Macrophages fine-tune the shape of pupils

Running title: Macrophages in the pupillary shape

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Highlights (maximum 85 characters, including spaces)

1. Absence of macrophages results in zig-zag shaped pupils.
2. Macrophages do not prune pupillary membrane vessels during postnatal first week.
3. Macrophages engulf pigmented debris in pupillary edges.
4. Irises protrude between blood vessels in the absence of macrophages.

Abstract

Tissue macrophages, ubiquitously presenting innate immune cells, play versatile roles during development and organogenesis. In particular they prune transient or unnecessary synapses and blood vessels for remodeling developing tissues properly. In this study, we show macrophages contribute to the formation of the pupillary shape. *Csf1*$^{op/op}$ mutant mice, which ocular macrophages are almost absent, have zig-zag shaped pupils. Macrophages located near the pupillary edge engulf pigmented debris, likely unnecessary protrusion of irises which emerges during smoothening of the pupillary edge. Indeed these macrophages phenotypically possess M2-like features indicating high activity of tissue engulfment and remodeling. Interestingly, protruded irises in *Csf1*$^{op/op}$ mice were only detected in the gap between regressing blood vessels, suggesting cooperation of macrophages and blood vessels in this process. Taken together, our data uncovered a new role of tissue macrophages; they fine-tune the shape of pupils by shaving the unnecessary portion of irises.

(**single PDF で 10MB 以下**)

Introduction

Tissue macrophages are mononuclear phagocytes with versatile roles in development, tissue homeostasis, and immunity. They mostly originate from yolk-sac-derived erythro-myeloid progenitors distinct from hematopoietic stem cells (Gomez Perdiguero et al., 2015), and subsequently acquire tissue-specificity after colonization into the whole embryo (Mass et al., 2016). Functionally, during embryonic and postnatal development, macrophages properly remodel various organs by removing transient or unnecessary tissues, in particular pruning synapses (Paolicelli et al., 2011) and blood vessels (Lang and Bishop, 1993). In addition, polarization of macrophages is known to influence their activity for tissue remodeling as well as inflammatory responses (Sica and Mantovani, 2012).

Pupillary membrane (PM) vessels and hyaloid vessels (HVs) form the temporary circulatory system in the fetal eyes that gradually regresses after birth in mice and by the late gestation in humans (Lang et al., 1993; Silbert et al., 2000). This programmed regression of feta-type vessels is controlled by macrophages (Lang and Bishop, 1993) as well as by neurons (Yoshikawa et al., 2016). However, the role of macrophages for remodeling of other ocular components has not been elucidated. Here, through analysis of *Csf1*$^{op/op}$ mutant mice (i.e., mice that possess a mutant *Csf-1* gene) (Marks and Lane et al., 1976; Yoshida et al., 1990; Cecchini et al., 1994) which eye balls almost lack macrophages, we report a novel developmental role of macrophages. They fine-tunes the shape of pupils through engulfment of unnecessary irises in collaboration with blood vessels.

Results

Visualization and characterization of pupillary membrane vessels.

