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# **Role of the chemokine receptor CX3CR1 in the mobilization of phagocytic retinal microglial cells**

William Raoul<sup>1,2,3</sup>, Nicole Keller<sup>1,2,3</sup>, Mathieu Rodéro<sup>4,5</sup>, Francine Behar-Cohen<sup>1,2,3,6</sup>, Florian Sennlaub<sup>1,2,3\*</sup> and Christophe Combadière<sup>4,5,7\*</sup>

<sup>1</sup> INSERM, UMR S 872, Centre de Recherche des Cordeliers, F-75005, Paris, France

<sup>2</sup> UPMC Univ Paris 06, UMR S 872, F-75005, Paris, France

<sup>3</sup> Université Paris Descartes, UMR S 872, F-75005, Paris, France

<sup>4</sup> INSERM U543, Laboratoire d'Immunologie Cellulaire, F-75005, Paris, France

<sup>5</sup> UPMC Univ Paris 06, F-75005, Paris, France.

<sup>6</sup> AP-HP, Hôtel Dieu, Service d'Ophtalmologie, Centre de Recherche Ophtalmologique, Paris, France.

<sup>7</sup> AP-HP, Groupe hospitalier Pitié-Salpêtrière, Service d'Immunologie, F-75005, Paris, France.

\* FS and CC contributed equally to this work.

To whom correspondence should be addressed at: 1) Dr William Raoul, INSERM UMR S 872, équipe 17, Centre de Recherche des Cordeliers, 15 rue de l'Ecole de Médecine, 75006 Paris, France. Tel: (33) 1 40 46 78 58, Fax: (33) 1 40 46 78 65, Email: [raoul@idf.inserm.fr](mailto:raoul@idf.inserm.fr) or 2) Dr Christophe Combadière, INSERM U543, Faculté de Médecine Pitié-Salpêtrière, 91 Boulevard de l'Hôpital, 75013 Paris, France. Tel: (33) 1 40 77 98 92, Fax: (33) 1 40 77 97 34, Email: [combad@ccr.jussieu.fr](mailto:combad@ccr.jussieu.fr)

Short Running Title: CX3CR1 in the mobilization of phagocytic retinal microglial cells

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## **Abstract**

We recently showed that subretinal CX3CR1-dependent microglial cell (MC) accumulation may lead to age-related macular degeneration. The fate of MC after engulfing retinal debris is poorly understood. Severe photoreceptor degeneration was observed 40 days after exposure to bright light in CX3CR1-deficient but not control mice, and more MCs accumulated in the subretinal space of the former than the latter. To study the fate of subretinal MCs in CX3CR1 competent animals we used a dystrophic rat model, in which abundant subretinal MC accumulation is observed secondary to primary retinal degeneration. In dystrophic rats, MCs containing rhodopsin or rod outer segment (ROS) debris were found outside the outer retina at sites suggesting choroidal and ciliary egress. In conclusion, our data indicate that MC accumulation at injury sites is independent of CX3CR1 and precedes photoreceptor degeneration. The ectopic presence of rhodopsin-positive MCs suggest that CX3CR1 participates in MC egress from the outer retina.

## **1. Introduction**

Age-related macular degeneration (AMD) is the leading cause of vision loss in the elderly throughout the industrialized world (Friedman et al., 2004). Its most prominent pathologic features are lesions involving the retinal pigment epithelium (RPE) and Bruch's membrane (BM), the degeneration of photoreceptors (Sarks, 1976) and, in the most aggressive cases, choroidal neovascularization. The causes of AMD are not well understood, but epidemiological studies and murine models have identified key factors in its pathogenesis. The main predisposing factors for its onset are age (Friedman et al., 2004) and family history (Bird et al., 1995). Increasing evidence suggests that retinal microglial cells (MCs) and alterations in chemokine pathways play a major role in its development (Ambati et al., 2003; Combadière et al., 2007; Pham et al., 2005).

Chemokines are well-described proinflammatory cytokines that coordinate the deployment and activation of leukocytes at injury sites by signaling through a family of G protein-coupled receptors (Luster, 1998). There is growing evidence that they are not restricted to leukocyte trafficking from the blood to tissues but rather are multifunctional mediators acting on various cell types, including resident cells such as epithelial, endothelial, and smooth muscle cells as well as neurons. Cell functions associated with chemokines are no longer limited to basic cell recruitment and now extend to angiogenesis, cell survival, cell proliferation and neurotransmission (Ransohoff et al., 2007). Drugs targeting them are among the most promising new anti-inflammatory treatments because they may be more selective than current anti-inflammatory drugs and less harmful than immunosuppressants.

