Caffeine administration prevents retinal neuroinflammation and loss of retinal ganglion cells in an animal model of glaucoma

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Glaucoma is the leading second cause of blindness worldwide, being characterized by progressive optic nerve damage and loss of retinal ganglion cells (RGCs), accompanied by increased inflammatory response involving retinal microglial cells. The etiology of glaucoma is still unknown, and despite elevated intraocular pressure (IOP) being a major risk factor, the exact mechanisms responsible for RGC degeneration remain unknown. Caffeine, which is an antagonist of adenosine receptors, is the most widely consumed psychoactive drug in the world. Several evidences suggest that caffeine can attenuate the neuroinflammatory responses and afford protection upon central nervous system (CNS) injury. We took advantage of a well characterized animal model of glaucoma to investigate whether caffeine administration controls neuroinflammation and elicits neuroprotection. Caffeine or water were administered ad libitum and ocular hypertension (OHT) was induced by laser photocoagulation of the limbal veins in Sprague Dawley rats. Herein, we show that caffeine is able to partially decrease the IOP in ocular hypertensive animals. More importantly, we found that drinking caffeine prevented retinal microglia-mediated neuroinflammatory response and attenuated the loss of RGCs in animals with ocular hypertension (OHT). This study opens the possibility that caffeine or adenosine receptor antagonists might be a therapeutic option to manage RGC loss in glaucoma.

Glaucoma is a group of progressive neurodegenerative multifactorial diseases, characterized by the loss of retinal ganglion cells (RGCs), optic nerve excavation, and axonal degeneration leading to irreversible vision loss¹. Although the etiology of glaucoma is still not completely elucidated, advanced age and elevation of intraocular pressure (IOP) are considered the main risk factors for the disease onset. Current available treatments for glaucoma are focused on the reduction of IOP, the only modifiable risk factor². However, in several patients the disease still progresses, despite the effective control of IOP. Therefore, it is urgent to develop novel therapeutic strategies focused on the neuroprotection of RGCs³.

It is currently recognized that degeneration of RGCs in human and experimental glaucoma is accompanied by a neuroinflammatory response, involving retinal microglial cells and increased production of inflammatory mediators, such as tumor necrosis factor (TNF) and interleukin-1β (IL-1β)⁴–⁷. In addition, early and exacerbated activation of retinal microglial cells has been described and proposed to contribute to the degenerative process⁸–¹⁰, suggesting that the control of microglia reactivity can prevent the glaucomatous loss of RGCs¹¹–¹³. The adenosine A₂A receptor (A₂AR) is an important drug target in the central nervous system (CNS), since its blockade has been shown to afford robust neuroprotection in different noxious brain conditions, namely through the control of microglia-mediated neuroinflammatory processes¹⁴. Recently, we showed that the blockade of the A₂AR affords protection to RGCs against damage induced by elevated hydrostatic pressure in retinal organotypic
mRNA levels (n = 7, p < 0.001). Caffeine administration per se did not alter the IOP.

OHT increases the expression of A₂₃R and does not affect A₁R expression. In the CNS, including the retina, the pharmacological actions of caffeine are exerted mainly by antagonizing adenosine receptors⁶⁶. It has been documented that brain noxious conditions downregulate A₁Rs and up-regulate A₂₃Rs. Therefore, we evaluated whether OHT could alter the expression of A₁Rs and A₂₃Rs. No significant alterations were detected in the mRNA expression of A₁R (Fig. 2) at the 2 time-points analyzed (3 and 7 days after inducing OHT). Nevertheless, at 3 days post-OHT we detected a significant increase in the expression of A₂₃R compared with control animals (2.3-fold change; n = 5, p < 0.05), which was maintained until 7 days post-OHT (2.4-fold change; n = 7, p < 0.05).

Caffeine inhibits the inflammatory response triggered by OHT. Since chronic inflammation plays an important role in the pathophysiology of glaucoma, we evaluated mRNA and protein levels of the pro-inflammatory markers TNF and IL-1β. As shown in Fig. 3A, 3 days with OHT significantly increased the mRNA levels of TNF and IL-1β (4.3 ± 0.6 and 6.8 ± 0.9 fold-change of the control, respectively; n = 7, p < 0.01). Similarly, at 7 days with OHT the mRNA levels of these cytokines were still significantly elevated, when compared with the control (2.8 ± 0.6 and 3.2 ± 0.3 fold change for TNF and IL-1β, respectively; n = 7, p < 0.05). The administration of caffeine to OHT animals significantly inhibited the OHT-induced up-regulation of TNF and IL-1β mRNA levels (n = 7, p < 0.05) in both time points, without altering the expression of both TNF and IL-1β in the retinas of water-drinking control animals (control group).

