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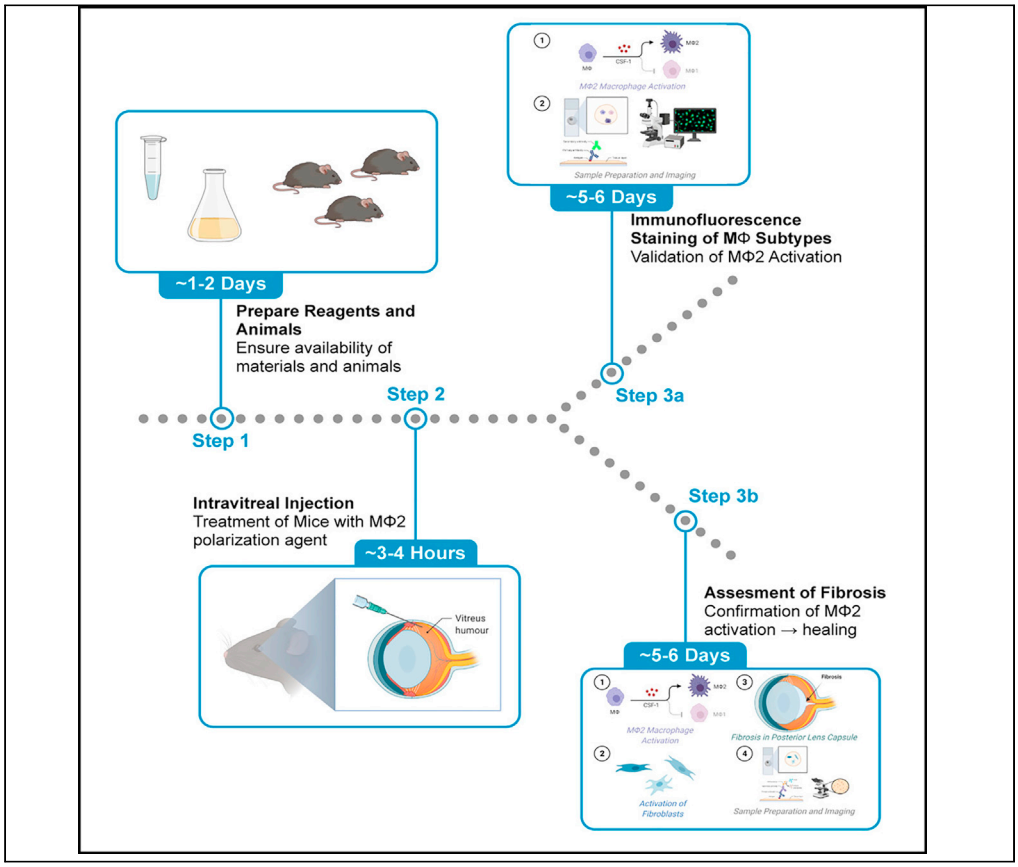
Studying macrophage activation in immune-privileged lens through CSF-1 protein intravitreal injection in mouse model



Macrophage (MΦ) activation and promotion of fibrosis are critical processes in lens capsule healing after injury. Here, we detail a protocol that induces MΦ2 formation within the vitreous body of the eye. Our procedure combines the use of an intravitreal injection of a growth factor (CSF-1) and immunofluorescence to confirm the presence of MΦ2 and fibrotic tissue formation. This protocol allows assessment of the distribution of macrophages and quantification of fibrotic tissue formation/sealing within the vitreous body of mouse eyes.

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Highlights

Use of intravitreal injection of CSF-1 protein to topically activate macrophages

Immunofluorescence assessment of different MΦ subtypes simultaneously

Study of macrophage activation in the vitreous body and lens

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Studying macrophage activation in immune-privileged lens through CSF-1 protein intravitreal injection in mouse model

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SUMMARY

Macrophage (MΦ) activation and promotion of fibrosis are critical processes in lens capsule healing after injury. Here, we detail a protocol that induces MΦ2 formation within the vitreous body of the eye. Our procedure combines the use of an intravitreal injection of a growth factor (CSF-1) and immunofluorescence to confirm the presence of MΦ2 and fibrotic tissue formation. This protocol allows assessment of the distribution of macrophages and quantification of fibrotic tissue formation/sealing within the vitreous body of mouse eyes.

For complete details on the use and execution of this profile, please refer to Li et al. (2021), Gerhardt et al. (2003), Kubota et al. (2009).

BEFORE YOU BEGIN

The protocol below describes the use of mice handled in accordance with the Association for Research in Vision and Ophthalmology (ARVO) Statement for the Use of Animals in Ophthalmic and Vision Research and institutional protocols. Assurance of appropriate animal models and reagent preparation and availability prior to the start of the experiment must be done. For studies with genetically modified mice, at least three animals from different litters should be used for each genotype. Although the preparation of colony-stimulating factor 1 (CSF-1), a hematopoietic growth factor known to promote proliferation, differentiation, and survival of monocytes and macrophages, and polarization to MΦ2 (Stanley et al., 1997; Jones and Ricardo, 2013; Mia et al., 2014), and AFS98, a monoclonal antibody that blocks CSF-1 receptor function (Hume and Macdonald, 2012; Kubota et al., 2009), for intravitreal injections are described here, animals can be treated with other activation/blocking agents for the study of MΦ activation in lens capsule healing.