Recently, we showed that the chemokine receptor CX3CR1 is expressed on all retinal MCs and that a defect in its function is associated with the risk of AMD (Combadière et al., 2007). CX3CR1-deficient mice developed cardinal features of AMD with resident MCs accumulating into the subretinal space. Photoreceptor cell degeneration occurred secondarily to the MC accumulation in the subretinal space, regardless of the retinal insult (aging and laser photocoagulation). The fate of MCs after they engulf retinal debris is poorly understood. We postulated that MCs in the subretinal space phagocytize degenerating rod outer segments (ROS) and may be then redistributed in the retina to clear debris into the vasculature. Here we used a model of bright light exposure to corroborate our previous findings in CX3CR1-deficient mice and then tracked the fate of subretinal MCs further in a well-known rodent model: the dystrophic Royal College of Surgeons rats (RCS).

## 2. Materials and methods

### 2.1. Animals

CX3CR1<sup>-/-</sup> and CX3CR1<sup>GFP/GFP</sup> mouse strains on C57BL/6 background and their CX3CR1<sup>+/+</sup> controls were generated as described before (Combadiere et al., 2003; Jung et al., 2000). The mice were maintained at the “Centre d’Exploration Fonctionnelle” animal facility (Pitié-Salpêtrière, Paris) under pathogen-free conditions. Control C57BL/6 wild type mice (here referred as CX3CR1<sup>+/+</sup>) were obtained from the Center d’Elevage Janvier (Le Genest St Isle, France). Royal College of Surgeon (RCS) rats were from our colony and were kept in specific pathogen-free conditions at the animal facility of University Paris V Descartes (Paris). All animals were housed in a 12/12 hour light/dark (100-500 lux) cycle with food and water available *ad libitum*. Animal experiments were approved by the Institutional Animal Care and Use Committee of the Faculté de Médecine Pitié-Salpêtrière (Paris).

### 2.2. Bright light exposure

Mice were anesthetized by intraperitoneal injection of ketamine (50 mg/kg) and xylazine (10 mg/kg) from Bayer (Puteaux, France). Pupils were fully dilated with 1% tropicamide or 1% Atropin (Novartis, Rueil Malmaison, France). The mice were then exposed to bright light (100 Klux/eye) from a KL2500 LCD Schott lamp (Schott, Clichy, France), with the light-conducting glass fibers close to the dilated eyes for 10 min.

### 2.3. Immunohistochemistry

Frozen sections were stained according to standard immunohistochemical procedures, as previously described (Checchin et al., 2006). The primary antibodies used were mouse anti-rhodopsin (Rho4D2, a gift from Dr. R.S. Molday, University of British Columbia, Canada), mouse anti-CD68 (ED1, Serotec, Cergy Saint Christophe, France) and mouse anti-GFP (Torrey Pines Biolabs, Houston, TX, USA). The lectins used were TRITC-conjugated BSA-1

(Sigma–Aldrich, Saint Quentin Fallavier, France) and rhodamine phalloidin (Molecular Probes, Leiden, Netherlands). **FKN antibodies** Primary antibodies were revealed with the corresponding Alexa or phosphatase alkaline-coupled secondary antibodies (Molecular Probes) (followed by fast-red retrieval), and sections were counterstained with 4-6-diamino-2-phenylindole (DAPI). Sections were viewed with a fluorescence microscope (BX51; Olympus, Rungis, France) or a confocal microscope (Zeiss LSM 510 laser scanning microscope, Le Pecq, France). Each immunostaining was repeated at least 3 times and staining omitting the primary antibody served as the negative control.

#### **2.4. Choroidal flatmounts and MC quantification**

Eyes were enucleated, fixed in 4% PFA for 15 minutes at room temperature, and sectioned at the limbus; the cornea and lens were discarded. The retinas were carefully peeled from the RPE/choroid/sclera. Retinas and choroids were fixed for additional 15 minutes in methanol at  $-20^{\circ}\text{C}$  and incubated with the indicated primary and secondary antibodies. The choroids and retinas were radially incised, flatmounted and viewed with a fluorescence microscope (BX51; Olympus). MCs were counted on whole RPE/choroidal flatmounts up to the ciliary body and on the outer segment side of the retina. The surface of the RPE was measured and MC density calculated.

