

Microglia, Inflammation and Glaucoma

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ABSTRACT

Glaucoma is a multi factorial disease characterized by loss of retinal-ganglion cells. The increase of the intraocular pressure is a major risk factor, but their control cannot always stop the glaucomatous damage. Recently the immune system involvement in glaucoma has been observed. Microglial cells, the resident immune cells in the retina and optic nerve, appear to be key players in the immune response. This chapter highlights the current knowledge on the role of microglia in glaucomatous neuro degeneration.

Keywords: Microglia; Retina; Glaucoma; Immune response; Inflammation; Neuron; Retinal ganglion cell

Abbreviations: CNS: Central Nervous System; ECM: Extracellular Matrix; HSP: Heat Shock Protein; INF- γ : Interferon Gamma; iONS: Inducible Nitric Oxide Synthase; NFL: Nerve Fiber Layer; IOP: Intraocular Pressure; NO: Nitric Oxide; OHT: Ocular Hypertension; RGC: Retinal Ganglion Cells; RPE: Retinal Pigment Epithelium

INTRODUCTION

Glaucoma, the second leading cause of blindness in the world, is a multifactorial neurodegenerative disease characterized by an irreversible decrease of Retinal-Ganglion Cells (**RCG**) of the retina and their axons, the functional impact of which leads to a visual-field loss [1]. The control of the Intraocular Pressure (**IOP**) as the only risk factor that allows action to be taken does not always stop the progression of the glaucomatous neuro degeneration.

During the initial phases of the glaucoma, among the alterations which actively occur in the progression of the damage, the reactivation of the glial cells is notable [2]. The aim of acute gliosis is the protection of the nervous tissue by reestablishing the extracellular medium and by supplementing neurons with factors that promote their survival. None the less, an uncontrolled response, as occurs in the glaucoma, has a negative impact on the tissue [3]. The over activation of microglia results in excessive production of inflammatory mediators which contribute to retinal neurodegeneration [4,5]. Thus, microglial cells have a key role in the onset and perpetuation of the inflammatory response [6].

In this chapter, we will therefore focus on the evidence available concerning the role of microglia in glaucomatous neurodegeneration. This neurodegenerative mechanism is not completely understood and thus the knowledge of the mechanisms regulating this chronic neuro inflammation in the retina and how they can be therapeutically modulated are of key importance in glaucoma.

Microglia in the Healthy Eye

Microglia, first described by del Río-Hortega [7], are Central Nervous System (**CNS**) resident innate immune cells endowed with sensor and effector functions as well as with phagocytic capacity [8-11]. Microglial cells come from precursor cells originating in the bone marrow (mesodermic). These cells, which enter the developing nervous system from the blood stream, are derived from monocytes and differentiate into microglia, maintaining numerous cellular antigens present in macrophages or monocytes [12-14]. In adult healthy retinas, non-activated microglial are tiled within the Nerve Fiber Layer (**NFL**) and adopt a mosaic-like distribution within the Inner Plexiform Layer (**IPL**) and Outer Plexiform Layer (**OPL**) (Figure 1) [10,11,15]. The non-activate state is maintained by intercellular contacts and soluble factors secreted by neurons, astrocytes and the Retinal Pigment Epithelium (**RPE**) [4]. In the healthy retina the resident microglia have very limited proliferation [16]. It has been demonstrated that blood-derived cells play a minor role in maintaining the microglia population in the healthy retina [17]. However, damage of the blood-retina barrier allows myeloid cells entry from blood stream [18].

