# Scavenger Function of Resident Autofluorescent Perivascular Macrophages and Their Contribution to the Maintenance of the Blood–Retinal Barrier

*Luísa Mendes-Jorge,*[1,2] *David Ramos,*[2] *Mariana Luppo,*[2,3] *Cristina Llombart,*[2,3] *Graça Alexandre-Pires,*[1] *Victor Nacher,*[2,3,4] *Verónica Melgarejo,*[2] *Miguel Correia,*[5] *Marc Navarro,*[2,3,4] *Ana Carretero,*[2,3,4] *Sabrina Tafuro,*[2,4] *Alfonso Rodriguez-Baeza,*[6] *José António Esperança-Pina,*[5] *Fàtima Bosch,*[2,4,7] *and Jesús Ruberte*[2,3,4]

**Purpose.** The retina contains two distinct populations of monocyte-derived cells: perivascular macrophages, and microglia. The present study was undertaken to evaluate the presence and function in mouse and human retinas of a subtype of resident perivascular macrophages with scavenger function, different from microglia, in physiological conditions and during retinopathy.

**Methods.** Perivascular macrophages were characterized by means of confocal microscopy, electron microscopy, and flow cytometry analyses. Two murine models of blood–retinal barrier breakdown and photoreceptor degeneration were used to analyze the role of these macrophages during retinopathy.

**Results.** The macrophages analyzed constituted a small population of resident perivascular cells different from microglia, since they were Iba-1 negative. Although these cells expressed F4/80 and CD11b antigens in common with microglia, they also expressed BM8 and MOMA-2 epitopes, which are macrophagic markers not expressed by microglia. Perivascular macrophages emitted autofluorescence due to cytoplasmic inclusions containing protein-bound oxidized lipids. They constitutively expressed the scavenger receptor class A and moved along blood vessels, providing an additional coating to thinner areas of the basement membrane. Moreover, they accumulated blood-borne horseradish peroxidase and acetylated low-density lipoprotein in healthy retinas. In addition, during blood–retinal barrier breakdown and photoreceptor degeneration, these cells migrated to the lesion site.

**Conclusions.** All these morphologic and functional features are consistent with those described for brain Mato cells. Thus, this study showed the presence of autofluorescent perivascular macrophages, different from microglia, with a scavenger function that may contribute to the maintenance of the blood–retinal barrier in healthy conditions and that are also involved in retinopathy. (*Invest Ophthalmol Vis Sci.* 2009;50:5997–6005) DOI:10.1167/iovs.09-3515

Autofluorescent perivascular macrophages different from microglia were described in the brain by Mato et al.[1] These cells contain autofluorescent inclusion bodies and are localized around blood vessels, between the blood vessel basement membrane and the astrocyte end feet.[1] They fulfill a major role in the scavenging of blood-borne proteins and lipids. In fact, the autofluorescent inclusion bodies have been categorized as lysosomes rich in hydrolytic enzymes that can efficiently concentrate and digest these substances.[1,2] Autofluorescent perivascular macrophages participate in blood–brain barrier (BBB) function, specifically in the exclusion of blood-borne molecules from the brain,[2] and are also implicated in hypertension and stroke.[3]

The blood–retinal barrier (BRB) is a homologue of the BBB,[4] since the retina is an outgrowth of the brain and exclusion of blood-borne molecules is also required for normal retinal function. Perivascular cells have been described in normal retina.[5] A small population of macrophages and resident microglial cells both acting as antigen presenting cells have been found surrounding retinal blood vessels.[6] However, no perivascular cells with scavenger function have been described in healthy retina. Microglia only express scavenger receptors when activated in response to injury.[7,8] In this report, we describe and characterize a retinal autofluorescent perivascular macrophage (APM) population. These macrophages moved along retinal blood vessels, coating protein low-density areas in the basement membrane, scavenging and accumulating blood-borne molecules without BRB disruption. In mannitol-induced BRB breakdown and in experimental and human retinopathy, these perivascular cells migrated to the lesion site.

## Materials and Methods

### Animals

One- to six-month-old ICR mice were used. C57Bl/6 mice present loss of 2F8 immunoreactivity as a consequence of scavenger receptor class A polymorphism[9] and their inability for recognize 2F8 antibody led us to use ICR mice to study scavenger function in the APMs. The mice were fed ad libitum with a standard diet (Panlab SL, Barcelona, Spain).

From the [1]Interdisciplinary Center of Research in Animal Health (CIISA), Faculty of Veterinary Medicine, Universidade Técnica de Lisboa, Lisbon, Portugal; the [2]Center of Animal Biotechnology and Gene Therapy, and Departments of [3]Animal Health and Anatomy and [7]Biochemistry and Molecular Biology, School of Veterinary Medicine, Universitat Autònoma de Barcelona, Bellaterra, Spain; [4]CIBER de Diabetes y Enfermedades Metabólicas Asociadas (CIBERDEM), Barcelona, Spain; the [5]Faculty of Medical Sciences, Universidade Nova de Lisboa, Lisbon, Portugal; and the [6]Faculty of Medicine, Universitat Autònoma de Barcelona, Bellaterra, Spain.

Supported by two grants from Instituto de Salud Carlos III (FIS PI061837 and CIBER de Diabetes y Enfermedades Metabólicas Asociadas) and the Interdisciplinary Centre of Research in Animal Health (CIISA) from the Faculty of Veterinary Medicine, Lisbon, Portugal (72.Retina).

Submitted for publication February 4, 2009; revised May 21, 2009; accepted September 8, 2009.

Disclosure: **L. Mendes-Jorge**, None; **D. Ramos**, None; **M. Luppo**, None; **C. Llombart**, None; **G. Alexandre-Pires**, None; **V. Nacher**, None; **V. Melgarejo**, None; **M. Correia**, None; **M. Navarro**, None; **A. Carretero**, None; **S. Tafuro**, None; **A. Rodriguez-Baeza**, None; **J.A. Esperança-Pina**, None; **F. Bosch**, None; **J. Ruberte**, None

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Corresponding author: Jesús Ruberte, Center of Animal Biotechnology and Gene Therapy (CBATEG), Edifici H, Campus UAB, Bellaterra 08193, Barcelona, Spain; jesus.ruberte@uab.es.

Animal care and experimental procedures were performed according to the ARVO Statement for Use of Animals in Ophthalmic and Vision Research.

## Human Retinas

Human retinas obtained from a 89-year-old female and an 83-year-old male donor were formalin fixed and used in the experiments.

## Lipid Solubility Experiments

Six mouse retinas were treated with 70% methanol (1 hour) and ethyl ether (20 minutes) for lipid extraction.[10] Human retinas were stained with oil red O to demonstrate the presence of lipids in perivascular macrophage cytoplasmic inclusions.[10]

## Analysis of Autofluorescence

Autofluorescence fingerprints of 10 wholemount, unstained retinas and their corresponding retinal pigment epithelium attached to the inner surface of choroids were analyzed. The lambda mode of a laser scanning confocal microscope (TCS SP2; Leica Microsystems, Wetzlar, Germany) with an excitation laser of 488 nm was used.

## Immunohistochemistry

The retinas were fixed in 10% neutral buffered formalin for 1 hour at room temperature. Each one of the antibodies was used for the six different mouse retinas. After they were washed in 0.01-M phosphate-buffered saline (PBS), wholemount retinas or paraffin-embedded sections were incubated over night at 4°C with the following markers: rabbit anti-mouse collagen IV (Chemicon- Millipore Iberica SAU, Madrid, Spain) at 1:200 dilution; rabbit anti-mouse glial fibrillary acidic protein (GFAP; Dako, Barcelona, Spain) at 1:500 dilution; rabbit anti-mouse Iba-1 (Wako, Neuss, Germany) at 1:3000 dilution; rat anti-mouse CD11b and rat anti-mouse anti-2F8 (Serotec, Raleigh, NC) at 1:100 and 1:50 dilutions, respectively; rat anti-mouse F4/80 (CI:A3-I; BMA Biomedicals, Augst, Switzerland) at 1:100 dilution; rat anti-mouse BM8 (eBioscience, San Diego, CA) at 1:50 dilution; rat anti-mouse MOMA-2 (Abcam, Cambridge, UK) at 1:50 dilution and also with the biotinylated *Lycopersicon esculentum* lectin (20 μg/mL; Vector Laboratories, Burlingame, CA). After they were washed in PBS, the retinas were incubated at 4°C over night with specific secondary antibodies: biotinylated anti-rabbit (1:100) and anti-rat mouse absorbed (1:100) IgGs (Vector Laboratories). After the retinas were washed in PBS, an infrared fluorochrome Cy5 (GE Healthcare Espana SA, Alcobendas, Spain) at 1:500 dilution was used to avoid interference with yellow autofluorescence capture; the incubation was made at 4°C overnight. Nuclear counterstaining with Hoechst stain solution (Sigma-Aldrich, Química, Spain) and iodide (To-Pro-3; Invitrogen SA-Molecular Probes, Barcelona, Spain) was performed for microscopic analysis with the laser scanning confocal microscope (TCS SP2; Leica Microsystems).