We then quantified the protein levels of TNF and IL-1β, by ELISA, in retinal extracts (Fig. 3B). In the retinas of control animals, the expression of TNF and IL-1β was 74.6 ± 8.6 and 150.5 ± 12.6 pg/μg of total protein, respectively. At 3 and 7 days after inducing OHT, IL-1β protein levels were significantly increased (314.7 ± 44.3 and 424.1 ± 51.2 pg/μg of total protein, respectively; n = 8–9, p < 0.01 and p < 0.001), when compared with control animals. In animals drinking caffeine, after 3 days of OHT, the levels of IL-1β in the retina slightly decreased to 194.9 ± 8.1 pg/μg of total protein, comparing with animals drinking water. Moreover, at 7 days of OHT the administration of caffeine significantly decreased IL-1β to 140.9 ± 7.1 pg/μg of total protein (n = 7, p < 0.001). After 3 days of OHT, the protein levels of TNF were not altered in retinal extracts both in water and caffeine drinking animals, comparing with control animals. Nevertheless, after 7 days of OHT the protein levels of TNF significantly increased to 127.4 ± 13.7 pg/μg of total protein (n = 9, p < 0.05), which was prevented by caffeine.
administration (29.9 ± 7.98 pg/μg protein; n = 8, p < 0.0001). Caffeine administration, per se, did not alter the protein levels of these two cytokines.

**Caffeine prevents OHT-induced microglia activation.** Microglia activation appears early in the retina of animal models of glaucoma, implicating a role for these cells in the early stages of the disease. Therefore, we evaluated whether caffeine administration could modulate microglia reactivity elicited by OHT.

By qPCR, we evaluated the mRNA levels of two markers of microglia reactivity, major histocompatibility complex class II (MHC-II) and translocator protein (18 kDa) (TSPO), as well as CD11b, a general marker of microglia and triggering receptor expressed on myeloid cells 2 (TREM2), which is associated with microglia phagocytic capacity. After 3 and 7 days of OHT, there was a significant increase in the mRNA levels of MHC-II (3.5 ± 0.8 and 19.8 ± 2.2 fold change, respectively; n = 7, p < 0.05 and p < 0.001). Caffeine administration significantly prevented the OHT-induced increase in MHC-II expression (1.2 ± 0.8 and 4.4 ± 0.3 fold change for 3 and 7 days of OHT, respectively; n = 7, p < 0.05 and p < 0.01). The immunoreactivity for MHC-II was mainly detected in the ganglion cell layer in OHT animals and co-localized with Iba1, a general marker of microglia.

The mRNA expression of TSPO was also up-regulated after 3 and 7 days of OHT (11.6 ± 1.5- and 19.8 ± 1.2-fold change, respectively; n = 7, p < 0.001). The administration of caffeine slightly decreased the levels of the mRNA coding for TSPO (6.7 ± 0.8-fold change) at 3 days in OHT animals, and the decrease reached statistical significance at 7 days in animals with OHT drinking caffeine (4.3 ± 0.8-fold change; n = 7, p < 0.05), compared with water drinking animals.

We then assessed the expression levels of CD11b and detected a significant up-regulation of the mRNA levels after 7 days of OHT (1.9 ± 0.2-fold change; n = 7, p < 0.05). This up-regulation was prevented by caffeine administration (1.1 ± 0.2-fold change; n = 7, p < 0.01). The expression of TREM2 was also assessed in order to estimate microglia phagocytic activity. After 3 and 7 days significantly increased the expression of TREM2 mRNA by 5.5 ± 1.0 and 2.3 ± 0.32 fold-change, respectively (n = 7, p < 0.05 and p < 0.01), and caffeine prevented the OHT-induced increase in TSPO expression (1.8 ± 0.4- and 0.9 ± 0.2-fold change, for 3 and 7 days, respectively; n = 7, p < 0.05 and p < 0.01). Administration of caffeine per se did not alter the expression of pro-inflammatory markers.

