

Retinal microglial activation in glaucoma: evolution over time in a unilateral ocular hypertension model

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Glaucoma is a neurodegenerative chronic pathology, characterized by the loss of retinal ganglion cells (RGC), which leads to an irreversible vision field loss. The increased intraocular pressure (IOP) constitutes its main risk factor. Nowadays, the main treatments for glaucoma are focused on decreasing IOP; nevertheless, the progression of the disease continues, despite IOP control. This fact shows the existence of other factors that could contribute to the advance of glaucomatous neurodegeneration. The early diagnostics, the use of neuroprotective therapies, and knowledge of the pathological processes are the major challenges associated with the management of this disease. Different pathogenic mechanisms have been proposed to be responsible for RGC death, including oxidative stress, mitochondrial dysfunction, glutamate excitotoxicity, and neuroinflammation (Casson et al., 2012).

As in other neurodegenerative diseases, it has been shown that the immune system may be involved in glaucomatous neurodegeneration. In the retina and the optic nerve, as in the rest of the central nervous system, microglial cells constitute the population of immune resident cells, and they play an important role in the physiology and survival of RGC (Neufeld, 1999; Yuan and Neufeld, 2001). The microglia are able to respond promptly to retinal damage, in order to maintain its immune privilege. Initially, a controlled response may be responsible for a neuroprotective role, but a state of tissue damage maintained over time is related to a chronic activation of microglial cells, resulting in an inflammatory process. Thus, in glaucomatous pathology, neuroinflammation could constitute a protection mechanism against retinal and optic nerve nerve damage. However, a prolonged state of inflammation could increase the death of the RGC, thus contributing to the progression of this pathology (Neufeld, 1999; Yuan and Neufeld, 2001; Tezel et al., 2009). In glaucoma, RGC death has been demonstrated both in humans and in experimental models (Vidal-Sanz et al., 2012).

The microglial activation phenotype

is characterized by alteration in the expression of different receptors (CD200R, CX3CR1, and P2Y12) and by an increased release of growth factors, pro-inflammatory cytokines (interleukin-1 β , tumor necrosis factor- α , interleukin-6, etc.), and cytotoxic substances (reactive oxygen and nitric oxide species), as well as by morphological changes (Ramírez et al., 2015).

Microglial activation has been demonstrated in different models of glaucoma (in rats or mice), both hereditary and induced, using different techniques for the induction of ocular hypertension (OHT) (Vidal-Sanz et al., 2012). One of the models in which microglial activation has been studied in detail is a laser-induced OHT mouse model, in which unilateral OHT is induced by photocoagulation of the limbal and episcleral veins (Vidal-Sanz et al., 2012). In this animals, OHT induces an alteration of retrograde axonal transport in RGCs and a subsequent degeneration of these cells. In this model, 15 days after OHT induction, the microglia exhibited different signs of activation, such as proliferation and migration to areas of damage, retraction, reorientation, and hyper-ramification of their processes, and the appearance of amoeboid- and rod-like microglia with macrophagic capacity, related to RGC degeneration (Gallego et al., 2012; de Hoz et al., 2013; Rojas et al., 2014). In this model, 15 days after OHT induction, the activation of the macroglia (astrocytes and Müller cells) has also been demonstrated, showing morphological changes, as well as an overexpression of glial fibrillary acidic protein (Gallego et al., 2012). In addition, in this model, numerous rounded Iba-1⁺ CD68⁺ cells have also been found close to the vessels. These cells could be monocytes that have been able to enter the retina by alterations produced in the blood-retinal barrier (BRB) due to the glaucomatous neuroinflammatory process (Stolp and Dziegielewska, 2009; Rojas et al., 2014). The infiltrated cells could induce the release of proinflammatory mediators that could activate the glial cells and stimulate the macrophages, thus provoking neuronal damage.

In glaucoma, gliosis is also associated with an overexpression of immune-related surface markers, such as the major histocompatibility type II complex (MHC-II). This molecule is essential for antigen presentation and the activation of T cells. In physiological conditions, the expression of MHC-II is restricted to some cells and at very low levels; however, in the context of glaucomatous pathology, some factors (tumor necrosis factor- α and interferon- γ) can lead to its increase (Tezel et al., 2009; Ramírez et al., 2015). In the laser-induced OHT model, 15 days after OHT induction, a MHC-II overexpression was observed in the microglia, astrocytes, and Müller cells, allowing these cells to induce a T-cell-mediated immune response (Gallego et al., 2012). The presence of a co-stimulating molecule is required for the presentation of the antigen to the T cells. However, most of the microglia were MHC-II⁺/CD86⁻, with only a few amoebic cells having the CD86⁺ immunolabeling. The absence of this co-stimulating molecule could produce apoptosis or anergy of the T cells, which could have entered from the bloodstream, thus causing the downregulation of the adaptive immune response.

