The retinal pigment epithelium as a gateway for monocyte trafficking into the eye

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Abstract

The choroid plexus epithelium within the brain ventricles orchestrates blood-derived monocyte entry to the central nervous system under injurious conditions, including when the primary injury site is remote from the brain. Here, we hypothesized that the retinal pigment epithelium (RPE) serves a parallel role, as a gateway for monocyte trafficking to the retina following direct or remote injury. We found elevated expression of genes encoding leukocyte trafficking determinants in mouse RPE as a consequence of retinal glutamate intoxication or optic nerve crush (ONC). Blocking VCAM-1 after ONC interfered with monocyte infiltration into the retina and resulted in a local pro-inflammatory cytokine bias. Live imaging of the injured eye showed monocyte accumulation first in the RPE, and subsequently in the retina, and peripheral leukocytes formed close contact with the RPE. Our findings further implied that the ocular milieu can confer monocytes a phenotype advantageous for neuroprotection. These results suggest that the eye utilizes a mechanism of crosstalk with the immune system similar to that of the brain, whereby epithelial barriers serve as gateways for leukocyte entry.

Keywords monocyte-derived macrophages; neuroprotection; optic nerve crush; retinal ganglion cells; retinal pigment epithelium

Subject Categories Neuroscience

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Introduction

The immune privileged status of the central nervous system (CNS), including the eye, brain and spinal cord, has led to the long-held view that immune activity within the CNS is a sign of pathology that should be mitigated. However, extensive research over the past two decades has revealed that the CNS can and should benefit from immune support, just like any other tissue in the body, both under health conditions (Kipnis *et al*, 2004; Ziv *et al*, 2006), and in the context of disease or injury (Rapalino *et al*, 1998; Moalem *et al*, 1999; Simard *et al*, 2006; Yin *et al*, 2006; Beers *et al*, 2008; Kigerl

et al, 2009; Shechter *et al*, 2009; London *et al*, 2011, 2013; Derecki *et al*, 2012).

In studies using animal models of spinal cord and retinal injuries, we found that monocyte-derived macrophages (mo-Mφ) are key players in the recovery from CNS insults (Shechter *et al*, 2009; London *et al*, 2011). Mo-Mφ are spontaneously recruited to the sites of injury, where they exhibit anti-inflammatory activities. Depleting blood monocytes impairs the repair process, whereas boosting their levels by adoptive transfer results in improved recovery (Shechter *et al*, 2009; London *et al*, 2011). Importantly, the anti-inflammatory features of the recruited mo-Mφ were found to be pivotal for their capacity to promote functional recovery after spinal cord injury (Shechter *et al*, 2009) and to provide neuroprotection to retinal ganglion cells (RGCs) after ocular glutamate intoxication (London *et al*, 2011).

The choroid plexus epithelium, which is located in the brain ventricles and forms the blood–cerebrospinal fluid barrier, has been identified as an active interface between the CNS and the immune system, acting both as a site of immunosurveillance and as a gateway that regulates the entry of immune cells to the brain and spinal cord upon need (Kunis *et al*, 2013, 2015; Shechter *et al*, 2013b; Baruch *et al*, 2014). Intriguingly, the infiltration of immune-resolving monocytes to the injured spinal cord was found to occur through this gateway, despite it being located remotely from the primary site of damage (Shechter *et al*, 2013b). In the present study, we explored whether leukocyte trafficking through an epithelial barrier is applicable to other sites of immune privilege and thus investigated whether a similar scenario takes place in the eye.

The interfaces between the retina and the blood circulation include barrier systems reminiscent of those that exist between the circulation and the brain. In the eye, these systems are paralleled by the inner and outer blood–retinal barriers (BRBs). The inner BRB (iBRB) is formed by the nonfenestrated endothelial capillaries within the inner layers of the retina and is similar in structure and function to the blood–brain barrier, whereas the outer BRB (oBRB) is comprised of the retinal pigment epithelium (RPE), Bruch's membrane, and the fenestrated capillaries of the choroid. The tight junctions within the RPE layer maintain the integrity of the oBRB (Kaur *et al*, 2008; Ambati *et al*, 2013; Shechter *et al*, 2013a). The RPE plays an active role in sustaining the immune privileged status of the eye (Holtkamp *et al*, 2001; Streilein, 2003; Ambati *et al*, 2013; Stein-Streilein, 2013) by producing and secreting a variety of

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immunoregulatory factors (Holtkamp *et al*, 2001; Zamiri *et al*, 2007; Ma *et al*, 2009; Detrick & Hooks, 2010; Shechter *et al*, 2013a), and skewing immune cells toward an immunoregulatory phenotype (Liversidge *et al*, 1993; Ishida *et al*, 2003; Sugita *et al*, 2008; Vega *et al*, 2010; Kawazoe *et al*, 2012). RPE supernatants were found to inhibit the production of IL-12 and to increase IL-10 secretion in a macrophage cell line (Zamiri *et al*, 2006), and the co-culturing of BM-derived monocytes with RPE cells was reported to induce their differentiation into myeloid-derived suppressor cells that reduce the severity of experimental autoimmune uveoretinitis (EAU) (Tu *et al*, 2012). Notably, the immunoregulatory microenvironment dictated by the RPE extends to the subretinal space (SRS) (Zamiri *et al*, 2007). The accumulation of macrophages in the SRS is reported to increase with age and in the context of retinal disease, such as age-related macular degeneration (AMD), apparently in association with defective immunomodulation by the RPE (Forrester, 2003; Xu *et al*, 2009; Sennlaub *et al*, 2013).

Here, we hypothesized that the RPE functions as a gateway for trafficking of monocytes into the retina upon damage to RGCs, regardless of whether the insult is inflicted directly on the RGCs or remotely from them. We found that the RPE responds to remote damage in the form of optic nerve crush (ONC), as well as to direct retinal damage, such as ocular glutamate intoxication, with the elevated expression of genes encoding leukocyte trafficking molecules, including VCAM-1, and that mo-M ϕ are recruited to this barrier, apparently *en route* to the retina. Blocking VCAM-1 decreased monocyte infiltration into the retina and resulted in a pro-inflammatory cytokine milieu in the injured eye. Monocytes injected intraocularly after glutamate intoxication localized to the RPE/SRS and conferred neuroprotection to RGCs.

