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PATHOLOGY

CD163+ macrophages infiltrate axon bundles of postmortem optic nerves with glaucoma

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Abstract

Purpose Prior research in animal models has shown that macrophages and microglia play an important role in pathogenesis of glaucoma, but the phenotype and distribution of macrophages in human glaucomatous tissue have not been sufficiently characterized.

Methods We analyzed H&E, CD68-, and CD163-immunostained slides from 25 formaldehyde-fixed, paraffin-embedded autopsy eyes: 12 control eyes and 13 eyes with glaucoma. The diagnosis of glaucoma was made based on a history of glaucoma as reported in the medical record and histological changes characteristic of glaucoma. Glaucoma cases and controls were matched in terms of age, sex, and race.

Results Qualitative analysis of the conventional outflow pathway and the optic nerve revealed that all eyes contained CD163+ cells but a negligible number of CD68+ cells. CD163+ macrophages infiltrated the trabecular meshwork and surrounded Schlemm's canal of normal eyes and eyes with glaucoma, but the pattern was variable and qualitatively similar between groups. In optic nerves of control eyes, CD163+ macrophages were present at low levels and restricted to septa between axon bundles. In glaucomatous optic nerves, the number of CD163+ cells was increased both qualitatively and quantitatively (glaucoma 5.1 ± 0.6 CD163+ cells/mm^2, control 2.5 ± 0.3 CD163+ cells/mm^2, $p < 0.001$), with CD163+ cells infiltrating axon bundles in cases of both mild and severe diseases.

Conclusions The increase in CD163+ cell number in eyes with mild and severe glaucoma is the first demonstration of macrophage infiltration in glaucomatous human optic nerves. This finding supports a role for macrophages in glaucoma pathogenesis and progression.

Keywords Glaucoma · Optic nerve · Macrophages · Microglia · Neurodegeneration · Neuroprotection

Introduction

Glaucoma is a chronic blinding disease characterized by progressive loss of retinal ganglion cells (RGCs) and cupping of the optic nerve (ON). Elevated intraocular pressure remains the only modifiable risk factor for glaucoma; currently, there are no clinically used treatments conferring RGC neuroprotection or reversing existing RGC/visual field loss. The immune system was initially proposed to play a role in the pathogenesis of normal-tension glaucoma [1]. More recently, neuroinflammation has been proposed to be critically involved in pathogenesis of all types of glaucoma [2–4], with cells of the innate immune system—recruited monocytes/macrophages and microglia, resident myeloid cells in neural tissue—playing an important role in disease development and progression [5].

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Previous studies have shown that macrophages are recruited to the trabecular meshwork of human eyes after selective laser trabeculoplasty and that intracameral infusion of macrophages increases aqueous outflow in a rabbit model [6]. In the ONs of human glaucomatous eyes, microglia become activated and redistributed to the parapapillary chorioretinal region [7] and colocalize with a variety of cytokines, including TGF-β2 and TNF-α [8]. In the DBA/2J mouse model of glaucoma, ON accumulation of Iba1+ [9, 10] and CX3CR1$^{+/GFP}$ [11] myeloid cells was present at an early disease stage, prior to detectable ON damage. Animals with reduced levels of microglial activation, either genetically through loss of MHCII RT1B protein [12] or pharmacologically (via administration of anti-inflammatory antibiotic minocycline [13, 14], TNF-α inhibitor etanercept [15, 16], cAMP phosphodiesterase type E4 inhibitor ibudilast [17], and adenosine receptor $A_{2A}R$ antagonist ZM241385 [18]), were protected from glaucomatous RGC neurodegeneration. Neuroprotection was also observed in DBA/2J animals in which monocyte/macrophage entry into the ON head was blocked with radiation treatment [10, 19]. Interestingly, serum level of MCP-1/CCL-2, a potent monocyte/macrophage chemoattractant, has been found to be elevated in otherwise healthy normal-tension glaucoma patients [20] and is associated with visual field progression [21], suggesting that peripheral macrophage recruitment may also be important in the pathogenesis of human glaucoma.

