Assessment of Retinal Function and Morphology in Aging Ccl2 Knockout Mice

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PURPOSE. The chemokine Ccl2, or monocyte chemotactant protein-1 (MCP-1), has previously been identified as playing a potential role in many ocular diseases; however, its role in mice is less clear. We sought to correlate changes in retinal pigment epithelium (RPE) and retinal morphology with changes in function in aging Ccl2−/− mice.

METHODS. Ccl2−/− mice on a C57BL6J background were genotyped for Crb1Rd8/Rd8 and were free of this mutation. Ccl2−/− mice and wild-type (WT) C57BL6J mice were investigated for changes in the retinal fundus and histology as a function of age. The function of the rod and cone pathways, and the rate of dark adaptation, was assessed using the electroretinogram (ERG) up to 15 months of age.

RESULTS. Fifteen-month-old Ccl2−/− mice had fundus lesions, more subretinal microglia/macrophages, and an increase in RPE cell size, indicative of RPE cell loss, when compared with WT mice. Within the retina, gross morphology was normal but there was an increase in Müller cell gliosis and microglial activation. These morphological changes in the Ccl2−/− RPE retina did not correlate with a change in either rod or cone ERG pathway function, or with the rate of dark adaptation.

CONCLUSIONS. These data show that Ccl2 is important for preserving RPE and glial morphology with age, yet retinal function and gross morphology are maintained. Altered signaling in this chemokine pathway may, however, increase RPE and retinal vulnerability to disease.

Keywords: retina, age-related macular degeneration (AMD), ARMD, microglia, macrophage, glia, electroretinogram (ERG), oscillatory potential, Müller cell, mouse, retinal pigment epithelium (RPE)

Chemokines are integral in the process of recruiting and trafficking microglia and macrophages, among having many other roles in the innate immune system.1 The Ccl2/Ccr2 pathway specifically aids in the recruitment of macrophages/microglia to sites of injury or inflammation.2,3 In the retina, Ccl2 is likely released from Müller glia and the retinal pigment epithelium (RPE) when the retina is under stress to attract microglia/macrophages expressing the Ccr2 receptor to sites of retinal damage.4,5 Upregulation of Ccl2 occurs with increasing age; suggesting that the retina requires a greater degree of microglial migration, particularly between the RPE and photoreceptor layers.6 One of the posited reasons for this may be to clear extracellular debris as RPE phagocytic function reduces with age.7,8 This concept is supported by the findings of El Khoury et al.9 where Ccl2 was found to be crucial in attracting microglia to Alzheimer’s plaques. Ccl2 has subsequently been shown to mediate the clearance of amyloid-β in Alzheimer’s disease.10,11 Ccl2 has been found to be upregulated in the aqueous humor of patients with exudative age-related macular degeneration (AMD), suggesting a role for this signaling pathway in this disease.12,13 However, genome wide screening suggests that there is little evidence for genetic association of mutations in Ccl2 conferring increased risk of AMD.14,15 Mice lacking Ccl2 or its cognate receptor, Ccr2, have been suggested to develop lesions that mimic AMD pathology. Despite apparent pathological processes occurring in the RPE and choroid of these mice,16 subsequent studies have failed to replicate these initial findings, reporting fundus lesions and an abnormal accumulation of macrophages and microglia within the subretinal space but no exacerbation of photoreceptor death with age.7,17 However, whether mice lacking Ccl2 display a definitive phenotype is unclear. A further issue confounding the interpretation of the Ccl2−/− phenotype is that this line may have a background retinal degeneration mutation in Crb1 (Crb1Ra/−)16–20 Recent work has shown that the pene- trance of the fundus and retinal morphology phenotype in mice with the Crb1Ra/− mutation is variable, depending on the C57BL6 background strain used.21 Thus, this variation may underlie the different phenotypes observed in the Ccl2−/− strain between research groups.

Here we extend on this work, using mice lacking Ccl2, free of the Crb1Ra/− mutation, to investigate retinal/RPE dys- function up to 15 months of age by using the electroretinogram (ERG) to determine gross retinal function and recovery of dark-adaptation following photoreceptor bleach in C57BL6J wild-type (WT) controls and Ccl2−/− mice. We sought to correlate any functional changes with fundus appearance and RPE/retinal morphology in the aging Ccl2−/− mouse.
Laboratories’ website. In light of recent studies showing the paradigm.
bourne. The tests were conducted in a nonrepeated measures ethics committee requirements of the University of Mel-
months of age due to increasing frailty in accordance with the under anaesthetic, animals were only assessed up to 15
least eight mice were investigated at each time point (Table).
Research Council, 1990) and the ethics committee standards of the University of Melbourne (Ethics ID: 0911158).