Results

ONC elicits an immune response in the retina and activates the RPE for leukocyte trafficking

To test whether the RPE is involved in monocyte recruitment to the retina when the primary insult is remote from the eye, we used an established model of ONC, in which extensive death of RGCs is seen within days, some of which could be rescued by immune-based neuroprotection (Yoles & Schwartz, 1998; Moalem *et al*, 1999; Levkovitch-Verbin *et al*, 2000; Fisher *et al*, 2001). First, we studied the local immune response at the lesion site. The injury site could be delineated by GFAP staining and by the accumulation of IB-4⁺ activated myeloid cells (Fig 1A). Analysis of optic nerve sections from [*Cx3cr1*^{gfp/+} → WT] BM chimeric mice, in which blood-derived macrophages carry a GFP label (Jung *et al*, 2000; London *et al*, 2011), confirmed that some of these activated myeloid cells represented GFP⁺ blood-derived macrophages (Fig 1B). T cells, depicted by CD3 immunostaining, were also detected at the injury site (Fig 1C), as previously described (Moalem *et al*, 1999). We next analyzed the immune response within the retina by flow cytometry. Immune cells in the retina were identified by pre-gating on the leukocyte marker CD45.2, and then divided into monocytes/macrophages and T cells, based on the expression of CD11b or TCR β , respectively (Fig 1D). Among the CD11b⁺ cells, infiltrating mo-M ϕ were regarded as CD45.2^{hi}, as

opposed to resident microglia, which have been shown to express low/intermediate CD45 levels (Sedgwick *et al*, 1991; Dick *et al*, 1995; Renno *et al*, 1995; Shechter *et al*, 2013b; Zhao *et al*, 2014; O'Koren *et al*, 2016). We verified this finding in [*Cx3cr1*^{gfp/+} → WT] BM chimeric mice, in which we detected the infiltration of CX3CR1-GFP⁺ mo-M ϕ into the retina only after ONC, and not in noninjured control eyes. Importantly, these GFP⁺ cells all expressed high levels of CD45.2 (Fig 1E). Using this gating strategy, we found an elevation in the total number of immune cells in the retina after ONC, including both macrophages and T cells (Fig 1F). Among the macrophages, an elevation was also seen in cells of the CD45^{hi} subset, representing mo-M ϕ (Fig 1F, middle graph).

To evaluate the RPE response to ONC in terms of its expression of genes encoding leukocyte trafficking molecules, RPE complex (consisting of the RPE, choroid, and sclera) was excised from non-injured eyes and from eyes 8 h, or 1, 3, and 7 days after ONC, and analyzed by quantitative real-time PCR. Results showed an early and transient increase in the expression of the integrin ligands *Icam1*, *Vcam1*, and *Madcam1* in the RPE complex after ONC, compared to RPE from the noninjured, contralateral eye (Fig 2A). The expression of ICAM-1 and VCAM-1 by the RPE was verified by immunofluorescence staining (Fig 2B). We also detected an elevation in the expression of chemokines relevant to monocyte chemoattraction and maturation, including the CCR2 ligands, *Ccl2* (MCP-1) and *Ccl12*, the transcript levels of which remained high as long as 1–3 days after the injury (Fig 2C). Kinetics similar to those of the adhesion molecules were seen for the expression of genes encoding for the chemokines *Mcsf*, *Cxcl10*, and *Cxcl12* (SDF-1) (Fig 2C and D), as well as for *Tnfr1* and *Ifngr1* (Fig 2E), receptors for the cytokines TNF- α and IFN γ , which were demonstrated to have synergistic effects in the activation of the brain's choroid plexus epithelium for leukocyte trafficking (Kunis *et al*, 2013).

Monocyte/macrophage dynamics in the RPE and retina after ONC

To gain insight as to whether the RPE could serve as the route of entry for monocytes into the retina after ONC, we followed the timing of appearance of these cells separately in the RPE and in the retina by flow cytometry, 1, 3, and 7 days after the injury. We observed a significant increase in total CD11b⁺ myeloid cells in both the RPE complex and the retina from ONC eyes at all three time points after the injury, as compared to noninjured control tissues (Fig 3A and B, left panels). However, while myeloid cell counts were higher in the RPE on d1 and d3 after the crush, and began decreasing by d7, within the retina these cells showed a distinct pattern, of accumulation over time (Fig 3A and B, left panels, filled bars). The finding that the kinetics of monocyte accumulation was delayed in the retina relative to the RPE supported our contention that the RPE is on the route of these cells into the retina.

To decipher the relative distribution of infiltrating mo-M ϕ as a function of time in the RPE and the retina, we next analyzed the myeloid cell population based on Ly6C expression levels; the levels of Ly6C expressed by infiltrating myeloid cells that are pro-inflammatory are higher than those expressed by infiltrating anti-inflammatory mo-M ϕ (Shechter *et al*, 2013b). The Ly6C^{-/lo} myeloid population may also include resident microglia (London *et al*, 2011, 2013; Butovsky *et al*, 2012; Zigmond *et al*, 2012; Shechter *et al*,

Figure 1. ONC elicits an immune response in the retina, including the infiltration of monocyte-derived macrophages.

- A Representative micrographs of optic nerve sections collected from mice 8 days after ONC, showing the accumulation of IB-4⁺ activated myeloid cells in the injury site, which is demarcated by GFAP staining. Scale bar, 50 μ m.
- B Representative micrograph of the lesion site in optic nerve sections 7 days after crush injury in [Cx₃cr1^{gfp/+} $\rightarrow$ WT] BM chimeric mice, immunostained for GFP (red), to depict monocyte-derived macrophages, and IB-4 (green) to label activated myeloid cells. Scale bar, 100 μ m. Inset scale, 20 μ m.
- C Representative micrograph of optic nerve section 7 days after crush injury, immunostained for CD3 (red, arrows). Scale bar, 100 μ m.
- D Representative dotplots showing the general gating strategy used in flow cytometry experiments for the analysis of immune cells in the retina. Leukocytes were identified by gating on CD45⁺ cells and were further gated on CD11b⁺ or TCR β ⁺ to detect monocytes/macrophages and T cells, respectively. Of the CD11b⁺ cells, infiltrating monocytes were regarded as CD45^{hi}.
- E Representative contour plot from a [Cx₃cr1^{gfp/+} $\rightarrow$ WT] BM chimeric mouse, verifying that infiltrating blood-derived macrophages (CX₃CR1-GFP⁺) are indeed CD45^{hi}, and showing that they are detectable only in retinas from eyes that underwent ONC, while absent in noninjured controls (red vs. blue line). Cells were pre-gated on CD11b. $n = 4$ per group.
- F Quantitative analysis of flow cytometric data, showing the induction of an immune response in the retina after ONC, evident by a general increase in leukocytes (CD45⁺), including CD11b⁺CD45^{hi} cells (representing infiltrating macrophages) and T cells (TCR β ⁺). $n = 4$ per group. Data shown are representative of six independent experiments. Graphs show mean $\pm$ SE of each group. *** $P < 0.001$, Student's t -test.

Data information: FSC, forward scatter; noninj, noninjured control; ONC, optic nerve crush.

Figure 2.

Figure 2. ONC elicits an early and transient elevation in the expression of leukocyte trafficking molecules in the RPE.