## Transmission Electron Microscopic Analysis

Retinal fragments from five different mice were fixed in 2.5% glutaraldehyde and 2% paraformaldehyde, postfixed in 1% osmium tetroxide, stained in aqueous uranyl acetate, dehydrated, and embedded in epoxy resin. Ultrathin sections (70 nm) were stained using lead citrate and examined by a transmission electron microscope (H-7000; Hitachi, Tokyo, Japan).

## Time-Lapse Imaging of Perivascular Cell Movement

Four retinas were cultured in fresh Dulbecco's modified Eagle's medium supplemented with 10% fetal calf serum (Sigma-Aldrich), 2 mM glutamine (Sigma-Aldrich) penicillin and streptomycin, in a $CO_2$/air (5% / 95%) humidified atmosphere at 37°C. Time-lapse imaging was performed in a laser-scanning confocal microscope (Leica; TCS SP2). Retinal cell viability was determined by neutral red staining (Sigma-Aldrich).

## Flow Cytometry

Each one of the antibodies was tested in 36 retinas in three different experiments. Each experiment was performed on 12 retinas to obtain enough cells for flow cytometry analysis. After cell suspension, the retinas were pooled to obtain three samples of four retinas each, to repeat the analysis. The retinas were mechanically disaggregated and further digested with collagenase B (Roche, Madrid, Spain), followed by filtration with 30-μm preseparation filters (Miltenyl Biotec, Madrid, Spain) to obtain a single cell suspension. For PBMC purification, blood was diluted 1:1 with PBS, layered over a density gradient (Histopaque-1.083; Sigma-Aldrich), and centrifuged. Mononuclear cells were collected from the interface. APC-conjugated anti-CD11b (BD Pharmingen, Madird, Spain), biotinylated anti-BM8 (eBioscience), and biotinylated anti-2F8 (Serotec) antibodies and Cy5-conjugated streptavidin (GE Healthcare) were used. Isotyped matched antibody served as the control. Samples were analyzed with a flow cytometer (FACScanto; BD Pharmigen). Autofluorescence was detected in the FLH2 channel.

## Protein Intravenous Administration Experiments

**Single Injection.** HRP type II (Sigma-Aldrich), 15 mg/mL, and DiI acetylated-low-density lipoprotein (DiI-ac-LDL; Molecular Probes Europe BV, Leiden, The Netherlands) 200 μg/mL, in physiological saline solution (PSS), were injected in two different groups of four animals. Six hours after HRP injection and 2 hours after DiI-ac-LDL injection, the retinas were fixed in formalin. HRP was revealed with 3′3′-diaminobenzidine (Sigma-Aldrich). Red fluorescence emitted by DiI showed the localization of DiI-ac-LDL. Images were acquired with an epifluorescence microscope (Eclipse E-800; Nikon Corp., Tokyo, Japan) with a digital camera (DXM 1200F; Nikon Corp.).

**Combination of Bis-benzimide and HRP Injections.** Ten minutes after tail injection of 250 μL of a 0.5% solution of Bis-benzimide (Hoechst stain, H33258) (Calbiochem, San Diego, CA) dissolved in PSS, a second injection of 250 μL of HRP type II (Sigma-Aldrich), 15 mg/mL in PSS, was performed in four mice. One hour after HRP injection, the mice were euthanatized and the retinas were formalin fixed at room temperature for 30 minutes. HRP was revealed with 3′3′-diaminobenzidine and nuclear counterstaining was performed with iodide (To-Pro-3; Molecular Probes).

**Combination of Mannitol, Bis-benzimide, and HRP Injections.** Four mice were intravenously injected with 250 μL of a 25% solution of mannitol (Sigma-Aldrich) dissolved in PSS. Ten minutes later, a combination of bis-benzimide and HRP injections was performed as previously described. The retinas were obtained 10 minutes later and HRP was revealed with 3′3′-diaminobenzidine. Nuclear counterstaining was performed with iodide (To-Pro-3; Molecular Probes).

Images in these experiments were acquired with an epifluorescence microscope (Eclipse E-800; Nikon Corp.) with a digital camera (DXM 1200F; Nikon Corp.) and a laser scanning confocal microscope (TCS SP2; Leica).

## Mouse Retinopathy Model Experiments

Four groups of eight mice each were intraperitoneally injected with 100 mg/kg of sodium iodate ($NaIO_3$; Sigma-Aldrich) in PSS and euthanatized immediately after injection or 1, 2, or 3 days after treatment. A group injected only with PSS was used as the control.

## Statistical Analysis

The number of autofluorescent cells present in the inner retina (ganglion cell layer+inner plexiform layer+inner nuclear layer) and the outer retina (outer plexiform layer+external nuclear layer+inner and outer photoreceptor segments) after sodium iodate injection was counted. Three wholemount retinas per group, half sectioned, and each half, with the inner or the outer retina facing up, were counterstained with iodide, to visualize the nuclei, and were analyzed by laser confocal microscopy, using the same settings used to reveal autofluorescence. Ten random fields at 63×, corresponding to a retinal area of

56,691 $\mu m^2$ each were counted immediately after injection and 24 and 48 hours after injection. The results are expressed as the mean ± SEM.

## Results

### Autofluorescent Perivascular Cells in Mouse Retina

Confocal microscopy analysis revealed the presence of autofluorescence in cells surrounding retinal blood vessels (Fig. 1). Autofluorescence was detected in granular inclusions close to the cellular nuclei (Figs. 1A, 1B, 1C). Oxidized lipids are known to produce fluorescence.[1] To determine whether perivascular cell autofluorescent inclusions contained protein-bound oxidized lipids, we treated the retinas with lipid solvents. Methanol and ethyl ether remove lipids from cells, whereas protein-bound lipids are insoluble to lipid solvents.[10] Autofluorescence was not released from perivascular cell inclusions treated with methanol and ethyl ether (Figs. 1B, 1C). These results suggest that protein-bound oxidized lipids are present in the autofluorescent inclusions. Fluorescence produced by lipofuscin accumulation has been described in perivascular microglia in aging mice.[11] Lipofuscin fluorescence is faded by lipid solvents[12]; therefore, autofluorescence in retinal perivascular cells was probably not due to lipofuscin. In addition, the emission spectrum of both perivascular cell autofluorescent inclusions and retinal pigment epithelium lipofuscin granules was evaluated. The fluorescence fingerprint of autofluorescent inclusions (Fig. 1D) was different from that of lipofuscin (Fig. 1E), which was consistent with the fact that perivascular cell autofluorescence was due to protein-bound oxidized lipids and not to lipofuscin.

### Topography of Autofluorescent Perivascular Cells in the Retina

Retinas immunohistochemically labeled for collagen IV, a basement membrane marker,[13] showed that autofluorescent perivascular cells were located externally to blood vessel basement membrane (Figs. 1F, 1H). Thus, these cells were not pericytes or smooth muscle cells, since these latter are components of the vascular wall and are completely surrounded by the basement membrane.[14] Retinas immunohistochemically labeled for glial fibrillary acidic protein (GFAP), a marker of neuroglial cells,[15] revealed that glial end feet did not separate autofluorescent perivascular cells from the blood vessel basement membrane (Figs. 1G, 1H). Transmission electron microscopic (TEM) analysis confirmed the localization of autofluorescent perivascular cells in the retinal perivascular space (Fig. 2). The location of retinal autofluorescent perivascular cells was frequently associated with endothelial cell tight junctions (Fig. 2B). Osmium tetroxide, used during postfixation in TEM studies, binds to lipids leading to electron density of protein-bound oxidized lipids.[10] Similarly to brain Mato cells, retinal autofluorescent perivascular cells presented lipidic electron-dense inclusion bodies (Figs. 2B, 2C, 2D). A schematic representation of retinal autofluorescent perivascular cell topography is presented in Figure 2E.