Preparation of reagents and materials – day 1–2

Timing: ~1–2 days

1. Preparation of glass micropipette for intravitreal injections. Glass capillaries (Sutter Instruments, B100-75-10, outer diameter (OD) 1 mm, inner diameter 0.75 mm, length 10 cm) are used to make glass micropipettes with a tip point and an OD of ~ 40 μm for intravitreal injection.
   a. Fix the borosilicate glass capillary to the rubber cushioned clips in the P-30 manual vertical micropipette puller (Sutter Instruments (Novato, CA, USA), Figure 1A).

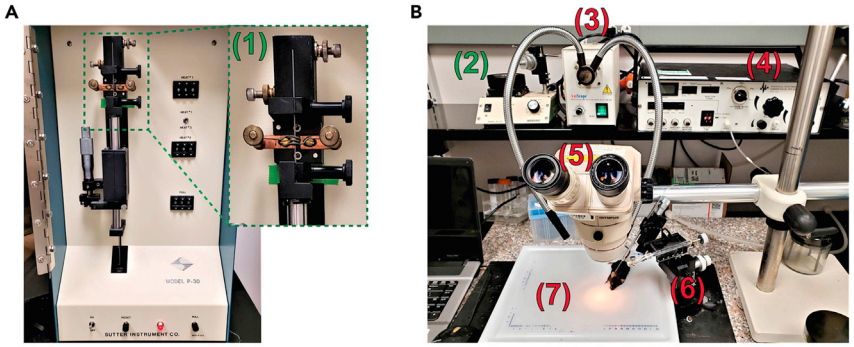

Figure 1. Instruments for intravitreal injection. instruments for intravitreal injection

(A) P-30 manual vertical microelectrode micropipette puller. (1) inset showing glass micropipette being pulled (B) (2) Micropipette grinder. Microinjection system including lighting equipment (3), Pico-Injector (4), dissecting microscope (5), drummond micromanipulator (6), animal dissection plate (7). Green = instruments for micropipette preparation. Red = instruments for performing the injection.

b. Set heat temperature (HEAT 1) to 950°C and preform pre-pulling to obtain a thinner and softer glass capillary.
c. Set heat temperature (HEAT 2) to 790°C and preform a secondary pulling to produce final micropipettes.
d. Sharpen the tip opening to an OD ~40 μm with a micropipette grinder (Narishige Model EG-40).
e. Keep the glass micropipettes in a sponge clamping pad (Sutter Instruments (Novato, CA, USA)) inside a glass jar for further experiments.

2. Coating of glass slides to increase adherence of cryosections.
   a. Prepare the cocktail described below of "glass slide coating solution" by first dissolving 2.5 g of gelatin in 500 mL of deionized warm water (final concentration 0.5%, ≤ 45°C).

   Note: Avoid surpassing 45°C to avoid degradation of gelatin

   b. Add 0.25 g of chromium potassium sulfate dodecahydrate into gelatin solution after gelatin has completely dissolved for addition of charge/promotion of adherence into solution.
   c. Filter the solution using a large pore mesh.
   d. Dip the slides 3× and incubate slides during the final dip in the coating solution for 3 min, drain, and then dry the slides on a rack in a dust-free hood at 22°C for 48 h.

   Note: Dried slides can be stored for a short period of time (~1 week) at 4°C and −20°C for long-term storage (≤ one year).

3. Preparation of CSF-1 protein solution.
   a. The stock solution is prepared by dissolving 10 μg of CSF-1 into 200 μL of saline (final concentration = 50 μg/mL) and kept at −20°C (≤ three months).
   b. Before use, the working solution is prepared by diluting the stock solution to a 1.8 μg/mL concentration.
4. Preparation of other solutions: Prepare other solutions following instructions in recipe table (Materials and Equipment). All reagents are purchased from Sigma Aldrich or Fisher Scientific unless stated otherwise.

Animal preparation

Timing: (dependent on treatment duration)

5. Ensure that mice being utilized have phenotype present of posterior capsule rupture (through confirmatory histology) or induction of injury is done.

Note: Mice discussed in this protocol "Cx50 and AQP0 double knockout ($Cx50^{-/-}$/$Aqp0^{-/-}$)) (Li et al., 2021), phenotypically have damaged posterior capsules in their eyes. However, other models, such as the lens injury model (Leon et al., 2000), cataract surgery model (Jiang et al., 2018) and optic nerve injury model (Leibinger et al., 2009) are all other scenarios where there is lens injury and macrophage activation.