Macrophages and microglia are thought to exist in a variety of activation states, both pro-inflammatory and anti-inflammatory, that are characterized by different surface markers, cytokine profiles, and biologic functions [22, 23]. Two macrophage-specific markers frequently used in paraffin-embedded human tissue samples are CD68 (a lysosomal marker) and CD163 (a scavenger receptor that binds hemoglobin-haptoglobin complexes) [24–26]. CD163 is commonly used as a marker for alternatively activated, anti-inflammatory macrophages that are involved in tissue repair and remodeling, cytoprotection, resolution of inflammation, and fibrosis [22, 24]. We have previously shown an abundance of infiltrating CD163+ cells in the retina of postmortem eyes with advanced dry and neovascular age-related macular degeneration [27]. Herein, we characterize the numbers and distribution of CD163+ and CD68+ cells in human postmortem eyes with and without glaucoma and demonstrate that CD163+ macrophages infiltrate structures critical for pathogenesis of glaucoma.

Materials and methods

Autopsy eyes from 25 patients (12 controls and 13 glaucoma patients) were analyzed (Supplementary Table 1). The use of autopsy eyes for research was approved by the Institutional Review Board of Duke University (Pro00083250) and followed the tenets of the Declaration of Helsinki. To be classified as having glaucoma, patients had to have the diagnosis of glaucoma (as listed in the autopsy report and abstracted from their medical record) and presence of histological changes characteristic of glaucoma. For 5 out of 13 glaucoma cases, we were able to obtain additional details about their glaucoma diagnosis from the ophthalmology clinic notes at our institution. Glaucoma cases and controls were matched in terms of age, sex, and race (as defined in the autopsy report). Eyes with evidence of secondary glaucoma, prior glaucoma surgery, or other significant ocular comorbidities (e.g., moderate or severe diabetic retinopathy, uveitis, advanced age-related macular degeneration) were excluded from the study. In addition, eyes with postmortem intervals to tissue fixation greater than 72 h were excluded.

Autopsy eyes were enucleated and fixed in 3.7% neutral-buffered formaldehyde. Eyes were sectioned in the horizontal (axial) plane, and following removal of the superior calotte, postmortem fundus examination and color photography were performed. Cross sections of the optic nerve were prepared leaving a 4–5-mm stump of nerve attached to the globe. The eyes were then embedded in paraffin and sectioned at 5 μm intervals to the center of the optic nerve. Five micrometers of cross sections of the optic nerve was stained with hematoxylin and eosin (H&E), Luxol fast blue (LFB), and Masson trichrome stains. H&E and periodic acid-Schiff reagent (PAS) stains were done on the pupil-optic nerve sections of the globes and slides evaluated for presence of glaucomatous changes and other ocular conditions. The histological diagnosis of glaucoma was made based on presence of optic nerve atrophy and retinal ganglion cell loss; the degree of glaucomatous damage was graded qualitatively as mild, moderate, or severe (Supplementary Fig. 1; all retinal images shown were taken through the macula, and optic nerve sections in the proximal optic nerve posterior to the lamina cribrosa). Mild glaucomatous damage was characterized by a normal number of retinal ganglion cells and mildly thickened fibrous trabeculae in the optic nerve. Moderate damage was manifested a decreased number of retinal ganglion cells, increased fibrotic thickening of the optic nerve trabeculae, and a diminished size and often paler staining of the optic nerve axon bundles. Eyes with severe glaucomatous damage had an even further reduction or a paucity of retinal ganglion cells and more advanced fibrosis and axon loss than the eyes with moderate damage.

For immunohistochemical studies of CD68 and CD163, horizontal sections through anterior chamber structures, retina, and proximal optic nerve were obtained. In addition, cross sections of the optic nerve (obtained 4–5 mm posteriorly from the outer edge of the scleral wall) were also stained for CD163. The IHC detection of CD68 and CD163 was performed using Leica Bond-MAX or Leica Bond-III automated

stainers (Leica Microsystems Inc., Buffalo Grove, IL, USA), a 20-min antigen retrieval with Novocastra Bond Epitope Retrieval 2 solution (Leica Microsystems) at 100 °C and pH 6.0, ready-to-use CD68 (clone 514H12) antibodies or 1:200 dilution CD163 (clone 10D6) antibodies (Leica Microsystems), and the Novocastra Bond Polymer Refine Red Detection system (Leica Microsystems). Negative controls employed a commercially available cocktail of mouse IgG1, IgG2b, IgG3, and IgM (Leica Microsystems). Human tonsil sections were used as positive external controls.

