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Aldose Reductase Mediates Retinal Microglia Activation

Kun-Che Chang[1], **Biehuoy Shieh**[1], and **J. Mark Petrash**[*,1]

[1]Department of Ophthalmology, School of Medicine, University of Colorado, Anschutz Medical Campus, Aurora, CO 80045, USA

Abstract

Retinal microglia (RMG) are one of the major immune cells in charge of surveillance of inflammatory responses in the eye. In the absence of an inflammatory stimulus, RMG reside predominately in the ganglion layer and inner or outer plexiform layers. However, under stress RMG become activated and migrate into the inner nuclear layer (INL) or outer nuclear layer (ONL). Activated RMG in cell culture secrete pro-inflammatory cytokines in a manner sensitive to downregulation by aldose reductase inhibitors. In this study, we utilized $CX3CR1^{GFP}$ mice carrying AR mutant alleles to evaluate the role of AR on RMG activation and migration *in vivo*. When tested on an AR^{WT} background, IP injection of LPS induced RMG activation and migration into the INL and ONL. However, this phenomenon was largely prevented by AR inhibitors or in AR null mice, or was exacerbated in transgenic mice that over-express AR. LPS-induced increases in ocular levels of TNF-α and CX3CL-1 in WT mice were substantially lower in AR null mice or were reduced by AR inhibitor treatment. These studies demonstrate that AR expression in RMG may contribute to the proinflammatory phenotypes common to various eye diseases such as uveitis and diabetic retinopathy.

Keywords

Aldose reductase; Retinal microglia; Inflammation

1. Introduction

Microglia were discovered as a novel cell population in central nervous system (CNS) in 1933 by a Spanish physician and histologist Pío del Río-Hortega [1]. In the eye, scientists used Hortega's staining technique to label the microglia in the retinal outer plexiform layer (OPL) in monkeys and rabbits [2], or in rats [3]. The origin of microglia has been debated for a long time. In the early 19 century, scientists hypothesized that microglia were derived

[*]Corresponding Author: J. Mark Petrash, Ph.D., Department of Ophthalmology, School of Medicine, University of Colorado Anschutz Medical Campus, 12800 East 19th Avenue, Mail Stop 8311, RC1-North, 5100, Aurora, CO 80045, Tel: 303-724-0681; Fax: 303-848-5014; mark.petrash@ucdenver.edu.

Conflict of Interest Statement

The authors declare that there are no conflicts of interest.

from mesenchyme and used the term “mesoglia” [1], which subsequently supported by other researchers in the late 19th [4] and early 20th centuries [5]. Other observations pointed to alternative origins for microglia, including the nervous system [6, 7], monocytes/macrophages [8, 9], or even yolk sac [10–12]. Retinal microglia (RMG) migrate from the vasculature [13] and reside in the inner or outer plexiform layers, respectively. However, RMG become activated in response to proinflammatory signals or retinal injury. RMG activation has been studied in a variety of disease models, including studies of choroidal neovascularization (CNV) in wet AMD [14–16], experimental autoimmune uveoretinitis (EAU) [17, 18], and mechanisms leading to diabetic retinopathy [19]. Given the devastating vision loss associated with robust inflammation in the retina, prevention of RMG activation is an important therapeutic goal for management of many ocular diseases.

Aldose reductase (AR), best studied as an enzyme of the polyol pathway, is involved in ocular complications of diabetes including diabetic cataract [20–22] and diabetic retinopathy [23, 24]. Recent studies also implicate AR in the pathogenesis of uveitis [25–27] and fibrotic changes associated with posterior capsular opacification [28, 29]. Genetic or pharmacological blockade of AR successfully alleviates inflammatory responses induced by endotoxin [25, 26, 30–32] or hyperglycemia [33–35]. Our previous study showed that genetic or pharmacological downregulation of AR prevents endotoxin-induced inflammatory responses in RMG primary cell cultures [26]. However, understanding of AR-regulated RMG behavior *in vivo* is still unclear. In this study, we took a dual approach to investigate the AR effect on RMG *in vivo* by using AR inhibitors or AR knockout mice (ARKO). Experiments were conducted in CX3CR1^{GFP} mice, where microglia express green fluorescent protein (GFP) [36], allowing us to easily track RMG localization and migration in the eye. By crossing the CX3CR1^{GFP} allele with our AR Transgenic (AR-Tg) mice, we additionally tested the effect of AR expression level on RMG activation in response to endotoxin exposure. Taken together, these studies implicate AR as an effective mediator of RMG activation in the mouse eye.