**Table 1. Animal fluid intake and weight.**

| Animals drinking water (n = 48) | Animals drinking caffeine (n = 49) |
|---------------------------------|-----------------------------------|
| Fluid intake (mL/day)           |                                   |
| Day 0                           | 23.7 ± 0.9                        |
| Day 1                           | 22.0 ± 0.7                        |
| Day 2                           |                                   |
| Day 3                           |                                   |
| Day 4                           |                                   |
| Day 5                           |                                   |
| Day 6                           |                                   |
| Day 7                           |                                   |
| Animal Weight (g)               |                                   |
| Day 0                           | 200.1 ± 1.1                       |
| Day 1                           | 201.5 ± 1.7                       |
| Day 2                           | 201.5 ± 1.7                       |
| Day 3                           | 234.4 ± 2.4                       |
| Day 4                           | 236.0 ± 1.8                       |
| Day 5                           | 253.4 ± 1.9                       |
| Day 6                           | 250.8 ± 5.7                       |
| Day 7                           |                                   |

![Figure 1. Caffeine administration reduces IOP in OHT animals.](image)

Water or caffeine (1 g/L) was administrated *ad libitum* to Sprague Dawley rats, during 2 weeks prior induction of OHT, and until the end of the experiment. IOP was measured with a rebound tonometer. Results are expressed in mmHg and represent the mean ± s.e.m of 22 to 37 independent experiments. **p < 0.0001, significantly different from control animals; +++p < 0.001, significantly different from OHT control animals; Two-way ANOVA, followed by Tukey’s multiple comparison test.**
Caffeine administration prevents microglia reactivity in contralateral retina induced by OHT. Microglia reactivity in the contralateral eye (without OHT) has been reported\(^{35,36}\). Therefore, we evaluated the ability of caffeine to modulate the microglia reactivity in contralateral eyes.

The expression of mRNA coding for MHC-II and TSPO (Fig. 5A,B) in the contralateral eye was significantly up-regulated after 7 days of OHT (3.8 ± 0.2- and 3.0 ± 0.3-fold change, \(p < 0.01\) and \(p < 0.001\), respectively; \(n = 7\)). Caffeine administration reduced the expression of MHC-II and TSPO in the contralateral eyes, significantly for MHC-II (1.5 ± 0.2-fold change, \(p < 0.05\); \(n = 6\)). By immunohistochemistry, similarly to the OHT eyes, MHC-II immunoreactivity is detected in the microglia (Iba1-positive cells) within the GCL (Fig. 5C). Caffeine administration inhibited the OHT-induced increase in MHC-II immunoreactivity in the contralateral eyes.
Caffeine administration does not ameliorate the OHT-induced impairment in the RGC retrograde transport.

Alterations in the structure of the optic nerve and in the retrograde axonal transport of RGCs have been described in glaucomatous animal models 24,37. Therefore, in OHT animals, we assessed the effect of caffeine administration in both the integrity of the optic nerve and the axonal transport in RGCs. The structural integrity of the optic nerve was assessed by transmission electron microscopy (Fig. 6A). We observed that OHT increased the incidence of axon degenerative profiles, with disorganized and abnormal myelin wrapping (Table 2). Interestingly, in animals drinking caffeine this effect appears to be partially attenuated, with caffeine-drinking OHT animals presenting a reduced number of disorganized myelin structures (Fig. 6A and Table 2).

Retrograde axonal transport was assessed after Fluorogold (FG) application in both superior colliculi, the targets of 98% of RGCs 38. Application of FG after induction of OHT is an established method to evaluate the impairment of the retrograde axonal transport of RGC, by counting the total number of FG-positive cells in whole-mounted retinas (FG+ labeled RGCs) 26. Isodensity maps allowed us to visualize the distribution of FG+ RGCs in the retina (Fig. 6B). In control animals, drinking water or caffeine, the total number of FG+ cells was 73,698 ± 1,611 and 78,125 ± 1,096 cells, respectively (n = 5) (Fig. 6C), similar to previous reports 24. This number was significantly reduced to 19,619 ± 3,990 cells (n = 4; p < 0.05) in animals with OHT for 7 days. In animals with OHT, caffeine administration did not prevent the OHT-induced reduction in the number of FG+ labeled RGCs (17,630 ± 2,102 cells; n = 4, p < 0.05).