#### **2.5. Histology and electron microscopy**

Eyes were fixed in 2.5% glutaraldehyde of cacodylate buffer (0.1 M, pH 7.4). After 1 hour, eye balls were dissected, fixed for another 3 hours, post-fixed in 1% osmium tetroxide in cacodylate buffer and dehydrated in graduated ethanol solution. The samples were included in epoxy resin and oriented. Semithin sections (1  $\mu\text{m}$ ), obtained with an ultramicrotome Reichert Ultracut E (Leica, Rueil Malmaison, France), were stained with toluidine blue and examined with a light microscope to measure photoreceptor layer thickness 300 – 800  $\mu\text{m}$  from the optic nerve . Ultrathin sections (80 nm) were contrasted with uranyl acetate and lead citrate and

observed in a JEOL 100 CX II electron microscope (JEOL, Tokyo, Japan) with 80 kV.

## **2.6. Microglial cell migration assay**

Brain glial cell suspensions were obtained from brains of 5 donor C57BL6/J or CX3CR1<sup>-/-</sup> mice. Brains were isolated, freed of meninges and put into a 5% CO<sub>2</sub>/ 37°C incubator for 15 min. The tissue was minced mechanically and flushed several times with a 1-ml pipette. Pooled minced tissue was harvested in PBS, and cells were centrifuged at 2000 rpm for 10 min. The pellet was resuspended in fresh medium, and floating cells were separated from debris by decantation. This process was repeated 3 times. Harvested cells were centrifuged again. Finally, 200 µl of cell suspension (1.5 million glial cells, about 1/10 microglial cells) was placed in each of 5 µm pore polycarbonate Transwell inserts with 5-µm pores (Corning, Avon, France). CX3CL1, CCL2 and CCL5 (R&D Systems, Lille, France) were used in 24-well plates at concentrations of 0.5 to 50 µM (dilution in DMEM medium, GIBCO BRL, Life Technology, Paisley, Scotland). Media in wells with chemokines (Peprotech, Rocky Hill, NJ) were harvested 1 day later and analyzed by flow cytometry (FACSCalibur, Becton Dickinson) for the CD11b marker (clone M1/70, BD Pharmingen, San Diego, CA, USA).

## **2.7. Statistical Analysis**

Graph Pad Prism 4 (GraphPad Software, San Diego, CA, USA) was used for the data analysis and graphic representations. All values are reported as means ± SEM. Statistical comparisons used unpaired two-sample *t*-tests for means and the Mann-Whitney U test. Significance was set at  $p < 0.05$ .

### 3. Results and Discussion

#### 3.1. MC accumulation precedes photoreceptor degeneration in CX3CR1<sup>-/-</sup> mice

We previously showed that photoreceptor cell degeneration occurred secondarily to the MC accumulation in subretinal space in both chronic (aging) and aggressive acute (laser photocoagulation) models of retinal insult. We sought to replicate these results in a less aggressive model of acute light insult that, unlike laser photocoagulation, does not promote angiogenesis. C57Bl/6 control and CX3CR1<sup>-/-</sup> mice were exposed to a short period of very bright light. After 40 days, the mice were euthanized and their retinas stained with toluidine blue as shown in Figure 1. A marked degeneration of photoreceptors was observed in the CX3CR1<sup>-/-</sup> mice exposed to bright light (Fig. 1B), compared with control mice (Fig. 1A) and control unexposed mice (data not shown). Measurements of photoreceptor cell layer thickness showed significant thinning (~50%) of the photoreceptor cell layer in the CX3CR1<sup>-/-</sup> mice compared with control whereas cell layer thickness were similar in unexposed CX3CR1<sup>-/-</sup> and CX3CR1<sup>+/+</sup> mice (Fig. 1C) modifier la figure 1C et réajuster par rapport a jour 40.. These data indicate that CX3CR1<sup>-/-</sup> mice are much more sensitive to light exposure and that CX3CR1 may participate in photoreceptor survival.