- A Changes in mRNA transcript levels of an array of adhesion molecules in retinas from ONC eyes compared with contralateral noninjured retinas, 8 h, and 1, 3, and 7 days after ONC. For the noninj group, $n = 13$ for all genes, except for *Madcam1*, where $n = 12$. For the 8 h group, $n = 6$. For the d1 group, $n = 7$. For the d3 group, $n = 6$, except for *Madcam1*, where $n = 5$. For the d7 group, $n = 7$.
- B Representative micrographs showing the expression of ICAM-1 and VCAM-1 (red) by the RPE (green) 12 h after ONC. Scale bar, 20 μ m.
- C–E Changes in mRNA transcript levels of an array of chemokines (C, D) and cytokine receptors (E) in retinas from ONC eyes compared with contralateral noninjured retinas, 8 h, and 1, 3 and 7 days after ONC. For the noninj group, $n = 13$ for all genes, except for *Cxcl9* and *Cxcl10*, where $n = 12$. For the 8 h group, $n = 6$, except for *Cxcl9* and *Cxcl10*, where $n = 5$. For the d1 group, $n = 7$, except for *Mcsf*, where $n = 6$. For the d3 group, $n = 6$. For the d7 group, $n = 7$, except for *Cxcl9*, where $n = 6$.

Data information: Data shown are representative of three independent experiments. Bar graphs throughout the figure show mean $\pm$ SE of each group. Asterisks above bars indicate significant differences compared with noninjured tissue; significant differences between different time points are indicated by asterisks between bars.

* $P < 0.05$; ** $P < 0.01$; *** $P < 0.001$, one-way ANOVA followed by Tukey's HSD. noninj, noninjured control; ONC, optic nerve crush.

2013b; Yona *et al*, 2013). We detected an early elevation in $\text{Ly6C}^{+/hi}$ mo-M ϕ in both the RPE and the retina (Fig 3A and B, middle panels). Subsequently, while the proportion of $\text{Ly6C}^{+/hi}$ myeloid cells decreased in both compartments, the proportion of $\text{Ly6C}^{-/lo}$ myeloid cells gradually increased in the retina (Fig 3B, right panel), in correlation with the $\text{CX}_3\text{CR1-GFP}^+\text{CD45}^{hi}\text{CD11b}^+$ infiltrating myeloid cells observed in chimeric mice at the same time point following the injury (Fig 1E).

Having observed that VCAM-1 is one of the integrin ligands that exhibited upregulated expression in the RPE after ONC (Fig 2A), we envisioned that the infiltration of immune-resolving mo-M ϕ to the

eye might occur through VCAM-1-VLA-4 interactions, in analogy to the entry of these cells via the remote brain choroid plexus epithelium to the injured spinal cord (Shechter *et al*, 2013b). Knowing that following glutamate intoxication, the infiltrating mo-M ϕ skew the milieu in the retina toward an anti-inflammatory one (London *et al*, 2011), we tested the effect of blocking VCAM-1 on the retinal cytokine milieu after ONC.

Mice were intravenously (i.v.) injected with anti-VCAM-1 antibody (clone M/K-2.7) or with an IgG isotype control antibody immediately after ONC. After 3 days, retinas were collected and analyzed for mRNA expression of *Tnf*, *Il12a*, *Il1b*, and *Tgfb2* by quantitative

Figure 3. Monocyte/macrophage dynamics in the RPE and retina after optic nerve crush.

- A, B Quantitative flow cytometric analysis of myeloid cells in the RPE complex (A) and retinas (B) from injured (filled bars) and contralateral noninjured eyes (empty bars), on days 1, 3, and 7 after ONC. Cells were pre-gated on total leukocytes (CD45^+) and analyzed for the expression of CD11b. CD11b⁺ cells were further discriminated based on their expression levels of Ly6C. $n = 7$ per group, except for d1 noninj retina, where $n = 6$ for $\text{Ly6C}^{+/hi}$, and d1 ONC RPE, where $n = 6$ for CD11b and $\text{Ly6C}^{+/hi}$. Bar graphs throughout the figure show mean $\pm$ SE of each group. Asterisks above bars indicate significant differences compared with noninjured tissue; significant differences between different time points are indicated by asterisks between bars. * $P < 0.05$; ** $P < 0.01$; *** $P < 0.001$, two-way ANOVA and Bonferroni post test. noninj, noninjured control; ONC, optic nerve crush.

Figure 4. Blocking VCAM-1 after ONC affects retinal monocyte infiltration and the local cytokine bias.

Mice were subjected to ONC and i.v.-injected with either an anti-VCAM-1 blocking antibody or an isotype control antibody. Eyes were collected after 2 or 3 days for flow cytometric or real-time PCR analysis, respectively.

A Flow cytometric quantification of myeloid cells in retinas from mice that were treated with anti-VCAM-1 antibody, as compared to isotype-treated controls. $n = 3$ per group. Asterisks above bars indicate significant differences compared with noninjured tissue; significant differences between different antibody treatments are indicated by asterisks between bars; analyzed by two-way ANOVA and Bonferroni post test.

B Expression ratios of *Tnf*, *Il12a*, and *Il1b*, compared to *Tgfb2*, in retinas of mice treated after ONC with anti-VCAM-1 antibody ($n = 13$) or isotype control antibody ($n = 12$). Each antibody treatment group was normalized to its relevant noninjured control. Data shown are representative of two independent experiments. Analyzed by Student's *t*-test.

Data information: Bar graphs throughout the figure show mean $\pm$ SE of each group. * $P < 0.05$; ** $P < 0.01$; *** $P < 0.001$. noninj, noninjured control; ONC, optic nerve crush.

real-time PCR. Blocking VCAM-1 resulted in decreased myeloid cell counts in the retinas of ONC mice, as compared to retinas from injured mice injected with an isotype control antibody (Fig 4A). The reduction in myeloid cells was accompanied by a pro-inflammatory bias in the retinal cytokine milieu in anti-VCAM-1-treated mice compared to mice treated with control IgG (Fig 4B). Overall, these results supported the involvement of VCAM-1 in the recruitment of monocytes that contribute to retinal immune homeostasis.

Live imaging of infiltrating monocytes in the eye

To further substantiate the RPE as an entry route of monocytes to the retina after injury, we took advantage of the optical properties of the retinal epithelium in the pigmented C57BL/6J mice and employed a double-labeling live imaging approach, in which CX₃CR1-GFP⁺ BM monocytes were additionally labeled *ex vivo* with a near-infrared lipophilic tracer, DiR, and injected i.v. into recipient mice several hours after ONC. In these mice, which were live-imaged through the lens of the eye, cells located posterior to the RPE could be detected in the near-infrared channel, but their GFP label was not visible beyond the pigmented layer; only upon the infiltration of these monocytes into the retina could their GFP tag be detected as well. Live *in vivo* imaging of these mice in the first days after the injury revealed the accumulation of DiR⁺ cell clusters in ONC eyes, but not in noninjured contralateral eyes (Fig 5A, left panels). Furthermore, in the injured eyes, unlike their noninjured controls, we found DiR⁺ clusters that were co-localized with GFP, indicating the infiltration of monocytes into the retina (Fig 5A, bottom right panel). In the noninjured eye, DiR⁺ cells could be seen circulating through blood vessels in the retina, but not adhering nor infiltrating the parenchyma (Movie EV1). Notably, at early time points after ONC, we could detect only DiR⁺ clusters and no double-labeled cells in the injured eye, in accordance with the flow

cytometry results that suggested the sequential recruitment of monocytes from the oBRB to the retina (Fig 3).