KEY RESOURCES TABLE

REAGENT or RESOURCE	SOURCE	IDENTIFIER
Antibodies		
Rat Anti-Mouse CD68 Monoclonal Antibody | Clone: FA-11	Bio-Rad Laboratories (Hercules, CA, USA)	MCA1957
Mouse Anti-Human Smooth Muscle Actin Monoclonal Antibody | Clone: 1A4	Agilent (Santa Clara, CA, USA)	M085129-2
Goat Arginase 1 (ARG1) Polyclonal Antibody	Novus Biologicals (Centennial, CO, USA)	NB10059740
Rabbit iNOS Polyclonal Antibody	Novus Biologicals (Centennial, CO, USA)	NB300605
Rat Anti-Mouse CSF1R (CD115) Monoclonal Antibody | Clone: AFS98	BioXCell (Lebanon, NH, USA)	BE0213
Chemicals, peptides, and recombinant proteins		
CSF-1 (mouse) recombinant protein	ABnova (Walnut, CA. USA)	P4588
Gelatin	MP Biomedicals, LLC, (Irvine, CA, USA)	ICN90177190
Chromium Potassium Sulfate Dodecahydrate	MP Biomedicals, LLC, (Irvine, CA, USA)	194031
Sudan Black	Acros Organics, (Carlsbad, CA,USA)	190160250
Donkey Serum	Jackson ImmunoResearch (West Grove, PA, USA)	017000121
Fish Skin Gelatin	MilliporeSigma (Burlington, MA, USA)	G7041
Triton-X	MilliporeSigma (Burlington, MA, USA)	X100
BSA	MilliporeSigma (Burlington, MA, USA)	A7906
Formaldehyde solution (37%)	MilliporeSigma (Burlington, MA, USA)	1.04003
Acetic acid glacial	MP Biomedicals, LLC, (Irvine, CA, USA)	AX0073-75
Ethanol (100%)	Decon Laboratories, Inc. (King of Prussia, PA, USA)	2705
Critical commercial assays		
VECTASTAIN® ABC Kits	Vector Labs (Burlingame, CA USA)	PK-6102
Experimental models: Organisms/strains		
Cx50 and AQP0 double knockout ($Cx50^{-/-}$/$Aqp0^{-/-}$) mouse; C57BL/6	University of Texas Health Science Center at San Antonio	Gu et al. (2019)
Cx50 knockout ($Cx50^{-/-}$) mouse; C57BL/6	Stony Brook University	(White et al., 1998)
AQP0 knockout ($Aqp^{-/-}$) mouse	Washington University School of Medicine	(Shiels et al., 2001)
Software and algorithms		
ImageJ	NIH ImageJ software	NIH
GraphPad Prism 7 Software	GraphPad Software	La Jolla, CA, USA
Other		
Microscope	Keyence (Itasca, IL, USA)	BZ-X710
Cryostat	Microm (Walldorf. Germany)	Microm HM 505
Micropipette Puller	Sutter Instrument (Novato, CA, USA)	P30
Micropipette grinder	Narishige (Amityville, NY, USA)	EG-40
Drummond Nanoject II Automatic Nanoliter Injector	Drummond Scientific (Broomall, PA, USA)	3-000-204

MATERIALS AND EQUIPMENT

Glass Slide Coating Solution		
Reagent	Final concentration	Amount
Gelatin	0.5%	2.5 g
Chromium potassium sulfate dodecahydrate	0.15%	0.25 g
Total (add ddH_2O to)	n/a	500 mL
Keep at 4°C when not in use (≤ 2 months)		

Sudan Black Solution		
Reagent	Final concentration	Amount
Sudan black	0.1%	50 mg
Total (add 70% ethanol to)	n/a	50 mL
Keep at 22°C		

Blocking Solution		
Reagent	Final concentration	Amount
Donkey serum	2%	1 mL
Fish skin gelatin	2%	1 mL
Triton	0.25%	0.0125 mL
BSA	1%	0.5 g
Total (add PBS to)	n/a	50 mL
Keep at 4°C for 3 weeks; keep at −20°C for long time storage (≤ six months)		

Fekete's Acid-Alcohol-Formalin Fixation Solution		
Reagent	Final concentration	Amount
Formaldehyde solution (37%)	3.7%	10 mL
Acetic acid glacial	5%	5 mL
Ethanol (100%)	70%	70 mL
Total (add ddH_2O to)	n/a	100 mL
Keep at 22°C		

Injection Solutions		
Injection solution (dosage)	Stock concentration	Working concentration
CSF-1 (0.1 mg/kg)	50 μg/mL	1.8 μg/mL
AFS98 (25 mg/kg)	8.47 mg/mL	245 μg/mL
Store at −20°C for several months or until the expiration of any component, whichever comes first. (See troubleshooting problem 2).		

Anesthesia Mix		
Injection solution (dosage)	Dosage	Working concentration
Xylazine	20 mg/kg	2 mg/mL
Ketamine	100 mg/kg for adult and 60 mg/kg for up to postnatal 15 days	10 mg/mL
Keep at 4°C when not in use		

⚠ CRITICAL: Ketamine is a controlled substance and should only be used in accordance with local laws.