We previously reported the mechanism for the programmed regression of HVs with a whole-mount immunostaining technique (Yoshikawa et al., 2016). In this study, we first established a procedure to visualize whole-mount structures of PM vessels, other fetal-type vessels in eye balls. Modifying the previous protocol (Poché et al., 2015), we isolated the PM en bloc with the iris as a template and stained immunohistochemically (**Fig. 1A**). This technique allowed us to examine the whole structure of PM vessels surrounded by macrophages, and found their endothelial cells (ECs) are actively proliferating (**Fig. 1B-E**) at postnatal (P) day 3, but not at P8 (data not shown). These data indicated PM vessels before P6 is still growing rather than regressing. Retinal vessels are also actively growing at this stage and the extent of the growth is robustly regulated by the vascular endothelial growth factor (VEGF) and Notch signaling (Benedito et al., 2012; Zarkada et al., 2015). Therefore we tested the effect of deletion of VEGF receptor 2 (VEGFR2) or Dll4 (**Fig. 1F**), well known endothelial ligand for the Notch signaling in neighboring ECs (Hellström et al., 2007). When we deleted *Vegfr2* in ECs from P2 using *Cdh5-BAC-Cre*^{ERT2} mice (*Cdh5–BAC–Cre*$^{ERT2+}$*Vegfr2*$^{flox/flox}$, hereafter referred to as *Vegfr2*$^{i\Delta EC}$), the *Vegfr2*$^{i\Delta EC}$ pups showed preterm regression of PM vessels at P6 (**Fig. 1G, H, J, K, M**). Conversely, mice with EC-specific *Dll4* deletion using the same mice (*Cdh5–BAC–Cre*$^{ERT2+}$*Dll4*$^{flox/flox}$, hereafter referred to as *Dll4*$^{i\Delta EC}$) showed increased density in PM vessels at P6 (**Fig. 1G, I, J, L, M**). These data suggest PM vessels before P6 are highly plastic and are vulnerable to cessation of the VEGF and Notch signalings like retinal vessels. However, deletion of *Vegfr2* in

neurons did not affect the density of PM vessels (data not shown) unlike HVs (Yoshikawa et al., 2016) suggesting the regression of PM vessels and HVs are regulated differently.

Absence of macrophages results in zig-zag shaped pupils.

Previously it was reported that lack of macrophages leads to the persistence of PM and its vasculature even two weeks after birth (Lang et al., 1993). However, the status of PM vessels during early neonatal stages, especially the first week after birth, has not been clear both in the existence or absence of macrophages. Moreover, it has been reported that macrophages contribute to bridging of tip cell filopodia leading to the new branch formation of growing retinal vessels (Fantin et al., 2010). Therefore we analyzed the PMs of *Csf1*$^{op/op}$ mutant mice in earlier stages than the previous report (Lang et al., 1993). In the bright field view, *Csf1*$^{op/op}$ mice interestingly showed pupillary edges with a lot of protruded irises, that is to say zig-zag shaped pupils et P6 (**Fig. 2A-C**). Furthermore, we followed the time course of this zig-zag shape, and measured the area protruding over pupillary edges. While no significant protrusion was detected by P2 in *Csf1*$^{op/op}$ mice, they had significant protrusion throughout the pupillary edges after P4, in contrast to smoothened edge of pupils in wild-type mice (**Fig. 2D-L**). This defect in *Csf1*$^{op/op}$ mice were normalized until weaning (data not shown), suggesting the existence of some compensatory mechanism. To test the involvement of the persistence of PM vessels in this phenotype, we thoroughly examined those vessels by immunostaining (**Fig. 2M-T**). Although PM vessels were persistent at P24 in *Csf1*$^{op/op}$ mice in agreement with a previous report (Lang et al., 1993), we found both vessel length and density were not significantly different between wild-type and *Csf1*$^{op/op}$ mice before P8 (**Fig. 2M-W**).

These data suggest the persistence of PM vessels in the absence of macrophages occurs two weeks after birth, and the pupillary phenotype in *Csf1*$^{op/op}$ mice is independent of persistence of PM vessels.

Macrophages engulf pigmented debris in pupillary edges.