To track the CX3CR1-expressing cells, we repeated these experiments in CX3CR1<sup>GFP/GFP</sup> mice in which the GFP is driven by the CX3CR1 promoter. Ten days after light injury, CX3CR1<sup>+GFP</sup> mice displayed only occasional GFP-positive MCs adjacent to the RPE monolayer (red fluorescence in Fig. 2A), compared with the CX3CR1<sup>GFP/GFP</sup> mice, which showed a massive MC accumulation (Fig. 2B). Before light injury, the number of subretinal MC were similarly low in two-months old CX3CR1<sup>+GFP</sup> and CX3CR1<sup>GFP/GFP</sup> mice (Fig.2C). In CX3CR1<sup>+GFP</sup> mice, MCs were present in the subretinal space as early as one day after light

exposure, and their number remained unchanged for 40 days (Fig. 2C). MC accumulation in CX3CR1<sup>GFP/GFP</sup> intensified early, on the other hand, peaking at day 10 and slowly returning to baseline by day 40. A more detailed analysis of MC morphology in bright light-exposed pigmented CX3CR1<sup>+GFP</sup> and CX3CR1<sup>GFP/GFP</sup> mice revealed small cell bodies with long, thin pseudopodia 1 day and 10 days after light exposure in the CX3CR1<sup>+GFP</sup> mice (Fig. 2D). In contrast, in the MCs of the CX3CR1<sup>GFP/GFP</sup> mice, the average cell body increased significantly in size by day 10 and the cells appeared bloated with intracellular inclusions (Fig. 2E). Quantification of the ratio of body size and pseudopodia length showed an increase in body size in the CX3CR1<sup>GFP/GFP</sup> mouse cells that was not observed in CX3CR1<sup>+GFP</sup> mice (Fig. 2F). Nevertheless, some MCs in CX3CR1<sup>GFP/GFP</sup> mice at day 10 had the slim body and long pseudopodia phenotype observed at day 1 and in control mice.

We previously showed that body growth in MCs may be attributed to ROS ingestion and lipid accumulation (Combadière et al., 2007). Our observations suggested that the average duration of time that CX3CR1<sup>GFP/GFP</sup> MCs spend in the subretinal space is longer than for controls. The accumulation of MCs in CX3CR1-deficient mice may therefore be due to their defective clearance from the subretinal space. This additional time may favor MC phagocytic functions and their transformation into bloated “foam cells”. The transient occurrence of MCs in this bright-light model and in our experiments with albino CX3CR1<sup>-/-</sup> mice (Combadière et al., 2007), together with the observation by Ng *et al.* (Ng and Streilein, 2001), suggests that retinal MCs may leave the subretinal space.

### **3.2. MC migrated in response to inflammatory chemokines**

To evaluate the role of the CX3CL1/CX3CR1 axis in MC redistribution, we studied CX3CL1 expression in 12-month-old CX3CR1<sup>+/+</sup> mice (Fig. 3A). CX3CL1 (red stain) was strongly expressed in the major vessels of the retina and choroid (arrows). When the primary antibody was omitted, this staining was not observed in adjacent sections (Fig. 3B). No or

very faint CX3CL1 staining was found in the photoreceptor layer, where the MCs accumulated in CX3CR1<sup>-/-</sup> mice. To assess the role of the chemokine pathway in MC redistribution, we performed an *in vitro* migration assay on single-cell suspensions of MCs from the brains of CX3CR1<sup>+/+</sup> and CX3CR1<sup>-/-</sup> mice (Fig. 3C). In cell suspensions from CX3CR1<sup>+/+</sup> brains, CD11b-positive MCs migrated specifically in response to CX3CL1, CCL2 and CCL5 at concentrations ranging from 0.1 nM to 50 nM (Fig. 3C and data not shown). As expected, MC migration in the CX3CR1<sup>-/-</sup> mice was totally blunted in response to CX3CL1 but remained unchanged in response to CCL2 and CCL5. These findings suggest that the CX3CR1/CX3CL1 axis is not involved in MC redistribution to the subretinal space but may be required for further movement in or outside the retina.

### **3.3. Rhodopsin-positive MCs exit the subretinal space by different routes**

The failure of studies in retinal degeneration models to detect TUNEL-positive cells in the subretinal space suggests that MCs, unlike photoreceptors, do not disappear by apoptosis (Tso et al., 1994). We hypothesized that MCs actively leave the subretinal space by migration. As MC phagocytize ROS, they become rhodopsin-positive and can be tracked outside the photoreceptor cell layer for as long as the intralysosomal antigen is recognized by the rhodopsin-specific antibody. In humans, MCs migrate into the inner retina after engulfing injured ROS in AMD (Gupta et al., 2003; Combadière et al., 2007). As subretinal MCs did not accumulate significantly in the light induced model in CX3CR1<sup>+/+</sup> mice this model, we found it not suitable to monitor the fate of subretinal in CX3CR1 competent MCs. Instead we used the model of dystrophic RCS rats, where MCs accumulate secondary to photoreceptor degeneration caused by a genetic defect (Roque et al., 1996). Dystrophic 35-day-old RCS rats displayed rhodopsin-positive cells (red fluorescence) expressing the microglial cell marker CD68 (green fluorescence) outside the photoreceptor cell layer — in the inner nuclear layer (Fig. 4A, B and C) and in the choroid (Fig. 4D, E and F). Furthermore, transmission electron