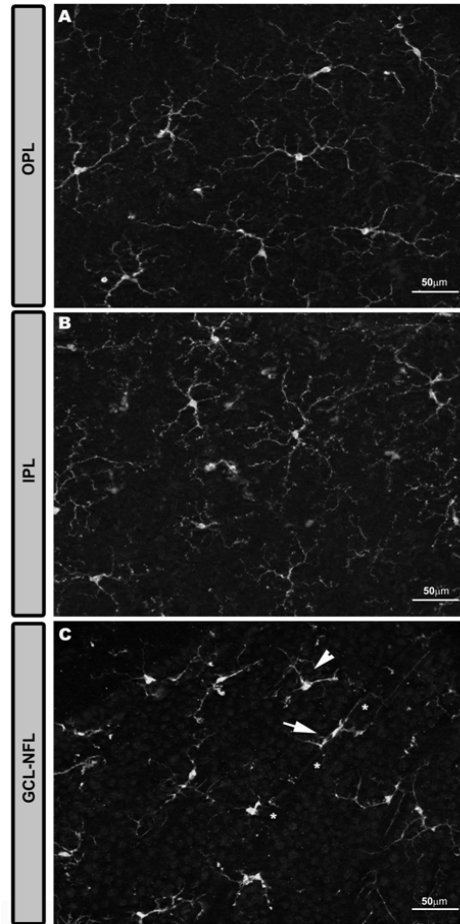


Figure 1: Retinal distribution of microglial cells in adult mouse retina. Iba-1 immunostaining. Retinal whole mount. Retinal microglia exhibit a ramified morphology and are distributed in different retinal layers: OPL (A), IPL (B), GCL-NFL (C). Microglial cells form a parallel mosaic of tiled cells that build networks throughout retina. In the plexiform layers, microglia cells form a mosaic-like pattern which is denser than in the GCL-NFL. In the latter, microglial cells are located on the retinal vessels (arrow in C) and in the intervacular space (arrowhead in C). [Asterisk: retinal vessel; **IPL**: Inner Plexiform Layer; **GCL-NFL**: Ganglion Cell Layer-Neve Fiber Layer; **OPL**: Outer Plexiform Layer].

When microglial cells are in a non-activated or resting state, they have various surface proteins including receptors for immune components such as complement, cytokines, chemokines, antibodies, and adhesion molecules that allow them to monitor the nervous-system environment [12]. This monitorization is realized by their long and motile processes which are rhythmically extended and retracted, allowing microglia to continuously scan the environment [19,20]. In the retina, the microglia population is capable of providing comprehensive surveillance of the entire

retina in a short time [21]. These surveying microglia are stationary (non-migratory) cells which have their cell body anchored in place, implying a strong interaction with the Extracellular Matrix (**ECM**). Therefore, in the uninjured adult, CNS microglia would be defined as motile and non-migratory cells, functioning to persistently survey the CNS in order to detect aberrations from homeostasis and to respond accordingly [22]. Thus, microglial cells combine a defensive role with homeostatic and neuro protective functions [13].

The immunological potential of microglia is comparable with blood monocytes and other tissue macrophages in terms of secretory functions. However, resting microglia express low levels of CD45 and costimulatory molecules, have a low Nitric Oxide (**NO**) production, and possess minimal migration and phagocytic activity [4,23].

Although traditionally microglia was recognized for their immune functions during disease, new roles are being attributed to these cells in the development and maintenance of CNS, including specific communication with neurons [22]. The microglia plays an essential role in the physiology and survival of neurons. Microglial cells secrete several neurotrophic factors, such as brain-derived neurotrophic factor, ciliary neurotrophic factor, glial-cell line-derived neurotrophic factor, nerve-growth factor, neurotrophin-3, and basic fibroblast-growth factor [4]. These factors have been shown to protect and regulate the survival of photoreceptors [24]. In addition, microglial cells make contact with neuronal synapses and respond to their functional status. Microglia also can modulate the neuronal activity in different ways. Microglial cells make transient physiological contact with neurons, involving homeostatic mechanisms to down regulate neuronal excitability. Moreover, these cells participate in establishing functional neuronal circuits through several molecular pathways, including fractalkine receptor, complement receptor and DAP12 [22].

