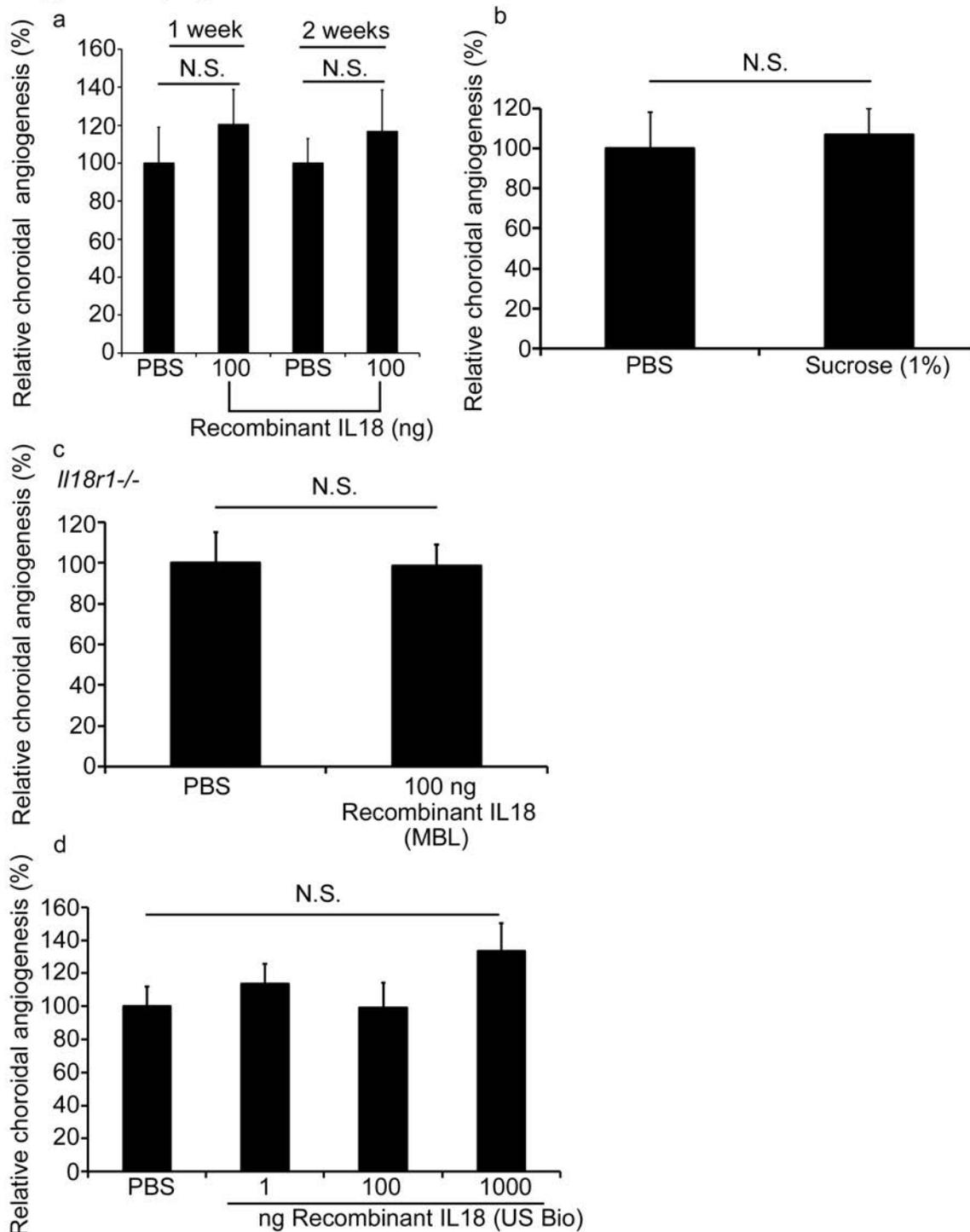


IL18 is not therapeutic for neovascular age-related macular degeneration

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Supplementary Figure 1



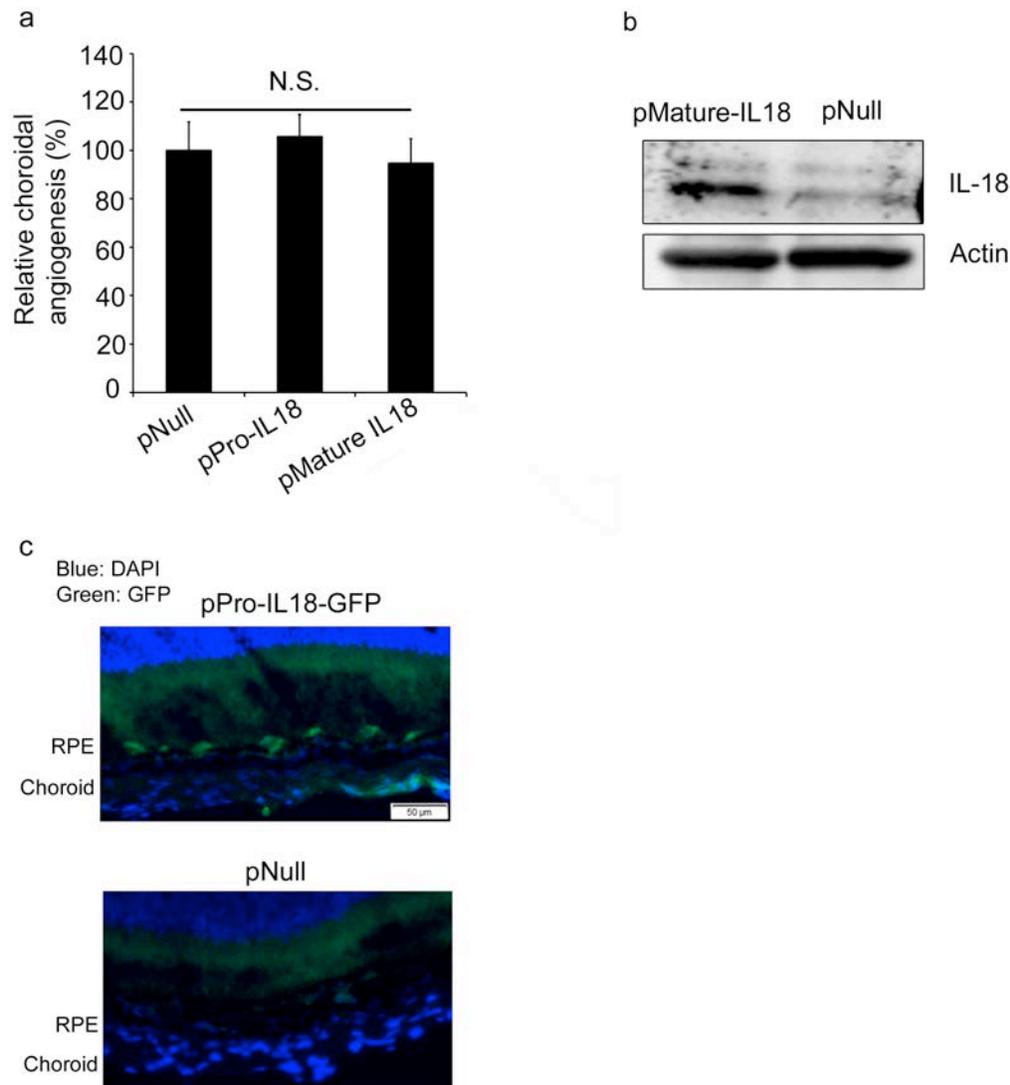
Supplementary Figure 1. IL18 administration does not affect choroidal angiogenesis. a.