microscopy of sections of dystrophic RCS rat retina (but not of control rat retina) showed the presence of cells in the lumen of choriocapillaries with the characteristic appearance of intralysosomal ROS remnants (Fig. 5A). Ultrastructural analysis confirmed the presence of both the early (Fig. 5B) and late (Fig. 5C) stages of ROS degradation, which are usually observed only in RPE cells. Taken together, our data show that cells positive for MC markers and containing ROS/rhodopsin can be found outside the photoreceptor cell layer, the exclusive site of physiological rhodopsin expression. The presence of rhodopsin-positive SrMCs in the inner retina suggests an exit via the retinal vessels, and their presence in the choroids points to an exit route through the choroid or ciliary body.

## **4. Conclusions**

MCs in CX3CR1-deficient mice after bright light injury migrated to the subretinal space where they accumulate in a CX3CR1 independent way. Secondly to this accumulation, photoreceptor cells degenerated in these mice. To investigate the fate of CX3CR1 competent subretinal MCs we used dystrophic RCS rats, where important subretinal MCs accumulations are found secondarily to primary photoreceptor degeneration. Detection of ROS debris containing mononuclear cells outside the photoreceptor cell layer in dystrophic RCS rats suggests that MCs actively migrate away from their subretinal location. The absence of these ectopic rhodopsin positive MCs in models of CX3CR1-deficient MC accumulation suggests a role of CX3CR1 in this process. These results describe a new clearance route that will necessitate further investigation to clarify and detail its mechanisms.

## **Acknowledgements**

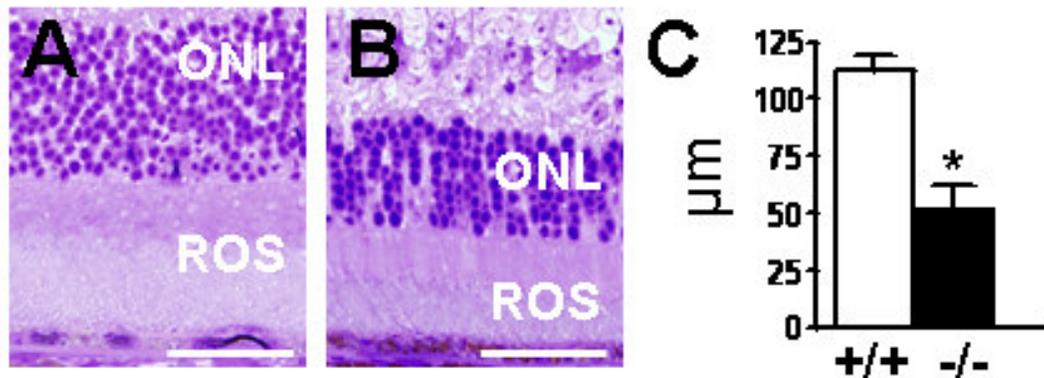
We thank Dr. D. Littman and Dr. S Jung for sharing the CX3CR1Kin GFP mice and Dr. R.S. Molday for the monoclonal anti-rhodopsin Rho4D2 antibody.

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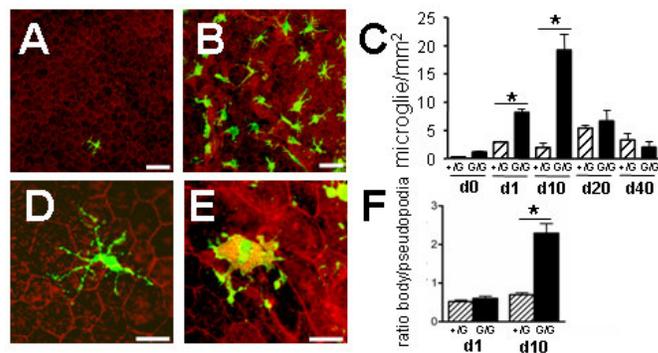
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## Figure Legends