In the healthy CNS the bidirectional microglia-neuron communication allows the activity of both cell types to be regulated. The neurons can regulate microglial activation states by specific chemokine, purinergic signaling and classic neurotransmitters [22]. Microglia possess receptors for the chemokine fractalkine (CX3CL1) (CX3CR1), CD200 (CD200R), and for purine nucleotides [4]. The secretion of neuronal factors including CD200 and fractalkine by healthy neurons may keep microglia in a quiescent or non-activated state (Figure 2) [25]. In the retina CD200 is expressed in several retinal cells including photoreceptors, ganglion cells, and the vascular endothelium [26,27]. CX3CR1 signaling in retinal microglia plays an important role in movements of microglial processes and cellular migration, thereby determining the level of microglial surveillance in the healthy retina [28]. The microglial purinergic receptor P2Y12 enables the detection of ATP and ADP release from damaged neurons and is required for early activation mechanisms [29]. In addition, neurotransmission plays an endogenous role in regulating the morphology and behaviour of “resting” microglia in the retina. Morphological parameters and process motility are increased by ionotropic glutamatergic neurotransmission and decreased by ionotropic GABAergic neuro transmission. These neurotransmitters influence retinal microglia mediated indirectly via extracellular ATP [30].

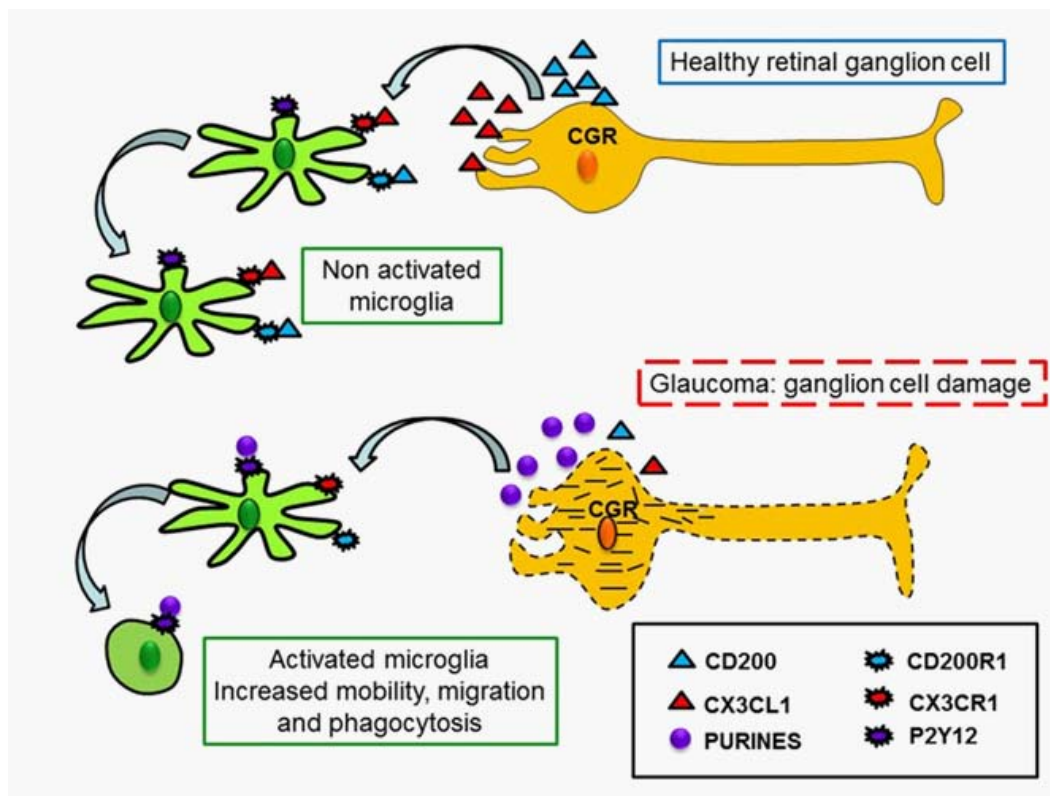


Figure 2: Scheme showing the microglia-neuron bidirectional communication in the healthy and glaucomatous retina. The neurons can regulate microglial activation states by receptors for the chemokine fractalkine (CX3CL1) (CX3CR1) and CD200 (CD200R), as well as for purine nucleotides. The secretion of CD200 and fractalkine by healthy neurons may keep microglia in a quiescent or non-activated state. The injured neurons increase the release of purines; this activates purinergic receptors in the microglia, which in turn strongly affects microglia motility and migration and triggers phagocytosis. In addition, deficiencies in the signaling pathways of the CX3CL1/CX3CR1 and the CD200/CD200R complexes results in greater microglial activity.