CNV volumes were unchanged at one and two weeks after CNV induction by recombinant

mouse IL18 (MBL; 100 ng) administration (JA, n = 32). **b.** Intravitreal injection of sucrose

(1%) in wild type mice did not affect CNV. n = 10–15. **c.** Intravitreal injection of 100 ng recombinant mouse IL18 (MBL) did not affect CNV in mice lacking the cognate receptor (*Il18rl^{-/-}*). n = 8–14. **d.** Intravitreal injection of a dose range (1 ng–1 µg) of recombinant mouse IL18 (USBiological Life Sciences) did not affect CNV in wild type mice. n = 5–14 (JA).

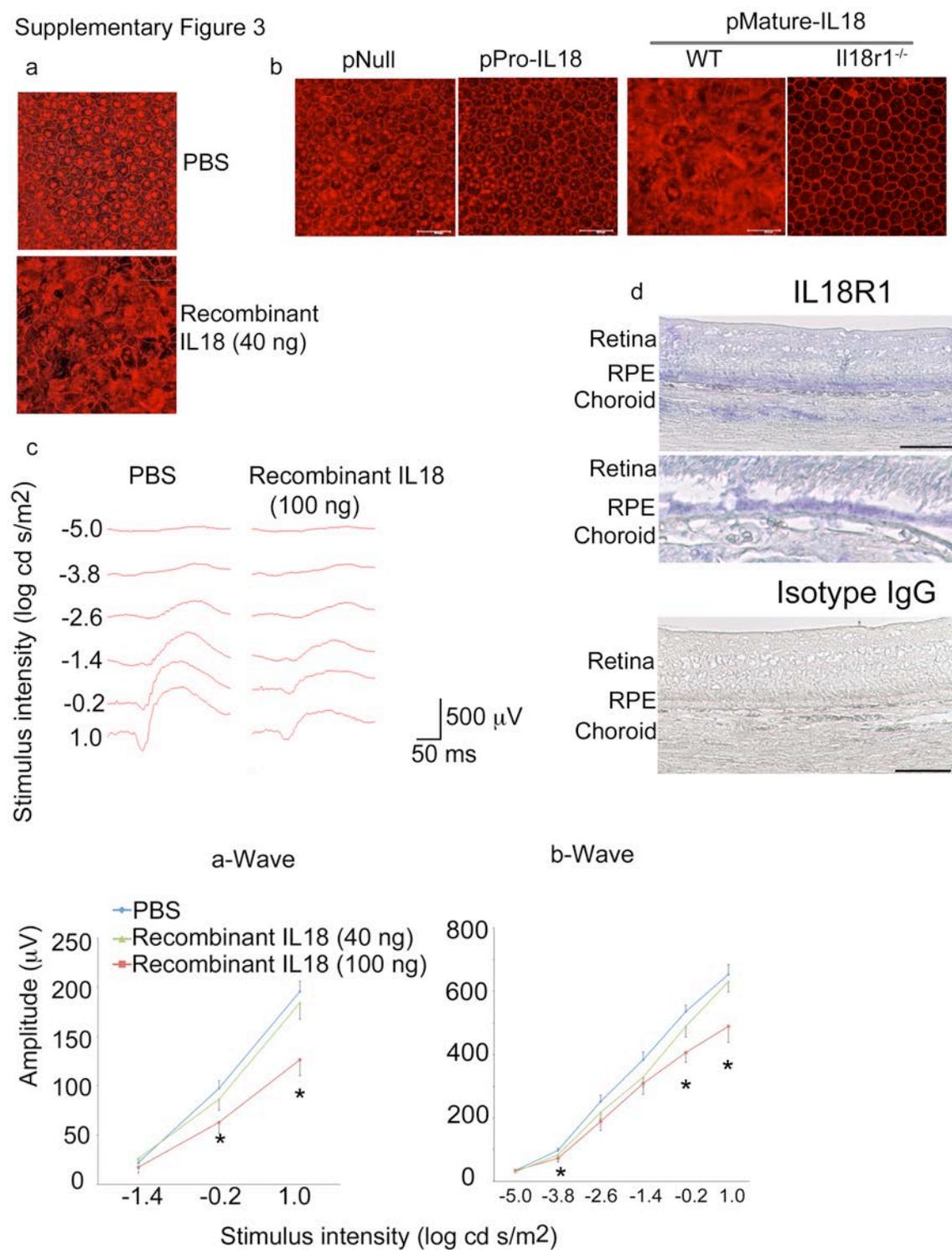
Supplementary Figure 2



Supplementary Figure 2. IL18 enforced expression does not affect choroidal angiogenesis.

a. Subretinal injection of expression plasmids encoding pro- or mature-IL18 did not affect CNV (NS) compared to empty vector control plasmid. JA, n = 18. **b.** Western blot of IL18 from RPE/choroid lysates of mice treated with plasmid expressing mature IL18 or pNull. **c.** Anti-GFP staining of wild type mice injected with a plasmid expressing pro-IL18-GFP, or pNull. Scale bars 50 μ m. n = number of eyes per condition.