### Cell Type of Retinal Autofluorescent Perivascular Cells

Immunocytochemistry and histochemistry revealed that retinal autofluorescent perivascular cells were not microglia, since they were Iba-1$^-$ (Fig. 3A) and only faintly marked by tomato lectin (Fig. 3B). Iba-1 is a calcium-binding protein of microglial cells present in rodents,[16] and recently has been considered the most reliable marker of microglia in mouse retina.[17] The tomato lectin, a lectin obtained from *Lycopersicon esculentum*, with affinity for poly-*N*-acetyl lactosamine sugar residues, has also been reported to mark microglia in mouse retina.[17] The tomato lectin labeling of autofluorescent perivascular cells was substantially different from that of microglial cells, which exhibit a characteristic ramified or ameboid cytoplasmic labeling with this marker (Fig. 3B). Immunocytochemistry also revealed that retinal autofluorescent perivascular cells were retinal macrophages since they expressed CD11b (Fig. 3C) and

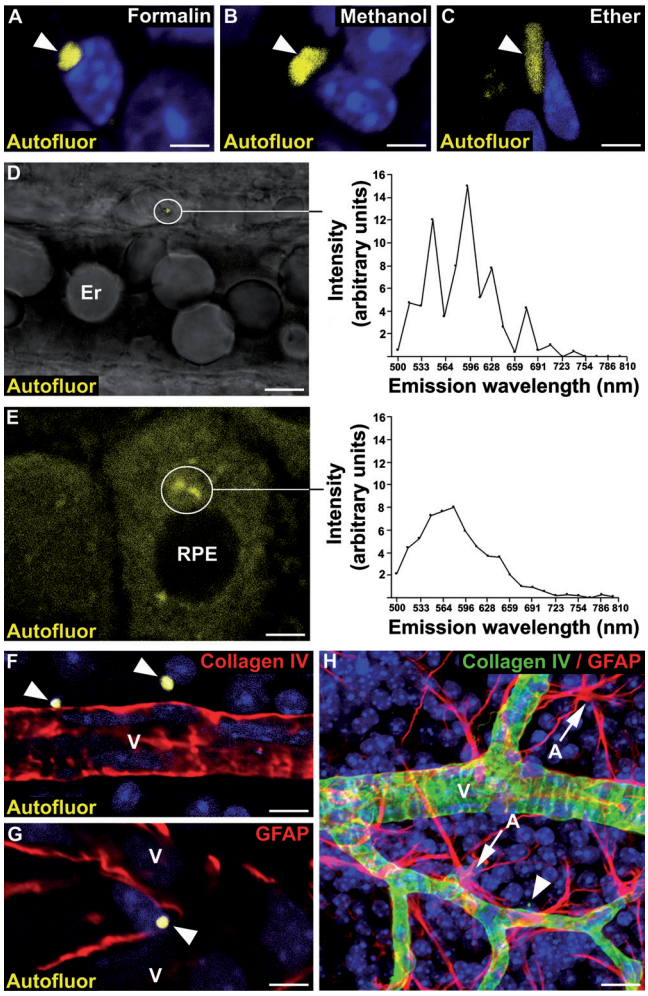


**Figure 1.** Autofluorescent perivascular cells in mouse retina. Laser confocal analysis of wholemount retinas showed autofluorescent granules (*arrowheads*) close to the cellular nuclei (**A–C**). Autofluorescence was not removed by methanol (**B**) or ethyl ether (**C**) treatment and was similar to that observed in formalin-fixed retinas (**A**), indicating that protein-bound oxidized lipids were probably present in the composition of autofluorescent inclusions. The emission fingerprints of perivascular cell autofluorescent inclusions (**D**, *circle*) and of retinal pigment epithelium lipofuscin (**E**, *circle*) were evaluated. They were markedly different. For instance, the maximum emission intensity for lipofuscin in retinal pigment epithelium cells corresponded to the lower levels of emission intensity of perivascular cell autofluorescent inclusion bodies. Retinas immunohistochemically marked for collagen IV (**F**, **H**) and GFAP (**G**, **H**) showed that retinal autofluorescent perivascular cells (*arrowheads*, **F**, **G**, **H**) were localized outside the blood vessel basement membrane and that glial end feet may cover these cells without separating them from the blood vessel wall. (**H**) A projection of several single confocal sections; in this image, the autofluorescence and the collagen IV signal were recorded in the same detection channel. Nuclei (*blue*) were counterstained with iodide (**A–C**) and Hoechst (**F–H**). Autofluor, autofluorescence; A, astrocyte; Er, erythrocyte; RPE, retinal pigment epithelium; V, blood vessel. Scale bars: (**A**) 8 $\mu$m; (**B**) 5 $\mu$m; (**C**, **D**) 6 $\mu$m; (**E**) 2.5 $\mu$m; (**F**) 9 $\mu$m; (**G**) 5.5 $\mu$m; (**H**) 19 $\mu$m.

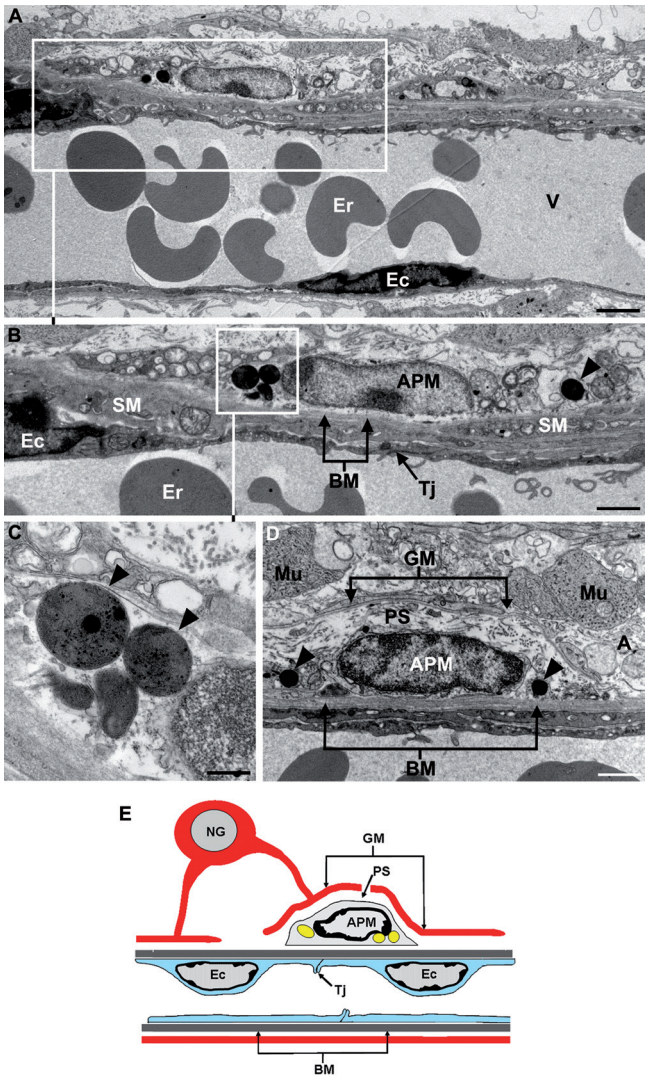


**FIGURE 2.** Topography of retinal autofluorescent perivascular cells. TEM studies revealed autofluorescent perivascular cells with electron-dense cytoplasmic inclusions (*arrowheads*) located in the perivascular space between the blood vessel basement membrane and the glial membrane (**A**–**D**). Autofluorescent perivascular cells were generally located in close contact with intraendothelial junctions (**B**). (**C**) A high magnification of the electron-dense cytoplasmic inclusions showing that they were not homogenously electron-dense and suggesting that they are secondary lysosomes containing osmiophilic lipids. (**E**) Schematic representation of autofluorescent perivascular cell topography. APM, autofluorescent perivascular macrophage; A, astrocyte; BM, basement membrane; Ec, endothelial cell; Er, erythrocyte; GM, glial membrane; Mu, Müller cell; NG, neuroglial cell; SM, smooth muscle cell; Tj, tight junction; V, blood vessel; PS, perivascular space. Scale bars: (**A**) 1.95 μm; (**B**) 1.38 μm; (**C**) 375 nm; (**D**) 870 nm.