In the retina, microglia also have specific communication with other retinal cells such as RPE and Müller cells. RPE influences the non-activated state of microglia through the secretion of TGF β . This cytokine contributes to microglial production of IL-10, which in turn down-regulates the expression of MHCII, CD86 and CD80 [23,31]. Müller cells can limit microglial reactivity and potentially transform activated microglia present during development into their ramified surveillance state by constitutive secretion of the diazepam-binding inhibitor, a ligand for the translocator protein [32]. Microglia can directly trigger the release of neurotrophic factors from Müller cells such as leukemia inhibitory factor, neurotrophin3l, basic fibroblast growth factor, neurotrophic growth factor, ciliary neurotrophic factor and glial derived neurotrophic factor, which are important for retinal physiology [24,33,34].

Retinal Microglia in Glaucoma

Microglial cells respond to neuronal stress or injury by adopting a so-called “activated” state [35,36]. In the retina, this activation can be triggered in response to autoimmune mechanisms or neuronal injury and ischemia, among other circumstances, all associated with progressive neurodegeneration [4] as occurs in glaucoma.

In the course of activation, microglial cells change their form become proliferative and motile, migrate to and accumulate around the damage sites, modify the expression of various enzymes and receptors, and begin to secrete potentially cytotoxic substances (oxygen-free radicals, proteases, and proinflammatory cytokines). In addition, they can act as an antigen-presenting cells and at the extreme end of the activation program, transform into phagocytes [32,37-39].

Several functional and phenotypic states are included within activated microglia. These microglial states are modulated by their relationship to the nerve cells of their environment and by immune-cell infiltration after injury. Usually, the morphology of “activated” microglia includes enlargement of the soma, retraction and shortening of processes, and increased expression of myeloid-cell markers [14].

It is currently known that reactive microgliosis associated with retinal damage takes place both in human glaucoma [40] as well as in animal models of Ocular Hypertension (**OHT**) [10,11,15,41-44]. In glaucoma, microglial cells become reactive prior to evidence of ganglion-cell loss [9,45]. These activated microglia first localize to the optic-nerve head and lamina cribosa [46,47] and then redistribute in the retina, optic nerve, and optic tract as early alterations, which may contribute to the disease onset or progression [6]. In a model of experimental glaucoma, the reduction of microglia reactivity caused by treatment both with minocycline, a tetracycline derivative known to inhibit microglial activation [48] as well as with a high dose of irradiation, is associated with decreased RGC degeneration and with improvement in the structural and functional integrity of RGC axons [49].

Reactive microglia cells can adopt different morphologies: hyper-ramified cells, stellate cells with thick processes, and rod like microglia (Figure 3) [10,50]. In the state of high reactivity, microglial cells acquire an amoeboid morphology (rounded cells with sparse processes) (Figure 3) and act as macrophages, engulfing and destroying cell debris [50]. All the aforementioned morphological types of activation have been observed in experimental models of glaucoma [10,11,15]. In addition, in eyes from glaucomatous patients, microglial cells present a more amoeboid morphology, clustering in the lamina cribosa, which is the site of initial axonal injury [51] and surrounding blood vessels. This peculiar localization of microglia suggests a protective role against Blood-Retinal Barrier (**BRB**) damage [6]. The morphological change from ramified cells to amoeboid phagocytes is accompanied by the expression of several surface markers such as F4/80, complement receptor 3 (CD11b/CD18, OX42), MHC-II (OX6), CD68, and Griffonia simplicifolia isolectin B4 [50,52].