Photomicrographs were acquired using a Vanox AHSB3 microscope (Olympus America, Center Valley, PA) and a D800 Nikon camera connected by a 2.5× MM-SLR adapter (Martin Microscope Co., Easley, SC). DigiCamControl software (www.digicamcontrol.com) was used to acquire images. Quantification was performed by manually counting CD163+ cells using ImageJ software (NIH, Bethesda, MD) in images of horizontal sections of the proximal optic nerve immediately posterior to the lamina cribrosa (× 20 objective). The quantification was performed in a masked fashion; one section was analyzed per eye, and one eye from each subject was included. We counted CD163+ entities that morphologically resembled a cell, regardless of whether the cell nucleus was included in the section. If overlapping cells could easily be distinguished as two separate cells based on size and morphology, then they were counted as two; otherwise, they were counted as one. Small isolated cell processes were not counted. All data analyses were performed using Microsoft Excel (Microsoft Corp., Redmond, WA) and GraphPad Prism 7 software (GraphPad Software, La Jolla, CA). Results are presented as mean ± one standard error of the mean (SEM). Statistical significance was determined using Mann-Whitney *U* test and Fisher's exact test. $P < 0.05$ was deemed to be statistically significant.

Results

Autopsy eyes of 25 patients (13 glaucoma cases and 12 controls) were analyzed in this study (demographic, ocular, and pathological data are presented in Supplementary Table 1). Glaucoma patients were 62% female, 54% white, and 46% black, with an average age of 79.4 years (range 50–92). Controls were 75% female, 58% white, and 42% black, with an average age of 83.7 years (range 70–97). There were no statistically significant differences between the two groups in terms of age, sex, or race. Furthermore, there was no statistically significant difference between glaucoma cases and controls in terms of average postmortem time (glaucoma 25 ± 4.2 h, control 22.6 ± 4.1 h).

Qualitative analysis of horizontal sections encompassing the anterior segment, retina, and the optic nerve revealed CD163+ cells infiltrating the trabecular meshwork and surrounding Schlemm's canal of control eyes (Fig. 1a), as well as infiltrating the optic nerve (Fig. 1c). CD163+ cells could also be seen in the iris, ciliary body, and choroid. The number of CD68+ cells was negligible in the conventional outflow pathway and the optic nerve of control eyes (Fig. 1b, d), as well as in glaucoma eyes (data not shown).

Comparison of CD163+ cell numbers in the conventional outflow pathway of control eyes and glaucoma eyes revealed marked variability. We identified control cases and glaucoma cases with scarce CD163+ staining in the trabecular meshwork and around Schlemm's canal (Fig. 2a, c), as well as glaucoma cases and controls with abundant CD163+ cell numbers in the same structures (Fig. 2b, d). The overall CD163+ staining pattern in the conventional outflow pathway was variable and qualitatively similar between groups. In the optic nerves of control eyes, CD163+ cells appeared enriched in the area of lamina cribrosa and in septa around axon bundles (Fig. 3a, b). In glaucomatous eyes, the number of CD163+ cells was significantly increased both qualitatively (Fig. 3c, d) and quantitatively (Fig. 3e; glaucoma 5.1 ± 0.6 CD163+ cells per mm^2, control 2.5 ± 0.3 CD163+ cells per mm^2, $p < 0.001$ using Mann-Whitney *U* test). There was no statistically significant relationship between the number of CD163+ cells and histological severity of glaucoma, as we could identify mild glaucoma cases with an abundance of CD163+ cells and severe glaucoma cases with lower numbers of CD163+ cells (Supplementary Table 1).