To rule out entry due to iBRB breakdown, which is known to be associated with inflammation in the eye (Parnaby-Price *et al*, 1998; Kerr *et al*, 2008a), we searched for GFP⁺ cells in association with retinal blood vessels; no such cells could be found (Fig 5A). To further verify that the cells did not enter the retina due to a breach in iBRB integrity, we excised ONC and control eyes and processed them for immunostaining for laminin and β -dystroglycan. As a positive control, we used sections of eyes from mice in which EAU was induced, as it has been shown that β -dystroglycan is lost at sites of leukocyte infiltration and blood–brain barrier breakdown in autoimmune inflammatory disease (Agrawal *et al*, 2006; Wolburg-Buchholz *et al*, 2009). We observed that the β -dystroglycan signal was indeed diminished in retinas from mice at the peak of EAU, but remained intact in retinas after ONC, in a manner comparable to noninjured controls (Figs 5B and EV1), indicating that ONC did not induce breakdown of the BRB.

To focus on the spatial association of peripheral immune cells with the RPE, we examined histological sections from the eyes of [*Actb^{egfp/+}* $\rightarrow$ WT] BM chimeric mice, whose peripheral immune cells express a GFP reporter. Sections from mice that had undergone ONC and from noninjured contralateral eyes were immunostained for the specific RPE marker, RPE65, and for GFP, to detect peripheral immune cells. Whereas only sporadic GFP⁺ cells could be seen around the choroid and RPE of control eyes, a clear accumulation of GFP⁺ cells was found in the injured eyes, both around the RPE and within the retina and vitreous (Fig 5C). Moreover, in the ONC eyes, we could detect single GFP⁺ cells that were in close contact with the RPE, and which extended processes toward this layer (Fig 5D). GFP⁺ cells that had infiltrated the retina could only be found within the injured eyes, where they localized near the ganglion cell layer (GCL), in the vicinity of RGC cell bodies (Fig 5E), as we have

previously observed in the case of glutamate intoxication (London *et al*, 2011).

Taken together, these results demonstrated that the recruitment of peripheral leukocytes to the retina after remote ocular injury involves the RPE and does not necessitate breaching of retinal endothelial barriers.

The ocular milieu can confer monocytes a neuroprotective phenotype

Having previously established the contribution of spontaneously recruited mo-Mφ to RGC protection after retinal glutamate intoxication (London *et al*, 2011), we analyzed the RPE for expression of leukocyte-homing determinants following this type of ocular insult as well. Transcript levels of the integrin ligands *Icam1*, *Vcam1*, and *Madcam1* were elevated in the RPE complex 1 day after glutamate intoxication (Fig 6A), as was the expression of *Ccl2* and *Mcsf* (Fig 6B), *Cxcl10* and *Cxcl12* (Fig 6C) and the cytokine receptor, *Tnfr1* (Fig 6D). Notably, *Ccl5* (RANTES), which was shown to be associated with intraocular inflammation and recruitment of Th1 cells into the eye (Crane *et al*, 2006), was not altered following glutamate intoxication (Fig 6B). The expression of ICAM-1 and VCAM-1 by the RPE was confirmed at the protein level by immunofluorescence staining (Fig 6E).

Considering the immunoregulatory capacity of the RPE/SRS milieu (Streilein, 2003; Zamiri *et al*, 2007; Detrick & Hooks, 2010; Shechter *et al*, 2013a; Stein-Streilein, 2013), we hypothesized that monocytes infiltrating the retina after injury may come in contact with this compartment on their route from the circulation into the retina, which could possibly affect them to acquire activities characteristic of myeloid cells that can contribute to inflammatory resolution (Zamiri *et al*, 2006; Tu *et al*, 2012). We adopted the protocol of i.v. injection of monocytes following glutamate intoxication (London *et al*, 2011), and searched within ocular sections for the injected CX₃CR1-GFP⁺ monocytes. Some of the i.v.-injected cells could be found in the SRS, in the vicinity of the RPE, and expressed the anti-inflammatory cytokine, IL-10 (Fig 7A), which was previously shown to mediate their neuroprotective effect after this type of insult (London *et al*, 2011). The presence of monocytes at the SRS, taken together with the upregulated expression of leukocyte trafficking molecules that we detected in the RPE after injury, reinforced the notion that this might reflect their natural homing route.

Next, to evaluate whether the ocular milieu has any role in conferring mo-Mφ a neuroprotective phenotype, we bypassed the

presumed gateway and directly injected CX₃CR1-GFP⁺ monocytes into the vitreous of glutamate-intoxicated eyes. Quantitative analysis of surviving RGCs, based on their immunoreactivity for the RGC marker, Brn3a (Nadal-Nicolas *et al*, 2009; London *et al*, 2011), revealed that RGC survival was significantly higher in injured retinas of monocyte-injected eyes compared to PBS-injected injured controls (Fig 7B and C), demonstrating that monocytes directly encountering the eye milieu could acquire a phenotype supportive of RGC survival. We next traced intravitreally (i.v.t.)-injected monocytes in ocular sections 7 days after the injury. The injected monocytes were found to be localized to the vitreous cavity, to which they were injected, as well as adjacent to the injured RGCs, within the GCL (Fig 7D and E, left panels), as previously described (London *et al*, 2011). Intriguingly, some of the cells were also detected in the SRS, in the vicinity of the RPE (Fig 7D and E, right panels). Immunofluorescence staining with the aim of characterizing the fate of the injected monocytes revealed that although the injected cells expressed the pro-inflammatory cytokines TNF-α and IL-1β (Fig EV2), most of them also expressed an array of anti-inflammatory cytokines and neurotrophic factors, including IL-10 (90.24 ± 5.96% of GFP⁺ cells), TGF-β (92.64 ± 9.33%), arginase-1 (92.84 ± 8.1%), IGF-1 (expressed by 100% of GFP⁺ cells detected) and BDNF (85.83 ± 8.66%), as well as the scavenger receptor, CD36 (92.74 ± 3.63%) (Fig 7D and E). Together, these findings suggested that the interaction of infiltrating monocytes with the RPE/SRS milieu might play a role in shaping their activities, even when the site of entry is bypassed.