As dark adaptation experiments involved long testing times under anaesthetic, animals were kept in the same animal room, housed on a 12-hour day-night cycle (light level, 230–275 lux source) at the Biomedical Sciences Animal Facility at The University of Melbourne. Standard mouse chow and water were provided ad libitum. All procedures conforms to the Association for Research in Vision and Ophthalmic statement for the Use of Animals in Ophthalmic and Vision Research (National Health and Medical Research Council, 1990) and the ethics committee standards of the University of Melbourne (Ethics ID: 0911158).

Mice were aged up to 15 months, and sample sizes of at least eight mice were investigated at each time point (Table). As dark adaptation experiments involved long testing times under anaesthetic, animals were only assessed up to 15 months of age due to increasing frailty in accordance with the ethics committee requirements of the University of Melbourne. The tests were conducted in a nonrepeated measures paradigm.

Genotyping
Small tail samples were collected from WT and Ccl2+/− mice when they were weaned for extraction of genomic DNA and genotyping. Standard PCR based genotyping for Ccl2 was completed according to the protocol provided on the Jackson Laboratories’ website. In light of recent studies showing the Rd8 (Crb1Rd8/Wt mutation, a single base pair deletion of a cytosine in exon 9 of the Crb1 gene) mutation in commercially available transgenic mice lines,19 the Crb1 gene from Ccl2+/− and WT mice was sequenced and found to be homologous, confirming that these mice do not carry the Rd8 mutation. Details of the sequencing strategy are available in a previous publication.22

Fundus Photography
Nine- and 15-month-old WT and Ccl2+/− mice were anaesthe-
tized using a mixture of ketamine (67 mg/kg) and xylazine (13 mg/kg). The cornea was anaesthetized with topical Alcaine (proparacaine hydrochloride 0.5%; Alcon Laboratories, Frenchs Forrest, NSW, Australia) and the pupil dilated with topical Mydriacyl (tropicamide 0.5%; Alcon Laboratories). The retinal fundi of mice were viewed and photographed using a Micron III fundus camera (Phoenix Research Laboratories, Inc., Pleasanton, CA, USA), and images were viewed and collected using specialty Micron III software (Phoenix Research Laboratories, Inc.).

Electroretinogram
Mice were dark-adapted overnight and under dim red illumination, general anesthesia was induced as for fundus photography. As the dark adaptation protocol could take up to 1 hour, a top-up of approximately two-thirds of the original dose of anaesthetic was administered subcutaneously via a butterfly needle at 25 to 30 minutes. Topical Alcaine, Mydriacyl, and corneal lubricant were administered. Animals were placed on a heat pad to maintain body temperature. The corneal (active) and mouth (reference) electrodes were constructed from pure silver/silver-chloride wire (Ag/AgCl).

The ERG stimulus and recording were coordinated using Scope software (version 3.6.10; ADInstruments Pty. Ltd., Castle Hill, NSW, Australia) on an Apple Macintosh computer (Apple, Inc., Cupertino, CA, USA), OS9. Electroretinogram responses were amplified with a gain of ×5000 (ML132 BioAmp, ADInstruments) and passed through an analogue-digital converter (ML785 Powerlab/8sp amplifier, ADInstruments) acquired at a 10 kHz sampling frequency over a 250-ms epoch. Line noise (~60 Hz) was filtered via a Humbug noise reduction system (Quest Scientific Instruments, Inc., North Vancouver, Canada). Two milliseconds were recorded before the stimuli onset to establish background noise levels. To elicit the ERG, a 2.1-log cd.s/m² full field flash was delivered using a commercial photographic flash unit (60CT4; Metz-Werke GmbH & Co. KG, Zirndorf, Germany) via a custom made Ganzfeld. Two flashes were delivered with a 0.8-second interstimulus interval, to elicit responses from the rod and cone pathways (mixed response) and the cone pathway (cone response) alone. The cone response was digitally subtracted from the mixed response to generate the rod response.23-24

Electroretinogram component analysis was completed using previously published equations and techniques.25-26 The rod photoreceptor responses (rod a-wave) were isolated and analyzed using a modified PII model, to derive the amplitude of the PII response (PII Rmax in µV) and the sensitivity, which represents the gain of phototransduction cascade (S in m²cd·s⁻²). The rod postphotoreceptoral function (rod b-wave) was isolated by subtraction of the rod PII from the raw rod waveform and then fitted using an inverted gamma function to generate the rod PII. From the rod PII fit, the amplitude of the PII response (rod PII Rmax in microvolts) and the time to peak (implicit time in milliseconds) were derived. To assess the oscillatory potentials (OPs), the fitted rod PII was subtracted from the raw b-wave, and the amplitude and time to peak of OPs 2, 3, and 4 were measured and summed.