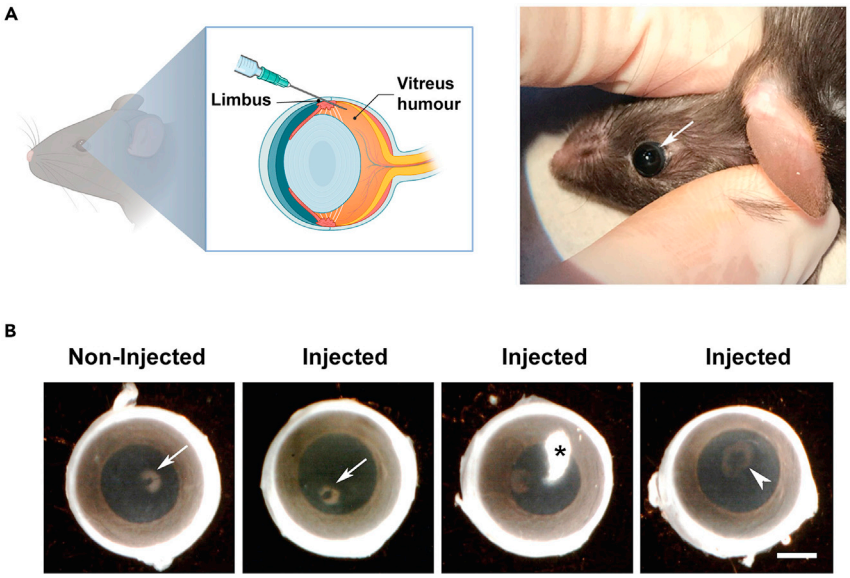

Figure 2. Injection position and effect of the injection

(A) Injections were made in the superior temporal quadrant and performed approximately 1 mm behind the limbus into the vitreous cavity

(B) The frontal images of the isolated eyeball 15 days after the injection. Arrows show the optic nerve through the pupil. Asterisk shows a leakoma. Arrowhead shows a lens injury. Scale bar = 500 μm

Alternatives: This protocol describes the use of $Cx50^{-/-}/Aqp0^{-/-}$ (dKO) mice, but other animal models or mice with injury induction to present rupture to the posterior capsule can be utilized. Alternate equipment/chemical agents serving the same function as described can also be used, with appropriate modifications.

STEP-BY-STEP METHOD DETAILS

Intravitreal injection – day 3

Timing: ~3–4 h (dependent on animal number)

The following experimental steps are used to promote the activation of MΦ2 by CSF-1 within the vitreous body of the eye utilizing an intravitreal injection. This step additionally describes the use of AFS98, a monoclonal antibody that blocks CSF-1 receptors, or saline as an experimental control.

Note: This protocol related to the injection into the vitreous cavity of the eye using needles and a micropipette is a modification and an optimization of previously published protocols (Gerhardt et al., 2003; Kubota et al., 2009).

Note: There are no pause points in this step, given the nature of the experiments. The whole process needs to be done without breaks due to the time limitation of animal anesthetization. Figure 2A for guiding critical steps.

1. Prepare equipment and laboratory space for injection (Figure 1B).
   a. Intravitreal injection should be performed under a dissecting microscope with a micropipette connected to a microsyringe.
   b. Connect a glass micropipette to a holder for the Drummond Nanoject II Automatic Nanoliter Injector (3-000-204, Drummond Scientific, Broomall, PA. USA) (Figure 1B).

c. Stretch laboratory parafilm on the surface of a 35 mm × 10 mm cell culture dish and add 1 μL of sterile saline on top of the film.
d. Press the fill-model to fill the sterile saline into a glass micropipette, and then use the inject-model to test the count of 1 μL of liquid. (See Troubleshooting problem 1).

2. Injection of MΦ2 activating or blocking agent into the vitreous body of the eye.
   a. Anesthetize mouse by intraperitoneal injection of the anesthetic mix (xylazine, 20 mg/kg, and ketamine, 100 mg/kg) 10 μL/g of the cocktail of 2 mg xylazine and 10 mg ketamine per mL is injected for adults and 6 μL/g of the cocktail for postnatal day 15 (i.e., for adult 200 μL for a 20 g mouse and 60 μL for a 10 g 15 day old mouse).
   b. Add a few levofloxacin drops on the surface of the eye to be injected to prevent infection.
   c. Insert a 33-gauge needle to make a hole at a point approximately 1 mm behind the limbus at the superior temporal quadrant into the vitreous cavity (Figure 2A).
   d. Fill a glass pipette with 0.5 μL of solution of CSF-1, CSF-1 receptor antagonist (AFS98), or saline, depending on the experimental group.
   e. Pull out the 33-gauge needle lightly and insert the filled glass micropipette, which is connected to the Drummond Nanoject II Auto-Nanoliter Injector, into the hole made by the 33-gauge needle and inject reagent into the vitreous cavity. (See Troubleshooting problem 1).
   f. Gently remove the glass micropipette.
   g. Reapply levofloxacin drops on the eye surface to keep it wet and prevent infection.
   h. After injection, place the mouse on a heating pad until the mouse is awake and alert.

Note: Build a channel for inserting a glass micropipette by using a 33-gauge needle to make a hole behind the limbus. Do not prick too deep and disturb the lens.

Note: Test the count of 1 μL volume liquid once more whenever changing the injection glass micropipette.

Note: Avoid touching the lens or retina, and the angle of injection should be ~ 40°–50°. Note: Best practice to choose littermate pups within a group. The animal number should be no less than 6; 2–3 pups for assessing macrophage subtypes 24 h after injection; 3–4 pups for analyzing fibrosis 15 days after injection. Every different injection solution needs at least three groups. Note: Use of right eye is described here, left eye can be used too, however, better to keep consistent for all animal groups.