F4/80 antigens (Fig. 3D), which are both markers for murine macrophagic cells.[18,19] Moreover, retinal autofluorescent perivascular cells were not microglia since they also expressed the BM8 antigen (Fig. 3E), a 125-kDa extracellular membrane protein sensitive to 2-mercaptoethanol, which is not expressed by microglial cells.[20,21] Flow cytometry analysis showed that autofluorescent CD11b- and BM8-positive cells constituted approximately 20% of retinal macrophages and that they were not found in blood (Figs. 3F, 3G), indicating that retinal autofluorescent perivascular cells were resident macrophages. Overall these findings suggest that retinal autofluorescent perivascular cells are a subset of the macrophagic cell lineage and do not constitute microglial cells.

## Movement of Retinal Autofluorescent Perivascular Cells

Perivascular cells migrate along brain blood vessels.[22] To investigate the retinal autofluorescent perivascular cells' dynamic behavior, we performed time-lapse laser scanning confocal imaging. Autofluorescent perivascular cells were identified by their characteristic autofluorescence, and blood vessels were identified in transmission mode after gelatinized India ink injection. Time-lapse sequences showed that retinal autofluorescent perivascular cells moved in an oscillatory manner along the external surface of the vascular wall at rates up to 15 μm/h (Fig. 4A). Retinal venules presented thinner areas in the basement membrane as a consequence of lower expression of collagen IV (Fig. 4B), similarly to what occurs in cremasteric venules.[23] Retinal autofluorescent perivascular cells were observed in close contact with collagen IV low-density areas in the basement membrane (Fig. 4B).

## Expression of Scavenger Receptor Class A and Accumulation of Blood-Borne Molecules by APMs in Healthy Conditions

In the brain, APMs are considered scavenger cells.[2] To determine whether retinal APMs expressed scavenger receptors, the anti-2F8 antibody, a specific marker for macrophage scavenger receptor class A (SR-A),[24] was used. Confocal microscopy and flow cytometry showed that retinal APMs expressed SR-A (Figs. 5A, 5B). These scavenger receptors appeared surrounding autofluorescent inclusions, suggesting that SR-A were localized in the lysosomal membrane. The percentage of autofluorescent 2F8-positive cells in retina was similar to that of autofluorescent BM8-positive cells (Fig. 3G), suggesting they are the same cellular population. Microglia only expresses scavenger receptors when activated in response to injury.[7,8] Therefore, these findings suggest that retinal autofluorescent perivascular cells are a perivascular macrophagic cell type with scavenger function in healthy conditions.

In the brain APMs accumulate blood-borne proteins under physiological conditions.[1,2] HRP is a tracer used to assess The BRB's integrity.[25,26] To investigate whether retinal autofluorescent perivascular cells accumulate blood-borne proteins without BRB disruption, HRP was intravenously injected in healthy mice. As expected, no HRP leakage from blood vessels was found in healthy retinas, even when a threefold higher concentration of HRP than that used in previous studies of BRB integrity was used.[27] However, 6 hours after intravenous injection of HRP, perivascular cells, in a position and distribution compatible with that of APMs, presented multiple HRP brownish inclusions in their cytoplasm (Fig. 6A). To examine whether retinal autofluorescent perivascular cells accumulate blood-borne low-density lipoproteins (LDLs), DiI-ac-LDL was intravenously injected in healthy mice. Two hours after injection, DiI cytoplasmic red granular fluorescence was observed in cells in a position and distribution compatible with that of APMs (Fig. 6B).

To rule out the possibility that APMs that take up HRP molecules in healthy retina are circulating monocytes/macrophages that, after taking up plasma HRP, migrate through the vessel wall, we performed an experiment with the combination of Bis-benzimide and HRP tail vein injections. Bis-benzimide interacts with DNA and stains nuclei of all cell types. In the retina, as this stain does not cross the BRB because the tight junctions between endothelial cells prevent access of the stain to extravascular elements, no nuclear staining is expected in the retinal parenchyma.[28] However, when the BRB is dis-

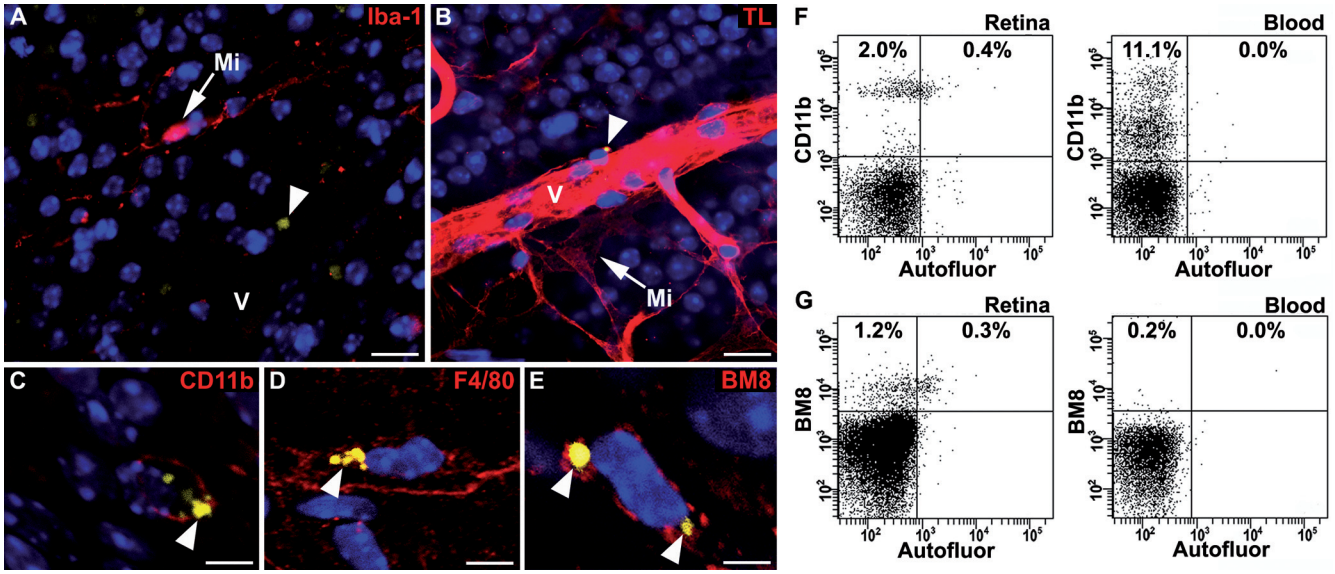


**FIGURE 3.** Characterization of autofluorescent perivascular cell immunophenotype. Retinal autofluorescent perivascular cells did not express Iba-1 (**A**) and were only faintly marked by tomato lectin. Tomato lectin was not specific for microglia and marked the blood vessels strongly (**B**). Retinal autofluorescent perivascular cells expressed CD11b (**C**), F4/80 (**D**), and BM8 (**E**) antigens. Representative flow cytometry dot plots of CD11b (**F**) and BM8 (**G**). Immunostaining for cells purified from mouse retinas and blood confirmed that autofluorescent perivascular cells were a subtype of resident macrophages. Percentages of single- and double-positive cells with respect to the total number of cells are shown. Autofluor, autofluorescence; Mi, microglia; v, blood vessel. *Arrowheads*: autofluorescent inclusions. Nuclei were counterstained with Hoechst. Scale bars: (**A**) 13 μm; (**B**) 19 μm; (**C**) 5 μm; (**D**) 8 μm; (**E**) 4 μm.