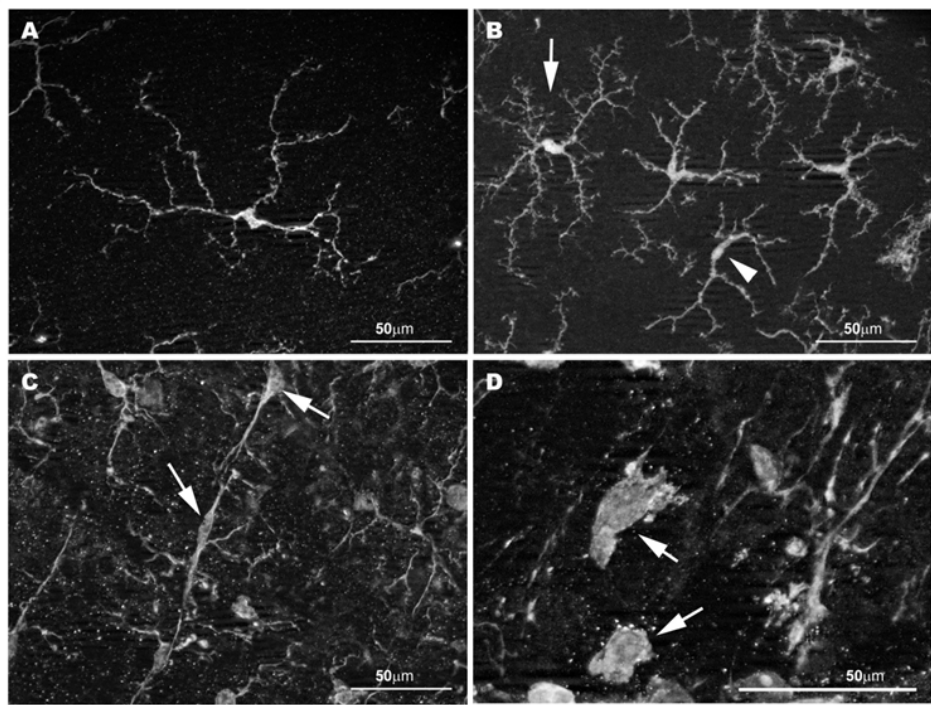


Figure 3: Morphological types of activated microglia in adult mouse retina with ocular hypertension. Iba-1 immunostaining. Retinal whole mount. In naïve retinas microglial cells show a ramified morphology (A). In OHT retinas the microglial cells can adopt a hyper-ramified morphology (arrow in B) (an intermediate state between quiescent and activated state) which is characterized by increased secondary, but not primary branching of microglial processes. Hyper-ramified microglia have the ability to reorient processes in response to changes in neural activity. Typically reactive microglial morphology, characterized by processes that are substantially shorter and wider (arrowhead in B), are also visible in OHT-eyes. Rod-like cells with elongated cell bodies and two processes prominently projecting from each pole (arrow in C) constitute another type of microglial activation that is restricted to the GCL-NFL of OHT-eyes. In the state of high reactivity, microglial cells acquire an amoeboid morphology (rounded cells with sparse processes) (arrow in D) and act as macrophages, engulfing and destroying cell debris [**GCL-NFL**: Ganglion Cell Layer-Neve Fiber Layer; **OHT**: Ocular Hypertension].

The mobilization of microglia after tissue injury, and their transition from a stationary to migratory amoeboid phenotype likely involves cell detachment from ECM, the up regulation of ECM degradation machinery being necessary [22]. Both migration and proliferation of microglial cells are regulated by either soluble factors or by changes in the ECM of damaged CNS tissues [53]. In human glaucomatous optic-nerve heads, microglia can reportedly express several MMPs and their inhibitors, suggesting that they could participate in the remodeling of the ECM, which is key in the physiopathology of glaucoma [40].

The injured neurons and macroglia increase the release of purines, thereby activating purinergic receptors in the microglia (Figure 2), which strongly affects microglia motility and migration [54,55]. Other factors such as vascular endothelial growth factor [56], nerve growth factor [57-59] and several chemokines [60] are also implicated in microglial migration. In experimental glaucoma, microglial activation is associated with cell migration which allows microglia to interact with and eliminate damaged or dead neurons [11,49].

In addition to phenotype change and mobilization, microgliosis also involves an increase in microglia-cell number, a fact that has been reported both in animal models of glaucoma [10,11,15,42-44] as well as in human glaucoma [40,61]. Several cytokines stimulate microglia division, such as Macrophage Colony-Stimulating Factors (**M-CSF**), granulocyte macrophage CSF (GM-CSF), IL-1 β , IL-4 and Interferon Gamma (**IFN- γ**). Also, neurotrophic factors such as brain-derived neurotrophic factor and neurotrophin-3 can act in a paracrine fashion, as microglial mitogens [53].