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Caffeine increases RGC survival in OHT animals. Taking into consideration the protective properties of caffeine administration 39, we evaluated the potential protective effect of caffeine against the degeneration of RGCs triggered by OHT. In whole-mounted retinas, RGCs were labeled with an antibody that recognizes Brn3a, a marker of RGCs 40 (Fig. 7A), and the total number was counted automatically (Fig. 7B). In control animals, the total number of RGCs per retina was 70,861 ± 1,258 (n = 5), similarly to previous works 24. The occurrence of OHT for 3 days triggered a reduction in the number of Brn3a-positive cells to 44,746 ± 6,151 cells (n = 4) and 50,021 ± 6,151 cells (n = 4) in animals drinking water and caffeine, respectively. Extension of OHT to 7 days resulted in a further significant loss of RGCs in animals drinking water (24,621 ± 3,443 Brn3a-positive cells, n = 7, p < 0.01). However, administration of caffeine to animals with OHT for 7 days significantly prevented the loss of
RGCs induced by OHT (44,027 ± 5,841 Brn3a-positive cells, n = 7, p < 0.05). Caffeine administration to animals with normal IOP did not significantly alter the number of RGCs (68,169 ± 1,840 Brn3a-positive cells, n = 6).

Discussion
The present work demonstrates that caffeine administration prevents retinal neuroinflammation, microglia reactivity and affords protection to RGCs in an animal model of glaucoma.

Similarly to what happens in chronic noxious brain conditions, OHT triggered A2AR upregulation, prompting the hypothesis that the manipulation of this receptor may control neurodegeneration. We previously reported that elevated hydrostatic pressure, to mimic OHT in vitro, increases A2AR expression, mainly in retinal microglia located within the ganglion cell layer. Since the actions of caffeine are exerted mainly by blocking adenosine receptors, including the high-affinity A1R and A2AR, the up-regulation of A2AR by OHT suggested that the effects of caffeine in OIT animals were mediated by A2AR antagonism.

Caffeine affords beneficial effects in animal models of conditions expected to impair memory performance such as Parkinson’s disease, chronic stress, type 2 diabetes, attention deficit and hyperactivity disorder, early life convulsions, or alcohol-induced amnesia. The effects of caffeine to ocular tissues have also been reported, like the prevention of diabetic cataract in galactose-fed animals and reduction of corneal thickness. The effects of consumption of caffeine in IOP are not yet clarified. While some authors suggest that caffeine consumption may increase IOP in patients with normotensive glaucoma or ocular hypertension, others have shown that caffeine does not significantly alter IOP in patients with glaucoma. Therefore, we regularly measured IOP in animals and found that administration of caffeine was able to reduce the IOP of OHT animals, without interfering with animals with normal IOP. Nevertheless, the IOP lowering effect of caffeine may not be enough to explain the effects exerted by caffeine, since IOP in caffeine-drinking OHT animals is still elevated (30% increase above basal IOP).

Glaucomatous damage is accompanied by early activation of microglia and increased expression of inflammatory mediators. It has been suggested that the control of microglia reactivity may represent a therapeutic strategy to manage glaucoma. Reduction of microglia reactivity by irradiation or pharmacological treatment, or the reduction of TNF expression, confers protection in an animal model of glaucoma. Several studies demonstrate that caffeine affords protection to the brain in models of neurodegenerative diseases and prevents microglia-mediated neuroinflammatory responses. Indeed, OHT animals treated with caffeine presented...
reduced microglia activation and lower levels of inflammatory mediators, demonstrating that caffeine prevents microglia-mediated neuroinflammation induced by OHT. Increased expression of MHC-II has been also detected in mice contralateral eyes\textsuperscript{35,36}. Remarkably, caffeine also reduced microglia reactivity in contralateral eyes (without OHT).

Although we did not assess the contribution of other cell types responsible for the inflammatory environment in the retina, we cannot discard the contribution of macroglial cells, which can also release inflammatory mediators\textsuperscript{50}. Nevertheless, caffeine administration did not prevent OHT-induced GFAP up-regulation, a marker...
of astroglial and Müller cell reactivity (supplementary data; Fig. 1), suggesting that caffeine appears to be preferentially modulating microglia responses. This is in line with previous studies reporting the inability of A2AR to modulate the activation of astroglial cells18,51.

Several studies have shown that axonal transport in the optic nerve is impaired in human glaucoma and in the OHT rat model, preceding the loss of RGCs24,26,38,52. In this model, OHT results in Wallerian-like degeneration53, which culminates in RGC death26,52. In fact, previous studies have shown that part of the surviving RGC population after one week of OHT exhibits impaired retrograde axonal transport, supporting evidence that not all RGCs die immediately upon impaired axonal transport25,26.