2. Materials and Methods

2.1. Materials and cell culture

LPS (*Salmonella enterica* serotype typhimurium) was purchased from Sigma-Aldrich (St. Louis, MO, USA). Sorbinil was generously provided by Pfizer Center Research (Groton, CT, USA. Primary RMG isolation was performed as previous described [26]. RMG were cultured in complete Dulbecco’s Modified Eagle Medium (DMEM/F12) supplemented with 4 mM L-glutamine, 10% (v/v) fetal bovine serum, 100 units/mL penicillin, and 100 μg/mL streptomycin.

2.2. Animals and treatments

This research was conducted in compliance with ARVO Statement for the Use of Animals in Ophthalmic and Vision Research. C57BL/6 wildtype (WT) mice and CX3CR1^{GFP} mice were obtained from The Jackson Laboratory (Bar Harbor, ME, USA). AR transgenic mice (AR-Tg) and AR knockout (ARKO) mice were generated from previous studies [37, 38]. Animal work was approved by Institutional Animal Care and Use Committee at University

of Colorado. Mice crossbred with CX3CR1^{GFP} mice carried one copy of the CX3CR1^{GFP} allele. All experimental mice were also genotyped as homozygous for the wild type allele of the retinal degeneration *rd8* mutation [39]. For our AR inhibitor study, WT mice were randomly assigned to different treatment groups (PBS+DMSO, $N = 3$; LPS+DMSO, $N = 3$; LPS+Sorbinil (10 mg/kg in DMSO), $N = 3$). For AR deficiency study, WT and ARKO mice were assigned to different groups (WT+PBS, $N = 3$; WT+LPS, $N = 3$; ARKO+PBS, $N = 3$; ARKO+LPS, $N = 3$). For AR overexpression study, WT and AR-Tg mice were assigned to two groups (WT, $N = 3$; AR-Tg, $N = 3$) for studies of RMG distribution in whole retina and ocular tissue cryosections.

2.3. Immunofluorescence quantification

RMG were visualized by GFP expression in cell as previously described [36]. Retinal vasculature was visualized by staining with Isolectin IB_4 Alexa Fluor conjugate (Invitrogen, Grand Island, NY, USA). In brief, enucleated eyes were embedded in Tissue-Tek optimal cutting temperature (OCT) compound (Sakura Finetek, Torrance, CA, USA) for freezing. Sections (10 μm thick) were prepared by cryostat (Micro HM 550, Thermo Scientific, Waltham, MA, US) and were mounted on SuperFrost Plus slides (Fisher Scientific, Pittsburgh, PA). Sections were washed in PBS for 15 min and mounted with DAPI-mounting buffer. RMG were identified according to their GFP autofluorescence and characteristic cellular morphology. To visualize the retinal vasculature, sections were stained with Isolectin IB_4 for 2 h at room temperature and then washed with PBS for 30 min. Images were obtained using a Nikon Eclipse 80i light microscope fitted to a Nikon DS Qi1Mc camera.

2.4. ELISA assay

Dissected retinas or whole eye globes were disrupted and sonicated in lysis buffer. TNF-α and CX3CL-1 amounts were determined using corresponding Mouse Cytokine and Growth Factor Immunoassays (ElisaTech, Aurora, CO). The optical density of ELISA reactions was measured using a BioTek Synergy™ 4 Hybrid Microplate Reader (Bio Tek) and the levels of each cytokine were deduced from the absorbance value by extrapolation from a standard curve generated in parallel.