Discussion

In this study, we characterized the RPE as a potential site that orchestrates the recruitment of monocytes to the retina under different “sterile” insults, including when the injury is remote from the eye, and demonstrated that neuroprotective mo-Mφ come in contact with the RPE/SRS milieu upon their infiltration to the retina.

Our live imaging results, together with the observed association of monocytes with the RPE in two distinct injury models that differ in their nature and location, both of which elicited an increase in the expression of leukocyte trafficking molecules by this epithelial tissue, lead us to suggest that the recruitment of monocytes that can potentially benefit the eye could take place via the RPE. Notably, the function of the RPE as a gateway between the circulation and the retina has mainly been studied in the context of retinal disease

Figure 5. Live imaging of infiltrating monocytes in the eye.

- A Live imaging of noninjured and ONC eyes, showing the presence of DiR⁺ monocyte clusters in injured eyes only. Some of the cell clusters are co-localized with GFP, indicating monocytes that have crossed the RPE and infiltrated the retina. Data shown are representative of two independent experiments, each involving the imaging of eyes from either 2 or 3 mice.
- B Representative micrographs of eye sections collected from mice 1 day after ONC, or 29 days after the induction of EAU, stained for β-dystroglycan (red, arrows) and laminin (green), showing a comparable structure of blood vessels in the inner retina of ONC eyes and noninjured controls, which is different from the appearance of blood vessels in EAU retina. Scale bar, 50 μm.
- C–E [*Actb*^{Cre/Cre} → WT] BM chimeric mice were generated and subjected to ONC. (C) Representative micrographs showing the abundance of GFP⁺ cells (green) in the RPE (RPE65, red) and retina of the ONC eye compared to noninjured contralateral eye. Retinal layers can be distinguished by Hoechst staining (blue; scale bar, 100 μm). (D) Higher magnification micrographs of ONC eyes, showing the association of blood-derived GFP⁺ cells (green, arrows) with the RPE (red); note processes extending from the cells to the RPE (scale bar, 20 μm). (E) Representative micrograph of ONC eye showing GFP⁺ cells (green) in proximity to the GCL, the site of dying retinal ganglion cells (scale bar, 20 μm). *n* = 4 per group.

Data information: EAU, experimental autoimmune uveoretinitis; GCL, ganglion cell layer; noninj, noninjured control; ONC, optic nerve crush; ONH, optic nerve head; RPE, retinal pigment epithelium.

Figure 5.

Figure 6. The expression of leukocyte trafficking molecules by the RPE is upregulated following ocular glutamate intoxication.

A–D Changes in mRNA transcript levels of a series of adhesion molecules (A), chemokines (B, C), and cytokine receptors (D) in injured retinas compared with contralateral noninjured retinas, assessed 1 day after GT intoxication. $n = 6$ per group. Bar graphs show mean $\pm$ SE of each group. * $P < 0.05$; ** $P < 0.01$; *** $P < 0.001$, Student's t -test.

E Representative micrographs showing the expression of ICAM-1 and VCAM-1 (red) by the RPE (green) 1 day after GT intoxication (scale bar, 20 μ m).

Data information: GT, glutamate; noninj, noninjured control.

A IL-10 / CX₃CR1-GFP / Hoechst

i.v. injection

B Brn3a **C** **D** IL-10 / CX₃CR1-GFP / Hoechst **TGF-β** / CX₃CR1-GFP / Hoechst **Arg-1** / CX₃CR1-GFP / Hoechst **E** IGF-1 / CX₃CR1-GFP / Hoechst **BDNF** / CX₃CR1-GFP / Hoechst **CD36** / CX₃CR1-GFP / Hoechst

Figure 7.

Figure 7. Neuroprotective monocytes localize to the RPE/SRS after glutamate intoxication.

- A Representative micrograph of an eye from day 7 after glutamate intoxication, from a mouse that was i.v.-injected with monocytes, showing monocyte localization to the SRS after this mode of application, and indicating a potential homing route for the cells (IL-10, red; CX₃CR1-GFP, green). Scale bar, 100 μ m; inset scale, 20 μ m.
- B–E Mice were injected i.v.t. with either monocytes or PBS 1 day after GT intoxication, and ocular sections from 7 days after the injury were analyzed. (B, C) Representative micrographs of noninjured and injured retinas stained for the RGC marker, Brn3a (red; scale bar, 100 μ m), and quantification of RGC survival ($n = 4$ per group). Arrows indicate Brn3a⁺ cells. Bar graph shows mean $\pm$ SE of each group. * $P < 0.05$, Student's *t*-test. Investigators were blinded to the identity of the groups during the quantification of RGC survival. (D, E) Representative micrographs of GT-intoxicated eyes that were i.v.t.-injected with monocytes, showing the expression of an array of cytokines and neurotrophic factors (red) by the injected monocytes (CX₃CR1-GFP, green). The injected cells were found in the vitreous and in proximity to injured RGCs (left panels), as well as in the SRS, adjacent to the RPE (right panels). Insets show higher magnification of representative cells (scale bar, 100 μ m; inset scale, 20 μ m). $n = 4$ per group.
- Data information: GCL, ganglion cell layer; GT, glutamate; mon, monocytes; RGC, retinal ganglion cell; RPE, retinal pigment epithelium; SRS, subretinal space; vit, vitreous.

and has therefore been associated with inflammation (Dua *et al*, 1991; Greenwood *et al*, 1994; Omri *et al*, 2011). Nevertheless, it has been proposed that even during inflammation, the RPE maintains an immunosuppressive role (Crane & Liversidge, 2008). In EAU, the early infiltration of immune cells was reported to occur through the inner retinal vessels, in a process involving perivascular cuffing, while the oBRB remains intact. At later stages of the disease, immune cells could also be seen in proximity to the RPE (Dua *et al*, 1991; Greenwood *et al*, 1994), possibly corresponding to the infiltration of cells with distinct properties necessary for the resolution of inflammation. In line with those findings, Kerr and colleagues more recently described the accumulation of alternatively activated macrophages in the SRS at the resolution phase of EAU (Kerr *et al*, 2008a).

It is important to emphasize that our results do not negate that leukocyte trafficking through the RPE can be destructive under certain conditions, nor that immune cells can enter the retina via additional sites, which do not involve the disruption of endothelial barriers. For instance, the ciliary body and optic nerve vessels were found to act as entry routes for blood-borne monocytes to the retina after light-induced injury, without obvious breakdown of the iBRB (Joly *et al*, 2009). In our present study, we found that following ONC blood-derived monocytes are found at the lesion site, as well as at the retina, indicating a potential migratory pathway for the cells between the optic nerve and the retina. Nevertheless, the close association of peripheral monocytes with the RPE, as shown in our results, including in areas remote from the optic nerve head, as well as the activation of this tissue for leukocyte trafficking, emphasizes the involvement of the RPE in monocyte trafficking into the retina in the models we studied.