In this work, we found that caffeine is not able to prevent the deficit in the axonal transport induced by OHT. Although caffeine-drinking animals with OHT presented a more preserved optic nerve structure, this was not sufficient to overcome the damage induced by OHT and it was not able to improve axonal transport. Nevertheless, there is a significant attenuation in the loss of RGCs induced by seven days of OHT in animals drinking caffeine. Glaucomatous injury to the optic nerve and optic nerve head appears to be related with the values of IOP, but the detailed mechanism remains to be elucidated26. In fact, previous reports using distinct models of IOP elevation have demonstrated that axonal damage in the optic nerve correlates with the magnitude and duration of IOP elevation37,54.

Although most of cell death occurs subsequently to the axonal degeneration, glial-mediated inflammatory response also contributes to the progress of the damage55. In fact, activation of microglia in a glaucoma animal model occurs prior the loss of RGCs8. In a model of LP-induced OHT, the presence of markers of microglia reactivity in the retina after 3 days of OHT is paralleled by a decrease in the number of RGCs56. Also, markers of reactive microglia are present in the optic nerve and optic tract after 7 days of induction of OHT56. Hence, caffeine, by blocking A2AR, might be attenuating microglia reactivity, thus protecting the soma of RGCs. Being the response of microglial cells in the optic nerve delayed, it seems plausible to speculate that the effects of caffeine might not be observed at the 7 days time-point. Also, adenosine receptors might not be directly related with the integrity of the retrograde axonal transport, and therefore caffeine might not be able to alter the functional damage induced by OHT. Indeed, as discussed above, functional impairment of retrograde axonal transport is directly correlated with the elevation of IOP37,54.

**Figure 7.** Caffeine administration inhibits Brn3a-positive cell loss triggered by OHT. Retinal whole-mounts were immunostained for Brn3a (red; RGC marker) and isodensity maps were generated to evaluate RGC survival. (A) Representative isodensity maps demonstrating the topological distribution of Brn3a-labelled RGCs, using a color code according to cell density value within a 28-step color scale range from 0 (dark blue) to 2500 or higher RGCs/mm² (red). (B) The graph represents mean ± s.e.m. of the number of Brn3a-positive cells, from 5 to 7 independent experiments. **p < 0.01, significantly different from control; *p < 0.05, significantly different from water drinking animals with 7 days OHT; Kruskall-Wallis test, followed by Dunn's multiple comparison test.
Notably, we have not observed any detrimental effect related with caffeine administration neither in animals with normal IOP nor with OHT. In fact, caffeine is able to reduce the neuroinflammatory response and increase the survival of RGCs in animals with OHT, in a mechanism independent of lowering IOP.

Herein, we demonstrate that caffeine consumption, as described in other neurodegenerative diseases, prevented the loss of RGCs associated with glaucoma. Taking in account the results obtained in this work, together with our previous works\textsuperscript{15,16}, selective antagonists of A\textsubscript{1}R, in combination with IOP lowering agents, might be envisaged as a potential therapeutic strategy to treat glaucoma.

**Methods**

**Animals.** All procedures involving animals were approved by the Ethical and Animal Studies Committee of the University of Murcia and were in accordance with the ARVO and European Union guidelines for the use of animals in research. Adult female albino Sprague Dawley rats, with 8 to 10 weeks, (Charles River Laboratories, L’Arbresle, France) were housed in the animal facilities of the University of Murcia, Spain, and were provided with standard rodent diet and water ad libitum, under a 12 h light/12 h dark cycle. Recent studies suggest that injury to one eye may produce significant molecular and structural changes in the intact contralateral eye\textsuperscript{35,36,57,58}. Therefore, comparisons were performed using a group of control animals.

**Caffeine administration.** Animals were randomly assigned to receive caffeine or normal drinking water. Caffeine (1 g/L, Sigma-Aldrich, St. Louis, MO, USA) was supplied in the drinking water for two weeks before the induction of OHT and was maintained until the end of the experiment. The chosen dose of caffeine was based in a previous a work that reported protective effects of caffeine intake\textsuperscript{39}.

The animals were divided into 6 experimental groups: 1) control (intact and water drinking); 2) caffeine (normal IOP and caffeine drinking); 3) water drinking + 3 days OHT; 4) caffeine drinking + 3 days OHT; 5) water drinking + 7 days OHT; 6) caffeine drinking + 7 days OHT.