Assessment of the distribution of various subtypes of macrophages – day 4–10

Timing: ~2–3 h (Day 4)

The following experimental steps are used to confirm the activation of MΦ by CSF-1 within the vitreous body of the eye utilizing an intravitreal injection. This step utilizes immunofluorescence to identify different MΦ subtypes using cryosections of the injected eyes.

Note: A previous report showed that macrophage numbers increased sharply 24 h after CSF-1 injection (Hume and Macdonald, 2012), in our mouse model this increase of macrophages, at this timepoint, was indeed observed. However, the real time point of interest for certifying macrophage activation may vary in different animal models.

3. Isolation of the Eye.
   a. Carefully isolate the eyeball under a dissecting microscope 24 h after CSF-1 intravitreal injection and put it in PBS.
   b. Use a sharp needle (BD Lo-Dose U-100 Insulin Syringes, Frankin Lake, USA) to make a hole for fixation at a location approximately 1 mm behind the limbus in PBS (same site of injection).

c. Fix the whole eyeball in 0.75% paraformaldehyde (PFA) in a 1.5 mL microcentrifuge tube for 24 h at 22°C. To enhance effect of fixation, the tube can be placed on a shaker shaking at the lowest speed (~60 rpm). (See Troubleshooting problem 3).

Note: Do not press or pull the eyeball directly when isolating the eyeball.

Note: Pierce through the same hole made during the injection step to accelerate fixation for keeping morphological structure and relative relationship of eye tissues in order.

Timing: ~48 h (Day 5–7)

4. Embedding and Sectioning of cryosections.
   a. Rinse off the PFA with PBS 3 times, incubating for 30 min each time.
   b. Cryoprotect the eyeball by incubating it sequentially in: 10% sucrose for 1 h at room temperature, 20% sucrose for 1 h at 22°C, and 30% sucrose 12 h at 4°C. (See Troubleshooting problem 4).
   c. Embed the eyeball in Tissue-Tek® optimal cutting temperature compound (O.C.T., Sakura, Torrance, CA, USA) in an aluminum foil mold in a sagittal position. To avoid potential tissue damage caused by the hole made for the injection and fixation, position the hole on the top of the eyeball using a microscope.
   d. Dip the embedding mold in liquid nitrogen for a short time and repeat dipping until the O.C.T. changes to a porcelain white color, indicating freezing of sample. Store at −20°C for at least 12 h before cutting sections. Long-term storage can be done at −80°C for ≤ 1 month.
   e. Cut sections in a sagittal orientation at a thickness of 12 μm and mount the sections on prepared gelatin-coated glass slides. (See Troubleshooting problem 5).
      i. Keep cut section at −20°C until use. Prior to staining, remove from freezer and let samples come to 22°C for ~30 min.

Pause Point: Staining Can be Continued the Next Day or later

Timing: ~2–3 h (Day 8–9)

5. Staining of Cryosections.
   a. Immerse the frozen tissue sections in cold acetone, which increases the permeability and fixation, at −20°C for 2 min, and then air-dry the sections.
   b. Use Hydrophobic Barrier PAP Pen (Cole-Parmer, Vernon Hills, IL, USA) to draw a liquid barrier around each tissue section. After drying, rinse the slide with PBST (0.1% Tween-20 in PBS) to remove residual O.C.T.. Wash 3 times with PBST, 5 min for each time.
   c. Add 0.1% Sudan Black solution at 22°C for 10 min to reduce the auto-fluorescence. Wash 3 times with PBST, 5 min for each time.
   d. Incubate with the blocking solution at 22°C for 1 h.
   e. To determine MΦ1.
      i. Add 1st primary antibody, Rabbit inducible nitric oxide synthase (iNOS) polyclonal antibody (1:100 dilution, Rabbit, NB10059740, Novus Biologicals, CO, USA) at 22°C and incubate 12 h at 4°C. (See Troubleshooting problem 6).

Timing: ~13–14 h (Day 9–10)

      ii. Wash 3 times with PBST, 5 min for each time.
      iii. Incubate with donkey-anti-rabbit 647 (1:400 dilution, Jackson Immuno Research, PA, USA) secondary antibody at 22°C for 1 h.
      iv. Wash 3 times with PBST, 5 min for each time.
   f. Incubate with the blocking solution 22°C for 1 h.