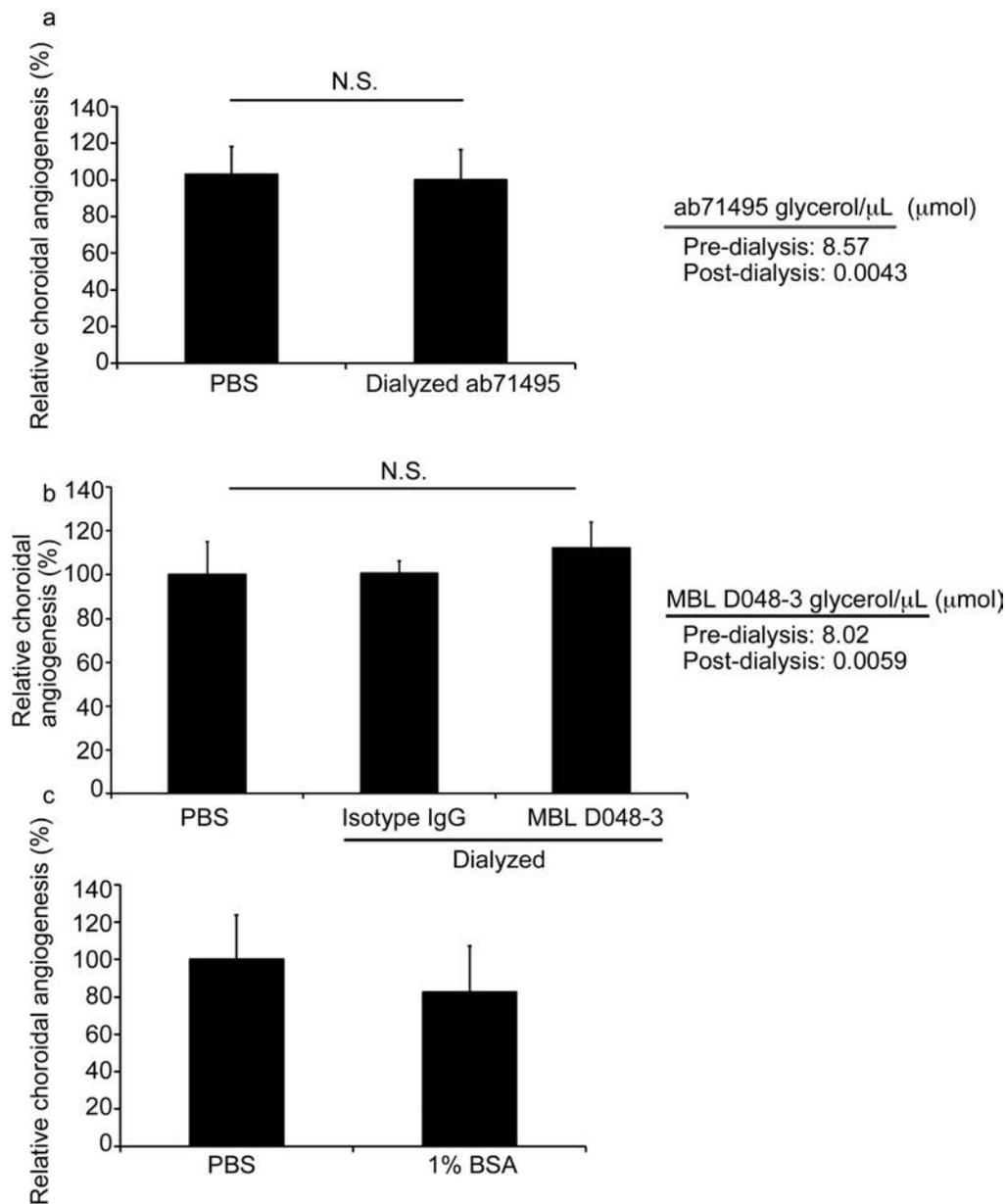
Supplementary Figure 3



Supplementary Figure 3. IL18 administration induces RPE degeneration and visual dysfunction. Flat mounts stained for ZO-1 (red) show RPE degeneration in wild-type mice with