Next, we examined the number and localization of macrophages in neonatal PMs. In wild-type, a large number of macrophages existed in the PMs, iris, and their marginal zone (**Fig. 3A, C**). Intriguingly, macrophages were present more abundantly in the periphery than in the center of PMs (**Fig. 3A**), suggesting some active function in the peripheral area. However, *Csf1*$^{op/op}$ mice lacked almost all of macrophages including the area around the protruded pupils (**Fig. 3B, D, E**). Macrophages in normal skin are capable of ingesting melanin, and called "melanophages" (Haniffa et al., 2009, 2012). Pigment uptake by dermal macrophages has been discussed in terms of tattoo removal (Kennedy et al., 1997; Baranska et al., 2018). Therefore, we suspected that macrophages on pupillary edges could also engulf pigmented debris. DAB (diaminobenzidine)/ Giemsa counterstaining of pupillary membrane showed that a lot of Iba1^{+} macrophages in wild-type mice included melanin granules in their cytoplasm suggesting active phagocytosis of pigmented tissues (**Fig. 3F, G**). The analysis by transmission electron microscopy (TEM) confirmed that pupillary macrophages had pigmented debris in their phagosomes (**Fig. 3H**). Similarly, use of transmission detector (TD) indicated that macrophages near the pupillary edges had pigmented granules (**Fig. 3I-K**). Indeed, *Csf1*$^{op/op}$ mice lacked such pigmented substances (**Fig. 3L, M**). The protruded iris observed in *Csf1*$^{op/op}$ mice was entirely located inside sphincter pupillae muscles stained with alpha smooth muscle actin (ASMA), and lacked nuclei (**Fig. 3N, O**), suggesting

those tissues are cellular debris which appear during the enlargement of pupils. TUNEL-positive pigmented cells were detected around sphincter pupillae muscles both in wild-type and *Csf1*$^{op/op}$ mice (**Fig. 3P-S**) suggesting apoptosis is actually involved in the physiological process of pupillary enlargement leading to the appearance of debris.

Macrophages in the pupillary membrane are M2-like.

Macrophages are typically classified into two states: M1 (classically) and M2 (alternatively) activated phenotypes (Biswas and Mantovani, 2010; Murray et al 2014) . M1 macrophages are the pro-inflammatory type, important in direct host-defense against pathogens through secretion of pro-inflammatory cytokines like *Il-6* and *Ifn-γ*. In contrast, M2 macrophages reduce inflammatory responses, but promote tissue remodeling and show abundant phagocytic activity (Biswas and Mantovani, 2010; Sica and Mantovani, 2012; Okuno et al., 2011). Several M2-specific markers have been identified like *Mrc1*, *Arg1*, *Igf1*, and *Csfr1* (Sica and Mantovani, 2012; Spadaro et al., 2017; Ohashi et al 2017). Considering the phenotypes of pupillary macrophages described above and the immune privilege in the anterior chamber of the eye, they were assumed to polarize toward M2 rather than M1. To test this, we quantified the relative expression of M1/M2 markers in pupillary macrophages, bone marrow macrophages, and retinal (resting) microglia. As expected pupillary macrophages expressed lower M1 and higher M2 markers than bone marrow macrophages, while retinal microglia showed the lowest expression in these macrophage markers except for *Nos2* (**Fig. 4A**). These data agree with the idea that pupillary macrophages are M2-like ones suggesting they are highly phagocytic but immunosuppressive. Immunohistochemical analysis also found pupillary macrophages were positive for Mrc1, and Mrc1^{+} macrophages were

particularly abundant around the pupillary edge (**Fig. 4B-G**).

Pupils protrude between blood vessels in the absence of macrophages.

If pupillary macrophages are to smoothe pupillary edges, they have to selectively shave protruding debris. Therefore, we analyzed exactly which parts of pupils protruded (**Fig. 5A**). The high magnification of pupillary edges revealed that irises protrude between blood vessels crossing pupils in *Csf1*$^{op/op}$ mutant mice. In addition, these peri-pupillary vessels looped around pupillary edges from the upper to lower surface of pupils (hereafter referred to as looping vessels) and appeared to drag in iris tissues (**Fig. 5B-D**). These data show that absence of macrophages leads to protrusion between looping vessels, where they failed to draw irises aside. To assess the blood flow known to be important for the regression of PM vessels (Morizane et al 2006; Meeson et al 1996), we applied *in vivo* imaging of VEGFR2-BAC-EGFP mice (Ishitobi et al., 2010). The result showed blood flow was abundant in looping vessels, but far less in the central vessels at P3, suggesting the programmed regression of PM vessels initially occurred in the central vessels in the later stages (**Fig. 5E; Supplementary Movie 1**). Staining erythrocytes with Ter119 antibodies confirmed the abundant blood flow in the looping vessels (**Fig. 5F**). As these results indicated the looping vessels were stable and physically stiff, we suspected the drug-in force of these vessels on the pupillary edge. Collectively, macrophages fine tune the shape of pupils likely in harmony with blood vessels.