2.5. Statistical analysis

Results are shown as the Means ± SEM of at least three experiments. Data were analyzed by Student's *t* test with *P* value of <0.05 considered significant.

3. Results

3.1. Pharmacological inhibition or genetic ablation of AR alleviates LPS-induced RMG activation

In response to inflammatory stimulation, RMG migrate from the outer plexiform layers (OPL) to the subretinal space where they release cytokines, which cause retinal damage [40]. Therefore, suppressing RMG migration is the potential strategy to protect the retina from cytokine-induced injury. Our previous study used primary cell cultures to demonstrate a role for AR in retinal inflammation. However, evaluation of the role for AR expression in RMG in the context of the complex cellular organization of the retina has not been

previously reported. In this study, we utilized WT-CX3CR1^{GFP} transgenic mice, which spontaneously express GFP in RMG in retina. At 24h following IP-injection of LPS, we observed that RMG become activated (Fig 1B) and migrate to the INL and ONL retinal layers (Fig 1E). In contrast, RMG were significantly less abundant in the INL and ONL ($p<0.005$) in mice treated with IP-injections of physiological saline as a control (Fig 1A and 1D). Treatment of mice with Sorbinil, a well-characterized AR inhibitor, attenuated both RMG activation (Fig 1C) and migration (Fig 1F). Cell migration in the absence or presence of AR inhibitor was combined in a bar chart (Fig 1G). As an additional test of the influence of AR on these responses to LPS stimulation, we included WT-CX3CR1^{GFP} and ARKO-CX3CR1^{GFP} mice for the study. In the absence of LPS, RMG in ARKO-CX3CR1^{GFP} (Fig 2C) group behave similarly to WT-CX3CR1^{GFP} (Fig 2A) group. In the presence of LPS, RMG migrate to INL and ONL regions of the eye (Fig 2B). However, LPS-induced RMG migration was substantially reduced in ARKO-CX3CR1^{GFP} mice (Fig 2D) suggesting that lack of AR prevents RMG from undergoing LPS-induced activation. Significant differences in cell migration between WT and ARKO groups can be appreciated when displayed graphically as shown in Fig 2E.

3.2. AR regulates cytokine and chemokine production in the eye

To understand the correlation of RMG activation and inflammatory modulator expression, we measured cytokine and chemokine secretion in retina and whole eye in our mouse model. We previously reported that AR modulates inflammatory product secretion in RMG [26]. In this *in vivo* study, we observed that genetic knockout or pharmacological inhibition of AR suppresses TNF-α production in either retina (Fig 3A) or whole eye (Fig 3B) of mice. Chemokine (C-X3-C motif) ligand 1 (CX3CL-1) is in charge of recruiting immune cells to sites of inflammation [41]. We found that LPS induces CX3CL-1 in the eye of WT mice but fails to induce CX3CL-1 elevation in the eye of ARKO mice (Fig 3C). These results are concordant with our observation of lowered LPS-induced RMG migration in the retina of ARKO mice (Fig 2).

3.3. AR overexpression elevates RMG activation in retina

To observe the AR overexpression effect on RMG and vasculature, we utilized 3 month old ARTg-CX3CR1^{GFP} mice in which AR is overexpressed in lens and retina. In comparison to WT mice (Fig 4A), we found an increase in number and brightness of RMG in the retinas of AR-Tg mice (Fig 4B), indicating that AR overexpression induces RMG activation. No obvious change in the retinal vasculature was noted. We observed very few RMG residing in the subretinal space (Fig 4C). However, an increased abundance of RMG was observed in inner or outer nuclear layers of retinas in AR-Tg mice (Fig 4D). Quantification of cell migration in the comparison of WT and AR-Tg groups are shown in Fig 4E. In addition, Western blots of whole retinas from WT and AR-Tg mice demonstrated that AR is elevated almost 3-fold in the AR-Tg group in comparison to WT (Fig 4F). While we do not yet know whether the elevated AR expression in retina has an endocrine rather than paracrine effect on RMG behavior, results from both genetic and pharmacological studies point to AR as playing a key role in ocular inflammation.