The integrin ligand VCAM-1, which was reported to facilitate leukocyte migration through the RPE *in vitro* (Devine *et al*, 1996), was elevated here in the RPE after injury. Thus, using an antibody against VCAM-1 to block monocyte trafficking resulted in skewing of the retinal cytokine milieu toward a pro-inflammatory one. Along the same lines, blocking VCAM-1 after spinal cord injury inhibits the recruitment of inflammation-resolving monocytes through the choroid plexus epithelium, resulting in worse functional outcomes (Shechter *et al*, 2013b). Collectively, these findings link VCAM-1-dependent entry of monocytes to the restoration of postinjury homeostasis in the neuronal tissue. Notably, we previously reported that a pro-inflammatory milieu persists in the injured neuronal tissue when monocyte infiltration is inhibited, vis-à-vis the ongoing accumulation of resident microglia (Shechter *et al*, 2009; London *et al*, 2011), suggesting that this

pro-inflammatory setting results, at least in part, from the uncontrolled activity of microglia in the absence of inflammation-resolving mo-M ϕ (Cohen *et al*, 2014).

This study highlights an aspect of the eye's immune privilege, which has hitherto been underappreciated; we propose that rather than serving as an immunosuppressive barrier that prevents entry into the eye, leukocyte trafficking involving the RPE/SRS milieu may provide a mechanism that allows infiltrating monocytes and perhaps other immune cells to acquire a phenotype favorable for repair. Importantly, in the present study, monocytes injected directly into the damaged eye after glutamate intoxication were effective in conferring neuroprotection to RGCs in a manner similar to monocytes trafficking from the circulation (London *et al*, 2011), substantiating that beyond the route of entry, the ocular milieu is also involved in controlling the activity of immune cells.

Our present results reinforce the recently reported roles of mo-M ϕ in supporting CNS recovery (Simard *et al*, 2006; Town *et al*, 2008; Kigerl *et al*, 2009; Koronyo-Hamaoui *et al*, 2009; Shechter *et al*, 2009, 2013b; London *et al*, 2011, 2013; Derecki *et al*, 2012; Koronyo *et al*, 2015). Mo-M ϕ can serve a source of a variety of molecules including cytokines, growth factors, and neurotrophins that benefit tissue repair. Accordingly, they can promote cell survival, renewal and regeneration, regulate matrix remodeling and remyelination, and resolve local inflammation (Barrette *et al*, 2008; Shechter *et al*, 2009, 2013b; London *et al*, 2011; Miron *et al*, 2013). Importantly, the spontaneous recruitment of these cells to the CNS after injury is suboptimal, but is amenable to boosting by systemic modulation or direct injection of monocytes to the CNS territory (Rapalino *et al*, 1998; Shechter *et al*, 2009; London *et al*, 2011; Baruch *et al*, 2016). As demonstrated in the present study, the eye is no exception to this rule. We found that the i.v.t.-injected monocytes expressed IL-10, which was previously reported to mediate the neuroprotective effect of mo-M ϕ after glutamate intoxication (London *et al*, 2011). The cells additionally expressed neurotrophic factors such as IGF-1 and BDNF, which may also account, in part, for their neuroprotective potential (Rocha *et al*, 1999; Kermer *et al*, 2000; Nakazawa *et al*, 2002; Morimoto *et al*, 2005). Notably, the expression of both pro- and anti-inflammatory cytokines by these cells highlights their heterogeneity and reflects their ability to respond to and affect the immunological milieu in the eye to best support repair (Mosser & Edwards, 2008; Sica & Mantovani, 2012; Novak & Koh, 2013).

The results of this study bear important implications to age-related degenerative diseases of the eye. For example, in AMD, the leading cause of blindness among the elderly in the Western World, RPE dysfunction is a critical factor. Animals that are impaired in

immune cell trafficking, such as mouse strains deficient in CCL2-CCR2 or fractalkine-CX₃CR1 signaling, show degenerative changes in the retina with age and are often used to model some of the pathological aspects of AMD (Ambati *et al*, 2003; Tuo *et al*, 2007; Chan *et al*, 2008; Luhmann *et al*, 2009). It was suggested that this local chemokine signaling is needed for immune-mediated retinal repair (Ambati *et al*, 2013), and specifically that an impaired monocytic response can tip the balance between a protective immune response and harmful inflammation at the retina–choroid interface (Chen *et al*, 2011). Along these lines, prevention of monocyte entry into the eye has been shown to promote choroidal neovascularization, one of the cardinal features of the exudative (“wet”) form of AMD (Apte *et al*, 2006). While the role of monocytes/macrophages in AMD is still a matter of debate (Espinosa-Heidmann *et al*, 2003; Skeie & Mullins, 2009; Sennlaub *et al*, 2013), it is clear that defective immune modulation is a key factor in the pathogenesis of this disease (Bird, 2010; Ambati & Fowler, 2012; Ambati *et al*, 2013). Thus, while the recruitment of immune cells may be a purposeful process in AMD, as in other pathologies in the CNS and the rest of the body, it may go awry if either the immune or the barrier systems dysfunction, as occurs, for instance, in aging or under neurodegenerative conditions (Baruch *et al*, 2014, 2015; Schwartz & Baruch, 2014; Robbie *et al*, 2016). Although the spontaneous response to CNS insult, in the absence of manipulation, is apparently insufficient to support significant repair, appreciation of the roles played by circulating immune cells in the healing process, and the importance of the route of their recruitment in shaping their activity may reveal targets for their augmentation in a controlled way toward better recovery.

Materials and Methods

Mice

Adult male (8–12 wk old) C57BL/6J mice, B6-EGFP and heterozygous *Cx₃cr1^{GFP/+}* transgenic mice (B6.129P-*Cx3cr1^{tm1Litt}/J*, in which one of the CX₃CR1 chemokine receptor alleles was replaced with a gene encoding GFP (Jung *et al*, 2000)) were used. Animals were supplied by Harlan Laboratories and the Animal Breeding Center of the Weizmann Institute of Science and were maintained and handled in compliance with the regulations formulated by the Institutional Animal Care and Use Committee of the Weizmann Institute of Science.

Preparation of BM chimeras

[*Cx₃cr1^{GFP/+}* → WT] BM chimeric mice were prepared as previously described (Rolls *et al*, 2008; Shechter *et al*, 2009). WT recipient mice received lethal whole-body irradiation (950 rad) while shielding the head, thus preventing any direct effects on the retina and/or infiltration of myeloid cells other than those related to the injury. On the next day, the mice were reconstituted with 5×10^6 *Cx₃cr1^{GFP/+}* BM cells. This protocol resulted in BM chimerism levels of 50–70%.