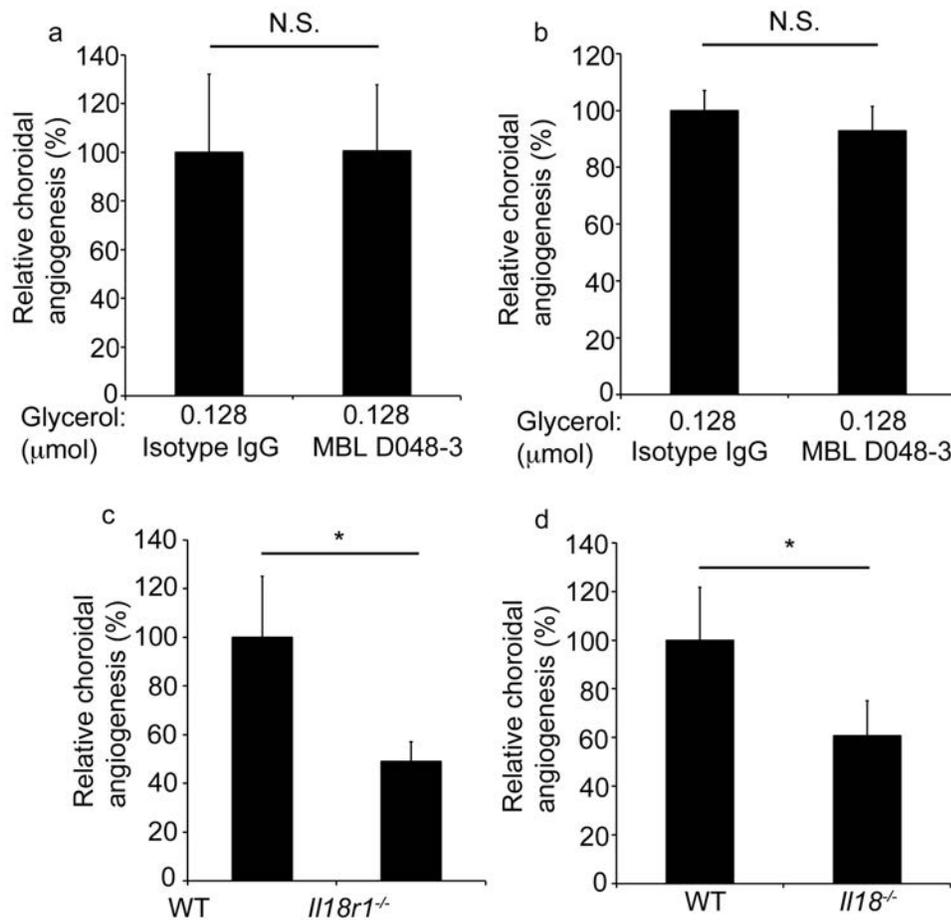
a. Intravitreal injection of recombinant IL18 (rIL18) (40 ng) compared to PBS-injected eyes (image representative of $n = 3-4$ per group), and **b.** Subretinal injection of an expression plasmid encoding mature IL18 (right panel), but not pro-IL18 or an empty vector control plasmid in wild type-mice, nor mature IL18 in *Il18r1*^{-/-} mice (images representative of $n = 3-4$. Scale bars 50 μm . **c.** Representative amplitude responses (top panel) and wave form (bottom panel) during scotopic flash electroretinography in mice are shown. Intravitreal injection of rIL18 (40 or 100 ng) decreased a- and b- wave responses compared to phosphate-buffered saline (PBS) control ($n = 25$ per group in 3-4 independent experiments, * $P < 0.05$, Mann Whitney *U* test, PBS vs. 100 ng; PBS vs. 40 ng, N.S. all data points). **d.** Immunolocalization of IL18 receptor (IL18R1; top) in non-diseased human retina, with robust expression in the RPE cell layer (middle panel). Bottom panel: Isotype IgG control. Scale bars 100 μm . $n =$ number of eyes per condition.

Supplementary Figure 4

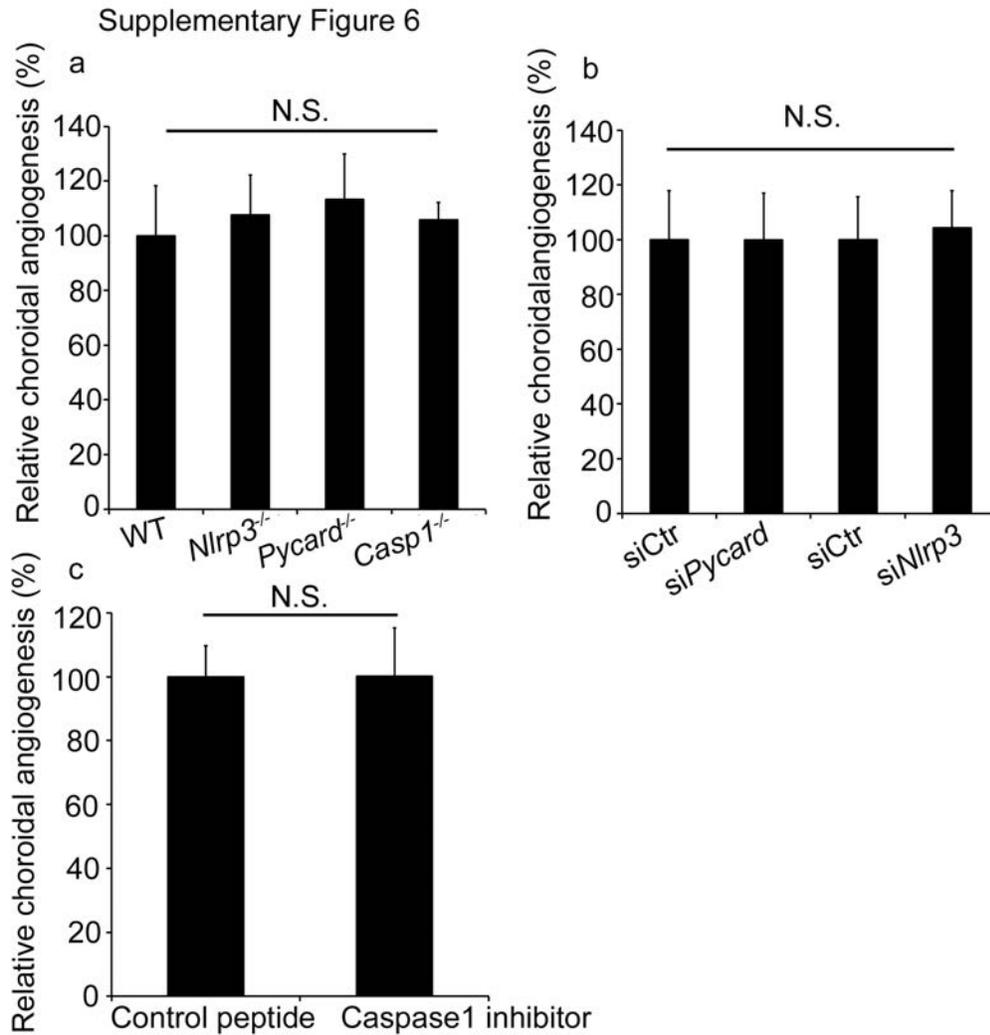


Supplementary Figure 4. Dialyzed ab71495 and BSA do not increase CNV. Neither (a) dialyzed anti-IL18 antibody (ab71495) (1 μ g, n = 15–21) (JA) nor (b) dialyzed anti-IL18 antibody (MBL D048-3) (1 μ g, n = 7–23) (JA) increased CNV in wild type mice. **c.** 1% BSA did not increase CNV in wild type mice (n = 12–14). n = number of eyes per condition.