We also examined cross sections of the optic nerve stained with CD163 antibody. In normal controls, CD163+ cells were scarce and restricted to septa around axon bundles (Fig. 4a, b), except rarely in the far periphery of the optic nerve, where mild, age-related nerve fiber degeneration was accompanied by CD163+ cell presence within axon bundles (data not shown). Next, we examined a case of mild glaucoma in a patient who had optic nerve thinning on optical coherence tomography (Fig. 4f) and a nearly intact visual field (Fig. 4e). We could detect an overall increase in CD163+ cell numbers (Fig. 4c), with CD163+ cells infiltrating axon bundles (Fig. 4d, arrows). This localization pattern, with CD163+ cells infiltrating axon bundles, was characteristic of glaucoma cases and was not seen in control cases except rarely in the far periphery of the optic nerve as described above. We also examined a case of moderate glaucoma with an inferior nasal step on Humphrey visual field testing (Fig. 4i) and a corresponding area of superotemporal optic nerve atrophy (Fig. 4g). CD163+ cells were abundant throughout the nerve but were especially enriched in the area of superotemporal atrophy (Fig. 4h). Finally, in a case of severe glaucoma (Fig. 4j, k), there was a marked increase in the number of CD163+ cells throughout the disorganized axon bundles. Thus, CD163+ cells were seen infiltrating axon bundles of eyes with all histological stages of glaucoma.

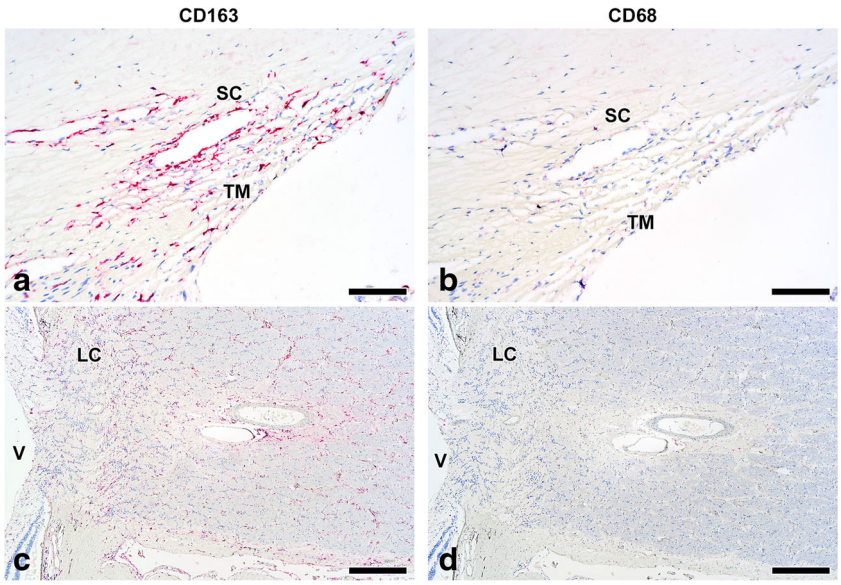

Fig. 1 Immunohistochemical localization of CD163+ and CD68+ cells in the conventional outflow pathway and the optic nerve of normal control eyes. **a** CD163+ cells infiltrate trabecular meshwork (TM) and surround Schlemm's canal (SC) in control eyes. **b** There is a negligible amount of staining with CD68 in the same eye as in **a**. **c** CD163+ cells are present at low levels in the optic nerves of control eyes and appear enriched in the area of the lamina cribrosa (LC). V vitreous cavity. **d** There is a negligible amount of staining with CD68 in the optic nerve of the same eye as in **c**. Scale bar for **a** and **b**, 100 μm; **c** and **d**, 500 μm

Discussion

In this study, we characterized the numbers and distribution of CD163+ and CD68+ macrophages in human postmortem eyes with and without glaucoma. We found that cells positive for CD163, a marker typically associated with anti-inflammatory, alternatively activated macrophages [22, 28], are abundantly present in structures critical for pathogenesis of glaucoma, while we detected only a negligible amount of staining with the lysosomal macrophage marker CD68. This is in agreement with prior publications that have found more limited CD68 staining compared to CD163 staining in aged human eyes, eyes with age-related macular degeneration, and in uveal melanoma [27, 29]. Since CD68 is a robust macrophage marker in other human tissues [26], this difference suggests different macrophage subpopulations in the human eye, with a CD163+ population predominating.