It has been reported that chronic tissue stress in glaucomatous eyes may lead to increased contact of the retina and optic-nerve-head tissues with systemic immune cells due to alterations in the perivascular barrier [2]. Chemokines derived from reactive astrocytes, including CCL2, CCL5, CCL20, CXCL10, CXCL12, CXCL1, CXCL2, and CX3CL1 [62], are involved in the recruitment of microglia, monocytes/macrophages, T-cells, and dendritic cells into the inflamed sites of the CNS [63]. At present, the role of infiltrating monocyte-derived macrophages in glaucoma is uncertain. The RGC response to monocyte-derived macrophages in the glaucomatous eyes is still poorly understood. In the DBA/2J model of glaucoma, circulating monocytes invade the retina and optic nerve whereas ocular irradiation that reduces the presence of these cells enhances RGC survival [64]. By contrast, in an experimental model of OHT induced by anterior chamber cannulation, an increase in the number of monocytes in the peripheral vasculature has been reported to be protective [65].

Activated retinal microglia can adopt a variety of distinct functional phenotypes in response to infections or injuries. These phenotypes are similar to the peripheral macrophages, and have been classified based on the expression of cell-surface markers, intracellular enzymes, and secreted molecules in M1 (classical activation) and M2 (alternative activation) [66,67]. It has been reported that, after injury, homeostatic microglia exposed to cell-derived cytokines such as IFN- γ become classically activated and acquire an M1-like phenotype. This phenotype includes: amoeboid shape, high motility, strong phagocytic capacity, up-regulation of Inducible Nitric Oxide Synthase (**iNOS**), and CD16/32. Also these cells produce several pro-inflammatory cytokines, including TNF- α , IL-1 β , IL-6, IL-12, NO, and proteolytic enzymes (metalloproteinases, collagenases and furin) among others, which promote sustained tissue inflammation [63,68,69]. Raised levels of the aforementioned inflammatory mediators are found in the retina and aqueous humor of patients and experimental glaucoma [6]. Upregulated iNOS and high NO levels were

found in the optic-nerve head of glaucomatous patients [70] and in the retina and optic-nerve head of experimental glaucoma [71,72]. Increased TNF α has been implicated as a mediator of RGC death in the glaucomatous retina [73,74]. By contrast, in an experimental model of glaucoma in rats, Lee et al. (2014) suggested that TNF α released by activated microglia stimulated macroglial cell to produce neuro protective factors, including nerve-growth factor, in response to a mid-hypertensive glaucomatous injury [75].

The functional effects of M1 activation are oriented to antigen presentation and the killing of intracellular pathogens. MHCII, CD86, and Fc γ receptors are up-regulated to allow for antigen-presenting activity of microglia and increased crosstalk with other immune cells [76,77]. MHC-II expression in glial cells is upregulated in the glaucomatous human retina and optic-nerve head [2,11,45,78]. Additionally, in a unilateral model of experimental glaucoma, increased expression of MHC-II was detected throughout all retinal layers (Figure 4) [10,11,15]. However, CD86 immunolabeling was found mainly in some amoeboid and rounded Iba-1+ cells (a marker of microglial cells) in the NFL and ganglion-cell layer [11]. The authors postulate that the fact that most microglial cells were CD86 negative could prevent the functional activation of T cells by their omission of co-stimulation, which may result in T-cell apoptosis or anergy, thus down regulating the immune response [79].

After acute activation, M1-like microglia can acquire two different chances in the recovery phase. In the first, M1-like microglia undergo uncontrolled activation, thus triggering chronic inflammation which results in the production of neurotoxic factors such as TNF- α , IL-1 α , IL-1 β , IL-6, NO, hydrogen peroxide, superoxide anion, chemokines and glutamate, leading to neuronal loss over time in different ways [39,80-83]. The second option becomes possible when the cells can return to the resting state, perhaps having transiently adopted the M2-activation state, to attenuate inflammation induced by M1-like microglia. The M2-activation state produces neurotrophic factors, ensuring tissue repair and restoration of functions [63,77,84,85].