[*Actb^{esfp/+}* → WT] BM chimeras were prepared as previously described (Cohen *et al*, 2014), by subjecting mice to lethal split-dose γ -irradiation (300 rad followed 48 h later by 950 rad with head protection). One day following the second irradiation, the mice were injected with 5×10^6 EGFP BM cells. Using this protocol, an average of 90% chimerism was achieved.

Chimeric mice were subjected to injury 8–12 wk after BM transplantation.

Glutamate intoxication injury

Mice were anesthetized and treated with local anesthesia (Localin, Dr. Fischer) applied directly to the eye, and were injected i.v.t. with a total volume of 1 μ l saline containing 400 nmol L-glutamate (Sigma), as previously described (Schori *et al*, 2002).

Optic nerve crush injury

Under a binocular operating microscope, the conjunctiva of the right eye of deeply anesthetized mice was incised, and the optic nerve was exposed. With the aid of cross-action forceps, the optic nerve of one eye was subjected to a severe crush injury 1–2 mm from the eyeball.

EAU induction

Mice were immunized subcutaneously with 500 μ g IRBP 1–20 peptide (GPTHLPQPSLVLDMAKVLLD) in a 1:1 emulsion with CFA containing *Mycobacterium tuberculosis* strain H37.RA (Difco) at 2.5 mg/ml (1.25 mg/ml final). Mice were co-injected intraperitoneally with 1 μ g *Bordetella pertussis* toxin (Sigma).

Adoptive monocyte transfer

BM cells were harvested from the femora and tibiae of mice and enriched for mononuclear cells on a Ficoll density gradient. The CD115⁺ BM monocyte population was isolated through MACS enrichment using biotinylated anti-CD115 antibodies and streptavidin-coupled magnetic beads (Miltenyi Biotec), according to the manufacturer's protocols. Following this procedure, monocytes were injected i.v. through the tail vein ($4\text{--}5 \times 10^6$ cells per mouse) or i.v.t. (1×10^5 per mouse).

DiR labeling of monocytes

For *in vivo* imaging experiments, purified monocytes were resuspended in 1 ml PBS and added to a labeling solution consisting of 9 ml PBS and 50 μ l of the near-infrared lipophilic dye, DiR (1,1'-di-octadecyl-3,3,3',3'-tetramethylindotricarbocyanine iodide, molecular probes; 0.75 mg/ml in 100% ethanol). The cells were incubated in the solution for 40 min at 37°C, with gentle agitation. After incubation, the cell suspension was added to a 50-ml tube containing 10% serum in PBS and centrifuged. Cells were resuspended in PBS and i.v. injected through the tail vein ($4\text{--}5 \times 10^6$ cells per mouse). Protocol was adapted from Kalchenko *et al* (2006).

Administration of blocking antibody

A rat antibody directed to VCAM-1 (clone M/K-2.7, BioXCell) or matching IgG1 isotype control (clone HRPN, BioXCell) was injected at 200 μ g per mouse through the tail vein, concurrently with ONC.

Histology and immunofluorescence

After intracardiac perfusion with PBS, eyes were removed, fixed in 2.5% paraformaldehyde (PFA) for 24 h, transferred to 70% ethanol,

and then embedded in paraffin, as previously described (Shechter *et al.*, 2007). Throughout the study, 6- μ m-thick paraffin sections were used. The following antibodies were used for immunolabeling: rabbit anti-glial fibrillary acidic protein (GFAP; 1:100; Dako), rabbit anti-CD3 (1:1,000; Dako), rabbit anti-GFP (1:100; MBL), biotinylated goat anti-GFP (1:100; Abcam), mouse anti-Brn3a (1:50; Santa Cruz Biotechnology, Inc.), goat anti-IL-10 (1:20; R&D Systems), mouse anti-TGF- β 1, 2, 3 (1:300; R&D Systems), mouse anti-arginase-1 (1:100; BD Biosciences), goat anti-TNF- α (1:20; R&D Systems), rabbit anti-IL-1 β (1:50; Santa Cruz Biotechnology, Inc.), mouse anti-CD36 (1:100; BD Biosciences), goat anti-IGF-1 (1:20; R&D Systems), rabbit anti-BDNF (1:200; Alomone Labs), mouse anti-RPE65 (1:100; Novus Biologicals), mouse anti- β -dystroglycan (1:50; Abcam), and chicken anti-laminin (1:500; Abcam). The M.O.M. immunodetection kit (Vector Laboratories) was used to detect mouse primary monoclonal antibodies. For labeling of activated myeloid cells, FITC-conjugated *Bandeiraea simplicifolia* isolectin B4 (IB-4; 1:40; Sigma-Aldrich) was added for 1 h to the secondary antibody solution. Secondary antibodies used included Cy2/Cy3-conjugated donkey anti-mouse, anti-rabbit, anti-goat, or anti-chicken antibodies (1:150–1:200; all from Jackson ImmunoResearch Laboratories, Inc.). Cy2-streptavidin was used for goat anti-GFP staining. The slides were exposed to Hoechst stain (1:2,000; Invitrogen) for 1 min for nuclear staining. For microscopic analysis, a fluorescence microscope (Eclipse 80i; Nikon) was used. The fluorescence microscope was equipped with a digital camera (DXM1200F; Nikon) and with either a 20 $\times$ NA 0.50 or 40 $\times$ NA 0.75 objective lens (Plan Fluor; Nikon). Recordings were made on postfixed tissues at 24°C using NIS-Elements, F3 (Nikon) acquisition software. Images were cropped, merged, and optimized using Photoshop, and arranged using Illustrator (both Adobe).

Isolation of RPE and retina and flow cytometry

Following intracardiac perfusion with PBS, eyes were gently dissected in HBSS under a binocular microscope to separately obtain eyecups (RPE, choroid and sclera) and retinas, which were processed to single-cell suspensions, as previously described (Kerr *et al.*, 2008b). The following fluorochrome-labeled mAbs were purchased from BioLegend or eBioscience and used according to the manufacturers' protocols: PE-conjugated anti-CD11b antibody; PerCP-cy5.5-conjugated anti-Ly6C antibody; allophycocyanin (APC)-conjugated anti-CD45.2 and TCR β antibodies; FITC-conjugated anti-CD45.2 and CD11b antibodies; and Pacific Blue/Brilliant Violet-conjugated anti-CD45.2, CD11b, and TCR β antibodies. Cells were analyzed on a FACS LSRII cytometer using FACSDiva software (BD Biosciences). Analysis was performed with FlowJo software (Tree Star, Inc.). In each experiment, relevant negative and positive control groups were used to determine the populations of interest and to exclude the others.