As immunomodulatory cells, microglia are able to trigger an immune response in the glaucoma, playing a neuroprotective or neurotoxic role by adopting different phenotypes, M1 (pro-inflammatory) or M2 (neuroprotective). These phenotypes are characterized as a continuous balance between two extreme states of activation: the M1 or cytotoxic state, due to the secretion of reactive oxygen species and pro-inflammatory cytokines, and the M2 or anti-inflammatory state, in which microglia secrete high levels of anti-inflammatory cytokines and neurotrophic factors (Parisi et al., 2018). At 15 days after OHT induction, only a few amoebic cells were marked with anti Ym1, which is a marker of the M2 phenotype. This indicates that most of the microglia are not exerting an M2 phenotype or are not neuroprotective in OHT eyes (Gallego et al., 2012; de Hoz et al., 2013).

Usually, the glaucomatous pathology is a bilateral disease that affects both eyes, but the alterations do not follow a symmetric evolution. First, one eye shows signs of neuronal damage, appearing afterwards in the contralateral eye. Experimental models of RGC damage, whether related to an increased IOP or to other mechanisms of neuronal damage, show the activation of glial cells in the uninjured contralateral eye (Vidal-Sanz et al., 2012). Morphological signs of microglial activation were detected in contralateral normotensive eyes at 15

days after OHT induction. In addition to morphological changes, the astrocytes and microglia in the contralateral eyes showed an increased expression of MHC-II. The reactive microgliosis and upregulation in the expression of MHC-II support the fact that an immune process was taking place in both the contralateral and OHT eyes (Gallego et al., 2012; de Hoz et al., 2013, 2018).

Considering the evidence of the involvement of the immune system in glaucomatous optic neuropathy and the important role of glial cell activation in RGC survival or death, an exhaustive study of how microglial activation progresses over time after OHT induction, in the laser-induced OHT model, would be a logical and interesting step. A study has examined the evolution of microglial activation after unilateral laser-induced OHT at different time points (1, 3, 5, 8, and 15 days) after OHT induction (Ramírez et al., 2020). Two markers have been used to identify microglia: Iba-1 and P2RY12. Iba-1 allows for the analysis of the morphological features of microglia, and the purinergic P2RY12 receptor allows for the differentiation of resident microglia (P2RY12⁺) from monocytes, infiltrating macrophages, or dendritic cells, which do not express this marker. In this study, an analysis is conducted on the different microglial morphological changes, such as changes of the soma size, the arbor area, the number of vertical processes, and the number of microglial cells, in all retinal layers in which microglia are present (the outer segment photoreceptor layer, and plexiform layers (outer and inner), and nerve fiber layer/ganglion cell layer (NFL-GCL)). Additionally, the P2RY12 expression of the microglia is analyzed (**Figure 1**). At 24 hours after unilateral laser OHT induction, the microglia showed signs of activation in all retinal layers where they were located (de Hoz et al., 2018; Ramírez et al., 2020). These signs included an increase in the soma size, shortening and reorientation of the processes, and the presence of macrophage amoebic cells, both in the OHT eye and in the normotensive contralateral eye, although it was more intense in the OHT eye. However, at this time, there was no increase in the number of microglial cells, although there was an increase in the area occupied by the microglia in the NFL-GCL layer, which could be due to migratory phenomena from other layers and an increase in the size of these cells, rather than an increase in their number (de Hoz et al., 2018). The activation of the microglial cells may be related to the

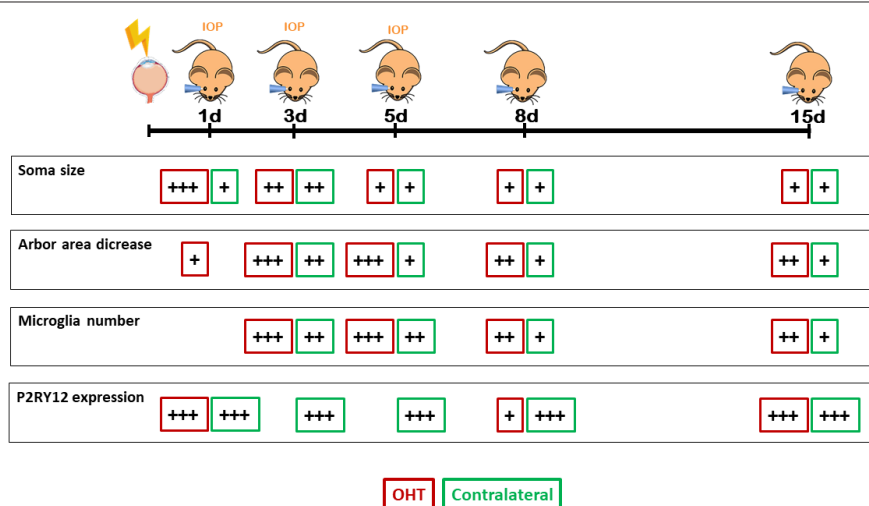


Figure 1 | Major changes observed in microglial cells at different time points after OHT induction in the unilateral laser-induced OHT mouse model.