4. Discussion

Many studies have indicated that RMG are involved in ocular diseases such as uveitis [42] and diabetic retinopathy (DR) [43, 44]. Therefore, understanding the mechanism of RMG activation is a necessity for preventing these ocular diseases. Ramana and colleagues conducted many studies showing that AR plays a key role in modulating inflammatory responses in macrophages [30–32]. In agreement with Ramana's group, we previously demonstrated that either genetic ablation or pharmacological inhibition of AR attenuates endotoxin-induced inflammatory responses in macrophages and primary RMG [25, 26]. In this study, we further demonstrated the *in vivo* effect of AR using AR mutant mice or studies involving AR blockade with Sorbinil, an effective AR inhibitor (Fig 1 and 2). LPS-induced RMG migration (Fig 1E and 2B) was attenuated by either Sorbinil (Fig 1F) or AR knockout (Fig 2D).

Expression levels of AR appear to correlate with the activation phenotype observed in RMG cell populations and abundance of ocular TNF-α and CX3CL-1. Levels of RMG activation and migration ability are reduced by AR inhibition (Fig 1) and gene inactivation (Fig 2). In addition, ocular TNF-α and CX3CL-1 are reduced by AR inhibition or genetic inactivation (Fig 3). The functional linkage between AR and RMG behavior is also apparent when AR levels are increased. Elevated levels of AR expression in transgenic mice are associated with increased RMG activation and migration to INL and ONL layers in the retina (Fig 4). Our observations point to AR as a drugable target for anti-inflammatory therapy in conditions such as uveitis. In addition, these studies suggest that blinding complications of diabetes, such as diabetic retinopathy and diabetic cataracts, which are associated with elevated inflammatory markers, may result from an AR-mediated mechanism [45].

AR is an enzyme involved in conversion of glucose into sorbitol using NADPH as a cofactor [46]. This AR polyol pathway generates sorbitol accumulation and NADPH depletion. NADPH participates in detoxification via glutathione (GSH) reductase pathway [47]. Therefore, reduction of NADPH attenuates removal of reactive oxygen species (ROS), suggesting that increased influx of AR polyol pathway causes oxidative stress. Diabetic patients have higher risks to develop organ complications such as nephropathy [48], cardiomyopathy [49] and peripheral neuropathy [50]. Previous data suggested that these diabetic complications could be due to AR elevation in these patients [51]. In the diabetic eye, cataract [52] and retinopathy [53] are major complications causing vision loss. Other than oxidative stress, AR polyol pathway also accelerates advanced glycation end-product (AGE) formation by producing fructose, which forms AGE much faster than glucose [54]. A study observed the increase of AGE in retina causes ocular inflammation by inducing RMG activation [19]. Accordingly, blockade of AR polyol pathway could play a protective role in retina by preventing AGE-induced inflammation.

NF-κB signaling pathway is one of the mechanisms that explain how AR induces inflammation. Many studies reported that down-regulation of AR either by genetic or enzymatic methods suppresses NF-κB activation in macrophages [30, 31] or in a mouse uveitis model [27]. Under cellular activation, NF-κB would be acetylated then translocated into nucleus to bind to transcriptional element of DNA, further inducing inflammatory

signaling [55]. However, the activity of acetyl-NF-κB could be attenuated due to deacetylation by sirtulin-1, which is an NAD^+-dependent deacetylase [56]. AR polyol pathway is involved in NAD+ consumption in conversion of sorbitol into fructose by sorbitol dehydrogenase (SDH) [46]. Increased influx of AR polyol pathway results in reduction of NAD^+, which would be expected to reduce sirtulin deacetylase activity. We, therefore, suspect that less deacetylase activity causes more acetyl-NF-κB accumulation in nucleus and remains higher inflammatory signaling production.