In the conventional outflow pathway, CD163 staining was prominent in the trabecular meshwork and around Schlemm's canal, but the number of cells was highly variable with no significant differences in localization patterns between control and glaucoma eyes. This is in agreement with a prior study by Coupland et al. that also found variability between glaucomatous and control trabecular meshwork in terms of staining for HLA-DR, CD45, and CD68 [30]. Of note, in our study, we examined single horizontal sections through

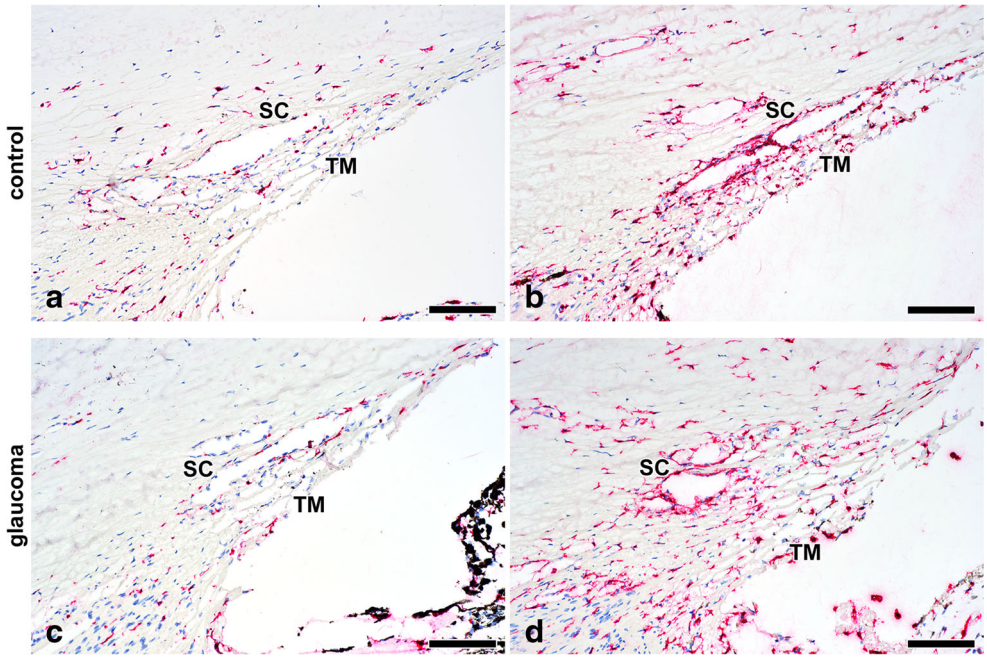

Fig. 2 The number of CD163+ cells in the conventional outflow pathway is highly variable in control eyes and eyes with glaucoma. CD163+ cells can be scarce (**a**) or abundant (**b**) in the trabecular meshwork (TM) or around Schlemm's canal (SC) in control eyes. Similarly, CD163+ cells can be rare (**c**) or abundantly present (**d**) in the conventional outflow pathway of glaucoma eyes. Scale bar = 100 μm