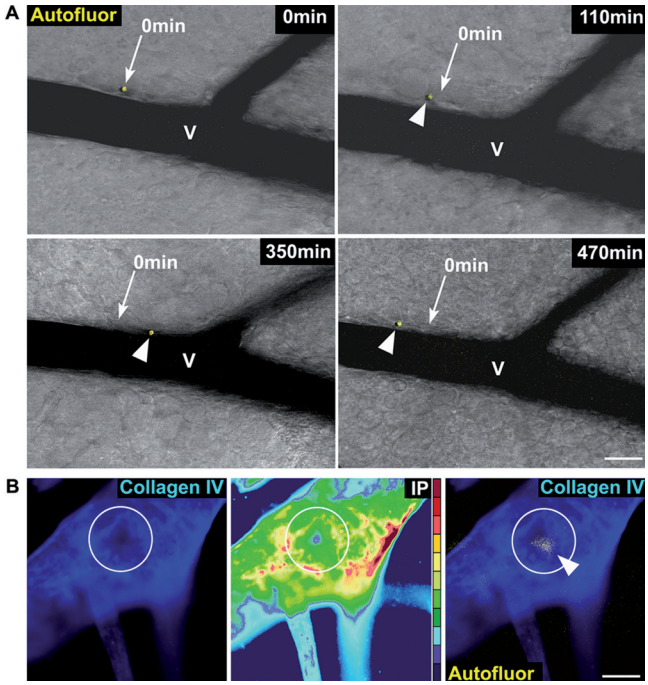


**FIGURE 4.** Kinetic behavior of retinal autofluorescent perivascular cell. (**A**) Time-lapse sequence showed that autofluorescent perivascular cells moved in an oscillatory manner along the external surface of retinal blood vessels (v). Autofluorescence (*arrowhead*) was used to detect autofluorescent perivascular cells by laser confocal microscopy. Blood vessels (v) were identified in transmission mode by India ink injection. (**B**) Autofluorescent perivascular cells (*arrowhead*) were preferentially found coating thinner areas of basement membrane with a low expression of collagen IV. IP, intensity profile. The intensity color scale is shown for reference (*right*). Scale bars: (**A**) 17 μm; (**B**) 6 μm.

rupted, the dye can access the retinal parenchyma labeling neurons and glia.[29] In our experiment Bis-benzimide was injected 10 minutes before HRP. This situation allows us to mark all intravascular monocytes/macrophages before the HRP injection. Thus, any HRP-loaded monocyte/macrophage that migrates through the vessel wall will be detected by both nuclear bright blue Bis-benzimide staining and HRP brownish cytoplasmic content.

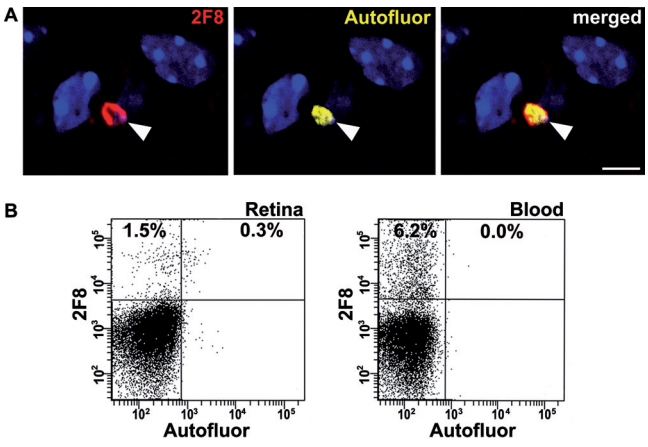


**FIGURE 5.** Retinal APMs expressed scavenger receptors. (**A**) The 2F8 signal (*red*) was observed specifically around the autofluorescent granular inclusions (*arrowhead*) of retinal APMs, suggesting that SR-A were localized in the membrane of granular inclusions. (**A**) Confocal images of single sections, with nuclei counterstained with Hoechst (*blue*). (**B**) Representative flow cytometry dot plots of 2F8 immunostaining of cells purified from mouse retinas and blood confirmed the presence of an autofluorescent cell population with SR-A in the retina. This suggests that autofluorescent perivascular cells are a subtype of resident macrophages that express SR-A. Percentages of single- and double-positive cells with respect to the total number of cells are shown. Scale bar, 6 μm.

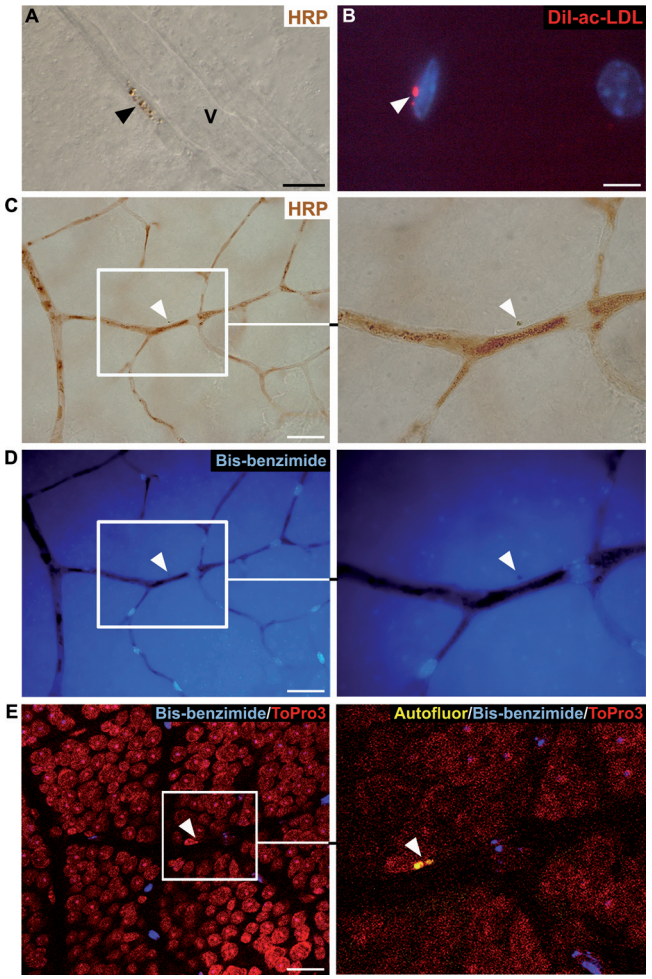


**FIGURE 6.** Retinal APMs have a scavenger function. (**A**) Six hours after HRP intravenous injection, retinas revealed with DAB showed that only perivascular cells, in a position and distribution compatible with that of APMs, presented intracytoplasmic *brown*-stained granules (*arrowhead*). (**B**) Two hours after intravenous injection of ac-LDL marked with the red fluorescent emitter DiI, *red* fluorescent intracytoplasmic granules (*arrowhead*) were observed in retinal perivascular cells, in a position and distribution compatible with that of APMs. Nuclei counterstained with Hoechst. (**C–E**) showed the same area of the inner retina 60 minutes after intravenous injection of Bis-benzimide and HRP. (**C**) Bright-field microscopic image. HRP was retained in the blood vessels, suggesting that the BRB was not broken. High magnification showed DAB *brown*-stained granules (*arrowhead*) in a position compatible with that of APMs. (**D**) Epifluorescence image. Only endothelial cell nuclei were stained with Bis-benzimide, confirming that the BRB was not broken. High magnification showed that the nucleus of the assumed APM is not stained by Bis-benzimide. *Arrowhead*: HRP granules. (**E**) Confocal laser microscopic single section. Iodide counterstaining confirmed that the nucleus of the cell containing the HRP granules (*arrowheads*) was not stained with Bis-benzimide. High magnification also confirmed that the cell containing the HRP granules was autofluorescent. v, blood vessel. Scale bars: (**A**) 12 $\mu$m; (**B**) 7 $\mu$m; (**C–E**) 18 $\mu$m.

The results obtained showed that, as expected, only the perivascular macrophages contained HRP (Fig. 6C), and, in addition, no Bis-benzimide nuclear staining of HRP loaded APMs was observed (Figs. 6D, 6E). This result suggests that APMs are not circulating cells that, after taking up plasma HRP, migrate through the vessel wall. Taken together, all these results suggest that retinal autofluorescent perivascular cells scavenge and accumulate blood-borne proteins in healthy retinas.

## Migration of Retinal APMs during BRB Breakdown

To better understand the scavenging function of APMs, we performed an experiment in which the BRB was disrupted with mannitol. Mannitol induces an osmotic opening of the BRB, with widening of the interendothelial tight junctions to an estimated radius of 200 Å.[30] Plasma HRP was seen to leak from the blood vessels to the retinal parenchyma after mannitol-induced BRB breakdown (Fig. 7A). As expected, cellular nuclei in the leakage area were marked with Bis-benzimide (Fig. 7B), confirming that the BRB was broken. The analysis of autofluorescence by laser confocal microscopy showed that autofluorescent cells quickly (30 minutes) migrate to the leakage area (Fig. 7C). Some of the migrating autofluorescent cells in the leakage area were not marked by Bis-benzimide (Fig. 7C), suggesting that these autofluorescent cells migrate from non-BRB–disrupted areas to the lesion site. These results reinforce the scavenging function of APMs during BRB breakdown.