The “+” indicates the intensity of the changes. The red color represents the OHT eye, and the green color represents the contralateral eye. IOP: Increased intraocular pressure; OHT: ocular hypertension.

increase in the IOP, which remains high in this model from day 1 to day 5, until it drops to normal values at 7 days. This IOP increase could produce damage in the RGCs, which would induce the activation of the microglial cells (Gallego et al., 2012; de Hoz et al., 2013, 2018; Rojas et al., 2014; Ramírez et al., 2020). The activated microglia would produce pro-inflammatory factors and chemokines that would induce the disruption of the BRB (Ramírez et al., 2015). At 24 hours after OHT induction, the microglia did not show an upregulation of MHC-II (de Hoz et al., 2018). However, there were MHC-II⁺ rounded cells, which were not microglia, because they did not express P2RY12, and could be monocytes infiltrated from the bloodstream due to the disruption of the BRB (Ramírez et al., 2020). These MHCII⁺ cells could induce the transformation of ramified microglial cells into amoeboid microglia, which would phagocytize the cells that had entered from the bloodstream (de Hoz et al., 2018). In addition, the microglia in both the OHT and contralateral eyes (more frequently in the OHT eyes) change their arrangement from parallel to the retinal surface to perpendicular, sending processes towards the neighboring microglial plexuses and connecting them. This could help in the transmission of information from the layers where the alteration of the BRB is taking place to the other retinal layers, thus contributing to microglial activation (de Hoz et al., 2018).

Most of the signs of microglial activation were increased at 3 and 5 days after OHT induction (Ramírez et al., 2020). The retraction of the microglial processes (measured by the arbor area) reaches

its maximum at these time points. The soma size remained large at 3 and 5 days, although it was smaller than it was at 24 hours. The increase in the number of microglial cells in all the layers where these cells are located reaches its maximum values 3 and 5 days after the induction of OHT (Ramírez et al., 2020). In addition, one of the most sensitive markers of the change from resting microglia to activated microglia is the down-regulation of P2RY12 (Haynes et al., 2006). The microglial cells of native animals express P2RY12. At 24 hours after the induction of OHT, the microglia express P2RY12. However, at 3 and 5 days, this expression is down-regulated and begins to increase slightly at 8 days, reaching similar values to those of naïve animals at 15 days (Ramírez et al., 2020). This also indicates that between 3 and 5 days after OHT induction is when a major inflammatory process occurs in the OHT eyes in this experimental model (**Figure 1**), coinciding with the highest IOP values (1, 3, and 5 days) (Ramírez et al., 2020). From 5 days until 15 days after OHT induction, the soma size decreased slightly, the arbor area increased, and the number of microglial cells decreased, although it did not reach the values of the naïve eyes at any time (Gallego et al., 2012; de Hoz et al., 2013; Rojas et al., 2014; Ramírez et al., 2020).

The activation of the microglial cells observed in the contralateral eye at 24 h after HTO induction persisted in the later time-points until 15 days (Gallego et al., 2012; de Hoz et al., 2013, 2018; Rojas et al., 2014; Ramírez et al., 2020) (**Figure 1**). This was independent of the increase in IOP, as the contralateral eye

was normotensive. Moreover, in these eyes, there was no down-regulation of P2RY12, and this expression was similar at all times and similar to that of the naïve eyes (Ramírez et al., 2020). In the contralateral eyes, the time point at which there was the highest microglial activation (soma area, arbor area, and cell number) was 3 days after OHT induction (Ramírez et al., 2020). The microglial activation in the contralateral eyes was less intense than that in the OHT eyes and remained fairly stable at all of the time points analyzed. The activation of the microglia in the contralateral eye in absence of RGC death could have been produced by an immune response as demonstrated by the up-regulation of MHC-II by microglial cells 15 days after OHT induction (Rojas et al., 2014; Ramírez et al., 2020).

Conclusions: In the unilateral glaucoma model induced by laser photocoagulation of the episcleral and limbal veins, IOP increase produces an activation of the retinal microglial cells. This activation is maintained over time, even when the IOP values become normal. This activation is most intense at 3 and 5 days after the OHT induction. The inflammatory response affects both eyes, even if the OHT occurs in only one of them, which indicates that the inflammatory response in the contralateral eye originates from the OHT eye. This fact confirms the involvement of the immune system in the glaucomatous pathology.

Thus, the involvement of microglia in the glaucomatous inflammatory process could constitute a new approach to the treatment of this pathology. Knowledge of the moment at which the microglial activation in this experimental model is at its highest can be used to identify factors that intervene in the inflammatory process related to glaucoma and could help in the development of new anti-inflammatory therapies that assist in neuroprotection.

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