The alternative M2-like phenotype plays an important role in resolving pro-inflammatory milieu [86]. This phenotype is characterized by thin cell bodies, branched processes, and up-regulation of specific molecular markers such as arginase-1, mannose receptor (CD206), Ym1, and CD68, among others. [68,69,86-88]. CD68 is a member of the scavenger-receptor family that clear away cell debris, promote phagocytosis, and mediate the recruitment and activation of macrophages. CD68 expression was observed in microglial cells throughout all the retinal layers in a unilateral model of experimental glaucoma (Figure 4) [11]. In this experimental model, the expression of Ym1 was restricted to a few amoeboid Iba-1+ cells in the NFL and ganglion cell layer of OHT eyes. The fact that only a few cells expressed a surface marker of the M2 phenotype could point towards most microglial cells in this OHT model exerting functions not related to the M2 activation pattern [11].

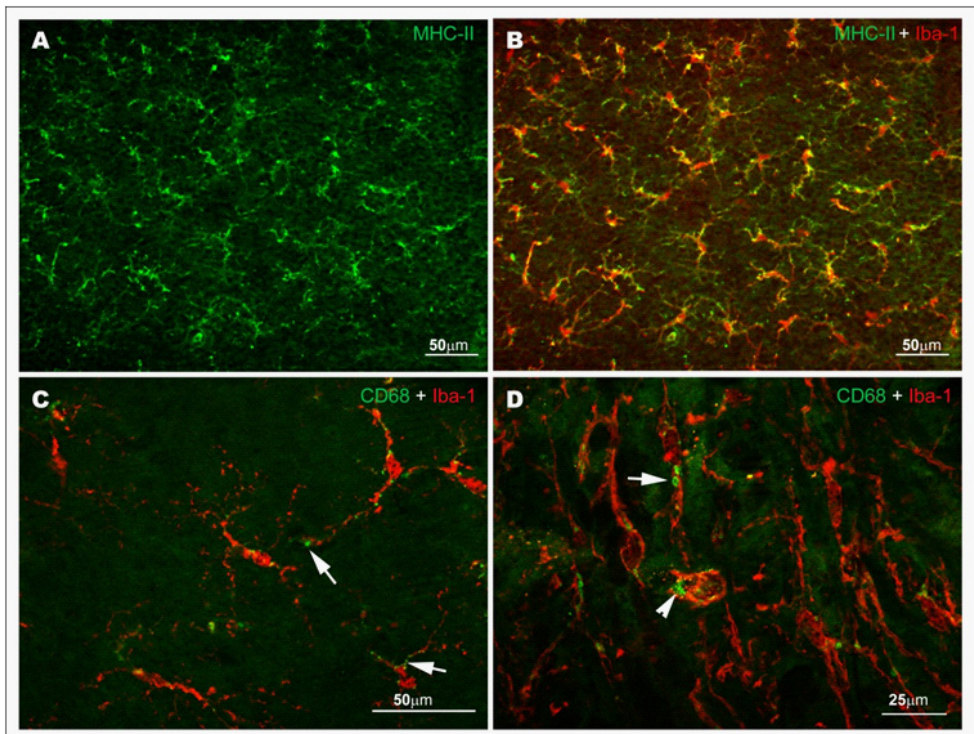


Figure 4: Activation markers of microglia in adult mouse retina with ocular hypertension. Iba-1, MHC-II and CD68 immunostaining. Retinal whole mount. Increased expression of MHC-II in the OPL (A,B). Such MHC-II up-regulation was detected throughout the retinal layers. Expression of CD68 in ramified microglia (C) and in rod-like microglia (D) in the GCL-NFL. As MHC-II, CD68 expression was detected in some microglial cells throughout the retinal layers. CD68+ ramified cell in the GCL-NFL (arrow in C). CD68+ rod-like microglia in the GCL-NFL (arrow in D). CD68+ ameboid microglia in the GCL-NFL (arrowhead in D). CD68 is a member of the scavenger-receptor family that clear away cell debris, promote phagocytosis, and mediate the recruitment and activation of macrophages. [GCL-NFL: Ganglion Cell Layer-Neve Fiber Layer; OPL: Outer Plexiform Layer].