Discussion

In the present study, we uncovered a novel role of macrophages in the ocular

development; they fine-tune the shape of pupils. Macrophages smoothe pupillary edges by engulfing enucleated pigmented debris of irises between looping blood vessels. Whereas in absence of macrophages, the debris which looping blood vessels have failed to drag in protrudes over pupillary edges, which results in zig-zag shaped pupils (**Fig. 5G**).

A number of studies reported that macrophages control formation or regression of ocular blood vessels (Lang and Bishop, 1993; Kubota et al., 2009; Fantin et al., 2010). Little information has been provided on the orchestration of macrophages and blood vessels to remodel another structure. In the present study, we demonstrated the cooperation of macrophages and blood vessels in fine-tuning the shapes of pupils. The normalization of the defects before weaning may occur few macrophages existing in *Csf1*$^{op/op}$ mice might be sufficient to dispose the debris. Another possibility is that the number of pupillary macrophages decreases after P6 (**Fig. 3E**), and the functional significance of pupillary macrophages taper after P6. Other cell types might compensate the role of macrophages in terms of the clearance of tissue debris.

The eye is often compared to a sophisticated camera. The iris, which surrounds the pupil, works as a shutter. A recent study has indicated a striking correlation between terrestrial species' pupillary shape and ecological niche (Martin et al., 2015). In this study we uncovered a new mode for shaping murine pupils. Macrophages fine tune the shape of pupils independently of well-known regressing effects on blood vessels. They phagocytose the unnecessary iris during the process of pupillary enlargement which is initially led by the drag-in effect of looping vessels located in the pupillary edge.

The zig-zag shaped pupils of *Csf1*$^{op/op}$ mutant mice looked similar to irregular pupils caused by posterior synechia, which is one of the major complications after intraocular

surgery, makes fundus examination difficult and causes photophobia, pupillary block and poor eyesight. Because inflammation in the anterior chamber provokes synechia between the iris and the peripheral structures, short-acting mydriatics and topical steroid injection etc. have been suggested to prevent the synechia (Lee et al., 2009; Shinoda et al., 2001). In this study, we showed that M2-like macrophages are responsible for the developmental remodeling of pupillary shapes. Modulation of macrophage polarization could serve as an effective treatment for posterior synechia.

Overall, the present results revealed a novel developmental function of macrophages; they fine tune the shapes of pupils in cooperation with blood vessels. This finding could contribute to further understanding of ocular autoimmune diseases.

Summary Statement

Tissue macrophages are mononuclear phagocytes with versatile functions in development, tissue homeostasis, and immunity. The present study demonstrated that macrophages engulf unnecessary pupillary tissues and contribute to fine tuning the pupillary shape in cooperation with blood vessels.

Materials and methods

Mice and analysis

All procedures involving animals were approved by the Institutional Animal Care and Use Committee of the Keio University or the Ethical Committee of Jichi Medical University, and performed in accordance with the guidelines of those universities. The C57BL/6 mice (Japan SLC, Inc.) and *Csf1*$^{op/op}$ mutant mice (The Jackson Laboratory; Stock No: 000231) were used. *Cdh5-BAC-Cre*^{ERT2} (Okabe et al., 2014), *Vegfr2-flox* (Okabe et al., 2014), *Dll4-flox* (Hozumi et al., 2008), and *Vegfr2-BAC-EGFP* (Ishitobi et al., 2010) mice were previously described. Mice of both sexes were included in analysis without exact determination. All mice were crossed with C57BL/6J mice more than eight times and maintained except for *Csf1*$^{op/op}$ mutant mice in the background of B6C3FeF1/J a/a.