## Migration of Retinal APMs in a Photoreceptor Degeneration Mouse Model

In the brain, APMs are implicated in encephalopathies. After cerebral injury, blood plasma infiltrating perivascular spaces is taken up by APMs that are the first cell type activated during encephalopathy.[3,31] We used a murine model of photoreceptor degeneration, to determine the role of APMs in retinopathy. Forty-eight hours after sodium iodate injection, alteration of photoreceptors and gliosis were observed (Fig. 8A), indicating that retinopathy was well established. In the altered retinas, autofluorescent cells positive for BM8 (Fig. 8B), MOMA-2 (Fig. 8C), and 2F8 (Fig. 8D) were detected within the outer nuclear layer, suggesting that retinal APMs migrate to lesion sites.

To provide quantitative data on the number of APMs cells through the retinal layers after sodium iodate injection, we counted the number of autofluorescent cells present in the inner retina (ganglion cell layer+inner plexiform layer+inner nuclear layer) and the outer retina (outer plexiform layer+external nuclear layer+inner and outer photoreceptor segments; Fig. 9). After injection, 2.2 $\pm$ 0.06 autofluorescent perivascular cells per field in the inner retina were observed. However, no autofluorescent cells were detected in the outer retina (Figs. 9A, 9B). Twenty-four hours after injection, the perivascular autofluorescent cells leave the inner retina and only 0.9 $\pm$ 0.13 cells per field were detected, whereas in the outer retina 3.3 $\pm$ 0.71 autofluorescent cells per field were counted (Fig. 9A). Finally, at 48 hours after injection autofluorescent perivascular cells nearly disappeared (0.2 $\pm$ 0.01) and a big population of autofluorescent cells (50.6 $\pm$ 7.25, per field) were observed in the outer retina (Figs. 9A, 9C). From the data it is clear that the population of autofluorescent cells that migrate to the outer retina do not represent only the APMs, and it is likely that activated microglial cells also migrate to the lesion site. To clarify this point, the Iba-1 microglial marker was used. As expected, many autofluorescent cells in the outer retina were microglial cells (Fig. 9C).

## Autofluorescent Perivascular Cells in Human Retinas

APMs in the brain were described not only in animals but also in humans.[3] To determine whether human retinas may also contain autofluorescent perivascular cells, wholemount retinas were analyzed. Similar to what was observed in mouse, perinuclear autofluorescent granular inclusions were detected in cells around the blood vessels (Figs. 10A–C). These perivascular cells accumulated lipids marked with oil red O (Fig. 10D). All these findings suggest that autofluorescent perivascular cells

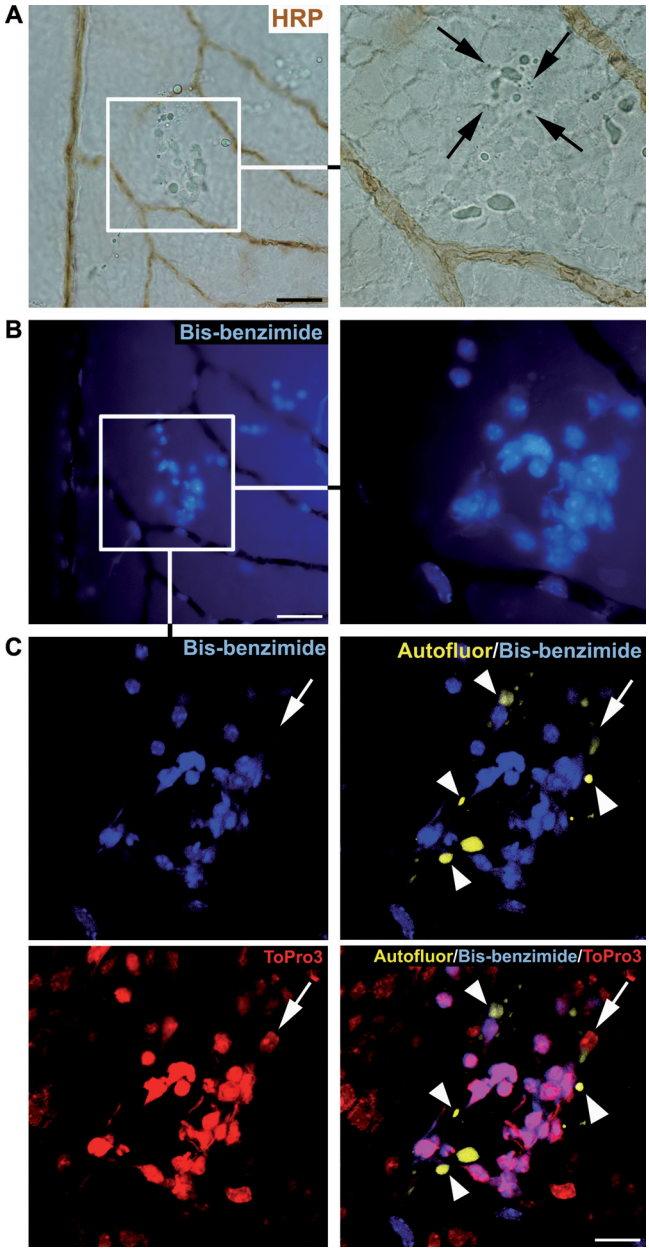


**FIGURE 7.** Retinal APMs migrate to the leakage areas during BRB breakdown. (**A**–**C**) The same area of the inner retina 30 minutes after intravenous injection of Bis-benzimide and HRP. The BRB was been broken previously with mannitol. (**A**) Bright-field microscopic image. Plasmatic HRP (*arrows*) was visible leaking from a blood vessel into the retinal parenchyma. (**B**) Epifluorescence image. As expected, cellular nuclei in the leakage area were marked with Bis-benzimide. (**C**) Confocal laser microscopic single section. Many autofluorescent cells (*arrowhead*) migrated to the leakage area. Some of the migrating autofluorescent cells were not marked by Bis-benzimide (*arrow*), suggesting that these cells migrate from non-BRB disrupted areas to the lesion site. v, blood vessel. Scale bars: (**A**, **B**) 38 μm; (**C**) 15 μm.

exist in human retina. Furthermore, in contrast with healthy retinas (Fig. 10E), autofluorescent cells were detected at lesions in a donor with marked gliosis (Fig. 10F) as occurred in the murine model of retinopathy induced by sodium iodate.

## DISCUSSION

Several terms are used to describe macrophages around retinal blood vessels, including perivascular macrophages and perivascular and paravascular microglia, which reflects the heterogeneity within this population.[5] Fluorescence, topography, immunophenotype, and scavenger activity, observed in the autofluorescent perivascular retinal cell population analyzed, are consistent with those described by Mato et al.[1] for brain APMs.

Autofluorescent perivascular cells found in the retina were different from perivascular retinal microglia due to the following features: (1) They expressed the BM8 and MOMA-2 antigens which are never expressed by microglial cells[20,21]; (2) they do not express Iba-1 protein, one of the most reliable markers of microglia[17]; (3) they constitutively expressed scavenger receptors class A; microglia express these scavenger receptors only when activated[7,8]; and (4) they emitted autofluorescence different from that of microglia.[11] In addi-