g. To determine MΦ2.
   i. Add 2nd primary antibody, goat Arginase-1 (Arg-1) polyclonal antibody (1:300 dilution, NB10059740, Novus Biologicals, CO, USA), and incubate at 22°C for 1 h.
   ii. Wash 3 times with PBST, 5 min for each time.
   iii. Incubate with donkey-anti-goat 488 (1:400 dilution, Jackson Immuno Research, PA, USA) secondary antibody at 22°C for 1 h.
   iv. Wash 3 times with PBST, 5 min each time.
h. To determine all MΦ.
   i. Add 3rd primary antibody, rat-anti-mouse CD68 polyclonal antibody (1:500 dilution, MCA1957, BIO-RAD, Hercules, CA, USA) at 22°C for 1 h.
   ii. Wash 3 times with PBST, 5 min for each time.
   iii. Incubate with donkey-anti-rat 594 (1:400 dilution, Jackson Immuno Research, PA, USA) secondary antibody at 22°C for 1 h.
   iv. Wash 3 times with PBST, 5 min for each time.
i. Add 4′,6-Diamidino-2′-phenylindole (DAPI) (1 ug/mL, Fisher Scientific, Hampton, NH, USA) for 10 min, followed by a PBST wash 3 times, 5 min each time.
j. Mount with Fluoromount-G (Fisher Scientific, Hampton, NH, USA) and seal with a cover slide (keep tissue hydrated throught this process). Then use nail polish to seal the edges of the cover slip carefully. After using the nail polish, put in a dark location for 10–15 min to allow the nail polish to dry.
k. Store the slides in a black plastic box at 4°C to prevent quenching of fluorescence signals until imaging is completed.

Note: After adding the secondary antibody, it is important to keep sections under dark conditions to prevent the quenching of fluorescence signals.

Note: Staining times for primary antibodies can be adjusted (at 4°C for 12 h or 1–2 h at 22°C), however 4°C for 12 h is suggested for 1st primary antibody (usually the one with the lowest affinity) in order to maximize the binding affinity.

Note: During immunofluorescence staining, tissue section slides need to be maintained hydrated. Dry samples may increase auto-fluorescence signals.

Note: All the antibodies, including primary and secondary, should be diluted before use. Keep all solutions on ice.

Assessment of fibrosis following MΦ2 activation in the posterior lens capsule – day 15–20

The following experimental steps are used to confirm that activated MΦ2, by CSF-1 injected in the vitreous body of the eye via intravitreal injection, induces the formation of fibrosis, which seals the injury site of posterior lens rupture. This step utilizes paraffin sections, to best preserve morphological details, and immunohistochemical staining to identify fibrotic tissue formation.

Note: After isolation of the eye, observe the refractive transparency on the injected eye (right eye) and compare it with the non-injected eye (left eye). The optic nerve is clearly shown through the pupil (white arrows, Figure 2B).

Note: If leakoma, a white corneal scar, on the cornea (black asterisk) or lens injury, a big opaque mass in the lens (solid white arrowhead), as showed in the Figure 2B, occurs, which can be caused by intravitreal injection, discard the sample.

Note: For our mouse model, we choose the $Cx50^{-/-}$/$AQP0^{-/-}$ knockout mouse, which has congenital cataracts. In such cases, it is hard to observe the refractive transparency. Special care must be taken to ensure the injective steps, especially the injection angle, are followed.

Timing: ~2–2.5 h for collecting samples

6. Isolation and Fixation of the Eyes
   a. Isolate the eyeball carefully under a dissecting microscope 15 days after CSF-1 intravitreal injection and place it in PBS.
   b. Use a sharp needle (BD Lo-Dose U-100 Insulin Syringes) to make a hole at a point approximately 1 mm behind the limbus.
   c. Place the entire eyeball into Fekete's acid-alcohol-formalin fixation solution in a 1.5 mL microcentrifuge tube and incubate for 24 h at 22°C. Place on a shaker to shake at the lowest speed (~60 rpm). (See Troubleshooting problem 7).
   d. Rinse off the fixation solution with 70% Ethanol directly.

Pause Point: Tissue Processing Can be Continued the Next Day

7. Dehydrate the eyeballs with ethanol and xylene, then embed in paraffin.
   a. Dehydrate the eyeballs with 70% Ethanol, 80% Ethanol, 95% Ethanol, 100% Ethanol (repeat 3 times) sequentially with 1 h each.
   b. Change to 100% Xylene (repeat 3 times) with 1 h each
8. Embed eyeball in paraffin wax.
   a. Incubate eyeball in 58°C paraffin wax for 2 h (repeat once)
   b. Embed eyeballs into paraffin blocks

Pause Point: Tissue Sectioning Can be Continued the Next Day

9. Prepare sagittal paraffin sections (~ 3-μm thickness)

Pause Point: Tissue Staining Can be Continued the Next Day

10. Immunohistochemistry-stain with Monoclonal mouse anti-human α-SMA antibody (1:200 dilution, M085129-2, Agilent, Santa Clara, CA, USA) and VECTASTAIN® ABC Kit (PK-6102, Burlingame, CA USA) following manufacturer's protocol, then image.

Note: Perform antigen retrieval prior to immunolabeling the paraffin sections. (See Troubleshooting problem 7).

EXPECTED OUTCOMES

Note: The double knockout (dKO) mice deficient in both Cx50 and AQP0, $Cx50^{-/-}/AQP0^{-/-}$, are generated by crossing C57BL/6 $Cx50^{-/-}$ mice with C57BL/6 $AQP0^{-/-}$ mice (Gu et al., 2019).

Note: To control for variability among our test groups, we set up the saline-injected and non-injected groups as controls. Additionally, we compare the differences between the injected right eye and the uninjected left eye in each mouse.