Preparation of whole-mount pupillary membranes

Enucleated eyes were fixed for 20 min in 4% paraformaldehyde in PBS and then dissected. A small hole was made in the edge of cornea using a 27-gauge needle, and a circular incision was made using fine scissors. Cornea and lens were isolated en bloc with the iris, which acted as a frame, followed by the dissection of the posterior side of lens. The whole-mount tissues were post-fixed overnight and then stained as described below.

Preparation of tissue sections

Enucleated eyes were fixed for 20 min in 4% paraformaldehyde (PFA) in phosphate-buffered saline (PBS), and then hemispheres were cut. After overnight

postfixation, samples were snap frozen in optimal cutting temperature compound (Sakura Finetechnical, Tokyo, Japan). All specimens were sectioned to a thickness of 14 μm.

Immunostaining

Immunohistochemistry (IHC) of whole-mount samples or tissue sections was performed as previously described (Kubota et al., 2009). The primary monoclonal antibodies used were hamster anti-CD31 (Chemicon, Temecula, CA, 2H8; 1:1,000), anti-Ter119 (R&D Systems, Minneapolis, MN, MAB1125; 1:1,000), α-smooth muscle actin-Cy3-conjugated (Sigma-Aldrich, Saint Louis, MO, USA, C6198; 1:500), and F4/80 (MCA497R; Serotec). The primary polyclonal antibodies used were anti-GFP (Alexa488-conjugated; Molecular Probes, Eugene, OR; 1:1,000), Erg (Abcam; Cambridge, UK, ab92513; 1:2,000), Iba1 (WAKO; Osaka, Japan; 1:1,000), and Mrc1 (R&D systems; Minneapolis, MN; AF2535; 1:100).

Secondary antibodies used were Alexa 488 fluorescence-conjugated IgGs (Molecular Probes), DyLight549/DyeLight649-conjugated IgGs, or HRP-conjugated IgGs (Jackson ImmunoResearch, West Grove, PA). For nuclear staining, specimens were treated with 4',6-diamidino-2-phenylindole (DAPI) prior to mounting. In some experiments, blood vessels and monocyte lineage cells were simultaneously visualized using biotinylated isolectin B4 (B-1205; Vector Laboratories) followed by fluorescent streptavidin conjugates (Molecular Probes). For TUNEL assays, tissue sections were stained using the ApopTag Fluorescein In situ Apoptosis Detection Kit (Chemicon) according to the manufacturer's instructions. To analyze cell proliferation *in vivo*, an EdU incorporation assay using Click-iT EdU Imaging Kits (Invitrogen) was performed

according to the manufacturer's instructions. Briefly, 50 μl of EdU dissolved in DMSO/PBS (final concentration, 0.5 mg/ml) was injected intraperitoneally into P8 mice 6 h before sacrifice. For Giemsa staining, Giemsa's Azur Eosin Methylene Blue solution (Merck; Temecula, CA) was used.

Quantitative PCR analysis

Ocular, retinal, and bone marrow tissues were incubated for 30 mins at 37°C in DMEM containing 1% collagenase D (from *Clostridium histolyticum*; Sigma-Aldrich, Saint Louis, MO), 1 U/ml dispase (ThermoFisher) and 1 U/ml DNase (Invitrogen, Carlsbad, CA) before cells were dissociated by gentle trituration. Cells were isolated using Dynabeads (Veritas; Tokyo, Japan), according to the manufacturer's instructions. To isolate macrophages, dissociated cells were incubated with an anti-F4/80 antibody pre-conjugated to to Dynabeads M-450 anti-Rat IgG (DB11035; Veritas); cells were positively selected. Reverse transcription was performed using Superscript II (Invitrogen). Quantitative PCR assays were performed on an ABI 7500 Fast Real-Time PCR System using TaqMan Fast Universal PCR master mix (Applied Biosystems) and TaqMan Gene Expression Assay Mix with mouse *Csf1r* (Mm00432689_m1), *Ifng* (Mm00801778_m1), *Igf1* (Mm00439560_m1), *Il6* (Mm00446190_m1), or *Nos2* (Mm00440502_m1). A mouse *β-Actin* (Mm00607939_s1) assay mix served as an endogenous control. Data were analyzed using 7500 Fast System SDS Software 1.3.1. Each experiment was performed with four replicates from each sample and the results were averaged.