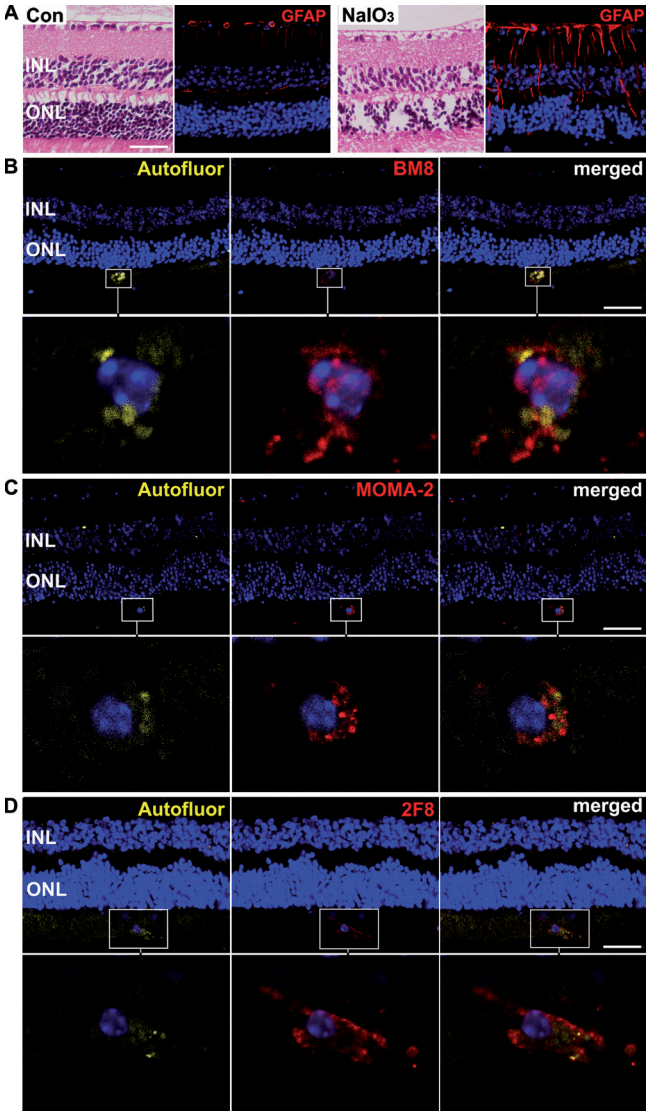


**FIGURE 8.** APMs migrated to the lesion site during retinopathy. (**B**, **C**, **D**, *second row*) Higher magnification of *boxed* areas in *first row*. In a photoreceptor degeneration murine model induced by sodium iodate (**A**), BM8$^+$ (**B**), MOMA-2$^+$ (**C**), and 2F8$^+$ (**D**) autofluorescent cells were observed within the photoreceptor layer. The gliosis observed in anti-GFAP antibody–labeled retinas revealed that retinopathy was well established in the photoreceptor degeneration model (**A**). Nuclei were counterstained with Hoechst (*blue*). Con, control retina; INL, inner nuclear layer; ONL, outer nuclear layer; Autofluor, autofluorescence. Scale bars: (**A**, **D**) 33 μm; (**B**) 25 μm; (**C**) 41 μm.

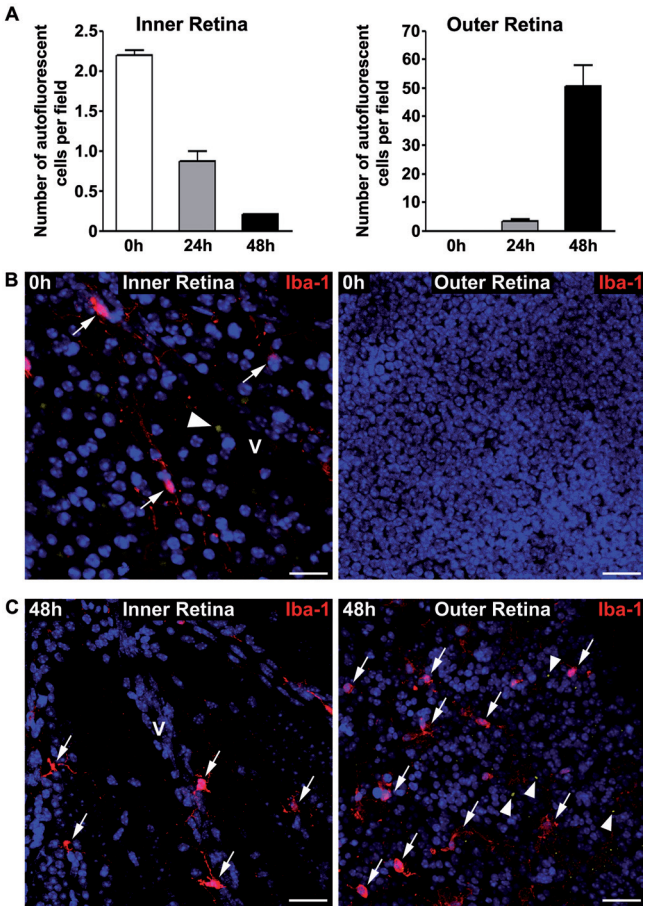


**Figure 9.** Quantification of migrating autofluorescent perivascular cells. (**A**) The number of autofluorescent cells present in the inner retina (ganglion cell layer+inner plexiform layer+inner nuclear layer) and the outer retina (outer plexiform layer+external nuclear layer+inner and outer photoreceptor segments) was counted in confocal laser microscopic single sections. Autofluorescent perivascular cells leave the inner retina and migrate to the outer retina in a model of photoreceptor degeneration induced by sodium iodate. (**B**, **C**) Representative areas of the inner and outer retina immediately after (0 hours) and 48 hours after injection of sodium iodide. Note that 48 hours after injection, a big population of autofluorescent macrophages (*arrowhead*) and Iba-1$^+$ microglial cells (*arrows*) were observed in the outer retina. v, blood vessel. Scale bars: (**B**, *left*) 21 $\mu$m; (**B**, *right*) 29 $\mu$m; (**C**, *left*) 32 $\mu$m; (**C**, *right*) 31 $\mu$m.

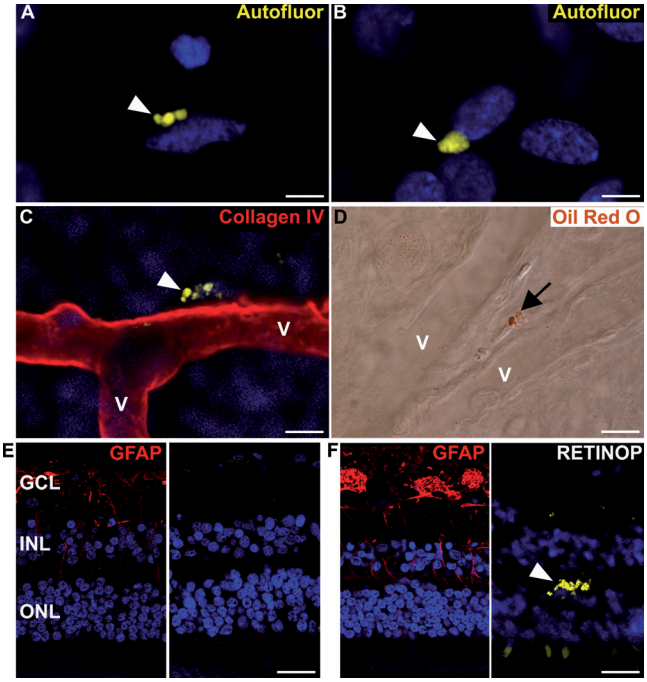


**Figure 10.** Autofluorescent perivascular cells were present in the human retinas. Laser confocal analysis of wholemount retinas confirmed the presence of perivascular cells with autofluorescent granules (*arrowheads*) close to their nuclei (**A**–**C**). Cells containing lipids stained with oil red O (*arrow*) were observed in a perivascular position compatible with that of APMs (**D**). Healthy retina (**E**). In human retinopathy, autofluorescent perivascular cells were found at the lesion site (**F**). Nuclei were counterstained with Hoechst (*blue*). Autofluor, autofluorescence; GCL, ganglion cell layer; INL, inner nuclear layer; ONL, outer nuclear layer; RETINOP, retinopathy. Scale bars: (**A**) 7.5 $\mu$m; (**B**) 7 $\mu$m; (**C**) 9 $\mu$m; (**D**) 18 $\mu$m; (**E**) 23 $\mu$m; (**F**) 24 $\mu$m.

tion, autofluorescence in perivascular microglia was observed only in aging mice, whereas retinal autofluorescent perivascular cells presented autofluorescent granules even in the first month of life. Table 1 summarizes the immunophenotype of the APMs in comparison with the previously published data for microglia.

Normal retinal function requires a strict control of the extracellular environment. The BRB regulates the extracellular environment by excluding blood-borne proteins from the retina.[32] This barrier is composed of the retinal vascular endothelium and the retinal pigment epithelium.[32] However, the data presented herein may suggest that small amounts of blood-borne proteins pass through the vascular endothelium in healthy retinas without BRB pathologic disruption. Autofluorescent perivascular cells found in mouse retina may act as scavenger macrophagic cells phagocytosing and accumulating blood-borne proteins (HRP and ac-LDL). This is also observed in BBB, where APMs phagocytose and accumulate these leakage proteins, thereby helping to prevent brain malfunction.[2,33] Therefore, retinal APMs, by their scavenger activity, may contribute to the BRB function in healthy conditions. Moreover, APMs migrate to the leakage areas during BRB breakdown.