Besides the microglial-derived neuro degeneration described above, neurons can induce an inflammatory response in microglial cells after an injury. The nucleotides released by damaged neurons can up-regulate purine receptors in microglia, triggering phagocytosis [89]. In an experimental mouse model of glaucoma, Wang et al. (2014) demonstrated an intercellular communication between retinal microglia and RGCs via activation of the fractalkine receptor CX3CR1. Deficiencies in this signaling pathway resulted in increased microglial activity, neurotoxicity and RGC death (Figure 2) [90]. The CD200/CD200R complex plays an important role in regulating microglia activity in relation to the surrounding neuroglial population. Changes in the expression of CD200R/CD200 occur early in experimental glaucoma and precede the peak in microglia infiltration and RGC death, suggesting that CD200R-positive microglia play an important

role in the onset of RGC death during OHT [91]. The protein High-Mobility Group Box 1 (**HMGB1**), which is released by dying neurons, binds the CD11b in the surface of microglia and stimulates the NF- κ B pathway and NADPH oxidase to induce the production of multiple inflammatory and neuro toxic factors [92]. The deletion of the phagocytic microglia receptor CD11b prevents microglial activation and has proven neuro protective in experimental glaucoma [74].

Finally, damaged neurons can release substances such as Head-Shock Protein (**HSP**) 60, which triggers an oxidative response in microglia [93]. High levels of HSP27, HSP60, and HSP72, as well as TLRs have been found in human glaucoma [94-98]. These HSPs contribute to the activation of the innate immune system via TLRs in this pathology [2,32]. In glaucoma patients, high concentrations of antibodies against HSPs have been found [99-101]. This has led to postulation that the onset of glaucoma could be mediated by an autoimmune mechanism [2]. In fact, antibody deposition in the sera and aqueous humor of patients with glaucoma has been described [102]. Similar to other autoimmune disorders, a serum alteration of the populations of T-cell repertoires and of interleukin-2 receptors has been detected in glaucoma [103]. These results support the contention that both innate and adaptive immune activity accompanies glaucomatous neuro degeneration [104].

Clinical and experimental evidence supports the idea of the involvement of the immune system in glaucoma. Higher autoantibody levels in the serum and in the aqueous humor, deposition of immunoglobulins and plasma cells in the retina [105-108], and chronic activation of resident immune regulatory glial cells and complement activation [2] have been reported in the context of glaucomatous degeneration.

Low levels of complement activation in the retina may be beneficial to the maintenance of retinal homeostasis. However, under inflammatory conditions, the presence of inflammatory cytokines such as TNF- α , INF- γ , and IL-6 promotes the synthesis of complementary constituents [32]. Under pathological conditions, the blood-retinal barrier can break, and the complement proteins can enter retinal parenchyma, resulting in local complement activation. However, it has been shown, that microglia may drive complement activation through local secretion of complement components [32]. In vitro studies have found that microglial cells synthesize components from classical pathway (C1q, C1r, C1s, C2, and C4), mannose-binding lectin (MASP1), and alternative pathway (CFB, CFH), express C3 and C5 [109], and synthesize complement regulatory factors (e.g. factor h, factor B, C59) [110,111]. The expression of the CR3 complex by microglia promotes opsonic phagocytosis of cellular constituents following complement activation, allowing microglia an effective cleaning of the retina of the noxious cell debris [32,112]. In the adult retina, C1q is upregulated in glaucomatous eyes [113,114]. An unknown stimulus triggers astrocytes to release a factor to which the RGCs respond by making C1q in the glaucomatous retina. C1q initiates a protease cascade that results in complement C3 deposition. C3 activates receptors on microglia that signal the cells to phagocytose any C3-coated elements. In glaucoma, microglia respond to C1q upregulation by eliminating the targeted synapses, reducing RGC connectivity.

Evidence for dendritic arbor shrinkage and loss [115-117] in glaucoma supports the veracity of this degeneration mechanism [118].

In conclusion, microglial cells and their complementary system, composed of the innate retinal immune system, may play an important role in retinal homeostasis. However, under inflammatory conditions, they may interact with the adaptive immune system, contributing to glaucoma.

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