Many studies showed that AR plays a role in eye pathogenesis including retinopathy. However, few studies mentioned that AR contributes to eye pathogenesis via immune cells. Here we report the first study regarding the role of AR in RMG activation *in vivo*. Our results point to AR as an attractive drugable target for suppressing inflammation in the eye. Future studies are required to determine if inhibition of AR is an effective strategy for prevention of ocular inflammation in a variety of disease settings such as diabetes and uveitis.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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Abbreviations

AR	aldose reductase
ARI	aldose reductase inhibitor
RMG	retinal microglia

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- AR inhibition prevents retinal microglial activation
- Endotoxin-induced ocular cytokine production is reduced in AR null mice
- Overexpression of AR spontaneously induces retinal microglial activation

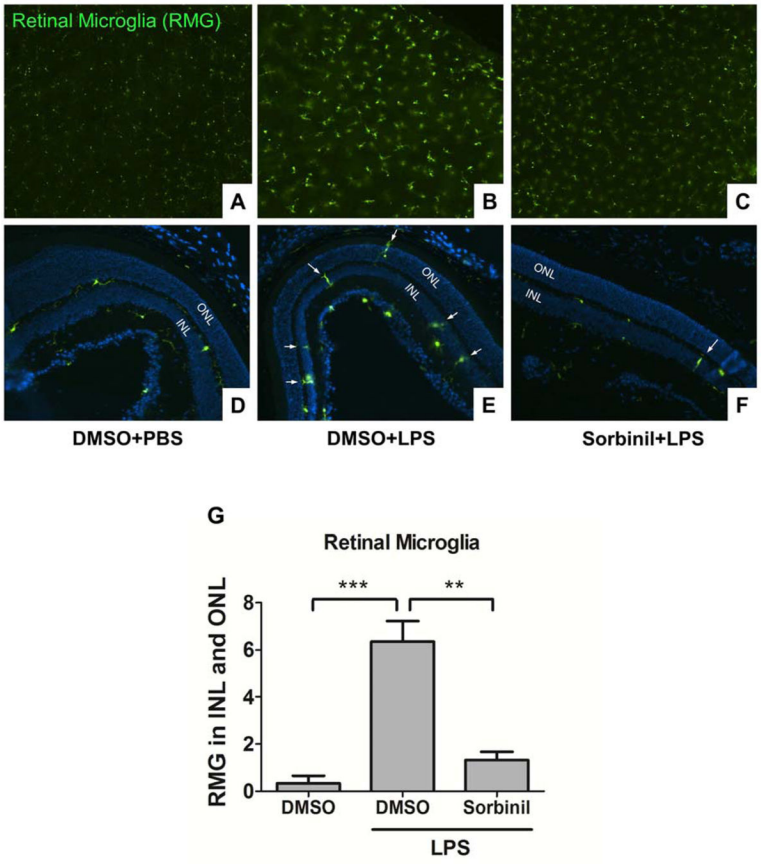

Fig. 1. Aldose reductase inhibitor prevents microglia from LPS-induced activation in the retina

WT-CX3XR1^{GFP} mice were injected with LPS (20 mg/kg body weight) in the presence of DMSO or Sorbinil (10 mg/kg body weight) for 24 h before sacrifice. Whole retinas from each group were mounted on slides (A–C) and cryo sections (D–F) were collected from each group stained with DAPI. GFP expression was used to indicate RMG. White arrows indicate migrated RMG in inner or outer nuclear layers. Statistic data was performed in a bar chart (G). Data shown are means ± SEM (N = 3). $^{**}P < 0.01$, $^{***}P < 0.005$. INL, inner nuclear layer; ONL, outer nuclear layer.