The localization of MΦ subtypes in the dKO lenses is analyzed using antibodies against CD68, iNOS, and Arg-1 to detect total, MΦ1, and MΦ2 macrophages, respectively. The numbers of total MΦ and Arg-1+ MΦ2 macrophages are markedly elevated 24 h after CSF-1 injection around the posterior lens region, while a minimal increase of iNOS+ MΦ1 macrophages is shown. In contrast, both MΦ1 and MΦ2 macrophages are detected in saline-injected and non-injected control groups in dKO lenses. However, there are fewer CD68+ MΦ compared to the CSF-1-injected group, and the predominant MΦ are the MΦ1 subtype. Furthermore, there is no change in WT CSF-1-injected group. (Figure 3).

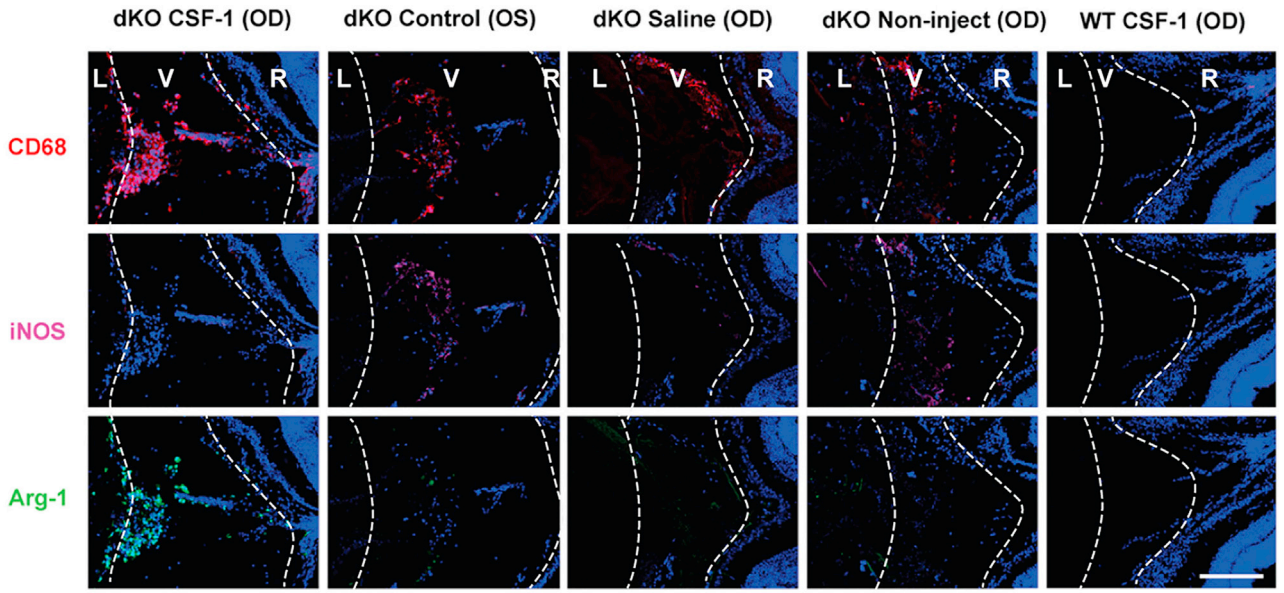

Figure 3. Subtypes of macrophages in the posterior lens 24 h after CSF-1 intravitreal injection

Cryosections of the whole eyeball from dKO and wild type mice were immunostained with antibodies against MΦ (CD68, red), MΦ1 (iNOS, purple), and MΦ2 (Arg-1, green) 24 h after CSF-1 intravitreal injection. OD (OculusDexter) indicates the right eye and OS (OculusSinister) indicates the left eye. Scale bar = 200 μm.

Fibrotic tissue formation in dKO lenses is analyzed using immunohistochemistry staining against α-SMA. The presence of α-SMA+ tissue in the posterior lens is clearly seen upon CSF-1 injection (Figure 4).

QUANTIFICATION AND STATISTICAL ANALYSIS

Twenty continuous sagittal sections (~12-μm thickness) around central regions of whole eyeball are prepared, and three sections with the largest lens areas are selected for immunofluorescence staining. After the staining, the fluorescence images are captured by Keyence BZ-X710 All-in-One fluorescence microscope and analyzed. Quantification is performed by using the counting number tools of NIH ImageJ software, and data is imported to GraphPad Prism software for graphical presentation and analysis. There is a significant increase of total and MΦ2 in dKO mice 24 h after CSF-1 injection (Figure 5).

LIMITATIONS

In this study, we use a dKO mouse model with posterior capsule ruptures. The lens posterior capsule rupture can be found in human diseases and animals. This model is rare genetic abnormality. We have not identified and compared our results with other animal models with similar phenotypes. This study focuses on the role of CSF-1 on priming MΦ development, other role(s) in the vitrous body needs to be further investigated.

TROUBLESHOOTING

Problem 1

The glass micropipette should be bumped by a tweezer gently under a microscope or grinded by the micropipette grinder in order to obtain a tip point with an OD of ~40 μm for intravitreal injection.

Over-wide opening of the micropipette can cause siphonage, resulting in backflow of injection solution and vitreous humor liquid. Meanwhile, the over-narrow opening of the micropipette can be clogged by the vitreous and ciliary body.