Quantitative real-time PCR

Total RNA was extracted separately from RPE complex and retinas using RNA MicroPrep kit (Zymo Research). mRNA was converted into cDNA using High Capacity Reverse Transcription Kit (Applied Biosystems; AB) for RPE, or qScript cDNA Synthesis Kit (Quanta Biosciences) for retinas. The expression of specific mRNAs was assayed using fluorescence-based quantitative real-time PCR with selected gene-specific primer pairs:

Gene Name	Primer Sequence
<i>Gapdh</i>	Forward: 5'-AATGTGTCCGTCGTGGATCTGA-3' Reverse: 5'-GATGCCTGCTTACCACCTTCT-3'
<i>Actb</i>	Forward: 5'-TCAGCAAGCAGGAGTACGATGA-3' Reverse: 5'-TGCGCAAGTTAGGTTTTGTCAA-3'
<i>Icam1</i>	Forward: 5'-AGATCACATTACGGTGCTGGCTA-3' Reverse: 5'-AGCTTTGGGATGGTAGCTGGAAGA-3'
<i>Vcam1</i>	Forward: 5'-TGTGAAGGATTACGAGGCTGGA-3' Reverse: 5'-CCATGTTTCGGGCACATTCCACA-3'
<i>Madcam1</i>	Forward: 5'-AGCACTCCGTGAAGATCCTTGTGT-3' Reverse: 5'-TAGCAGGGCAAGGAGAGACTGTT-3'
<i>Ccl2 (Mcp1)</i>	Forward: 5'-CATCCAGTGTTGGCTCA-3' Reverse: 5'-GATCATCTTGCTGGTGAATGAGT-3'
<i>Ccl12</i>	Forward: 5'-CCTCAGGTATTGGCTGGAC-3' Reverse: 5'-GACACTGGCTGCTTGTGATT-3'
<i>Ccl5</i>	Forward: 5'-GTGTCCTCAATCTTGACAGTCGTGT-3' Reverse: 5'-ACTTCTTCTCTGGGTGGCACACA-3'
<i>Mcsf</i>	Forward: 5'-CCACATGATTGGGAATGGAC-3' Reverse: 5'-GTAGCAACAGGATCATCCA-3'
<i>Cx3cl1 (Fractalkine)</i>	Forward: 5'-ATGTGCGACAAGATGACCTCACGA-3' Reverse: 5'-TTTCTCCTTCGGGTGAGCAGACAGAA-3'
<i>Cxcl9</i>	Forward: 5'-GAGTTCGAGGAACCTTAGTG-3' Reverse: 5'-AACTGTTTGAGGTCTTTGAGG-3'
<i>Cxcl10</i>	Forward: 5'-AACTGCATCCATATCGATGAC-3' Reverse: 5'-GTGGCAATGATCTCAACAC-3'
<i>Cxcl12</i>	Forward: 5'-CATCAGTGACGGTAAACCAG-3' Reverse: 5'-TTTCAGATGCTTGACGTTGG-3'
<i>Ifngr1</i>	Forward: 5'-GTCTTATAACTTGAAACCTGTCTC-3' Reverse: 5'-CCAGGAACCCGAATACAC-3'
<i>Ifngr2</i>	Forward: 5'-TCCCACACCCATTACAG-3' Reverse: 5'-AGGTCCAACAGTAACATTCTC-3'
<i>Tnfr1</i>	Forward: 5'-CTTCATCAGTTAATGTGCCGA-3' Reverse: 5'-AGCCTCTCTCTTTGACAG-3'
<i>Tgfb2</i>	Forward: 5'-AATTGCTGCCTTCGCCCTTTTAC-3' Reverse: 5'-TGACAGGCTGAGGACTTTGGTGT-3'
<i>Tnf</i>	Forward: 5'-AAATGGCCTCCCTCTCATCAG-3' Reverse: 5'-GCTTGCTACTCGAATTTGAGAAG-3'
<i>Il12a (Il12-p35)</i>	Forward: 5'-TCACCTGTGATGGTACAG-3' Reverse: 5'-AAATGAAGCTCTGCATCTGTC-3'
<i>Il1b</i>	Forward: 5'-ACAACAAAAAGCCTCGTGCTG-3' Reverse: 5'-CCATTGAGGTGGAGAGCTTCA-3'

Reactions were performed using AB Fast SYBR[®] Green PCR Master Mix. Each sample was run in triplicate. Amplification conditions were as follows: 95°C for 20 s, followed by 40 cycles of 95°C for 3 s, 60°C for 30 s. Dissociation curves showed a single species of amplicon for each primer combination. The relative amounts of mRNA were calculated using the standard curve method and normalized to either *Actb* (β -actin; RPE complex) or *Gapdh* (retinas). All quantitative real-time PCRs were performed and analyzed using the StepOnePlus Real-Time PCR System (AB).

In vivo fluorescence imaging

Mice were anesthetized and gently immobilized using a plastic apparatus. For visualization of the retina, a drop of 1% atropine sulfate

followed by 10% phenylephrine (both from Dr. Fischer) were used to dilate the pupil, and a drop of ophthalmic lubricant (Celluspan, Dr. Fischer) was used to allow placement of a glass coverslip on the eye.

Mice were placed under a Mono Zoom Microscope MVX10 (Olympus, Japan) equipped with a fluorescence illuminator and a Pixelfly QE charge-coupled device (CCD) camera (PCO, Kelheim, Germany). The excitation and emission for the near-infrared (NIR) filter set was 710/50 nm and 810/90 nm (long pass), respectively. The green filter set was 475/30 nm for excitation and 530/40 nm for emission. Fluorescence exposure time was 50 ms. Images were acquired using the Camware camera-controlling software program (PCO). Image analysis was performed using Fiji/ImageJ software (Schindelin *et al*, 2012).

Statistical analysis

Sample sizes were chosen with adequate statistical power on the basis of the literature and past experience. Levene's test was used to check equality of variance. In the case of equal variances, data were analyzed using unpaired Student's *t*-test to compare between two groups, or by one- or two-way ANOVA to compare several groups. Tukey's HSD or Bonferroni post test were used for follow-up pairwise comparison of groups after the null hypothesis was rejected ($P < 0.05$). In the case of unequal variances, data were log-transformed to achieve equal variances when possible; otherwise, the Mann–Whitney *U*-test was used to compare between two groups and the Kruskal–Wallis test was used to compare several groups, followed by Dunn's test. Results are presented as mean $\pm$ SE, and *y*-axis error bars in the graphs represent SE. *n* represents the number of animals/biological replicates. Animal inclusion and exclusion criteria were pre-established according to the IACUC. Data points were excluded from analysis if they were more than 2 standard deviations away from the mean.

Expanded View for this article is available online.

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Author contributions

IB, under the mentoring of MS, conceived the general ideas of this study, designed and performed the experiments, analyzed and interpreted the data, and prepared it for presentation. KR performed and analyzed some of the experiments and provided critical discussion. VK assisted with designing and performing the live imaging experiments and their analysis. The manuscript was written by IB and MS.

Conflict of interest

The authors declare that they have no conflict of interest.

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