Supplementary Figure 5

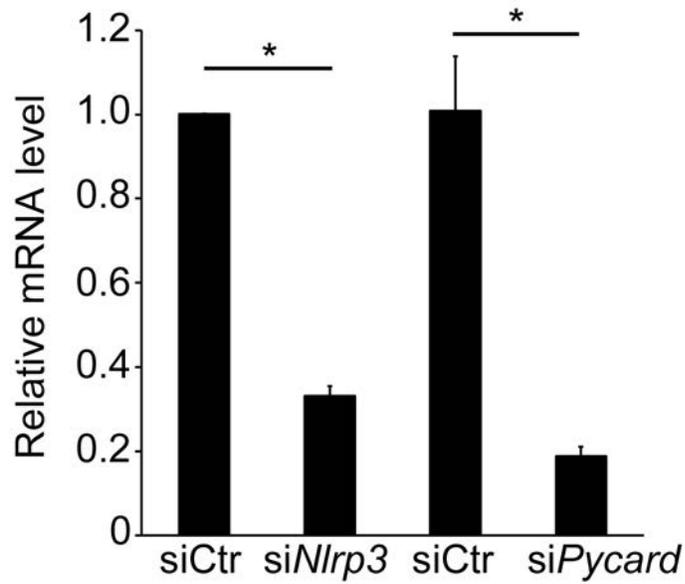


Supplementary Figure 5. IL18 deficiency does not increase choroidal angiogenesis. a,b. The anti-IL18 neutralizing antibody MBL D048-3 (30 ng) did not increase CNV compared to isotype IgG (30 ng) in PBS in wild-type mice. **a:** HT (n = 9); **b:** YO (n = 12). Mice lacking the **(c)** IL18 receptor (*Il18r1^{-/-}*) (n = 16) or **(d)** IL18 (*Il18^{-/-}*) (n = 16) exhibit decreased CNV compared to wild-type mice. **a,b,** final glycerol concentration of injections shown in μmol; NS, not significant. **c,d ***, $P < 0.05$, Mann Whitney *U* test. CNV volumes were measured at one week after laser injury. All error bars indicate mean ± s.e.m. Data from laboratory of: HT (Hiroki Terasaki); YO (Yuichiro Ogura); unless specified data from lab of JA (Jayakrishna Ambati). n = number of eyes per condition.



Supplementary Figure 6. Inflammasome deficiency does not increase choroidal angiogenesis. Mice deficient in **a.** *Nlrp3* (n = 29), *Pycard* (n = 39) or *Caspase1* (n = 16) do not exhibit increased CNV compared to wild-type mice. **b.** siRNA-mediated knockdown of *Pycard* or *Nlrp3* did not increase CNV compared to control siRNA in wild-type mice (n = 23–29). **c.** A Caspase-1 inhibitory peptide did not increase CNV compared to a control peptide in wild-type mice (n = 32). NS, not significant. CNV volumes were measured at one week after laser injury. All error bars indicate mean \pm s.e.m. n = number of eyes per condition.

Supplementary Figure 7



Supplementary Figure 7. Inflammasome genes knockdown. Confirmation of siRNA-mediated knockdown of *Nlrp3* and *Pycard* in primary mouse RPE cells by real-time quantitative PCR (n = 3). *, $P < 0.05$, Mann Whitney U test. All error bars indicate mean \pm s.e.m.

Supplementary Table.

Tabulation of CNV volumes

	Mean CNV Volume (μm^3)	S.E.M. (μm^3)
Figure		
1A		
PBS	6.68E+05	1.88E+05
IL18 (1000 ng)	5.77E+05	1.31E+05
1B		
PBS	3.62E+05	3.66E+03
IL18 (1 ng)	3.85E+05	6.26E+03
IL18 (100 ng)	3.75E+05	1.29E+04
IL18 (1000 ng)	3.88E+05	1.54E+03
1C		
PBS	3.82E+05	2.33E+04
IL18 (150 pg)	3.56E+05	4.04E+04
IL18 (10 ng)	3.32E+05	1.70E+04
IL18 (100 ng)	3.78E+05	2.57E+04
IL18 (1000 ng)	3.56E+05	2.71E+04
1D		

PBS	3.41E+05	3.18E+04
IL18 (1 ng)	3.35E+05	3.12E+04
IL18 (100 ng)	3.92E+05	5.95E+04
IL18 (1000 ng)	3.76E+05	3.99E+04

1E

PBS	5.36E+05	3.84E+04
IL18 (30 pg)	5.47E+05	2.63E+04
IL18 (150 pg)	5.61E+05	2.91E+04
IL18 (10 ng)	6.07E+05	3.16E+04

2A

Isotype IgG	3.61E+05	6.65E+04
anti-IL18 (ab71495)	5.56E+05	3.59E+04

2B

PBS	5.54E+05	1.03E+05
Glycerol (0.54 umol)	8.20E+05	1.75E+05
Glycerol (2.14 umol)	1.14E+06	1.17E+05

Glycerol (8.57 umol)	1.34E+06	4.45E+05
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2C