**Induction of OHT and IOP measurements.** To avoid the IOP-lowering effect of anesthetic agents, IOP measurements were performed in non-sedated rats that were trained as previously described\textsuperscript{40}. The IOP was measured in both eyes before surgery (basal) and at days 1, 2, 3, 5 and 7 post-surgery with a rebound tonometer specifically designed for rodents (Tonolab\textsuperscript{9}, Icare, Espoo, Finland). IOP was considered elevated when the mean of IOP measurements collected increase by 30% compared with basal IOP\textsuperscript{41}.

OHT was induced in the left eyes of anesthetized [intraperitoneal injection of xylazine (10 mg/kg) and ketamine (60 mg/kg)] rats in a single session of diode laser burns (Viridis Ophthalmic Photocoagulator-532 nm, Quantel Medical, Clermont-Ferrand, France), as we described previously\textsuperscript{24,27,38}. During recovery, topical ointment containing Tobramycin was applied to the eyes to prevent corneal desiccation.

**Retrograde tracing of retinal ganglion cells.** One day after laser photocoagulation, 3% fluorogold (FG; Fluorochrome Inc., Engelwood, CO, USA) diluted in 10% in DMSO saline was applied onto the surface of both superior colliculi (SCi), as previously described\textsuperscript{24,38,61}. Retinas were analyzed 6 days after the tracing, 7 days after the induction of the OHT.

**Immunodetection.** All animals were euthanized with an intraperitoneal overdose of 20% sodium pentobarbital and then transcardially perfused with saline followed by 4% (w/v) paraformaldehyde (PFA).

**Flat-mounted retinas.** Retinas from both eyes were dissected and then permeabilized with 0.5% Triton X-100 in PBS, followed by freezing at −70 °C for 15 min, and then a new rinse in 0.5% Triton X-100. Brn3a was detected by incubation with a goat anti-Brn3a antibody (1:750; C-20; Santa Cruz Biotechnology, Heidelberg, Germany), prepared in PBS with 2% Tween-20 and 2% normal donkey serum, overnight, at 4 °C. Secondary detection was performed with Alexa Fluor 568 donkey anti-goat conjugated secondary antibody (1:500, Life Technologies, Thermo-Fisher, Madrid, Spain). Finally, retinas were thoroughly washed in PBS and mounted with the RGC layer facing up and covered with anti-fading solution.

**Retinal cryosections.** The eyes were enucleated and post-fixed in 4% PFA for 1 h. Then, the cornea was carefully removed and the eye cup was fixed for an additional 1 h in 4% PFA. After washing in PBS, the tissue was cryopreserved in 15% sucrose in PBS for 1 h, followed by 30% sucrose in PBS for 1 h. The eye cups were embedded in tissue-freezing medium with 30% of sucrose in PBS (1:1), and stored at −80 °C. The tissue was sectioned on a cryostat (18 μm thickness) and the sections were mounted on Superfrost Plus glass slides.

Retinal slices were permeabilized with 0.1% Triton X-100 in PBS, followed by blockade with 10% normal goat serum and 1% bovine serum albumin (BSA) for 1 h. The slices were then incubated with primary antibodies, as follows: rabbit anti-Iba1 (1:1000; Wako, Osaka, Japan), rabbit anti-MHC-II (1:200; AbDSerotec, Oxford, UK), or mouse anti-GFAP (1:500; Merck Millipore, Billerica, MA, USA), in PBS with 1% BSA, overnight, at 4 °C. Secondary detection was performed with Alexa Fluor 568 goat anti-mouse and Alexa Fluor 488 goat anti-rabbit (all 1:500; Life Technologies, Carlsbad, USA). Nuclei were counterstained with DAPI (Life Technologies, Carlsbad, USA) and the slices were mounted with Glycergel (DAKO, Glostrup, Denmark).

The preparations were observed with a confocal microscope (LSM 710, Zeiss, Germany) on an Axio Observer Z1 microscope using an EC Plan-Neofluar 40x/1.30 Oil DIC M27 objective.