APMs in the brain constitute the first type of macrophage activated during encephalopathy.[2,3] In experimental retinopathy, macrophagic 5D4-negative cells migrate early to the lesion site, whereas 5D4-positive microglial cells appear only in the late phase of photoreceptor degeneration,[34,35] suggesting that macrophages different from microglial cells are involved earlier in retinopathy. Similarly, our results indicated that APMs dif-

**Table 1.** Immunophenotypes of APMs

| | Iba-1 | TL | CD11b | F4/80 | BM8 | MOMA-2 | 2F8 |
|---|---|---|---|---|---|---|---|
| APM | − | + | +++ | +++ | +++ | +++ | +++ |
| Microglia | +++[17] | +++[17] | +++[18] | +++[19] | −[20,21] | −[21] | −[7,8,24] |

Superscript numbers are reference sources. − no expression, + low expression, ++ moderate expression, +++ strong expression.

ferent from microglia appeared early at the lesion site in a murine model of experimental retinopathy.

In summary, this study describes a macrophagic, nonmicroglial, autofluorescent perivascular cell type in healthy retinas that is similar to the APMs found in brain. Retinal autofluorescent perivascular cells may contribute to the BRB function, scavenging and accumulating blood-borne proteins. Furthermore, their migration to the lesion site in BRB breakdown and in experimental and human retinopathy may suggest a key role of retinal APMs during retinopathy.

## *Acknowledgments*

The authors thank Carlos Andrade Fontes for critical revision of the manuscript and Pedro Otaegui and Lorena Noya for technical assistance.

## *References*

1. Mato M, Ookawara S, Mato TK, Namiki T. An attempt to differentiate further between microglia and fluorescent granular perithelial (FGP) cells by their capacity to incorporate exogenous protein. *Am J Anat.* 1985;172:125–140.
2. Mato M, Ookawara S, Sakamoto A, et al. Involvement of specific macrophage-lineage cells surrounding arterioles in barrier and scavenger function in brain cortex. *Proc Natl Acad Sci USA.* 1996;93:3269–3274.
3. Williams K, Alvarez X, Lackner AA. Central nervous system perivascular cells are immunoregulatory cells that connect the CNS with the peripheral immune system. *Glia.* 2001;36:156–164.
4. Janzer RC. The blood-brain barrier: cellular basis. *J Inherit Metab Dis.* 1993;16:639–647.
5. Provis JM, Penfold PL, Edwards AJ, vanDriel D. Human retinal microglia: expression of immune markers and relationship to the glia limitans. *Glia.* 1995;14:243–256.
6. Gregerson DS, Yang J. CD45-positive cells of the retina and their responsiveness to in vivo and in vitro treatment with IFN-gamma or anti-CD40. *Invest Ophthalmol Vis Sci.* 2003;44:3083–3093.
7. Gehrmann J, Mies G, Bonnekoh P, et al. Microglial reaction in the rat cerebral cortex induced by cortical spreading depression. *Brain Pathol.* 1993;3:11–17.
8. Lewis GP, Sethi CS, Carter KM, Charteris DG, Fisher SK. Microglial cell activation following retinal detachment: a comparison between species. *Mol Vis.* 2005;11:491–500.
9. Daugherty A, Whitman SC, Block AE, Rateri DL. Polymorphism of class A scavenger receptors in C57BL/6 mice. *J Lipid Res.* 2000; 41:1568–1577.
10. Bancroft JD, Gamble M. *Theory and Practice of Histological Techniques.* 5th ed. New York: Churchill Livingstone; 2002.
11. Xu H, Chen M, Manivannan A, Lois N, Forrester JV. Age-dependent accumulation of lipofuscin in perivascular and subretinal microglia in experimental mice. *Aging Cell.* 2008;7:58–68.
12. Feeney L. Lipofuscin and melanin of human retinal pigmented epithelium. Fluorescence, enzyme cytochemical, and ultrastructural studies. *Invest Ophthalmol Vis Sci.* 1978;17:583–600.
13. Hashizume H, Ushiki T. Three-dimensional cytoarchitecture of angiogenic blood vessels in a gelatin sheet implanted in the rat skeletal. *Arch Histol Cytol.* 2002;65:347–357.
14. Hughes S, Gardiner T, Hu P, Baxter L, Rosinova E, Chang-Ling T. Altered pericyte-endothelial relations in the rat retina during aging: implications for vessel stability. *Neurobiol Aging.* 2006;27:1838–1847.
15. Eng LF, Ghirnikar RS, Lee YL. Glial fibrillary acidic protein: GFAP-thirty-one years (1969–2000). *Neurochem Res.* 2000;25:1439–1451.
16. Ito D, Imai Y, Ohsawa K, Nakajima K, Fukuuchi Y, Kohsaka S. Microglia specific localisation of a novel calcium binding protein, Iba 1. *Mol Brain Res.* 1998;57:1–9.
17. Santos AM, Calvente R, Tassi M, et al. Embryonic and postnatal development of microglial cells in the mouse retina. *J Comp Neurol.* 2008;506:224–239.
18. Springer T, Galfré G, Secher DS, Milstein C. Mac-1: a macrophage differentiation antigen identified by monoclonal antibody. *Eur J Immunol.* 1979;9:301–306.
19. Inoue T, Plieth D, Venkov CD, Xu C, Nielson EG. Antibodies against macrophages that overlap in specificity with fibroblasts. *Kidney Int.* 2005;67:2488–2493.
20. Malorny U, Michels E, Sorg C. A monoclonal antibody against an antigen present on mouse macrophages and absent from monocytes. *Cell Tissue Res.* 1986;243:421–428.
21. Kraal G, Rep M, Janse M. Macrophages in T and B cell compartments and other tissue macrophages recognized by monoclonal antibody MOMA-2: an immunohistochemical study. *Scand J Immunol.* 1987;26:653–661.
22. Grossmann R, Stence N, Carr J, Fuller L, Waite M, Dailey ME. Juxtavascular microglia migrates along brain microvessels following activation during early postnatal development. *Glia.* 2002;37: 229–240.
23. Wang S, Voisin MB, Larbi KY, et al. Venular basement membranes contain specific matrix protein low expression regions that act as exit points for emigrating neutrophils. *J Exp Med.* 2006;203:1519–1532.
24. Fraser I, Hughes D, Gordon S. Divalent cation-independent macrophage adhesion inhibited by monoclonal antibody to murine scavenger receptor. *Nature.* 1993;364:343–346.
25. Caldwell RB, McLaughlin BJ. Permeability of retinal pigment epithelial cell junctions in the dystrophic rat retina. *Exp Eye Res.* 1983;36:415–427.
26. Bamforth SD, Lightman SL, Greenwood J. Ultrastructural analysis of interleukin-1 beta-induced leukocyte recruitment to the rat retina. *Invest Ophthalmol Vis Sci.* 1997;38:25–35.
27. Hikishima H, Mato M. Studies of age-related changes in intracerebral small vessels of rat: do all cerebral blood vessels get aging concurrently? (in Japanese) *No To Shinkei.* 1990;42:929–944.
28. Chang-Ling T, Neill AL, Hunt NH. Early microvascular changes in murine cerebral malaria detected in retinal wholemounts. *Am J Pathol.* 1992;140:1121–1130.
29. Hu P, Pollard JD, Chang-Ling T. Breakdown of the blood-retinal barrier induced by activated T cells of nonneural specificity. *Am J Pathol.* 2000;154:1139–1149.
30. Rapoport SI. Osmotic opening of the blood-brain barrier: principles, mechanism, and therapeutic applications. *Cell Mol Neurobiol.* 2000;20:217–230.
31. Fukuda S, Mato M. Studies on ultrastructural changes in perivascular cells (F.G.P.) of small cerebral vessels at the initial stage after cold injury: chronological observations on F.G.P. within 24 hours. *No To Shinkei.* 1985;37:349–357.
32. Vinores SA. Assessment of blood-retinal barrier integrity. *Histol Histopathol.* 1995;10:141–154.
33. Mato M, Ookawara S, Sano M, Fukuda S. Uptake of fat by fluorescent granular perithelial cells in cerebral cortex after administration of fat rich chow. *Experientia.* 1982;38:1496–1498.
34. Ng TF, Streilein JW. Light-induced migration of retinal microglia into the subretinal space. *Invest Ophthalmol Vis Sci.* 2001;42: 3301–3310.
35. Zhang C, Lam TT, Tso MO. Heterogeneous populations of microglia/macrophages in the retina and their activation after retinal ischemia and reperfusion injury. *Exp Eye Res.* 2005;81:700–709.