In vivo imaging

In vivo analysis for the PM of P3 pups was performed as described previously (Nishimura et al., 2012). In brief, P3 mice were injected with Texas-Red Dextran (MW 70 kDa) (Termo Fisher Scientific, Waltham, MA) intra-venously, and thereafter time-lapse images of ocular surfaces were acquired using a resonance-scanning confocal microscope (Nikon A1R System). The collected images were analyzed by observers blinded to the protocol using NIS-Elements software (Nikon).

Transmission electron microscope (TEM) analysis

Ocular samples were prepared for TEM observation as described previously (Shibata et al., 2015). Briefly, the tissues were dissected out from 2-days-old mice (n = 8), and fixed with 2.5% glutaraldehyde in 0.1M phosphate buffer (PB, pH 7.4, Muto Pure Chemicals, Tokyo, Japan) for 24 hours at 4°C. After 2 hours of post-fixation with 1.0% OsO4 (TAAB Laboratories Equipment Ltd, England, UK), samples were dehydrated in a series of increasing concentrations of ethanol (70 – 100 %), in acetone (Sigma, MO, USA), in n-butyl glycidyl ether (QY1, Oken-shoji Co. Ltd., Tokyo, Japan), in graded concentration of Epon by QY-1, and were incubated in 100% Epon (100 g Epon is composed of 27.0 g MNA, 51.3 g EPOK-812, 21.9 g DDSA and 1.1 ml DMP-30, all from Oken-shoji Co. Ltd., Tokyo, Japan) for 72 hours at 4 °C to enhance the infiltration of the resin. For 72 hours of polymerization in 100% Epon at 60°C, semithin-sections (1μm thickness) were prepared with a glass knife and were stained with toluidine blue. Ultrathin-sections (70nm thickness) were prepared by ultramicrotome (Leica UC7, Leica Biosystems, Wetzlar, Germany) with a diamond knife, were collected every 10 slices on the cupper grids, and stained with uranyl acetate and lead citrate for 10

minutes, respectively. The sections were examined under a TEM (JEM-1400plus, JEOL, Tokyo, Japan).

Confocal microscopy

Fluorescent images were obtained using a confocal laser scanning microscope (FV1000; Olympus, Tokyo, Japan). Multiple slices horizontally imaged from the same field of view at 0.5-μm intervals were integrated to construct three-dimensional images using an FV10-ASW Viewer (Olympus).

Statistics

Results are expressed as mean ± S.D. Comparisons between the averages of two groups were evaluated using a two-tailed Student's *t*-test. *P* values <0.05 were considered statistically significant.

Conflicts of interests

The authors declare no competing or financial interests.

Author contributions statement

Designed experiments: Y.K. Performed experiments: M.T., M.M., S.S., I.T-N., M.O., and S.N. Analyzed the data: M.T., M.M., S.S., T.I., K.O., and Y.K. Provided experimental materials: T.M. and M.E. Edited the manuscript: T.N. Wrote the paper: M.M., and Y.K.

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Figure Legends

Figure 1. ***Visualization and characterization of pupillary membrane vessels.***

(**A**) Schematic diagram depicting the technique used to visualize the pupillary membrane (PM) and associated blood vessels. (**B-E**) Bright-field view and whole-mount specimens of P3 or P4 PMs. Endothelial cells in PM vessels actively proliferate (arrowheads) at P3. (**F**) Protocol for 4-hydroxytamoxifen (4OHT) injection in neonates. P, postnatal. (**G-M**) Whole-mount specimens of PMs at P6 stained with Isolectin B4 and quantification (n ≧ 3). Scale bars, 500 μm (**B, C; G-I**); 50 μm (**D**, **E**, **J-L**). **P <0.01; *P <0.05; data are presented as mean ± SD.