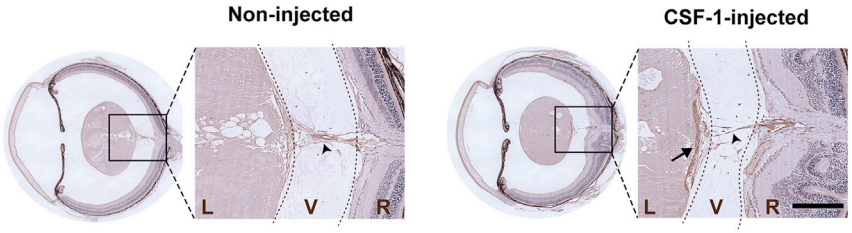

Figure 4. Fibrotic tissue formation in the posterior lens 15 days after CSF-1 intravitreal injection
Paraffin sections of the whole eyeball from dKO mice were stained using immunohistochemistry with α-SMA antibody to identify fibrotic tissue formation. Scale bar = 200 μm.

Potential solution
Move the tweezer to the glass micropipette, which is already secured into the Drummond Nanoject II Auto-Nanoliter Injector holder. Best to test the count of 1 μL volume liquid around 12–14 times with inject-model.

Problem 2
For most published studies, CSF-1 is administered via intraperitoneal injection. For intravitreal injection, the concentration of CSF-1 needs to be tested.

Potential solution
We estimated the volume of the entire eyeball and set up two groups of CSF-1 injection with differing concentrations. Both observed fibrosis, with the highest concentration demonstrating to be the most effective.

Problem 3
Due to the fragility of the eye tissue, multiple steps in the immunofluorescence staining protocol may cause tissue detachment and loss.

Potential solution
Use 0.75% PFA fixation and gelatin-coated slides, and later immerse the sections in cold acetone during staining steps.

Problem 4
Over-immersion in PBS and low concentration of fixation solution may induce detachment of retinal tissues or destroy morphology of the eyeball on the slides.

Potential solution
The eyeballs from the different developmental stages of the mice have various degrees of tissue fragility. Adjust times for PFA and cryoprotect in 10% sucrose.

Problem 5
Distorted morphology when preparing cryo-sagittal tissue sections with a fixed eyeballs using cryostat.

Potential solution
Though we typically prepare 12-μm thick frozen tissue sections, however you can decrease or increase thickness from 5–22 μm to meet the needs of your study.

Problem 6
Co-immunostaining is used to identify MΦ1 and MΦ2 macrophages with various antibodies against marker proteins; MΦ1: TNF-α, Nos2, and IL-12a and MΦ2: CD115, CD206, and PPARG. However,

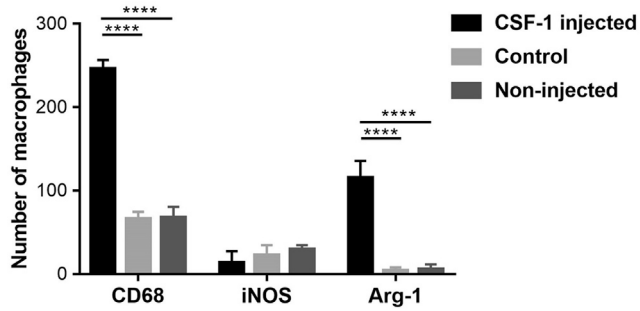

Figure 5. Quantification of macrophages in posterior lens 24 h after CSF-1 intravitreal injection
Total numbers (CD68+), MΦ1 (iNOS+), and MΦ2 (Arg-1+) in the posterior regions were quantified 24 h after CSF-1 injection. All the data are presented as mean ± SD. n = 3 per group. ****, P < 0.0001.

the co-immunostaining with two or more antibodies simultaneously may compromise individual antibody activity compared to staining with a single antibody.

Potential solution

First, perform single immunofluorescence staining to optimize staining conditions and followed by co-immunofluorescence staining. A useful strategy is to incubate primary antibodies separately, starting with the one with the weakest reactivity. Corresponding secondary antibodies can be applied together.

Problem 7

Fekete's acid-alcohol-formalin is a good fixation solution to maintain tissue morphology but may be too strong, causing damages to the antigen.

Potential solution

Treat slides with citrate-based solution (0.294%) at 37°C for 4–8 h to retrieve antigen-binding sites. If this method does not work, fix slides in 4% PFA for 2 h instead of Fekete's acid-alcohol-formalin fixation for 24 h.

RESOURCE AVAILABILITY

Lead contact

Further information and requests for resources and reagents should be directed to and will be fulfilled by the lead contact, Dr. Jean X. Jiang (jiangj@uthscsa.edu).

Materials availability

This study did not generate new unique reagents.

Data and code availability

The published article includes all datasets generated or analyzed during this study.

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AUTHOR CONTRIBUTIONS

Y.L., S.G., and J.X.J. designed the study. Y.L. mainly conducted studies and performed experiments; Z.L. and Y.L. devolped the protocol. Y.L. and Y.Q. performed statistical analysis; Y.L., F.M.A., and J.X.J. wrote the manuscript; all authors critically revised and approved the manuscript.

DECLARATION OF INTERESTS

The authors declare no competing interests.

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