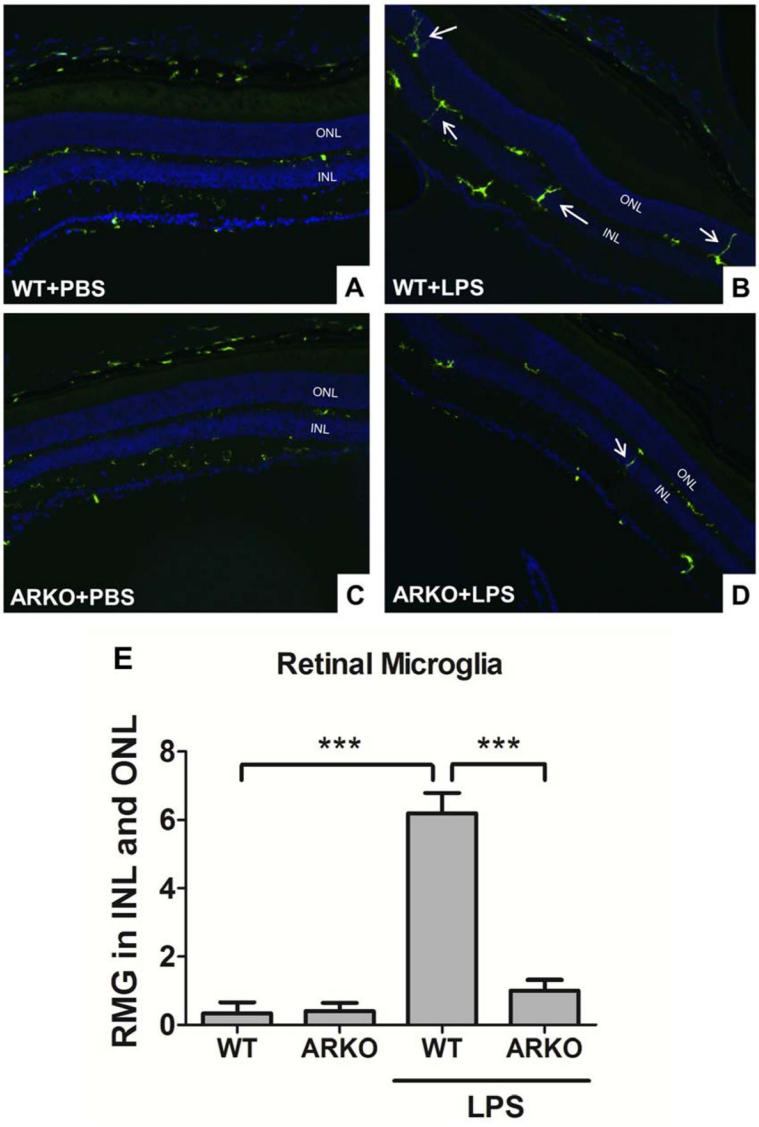

Fig. 2. Genetic ablation of aldose reductase prevents microglia from LPS-induced activation in the retina

WT-CX3XR1^{GFP} mice (A and B) and ARKO-CX3XR1^{GFP} mice (C and D) were injected with LPS (20 mg/kg body weight) for 24 h before sacrifice. Cryo sections were collected from each group and stained with DAPI. GFP expression was used to indicate RMG. White arrows indicate migrated RMG in inner or outer nuclear layers. Statistic data was performed in a bar chart (F). Data shown are means ± SEM (N = 3). ****P* < 0.005. INL, inner nuclear layer; ONL, outer nuclear layer.

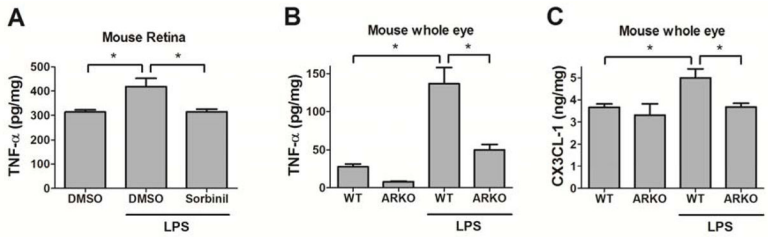

Fig. 3. Pharmacological inhibition or genetic ablation of aldose reductase reduces LPS-induced cytokines expression in the retina and whole eye

WT mice (A) and ARKO mice (B and C) were injected with LPS (20 mg/kg body weight) for 24 h before sacrifice. Mouse retinas (A) or whole eyes (B and C) were lysed in RIPA buffer. TNF-α (A and B) and CX3CL-1 (C) were measured using ELISA kit. Data shown are means ± SEM (N = 3). $*P < 0.05$.