Sham	5.14E+05	5.69E+04
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PBS	5.87E+05	3.84E+04
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Abcam diluent	1.31E+06	1.35E+05
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anti-IL18 (Ab71495)	1.07E+06	1.18E+05
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Isotype IgG in abcam diluent	1.22E+06	1.62E+05
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Isotype IgG in PBS	6.00E+05	6.38E+04
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2D

Il18^{-/-}

PBS	5.36E+05	5.10E+04
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anti-IL18 (Ab71495)	7.83E+05	7.25E+04
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IgG in PBS	5.31E+05	6.09E+04
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IgG in glycerol	8.67E+05	6.28E+04
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2E

Aqp1^{-/-}

PBS	4.72E+05	8.34E+04
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Abcam diluent	4.47E+05	4.75E+04
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anti-IL18 (Ab71495)	3.86E+05	7.48E+04
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Isotype IgG in abcam diluent	3.57E+05	5.57E+04
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2F

PBS	5.87E+05	3.84E+04
anti-IL18 (MBL)	4.50E+05	6.78E+04
Isotype IgG	4.92E+05	8.71E+04

S1A

1 week

PBS	6.87E+05	1.29E+05
IL18 (100 ng)	8.27E+05	1.53E+05

2 weeks

PBS	5.84E+05	7.58E+04
IL18 (100 ng)	6.81E+05	1.50E+05

S1B

PBS	4.24E+05	8.20E+04
Sucrose (1%)	4.53E+05	5.61E+04

S1C

Il18r1-/-

PBS	4.83E+05	7.19E+04
IL18	4.77E+05	4.89E+04

S1D

PBS	5.55E+05	6.55E+04
IL18 (1 ng)	6.31E+05	7.64E+04
IL18 (100 ng)	5.50E+05	8.28E+04
IL18 (1000 ng)	7.40E+05	1.26E+05
S2A		
Null plasmid	5.92E+05	6.91E+04
p-pro-IL18	6.26E+05	5.65E+04
p-mature-IL18	5.60E+05	5.68E+04
S4A		
PBS	6.03E+05	9.09E+04
Dialyzed Ab71495	5.87E+05	9.63E+04
S4B		
PBS	4.85E+05	7.15E+04
Dialyzed isotype	4.87E+05	2.76E+04
Dialyzed MBL	5.43E+05	6.40E+04
S4C		
PBS	5.66E+05	1.34E+05
BSA (1%)	4.67E+05	1.16E+05
S5A		
Isotype IgG	4.82E+05	1.54E+05

anti-IL18 (MBL)	4.84E+05	1.32E+05
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S5B

Isotype IgG	4.92E+05	8.71E+04
anti-IL18 (MBL)	4.50E+05	6.78E+04

S5C

WT	5.97E+05	1.50E+05
<i>Il18r1</i> ^{-/-}	2.93E+05	2.37E+04

S5D

WT	5.94E+06	1.29E+06
<i>Il18</i> ^{-/-}	3.61E+06	5.23E+05

S6A

Caspase1 ko	5.38E+05	7.27E+04
NLRP3 ko	6.15E+05	8.95E+04
Pycard ko	6.46E+05	1.08E+05
WT	5.71E+05	1.04E+05

S6B

siCtr	6.41E+05	1.14E+05
siASC	6.40E+05	1.09E+05

siCtr	6.35E+05	9.95E+04
siNLRP3	6.63E+05	8.92E+04
S6C		
Control inhibitor	6.28E+05	6.07E+04
Casp1 inhibitor	6.29E+05	9.51E+04

Supplemental Methods:

Animals

B. Ambati Lab: Experiments were approved by the University of Utah Institutional Animal Care and Use Committee and conformed to the Association for Research in Vision and Ophthalmology (ARVO) Statement on Animal Research. Male wild-type C57BL/6 were used between 6–8 weeks of age. Mice were placed under general anesthesia with an intraperitoneal injection of ketamine/xylazine (90 mg/10 mg per kg body weight). Pupils were dilated with topical application of 1% tropicamide.

J. Ambati Lab: All animal experiments were in accordance with the guidelines of the University of Kentucky Institutional Animal Care and Use Committee, and the ARVO Animal Statement for the Use of Animals in Ophthalmic and Vision Research. Male wild-type C57BL/6, *Il18^{-/-}*, and *Il18r1^{-/-}* mice (The Jackson Laboratory;), *Aqp1^{-/-}* mice¹ (gift of J. Schnermann), and *Nlrp3^{-/-}*, *Pycard^{-/-}*, and *Casp1^{-/-}* mice (G. Núñez; backcrossed at least 8 times to C57BL/6J mice) were used between 6–8 weeks of age. The mice were anesthetized for all procedures with an intramuscular injection of 0.2 mL of a 50:50 mixture of ketamine hydrochloride (20 mg/mL) and xylazine hydrochloride (100 mg/mL; both from Phoenix Pharmaceutical Inc., St. Joseph, MO). The pupils were dilated with 5% phenylephrine hydrochloride and 0.8% tropicamide.

Hinton Lab: The animal study protocol was approved by USC's Animal Care and Use Committee and followed the statement of the ARVO Statement for the Use of Animals in Ophthalmic and Vision Research. CNV was induced in male C57BL/6 mice (6–8 weeks old) by

laser photocoagulation. For all procedures, mice were anesthetized with ketamine (80 mg/kg) and xylazine (8 mg/kg), and pupils were dilated with topical 1% tropicamide.

Ogura Lab: Male wild-type C57BL/6J mice (Japan SLC, Shizuoka, Japan) between 6 and 8 weeks of age. Animals were anesthetized with Avertin (2.5% 2,2,2-tribromoethyl and tertiary amyl alcohol; Sigma, Tokyo, Japan; 10 μ L/g, 400 mg/kg intraperitoneal injection) and pupils were dilated with topical 1% tropicamide (Santen, Osaka, Japan). The study protocol was approved by the Nagoya City University Animal Care and Use Committee. All animal experiments were performed in accordance with the guidelines of the ARVO Statement for the Use of Animals in Ophthalmic and Vision Research.