**Figure 1: Photoreceptor degeneration after bright light injury in CX3CR1<sup>-/-</sup> mice.**

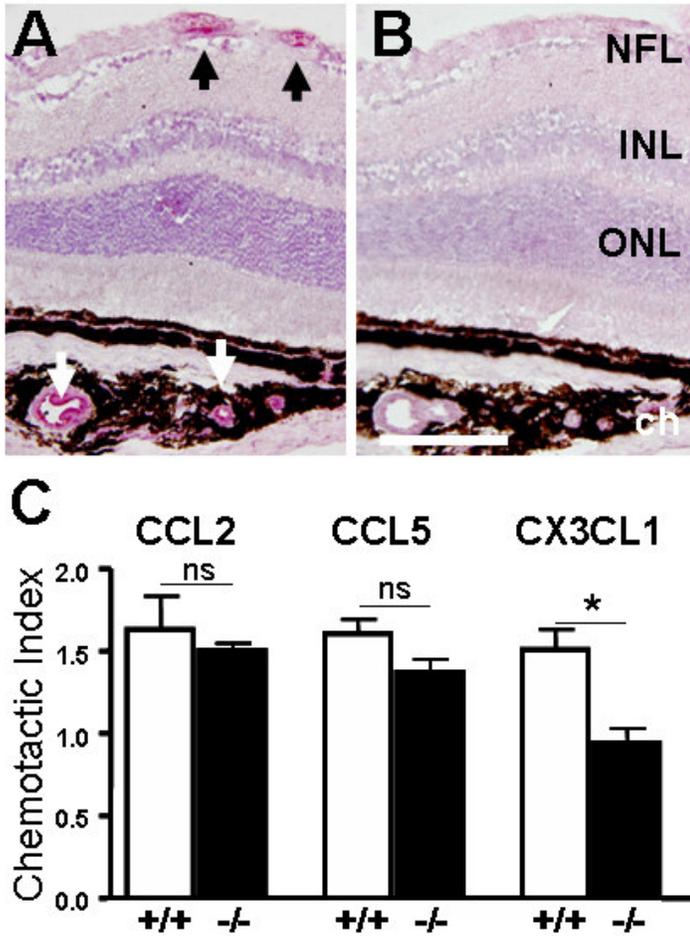
Toluidine blue-stained epoxy retinal semithin sections of CX3CR1<sup>+/+</sup> (A) and CX3CR1<sup>-/-</sup> (B) show degeneration of photoreceptors in CX3CR1<sup>-/-</sup> bright light-exposed mice. (C) Measurements of photoreceptor cell layer thickness show significant thinning of the photoreceptor cell layer in CX3CR1<sup>-/-</sup> mice. Experiments were performed on 4 to 8 eyes from different mice for C57BL/6 mice (+/+, empty columns) and CX3CR1<sup>-/-</sup> (-/-, plain columns). \*,  $P < 0.05$ . ONL: outer nuclear layer; ROS: rod outer segments. Scale bars: 50 µm.



**Figure 2: MC accumulate in the subretinal space after bright-light injury in CX3CR1<sup>-/-</sup> mice.**

RPE flatmounts of CX3CR1<sup>+/GFP</sup> (A) and CX3CR1<sup>GFP/GFP</sup> (B) show a substantial accumulation of subretinal MCs in CX3CR1<sup>GFP/GFP</sup> mice in the bright light-exposure model.

(C) Quantification of subretinal GFP cells on RPE flatmounts reveals a substantial accumulation of subretinal MC in CX3CR1<sup>-/-</sup> mice. Magnification of individual cells of bright light-exposed eyes reveals that subretinal MCs at day 10 after exposure of CX3CR1<sup>GFP/GFP</sup> (D) have large cell bodies and short pseudopodia compared with cells of CX3CR1<sup>+GFP</sup> mice (E). (F) Measurements of the ratio of the cell body versus the pseudopodia at d1 and d10 reveal that the bodies of CX3CR1<sup>GFP/GFP</sup> subretinal cells increase in size with time after bright light exposure. Experiments were performed on 4 to 8 eyes from different mice for CX3CR1<sup>+GFP</sup> (+/G, hatched columns) and CX3CR1<sup>GFP/GFP</sup> (G/G, plain columns). \*,  $P < 0.05$ . Scale bars: 50  $\mu\text{m}$ .