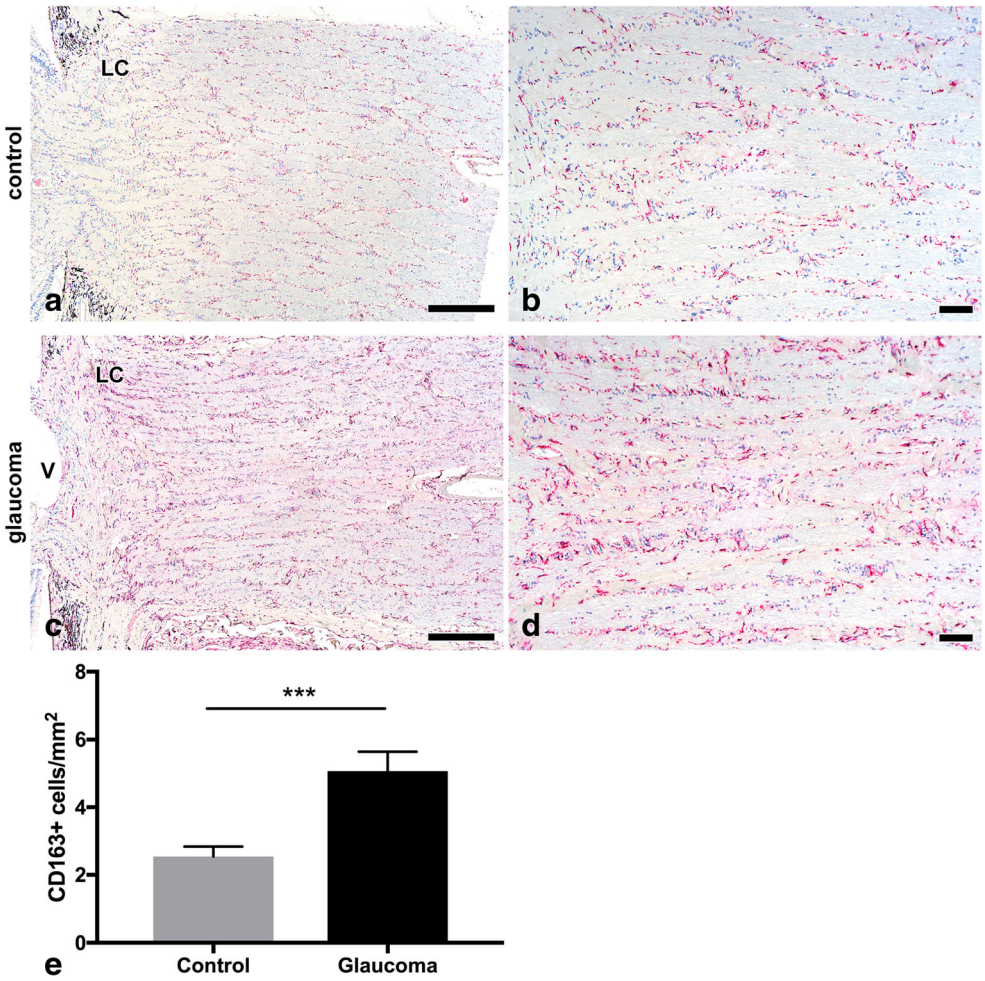

Fig. 3 Glaucomatous optic nerves contain more CD163+ cells compared to controls. **a**, **b** CD163+ cells are present at low levels in control optic nerves and appear enriched in the area of the lamina cribrosa and between optic nerve bundles. **c**, **d** CD163+ cells are increased in number in optic nerves of eyes with glaucoma. **e** There are significantly more CD163+ cells in glaucomatous optic nerves then in controls. Error bars represent one standard error of the mean; $***p < 0.001$ on Mann-Whitney *U* test. LC lamina cribrosa, V vitreous cavity. Scale bar for **a** and **c**, 500 μm; **b** and **d**, 100 μm

the anterior segment and were therefore sampling only a small region of the conventional outflow pathway in any given eye. It is possible that a comprehensive analysis of CD163+ staining in the entire trabecular meshwork may yield differences between control and glaucoma eyes, especially in the light of past work showing that macrophages can regulate aqueous outflow and are recruited to the trabecular meshwork after selective laser trabeculoplasty [6, 31].

The major finding of our study is a significant increase in CD163+ macrophages in optic nerves of eyes with both early and advanced glaucoma. In the optic nerves of control eyes, CD163+ cells were present at low levels and largely restricted to septa between axon bundles, while in glaucomatous eyes, the number of CD163+ cells was significantly increased, with cells infiltrating axon bundles. Optic nerve macrophage and microglia accumulation/activation has previously been demonstrated in animal models of glaucoma and suggested to be an important early step in glaucoma pathogenesis [9–11] that can be targeted therapeutically to prevent RGC degeneration [13, 14, 16–18]. To the best of our knowledge, ours is the first study to demonstrate macrophage infiltration in human glaucomatous optic nerves.