Terasaki Lab: Male wild-type C57BL/6J mice (CLEA Japan, Japan) between 6 and 8 weeks of age were used. For all procedures, the animals were anesthetized with intraperitoneal injection of 400 mg/kg Avertin (Sigma-Aldrich, USA) and the pupils were dilated with combination of tropicamide 0.5% and phenylephrine 0.5% (Mydrin-P; Santen, Japan). The use of animals in the experimental protocol was approved by Nagoya University Animal Care Committee. All animal experiments were performed in accordance with the guidelines of the ARVO Statement for the Use of Animals in Ophthalmic and Vision Research.

CNV experiments

B. Ambati Lab: Laser photocoagulation (OcuLight GLx, IRIDEX) was performed on both eyes of mice. The treatment parameters were chosen to produce a cavitation bubble in the choroid without hemorrhage (100 mW, 100 msec, 100 μ m). Recombinant mouse IL-18 (MBL; Catalog

No. B004-5; BSA-Free); was injected into the vitreous humor of mice using a 33-gauge double-caliber needle (Ito Corporation) once immediately after laser injury. Choroidal angiogenesis volumes were measured by scanning laser confocal microscopy (Olympus America) 1 week after injury using Alexa 488 conjugated isolectin GS-IB4 (Invitrogen) in masked fashion.

J. Ambati Lab: Laser photocoagulation (OcuLight GL, IRIDEX) was performed on both eyes of mice to induce CNV as previously described². Drugs (anti-IL-18 antibody (Abcam or MBL); isotype control antibodies; recombinant mouse IL-18 (MBL; Catalog No. B004-5; BSA-Free); recombinant mouse IL-18 (USBiological Life Sciences; Catalog No. 155389); Caspase-1 inhibitor; Caspase control inhibitor (R&D Systems)) were injected into the vitreous humor of mice using a 33-gauge double-caliber needle (Ito Corporation) once, immediately after laser injury as previously described. Choroidal angiogenesis volumes were measured by scanning laser confocal microscopy (TCS SP5, Leica) 7 or 14 days after injury as previously reported with 0.7% FITC-conjugated Isolectin B4 (Vector) in masked fashion^{2,3}.

Hinton Lab: Three photocoagulation lesions were delivered with a diode green laser (100 mW, 0.05 s, 75 μ m) between the retinal vessels in a peripapillary distribution. Production of a subretinal bubble at the time of laser treatment confirmed the rupture of Bruch's membrane. After laser application, anti-IL-18 antibody (Abcam), isotype control antibody, or recombinant mouse IL-18 (MBL; Catalog No. B004-5; BSA-Free) was injected intravitreally by passing through posterior sclera using a 33-gauge needle under surgical microscopy. Laser and injection were applied to both eyes in each mouse. Eyes from laser-induced CNV mice were enucleated at Day 7 after laser treatment; the cornea, lens and neuroretina were removed. Dissected eyecups

were fixed with 4% paraformaldehyde overnight. After being stained with isolectin B4 (an endothelial cell specific marker), flat mounts were visualized with a laser scanning confocal microscope (LSM510; Carl Zeiss) using a 25× objective lens. Z stack images of CNV lesion were taken. The image stacks were rendered in 3D using Volocity imaging software (Improvision Inc., Waltham, USA) and processed to digitally extract the fluorescent lesion volume. CNV volume was measured in micrometers cubed.

Ogura Lab: Laser photocoagulation (532 nm, 200 mW, 100 ms, 100 μm; Elite; Lumenis, Salt Lake City, UT) was bilaterally performed in each animal on day 0 by one individual masked to the drug group assignment as described previously^{4,5}. The laser spots were created in a standard fashion around the optic nerve using a slit lamp delivery system and a coverslip as a contact lens. The morphologic end-point of the laser injury was the appearance of a cavitation bubble, which correlates with disruption of Bruch's membrane. Mice were injected with anti-IL18 antibody (MBL) or the same dose of isotype IgG, or recombinant mouse IL18 (MBL; Catalog No. B004-5; BSA-Free) into the vitreous humor using a 33-gauge needle (Ito Corporation, Tokyo, Japan) immediately after the laser injury. At 1 week after the laser injury, the eyes were enucleated and fixed with 4% paraformaldehyde. The eyecups obtained by removing the anterior segments were incubated with 0.5% fluorescein-isothiocyanate (FITC)-isolectin B4 (Vector Laboratories, Burlingame, CA). CNV was visualized using a blue argon laser wavelength (488 nm) and a scanning laser confocal microscope (LSM 5 Pascal; Carl Zeiss Meditec GmbH, Oberkochen, Germany). Horizontal optical sections were obtained at 1-μm intervals from the surface of the RPE/choroid/sclera complex. The deepest focal plane in which the surrounding choroidal vascular network connected to the lesion could be identified was judged to be the floor of the

lesion. Any vessel in the laser-treated area and superficial to this reference plane was judged as CNV. The images of each section were digitally stored. The area of CNV-related fluorescence was measured using ImageJ software (U.S. National Institutes of Health). The summation of the whole fluorescent area in each horizontal section was used as an index for the volume of CNV, as described previously^{4,5}. The average volume obtained from all four to six laser spots per eye was generated (n = number of eyes). Imaging was performed by an operator masked to the treatment group assignments.