**Transmission electron microscopy of optic nerves.** Optic nerve samples were collected at approximately 1 mm from the optic chiasm and fixed with 2.5% glutaraldehyde in 0.1 M sodium cacodylate buffer (pH 7.2), supplemented with 1 mM calcium chloride for 2 h. Following rinsing in the same buffer, post-fixation was performed using 1% osmium tetroxide for 1 h. Samples were then washed in buffer and dehydrated in a graded
Primers (Table 3), as described previously. SYBR Green-based real-time quantitative PCR (qPCR) was performed using StepOnePlus, as previously described. Ct values were converted to “Relative quantification” using the 2−ΔΔCt method previously described. Four candidate housekeeping genes (Tbp, Hprt, Ywhaz and Rhodopsin) were evaluated using NormFinder (a Microsoft Excel Add-in) and Yhwaz was identified as the most stable gene.

TNF and IL-1β protein levels quantification by Enzyme-Linked Immunosorbent Assay (ELISA). Protein levels of IL-1β and TNF were quantified by ELISA, according to the instructions provided by the manufacturer (Peprotech, London, UK). Briefly, total retinas were lysed in 20 mM imidazole-HCl, 100 mM KCl, 1 mM MgCl₂, 1 mM EGTA, 1 mM EDTA, 10 mM NaF, 1% Triton X-100, supplemented with protease and phosphatase inhibitors (Roche, Basel, Switzerland). Then, lysates were sonicated and centrifuged at 16,000 g for 10 min at 4 °C and at 10,000 g for 5 min at 4 °C, respectively. The supernatant was collected and stored at −80 °C until use.

The cytokine concentration of each sample was normalized to the total protein concentration (determined by the bicinchoninic acid protein assay).

Statistical analysis. The results are presented as mean ± standard error of the mean (s.e.m.). The normality of the data was assessed with Shapiro-Wilk normality test. The data were analyzed using Kruskall-Wallis test, followed by Dunn’s multiple comparison test, or Two-Way ANOVA followed by Tukey’s Multiple Comparison Test, as indicated in the figure legends. The statistical analysis was performed in Prism 6.0 Software for Mac OS X (GraphPad Software, Inc).

| Gene   | GenBank number | Forward                        | Reverse                        | Amplicon Size (bp) |
|--------|----------------|--------------------------------|--------------------------------|--------------------|
| Adora 1| NM_017155.2    | 5′-TGAGTTGTGAGAAGCAGAC-3′      | 3′-CAGACGAAAGATGGGGTAAG-5′     | 118                |
| Adora2a| NM_053294      | 5′-GGGTTATGAGGAGCAACAAC-3′     | 3′-TTTTCGACGATAGGTGT-5′        | 106                |
| tnf    | NM_012675      | 5′-GCCAATCTGTGCTAATCTCT-3′     | 3′-TTTTCGACGATAGGTGT-5′        | 90                 |
| il-1β  | NM_031512      | 5′-ATAGAAGTCAACACAAAGTGA-3′    | 3′-GACATGTTGTCCTCTAG-5′        | 109                |
| mhc-ii | NM_013069.2    | 5′-CCACCTGAAGGACGCTGGGA-3′     | 3′-AGAGCTGGTTCTCTGCTC-5′       | 101                |
| Tspo   | NM_012515.2    | 5′-TGTATCGGAGTGGGTGATG-3′      | 3′-GGACAGCTGACAGTCTAG-5′       | 105                |
| trem2  | NM_00116884.1  | 5′-AACCTGACTGCTCACTGAGC-3′     | 3′-CCTGAGCCTTGAAGCTGGT-5′      | 90                 |
| yhwaz  | NM_013011.3    | 5′-CAAGAGTACACAGAAGCATGGTG-3′  | 3′-GGGACAGGCCAGGCATTG-5′       | 76                 |
| Aclb   | NM_031144      | 5′-GGTCTCCTGAGGCAAG-3′         | 3′-CATCTGCTGGAAGGTGGACA-5′     | 75                 |

Table 3. Primers used in qPCR and RT-PCR.
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M.H.M., M.V.S., A.F.A., M.A.B. and A.R.S. conceived and designed the experiments. M.H.M., A.O.M. and E.N.N. performed the experiments. M.H.M., A.O.M., E.N.N., M.V.S., A.F.A., M.A.B. and A.R.S. analyzed the data. M.V.S., A.F.A., M.A.B. and A.R.S. contributed with reagents/materials/analysis tools. M.H.M., A.O.M., E.N.N., M.V.S., A.F.A., M.A.B. and A.R.S. wrote the paper. All contributing authors have read and approved the final version of the manuscript.

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