**Figure 3: Localization of the chemokine CX3CL1 and *in vitro* migration assay.**

(A) In C57BL/6 mice, CX3CL1 (red staining) localizes to the major vessels of the retina and choroid (arrows). (B) Negative control omitting the primary antibody. NFL: nerve fiber layer; INL: inner nuclear layer; ONL: outer nuclear layer; ch: choroids. Scale bar 100  $\mu$ m. (C) *In vitro* migration assays of brain microglial cells compare chemotactic responses to 10 nM of the indicated chemokines in CX3CR1<sup>-/-</sup> (plain columns) to C57BL/6 (empty columns) groups of mice. Results are representative of at least three independent experiments.

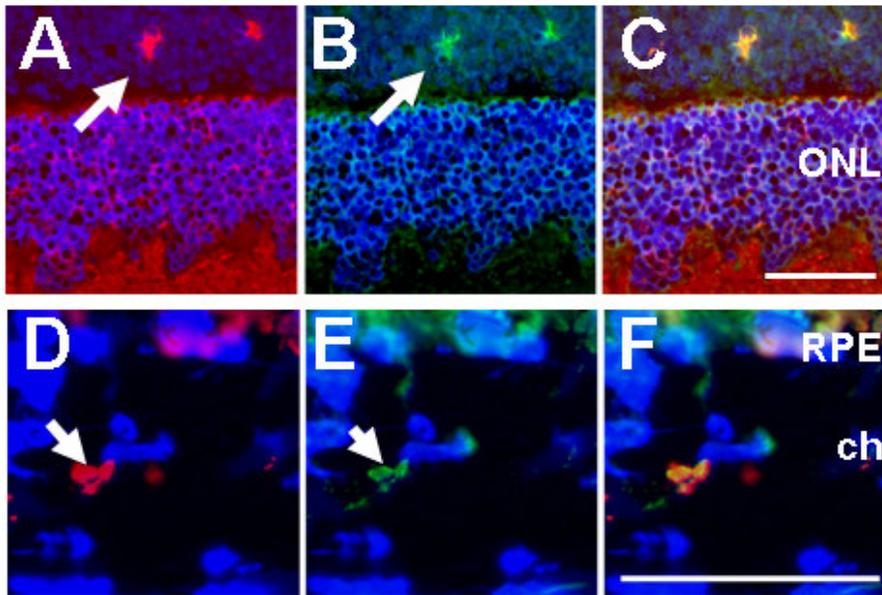
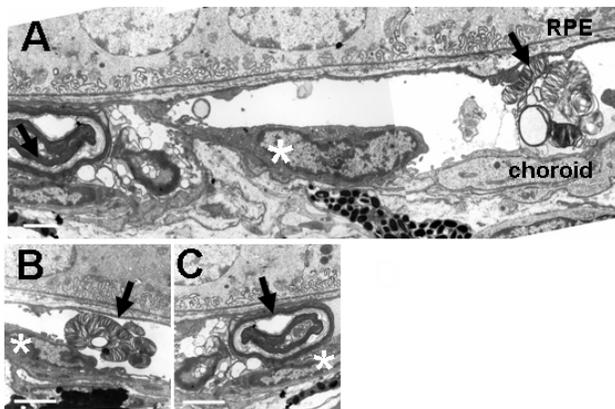


Figure 4:

**Rhodopsin-positive cells are found outside the PRL.**

Dystrophic 35-day-old RCS rats display rhodopsin-positive cells (A and D, red fluorescence) expressing the microglial cell marker CD68 (B and E, green fluorescence) outside the photoreceptor cell layer in the inner retina (A, B and C for overlay) and in the choroid (D, E and F for overlay). Results are representative of at least three independent experiments. ONL: outer nuclear layer; RPE: retinal pigment epithelium. Ch: choroid. Scale bars : 50  $\mu$ m.



**Figure 5 : ROS remnants in electron microscopy.**

(A) Electron microscopy of choroidal vessels of dystrophic rat retina show intraluminal cells (stars) with degrading intralysosomal outer segments (arrows). Ectopic MC show intracellular

intralysosomal remnants of the outer segment at early (B) and late (C) stages of degradation.

Results are representative of at least four independent experiments. RPE: retinal pigment

epithelium. Ch: choroid. Scale bars: 2  $\mu\text{m}$ .