Terasaki Lab:

Four spots of laser photocoagulations (532 nm, 180 mW, 100 ms, 75 μ m; Novus Verdi; Coherent Inc., USA) was performed in each fundus of the eye on day 0 by one individual masked to the group assignment to induce laser-induced choroidal neovascularization (laser-CNV). The laser spots were created around the optic nerve using a slit lamp delivery system and coverslip was used as a contact lens. For IL18 experiments, recombinant mouse IL18 (MBL; Catalog No. B004-5; BSA-Free) dissolved in ultrapure PBS or the same volume of PBS was administrated intravitreally at day 0 immediately after the laser injury in the wild-type mice eyes. Anti-mouse IL18 neutralizing antibody (MBL D048-3, MBL) or isotype IgG antibody was intravitreally administrated after inducing laser-CNV. Intravitreal injection was performed by 33-gauge needle (Ito Corporation, Japan) under surgical microscope. Laser-CNV volume was measured with the similar method as previously described^{2,6}. Briefly, one week after the laser injury, the eyes were enucleated and fixed with 4% paraformaldehyde. The eyecups obtained by removing the anterior segments were incubated with 0.5% fluorescein-isothiocyanate (FITC)-isolectin B4 (Sigma-Aldrich). CNV was visualized using a blue argon laser wavelength (488 nm) and a

scanning laser confocal microscope (Eclips C1 confocal, Nikon, Japan). Horizontal optical sections were obtained at 1µm intervals from the top of the CNV to the surface of the RPE. The images of each layer were digitally stored, and the area sizes were measured. The area of CNV-related fluorescence was measured using ImageJ software. The summation of the whole fluorescent area in each horizontal section was used as an index for the volume of CNV. Imaging was performed by an operator masked to the group assignments.

In all laboratories, laser lesions were excluded in masked fashion from analyses (5% incidence) if laser photocoagulation did not induce a bubble, if it induced hemorrhage, or if lesions became confluent with one another.

Human tissue. Donor eyes were obtained from eye banks in the United States of America. The study followed the guidelines of the Declaration of Helsinki. Institutional review boards granted approval for allocation and histological analysis of specimens.

Immunolabeling and histology. Human eyes fixed in formalin were prepared as eyecups, embedded in paraffin and sectioned into 5 µm sections. Depigmentation was achieved using 0.25% potassium permanganate and 0.1% oxalic acid. Immunohistochemical staining was performed with a rabbit antibody against IL18R (1:500, MD Bioproducts), or control IgG (1:500, MD Bioproducts) to assess the specificity of the staining. Bound antibody was detected with biotin-conjugated secondary antibodies, followed by incubation with ABC reagent and visualized by Vector Blue (Vector Laboratories). Levamisole (Vector Laboratories) was used to block endogenous alkaline phosphatase activity. Slides were washed in PBS and mounted in

Vectamount (Vector Laboratories). Mouse RPE/choroid flat mounts were fixed with 2-4% paraformaldehyde and stained with a rabbit antibody against zonula occludens-1 (ZO-1; Invitrogen), as previously described⁷. Two days after *in vivo* transfection of subretinally injected plasmid coding for GFP-tagged mouse IL-18 (Origene) in wild-type mice, mice were euthanized, eyes were enucleated, fixed in 4% paraformaldehyde, embedded in optimum cutting temperature compound (O.C.T., Tissue-Tek) and frozen in isopentane cooled by liquid nitrogen. 10 μ m cryosections were stained with rabbit anti-GFP (Life Technologies) overnight followed by secondary anti-rabbit-Alexa-594 antibody (Life Technologies). Slides were mounted in DAPI containing medium (Pro-long anti-fade, Life Technologies), and images were acquired on a Nikon Eclipse Ti inverted fluorescent microscope.

Electroretinography. Mice were dark adapted overnight, anesthetized, and both eyes were positioned within a ColorBurst Ganzfeld stimulator (E2; Diagnosys, Lowell, MA). After placing corneal and ground electrode, Espion software (Diagnosys) was used to deliver a fully automated flash intensity series from which retinal responses were recorded. Maximal a- and b-wave values were identified for each flash intensity, and mean values were compared for statistical analysis.

Real-time quantitative PCR. Total RNA was extracted from cells using TRIzol reagent (Invitrogen) according to manufacturer's recommendations and was reverse transcribed using qScript cDNA SuperMix (Quanta Biosciences). cDNA was amplified by real-time quantitative PCR (Applied Biosystems 7900 HT Fast RealTime PCR system with Power SYBR green Master Mix).

Drug treatments. siRNAs, Caspase-1 inhibitor Z-WEHD-FMK (R&D Systems), or Caspase control inhibitor Z-FA-FMK (R&D Systems) were dissolved in PBS (Sigma-Aldrich) and injected into the vitreous humor in a total volume of 1 μ l with a 33-gauge Exmire microsyringe (Ito Corporation). All labs used recombinant mouse IL-18 from MBL (B004-5); JA also used recombinant mouse IL18 from USBiological. For dialysis, antibodies were dialyzed (Slide-A-Lyzer Dialysis Cassettes, 3.5 kDa molecular weight cutoff; Thermo Scientific) thoroughly against PBS overnight. Glycerol measurements were performed using Glycerol Colorimetric Assay Kit (Cayman Chemical). To assess the effect of Nlrp3 or Pycard blockade, 1 μ l (2 μ g/ μ l) of 17+2 nt cholesterol (chol) conjugated mouse *Nlrp3* siRNA (5'-GUUUGACUAUCUGUUCUdTdT-3') or mouse *Pycard* siRNA (5'-GAAGCUCUUCAGUUUCAdTdT-3') was intravitreally injected. As a control, Luc siRNA-chol (17+2 nt) was used with identical dosages.

Plasmids. Plasmid encoding mouse pro-IL18 and mouse pro-IL18-GFP were obtained from OriGene Technologies, Inc (Rockville, MD). An expression plasmid encoding the secreted form of mouse mature IL18 was cloned into pCR31 expression vector (Invitrogen) as described earlier⁴⁴. *In vivo* plasmid transfection was achieved using 10% Neuroporter (Gene Therapy Systems Inc., San Diego, CA) as described earlier⁴ and injected into subretinal space using a microthin 33-gauge Exmire microsyringe (Ito Corporation).

Western blotting. IL-18 expression was assessed in the RPE/choroid protein lysates, 3 days after *in vivo* transfection of a subretinally injected plasmid encoding mature mouse IL18 in wild-type mice, using anti-mouse IL-18 antibody (1:200; MBL international).

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