Figure 2. ***Absence of macrophages results in zig-zag shaped pupils.***

(**A-K**) Bright-field views of the pupils at P1, P2, P3, P6 or P8. *Csf1*$^{op/op}$ mutant mice show zig-zag shaped pupils (arrowheads). (**L**) Quantification of the protruding iris showed in yellow (n ≧ 3). (**M-W**) Immunohistochemistry on whole-mount PMs at P2, 4, 6, 24 and quantification (n ≧ 3). Scale bars, 500 μm (**A, M-T**); 200 μm (**B-K, U, V**); 50 μm (**U, V**). **P <0.01; *P <0.05; NS, not significant. Data are presented as mean ± SD.

Figure 3. ***Macrophages engulf pigmented debris in pupillary edges.***

(A-D) Immunohistochemistry of whole-mount PMs at P6. *Csf1*$^{op/op}$ mice lack macrophages around zig-zag shaped pupillary edges (arrowheads). **(E)** Quantification of macrophages per pupil (n ≧ 3). (**F, G**) Immunohistochemistry of whole-mount PMs at P2. Macrophages around the pupillary edge had pigmented granules in their cytoplasm

(arrowheads). (**H**) Electron microscopic analysis of pupillary macrophages loaded with a pigmented granule. (**I-K**) Immunohistochemistry of whole-mount PM samples at P2. Macrophages had pigmented granules in their cytoplasm (arrowheads). (**L, M**) Immunohistochemistry of Iba1 combined with Giemsa counterstaining in whole-mount pupils at P2. Wild-type macrophages had pigmented granules in their cytoplasm (arrowheads). (**N, O**) Immunohistochemistry merged with images by transmission detector (TD) in whole-mount PMs at P6. The protruded iris observed in *Csf1*$^{op/op}$ mice is located inside sphincter pupillae muscles, and lack nuclei (arrowheads). (**P-S**) TUNEL staining combined with immunohistochemistry in eyeball sections at P6. Apoptotic cells are located on the iris (arrows), but not in protruding irises in *Csf1*$^{op/op}$ mutant mice (arrowhead). Scale bars, 500 μm (**A, B**); 200 μm (**C, D, N-Q**); 50 μm (**F, G, L, M, R, S**); 5 μm (**H, I-K**). Data are presented as mean ± SD.

Figure 4. ***Macrophages in the pupillary membrane are M2-like.***

(**A**) Quantitative PCR analysis in pupillary macrophages, bone marrow macrophages, and retinal microglia (n = 3). (**B-D**) Immunohistochemistry merged with images by transmission detector (TD) in whole-mount PMs at P3. Mrc1^{+} macrophages are present around the pupillary edge (arrowheads). Scale bar, 50 μm. **P <0.01; *P <0.05; data are presented as mean ± SD.

Figure 5. Pupils protrude between blood vessels in the absence of macrophages.

(**A**) Immunohistochemistry of whole-mount PMs at P6. Protruded irises (arrowheads) are detected between blood vessels (arrows) in *Csf1*$^{op/op}$ mutant mice. (**B-D**) Immunohistochemistry merged with images by TD in whole-mount pupils at P6. Each

image shows a slice indicated in the schema in **B**. Blood vessels appeared to loop over pupillary edges (arrow). (**E**) A snapshot of *in vivo* imaging for the PM of P3 VEGFR2-BAC-EGFP mice. Arrows indicate abundant blood flow in looping vessels around the pupillary edges (white line). (**F**) Immunohistochemical analysis of PMs at P3. A number of erythrocytes are detected in looping vessels (arrows) around pupillary edges (white line). Scale bars, 50 μm (**A, C-F**).

Supplementary Figures

Supplementary Movie 1. Active blood flow in the looping vessels near the pupillary margin.

In vivo imaging of the ocular surface in VEGFR2-BAC-EGFP mice injected with TexasRed Dextran intra-venously.