and complement gene expression,⁵⁰ elevations in choroidal macrophage density,^{51,52} and altered macrophage polarization.⁵³ We demonstrate here that aging mouse choroid demonstrates vascular alterations similar to those seen in the human in the forms of greater attenuation and tortuosity of choroidal arteries and decreased choriocapillaris density. Also, these vascular changes occur locally in areas with significant increases in the density of dendritiform resident myeloid cells. How changes in the resident myeloid cell population can induce vascular alterations in the choroid is an interesting area of inquiry that may be relevant to AMD pathogenesis.

In conclusion, we have shown in this study that dendritiform myeloid cells resident in the choroid demonstrate variations in the morphologies and associations with choroidal vessels that vary according to their position in the depth of the vascular tree. These dendritiform cells, likely consisting of a heterogeneous population of DCs and resident tissue macrophages, exhibit a marked dynamism in their processes that can provide extensive perivascular coverage which likely subserve immunosurveil-

lance functions. These descriptions of the morphology, distribution, and behavior of these resident cells provide a foundation for understanding immune function in the young healthy choroid and a basis for interpreting age-related and pathological changes in eye disease in future studies.

Acknowledgments

Supported by the Intramural Research Program of the National Eye Institute, National Institutes of Health.

Disclosure: **A. Kumar**, None; **L. Zhao**, None; **R.N. Fariss**, None; **P.G. McMenamin**, None; **W.T. Wong**, None

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