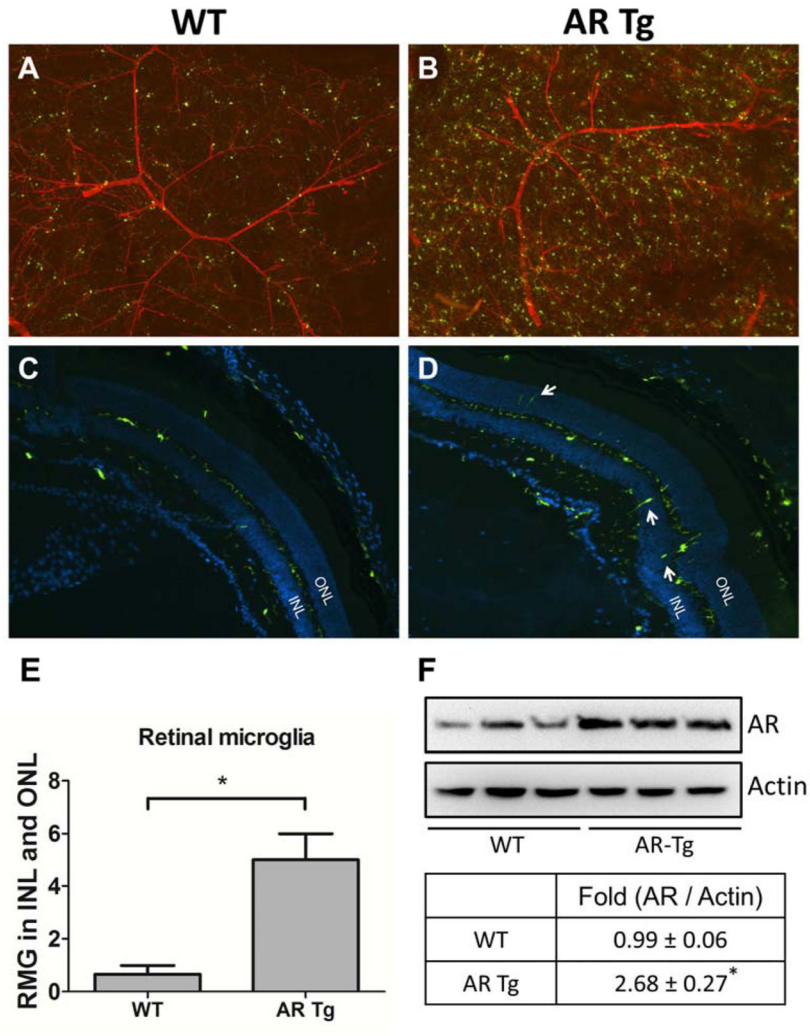

Fig. 4. Overexpression of aldose reductase causes microglia activation in the retina

WT-CX3XR1^{GFP} mice (A and C) and ARTg-CX3XR1^{GFP} mice (B and D) were sacrificed at 3 months of age. Whole retinas from each group were mounted on slides with Isolectin IB_4 staining (A and B) and cryo sections (C and D) were collected from each group stained with DAPI. GFP was spontaneously expressed indicating RMG. White arrows indicate migrated RMG in inner or outer nuclear layers. Statistic data was performed in a bar chart (E). Level of AR in retinas was measured by Western blot (F). Data shown are means ± SEM (N = 3). *$P < 0.05$. INL, inner nuclear layer; ONL, outer nuclear layer.