In our study, we could see an increase in CD163+ macrophage numbers in optic nerves with both mild and advanced glaucoma, implicating CD163+ macrophages both early and late in the glaucoma disease process. CD163+ macrophage number did not correlate with histologic severity of glaucoma (Supplementary Table 1 and data not shown), although the number of cases in each severity subgroup was small. It is possible that analysis of a larger number of eyes may detect a significant difference in the number of CD163+ cells in various stages of glaucoma. While the presence of CD163+ macrophages in glaucomatous optic nerves suggests they are active responders to various stages of RGC damage, whether they play a beneficial or a harmful role in glaucoma remains unknown. Examining colocalization of CD163 and a panel of pro-inflammatory and anti-inflammatory cytokines/chemokines [8] in glaucomatous human tissues may shed light on this important issue. Given that CD163 has classically been used as a marker for alternatively activated, anti-inflammatory macrophages [22, 28], our observations raise the intriguing possibility that this macrophage subtype may be playing a neuroprotective role in glaucoma.

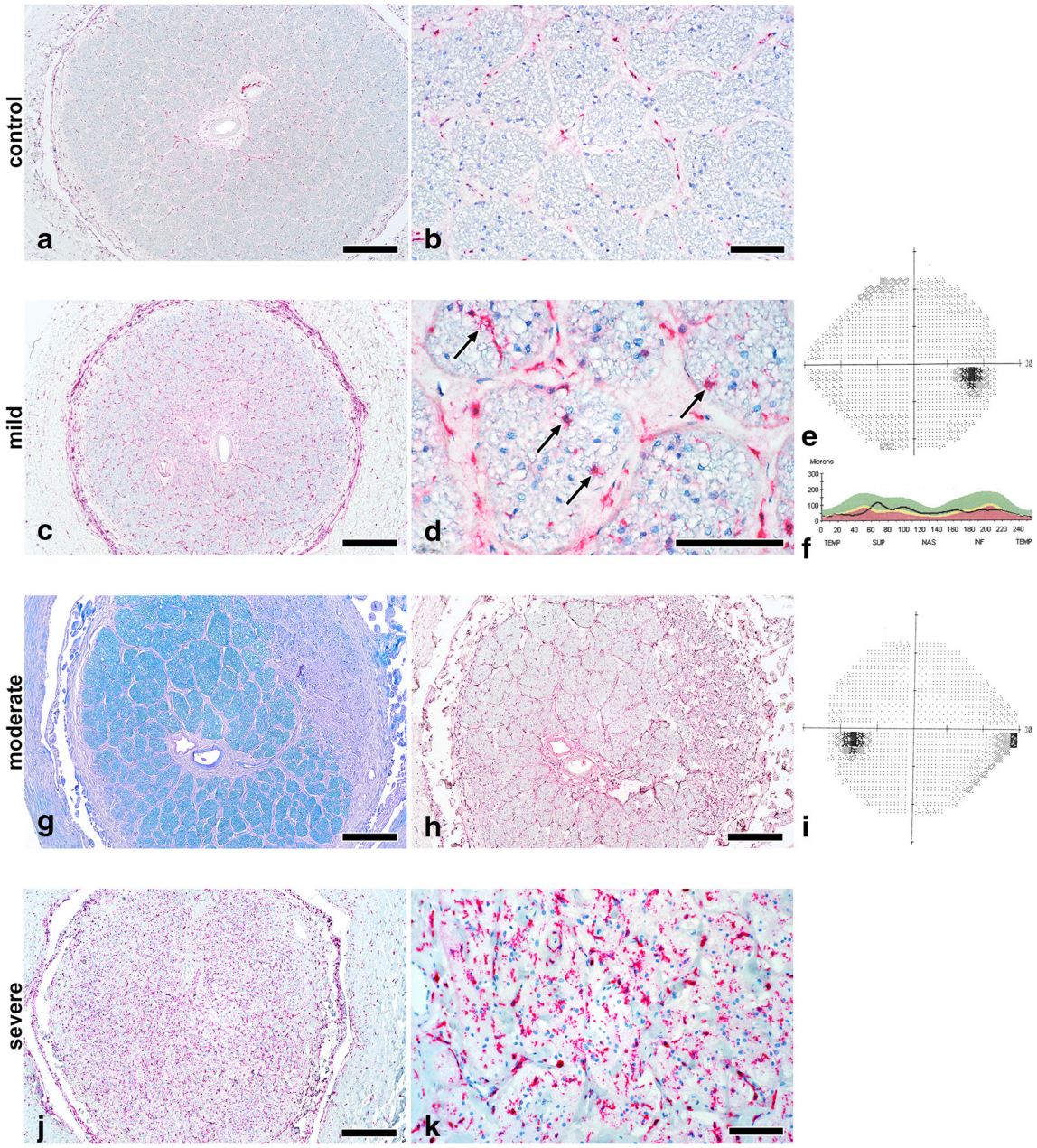

Fig. 4 CD163+ cells are restricted to septa between optic nerve axon bundles in controls and infiltrate axon bundles in both mild and severe glaucomas. **a**, **b** CD163+ cells are scarce and found between axon bundles in control eyes. **c**–**f** A case of mild glaucoma. There is an increased number of CD163+ cells (**c**, **d**) that infiltrate axon bundles (marked with arrows in **d**). The patient's glaucoma testing revealed areas of optic nerve thinning on optical coherence tomography (**f**) with a nearly intact visual field (**e**). **g**–**i** A case of moderate glaucoma. Please note the area of superotemporal optic nerve degeneration (easily visible on Luxol fast blue stain in **g**) and corresponding superotemporal infiltration of CD163+ cells (**h**), which match the patient's visual field deficit (inferior nasal step; **i**). **j**, **k** A case of severe glaucoma. There is a marked increase in the number of CD163+ cells throughout the disorganized axon bundles. Scale bars **a**,**c**, **g**, **h**, **j** 500 μm; **b**, **d**, **k** 100 μm

Our study has several limitations. The postmortem eyes we examined had incomplete clinical histories and largely unknown timelines of glaucomatous damage. Given that these are autopsy eyes and not donor eyes dedicated to research, we could examine a relatively small number of eye sections as dictated by standard-of-care autopsy protocols and therefore could not completely characterize CD163+ cell numbers in the conventional outflow pathway, inner retina, or the optic nerve. However, it is notable that even with limited sampling, we detected a difference in CD163+ cell numbers between control and glaucomatous optic nerves, which speaks to the strength of the association between CD163+ macrophages and glaucoma.

Although CD163 has classically been described as a macrophage marker [25, 26] that is not expressed by human CNS-derived microglia in culture [32], we cannot rule out that it is

not expressed by a subset of microglia in vivo. For example, Satoh and colleagues found that a small proportion of cells expressing Tmem119, a recently developed microglia-specific marker [33], also expresses CD163 [34]. Further immunohistochemical analysis utilizing microglia-specific antibodies [33, 35] and additional macrophage markers [22, 24] may yield insight into relative contributions of microglia and infiltrating macrophages to the glaucomatous disease process and allow us to subtype myeloid cells into pro-inflammatory and anti-inflammatory classes based on cellular markers and cytokine expression [22, 36].

Our observation that there is a quantitative increase in CD163+ cells in optic nerves of human eyes with early and advanced glaucoma supports a role for macrophages in glaucoma pathogenesis. Understanding and validating the role of neuroinflammation in glaucomatous human tissues may have important diagnostic and prognostic implications and lead to development of novel neuroprotective therapies for this blinding disease.

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Compliance with ethical standards

Conflict of interest The authors declare that they have no conflict of interest.

Ethical approval All procedures performed in studies involving human participants were in accordance with the ethical standards of the institutional research committee at Duke and with the 1964 Helsinki declaration and its later amendments or comparable ethical standards. For this type of study formal consent is not required.

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