Dark Adaptation Protocol
The dark adaptation protocol was designed to determine the rate of recovery of ERG amplitudes and sensitivity after exposure to a preadapting light stimulus. After dark adaptation for 12 hours, an average of two baseline twin-flash ERG recordings were sampled in response to a 2.1-log cd.s/m² flash with a 3-minute interval between samples, to establish a baseline response prior to the application of the bleaching light. Bleaching of the retina was achieved by six successive flashes at an exposure setting of one-fourth at an individual flash duration of 4.2 ms, which gave a stimulus exposure of 3.3 log cd.s/m² each time (19.8 log cd.s/m²) to achieve a total rhodopsin bleach of 14%.27 A standard twin-flash recording (using flash light intensity of 2.1 log cd.s/m²) immediately followed this bleaching, and thereafter a twin-flash recording

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**Materials and Methods**

**Animals**
Ccl2+/− mice on a C57BL/J6 background were obtained as a gift from J Wilkinson-Berka, Monash University, and were originally sourced from Jackson Laboratories (Bar Harbor, ME, USA; stock name B6.1298+−/Ccl2tm1Rol/J, stock number 004434). Control C57BL/J6 mice (WT) were obtained from the Animal Resource Center (ARC; Murdoch, WA, Australia). Ccl2+/− mice were recropped onto the C57BL/J6 background from the ARC and then kept as a homozygous line. Age and sex-matched Ccl2+/− and WT animals were kept in the same animal room, housed on a 12-hour day-night cycle (light level, 230–275 lux source) at the Biomedical Sciences Animal Facility at The University of Melbourne. Standard mouse chow and water were provided ad libitum. All procedures conformed to the Association for Research in Vision and Ophthalmology statement for the Use of Animals in Ophthalmic and Vision Research (National Health and Medical Research Council, 1990) and the ethics committee standards of the University of Melbourne (Ethics ID: 0911158).

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was taken every 3 minutes for approximately 45 minutes (15 responses after the initial preadapting light stimulus).

Preliminary experiments were performed to determine the highest level of photoreceptor bleaching that could be used to quantify a recovery response. With a bleach of 14%, a 75% to 80% recovery of the prebleach response could be ascertained within 45 minutes. This level of bleach was selected because a similar paradigm has been successfully used previously to detect changes in dark adaptation in rodents with disease.28 and consistent ERG recordings could be obtained over this time under anaesthesia in aged animals. If higher levels of bleach, up to 50%, were used the response did not recover reliably. This finding is consistent with the work of Wenzel et al.,29 who showed that following 50% bleach, recovery to a prebleach amplitude takes 5 hours in the C57BL6J mouse.

The following parameters were assessed as a factor of time for the isolated rod response: minimum amplitude (baseline to trough: a-wave microvolt) and time to minimum amplitude (milliseconds). A graph was plotted for each animal and fit in Prism software (GraphPad Software, Inc., La Jolla, CA, USA) using a one-phase exponential equation. The analysis did not take into account potential rebleaching that could have occurred with each subsequent test flash in this paradigm.

\[
Y = Y_{MAX} \times (1 - \exp(-K \times X)).
\]

This equation describes the first order exponential kinetics of recovery from bleaching stimulus where \( Y \) starts out equal to zero (no a-wave) and decreases to a maximum plateau (new a-wave at equilibrium after bleaching), equal to \( Y_{MAX} \) at a rate of change, \( K \). The parameters compared were: Plateau amplitude, half-life (time to reach half the plateau amplitude response), and \( K \) (rate of change).

**Gross Histology**

For all procedures involving tissue collection, mice were first deeply anesthetized (as above) and euthanized by cervical dislocation. The eyes were isolated and the anterior portions of the eye and lens were removed by dissection. Eyecups were fixed overnight in 1% paraformaldehyde, 2.5% glutaraldehyde, 3% sucrose, and 0.01% calcium chloride in 0.1 M phosphate buffer (PB) for 30 minutes. The tissues were then washed with PB (three times, 10 minutes each time). Cryoprotection was achieved by exposing to three freeze/thaw cycles in 30% sucrose in PB, before rapid freezing into blocks in isopentane cooled by liquid nitrogen. Transverse sections of the eyecup were cut with a Microm HM 550 cryostat (Microm Pty. Ltd., Walldorf, Germany) at −20°C, at 14 μm and placed on Poly-sine glass slides (Poly-sine adhesion slides; Thermo Scientific; Scoresby, VIC, Australia) before storage at −20°C.

For labeling of frozen retinal sections, the slides were thawed, rinsed three times for 10 minutes in PB and then incubated with 10% normal goat serum (NGS), 1% bovine serum albumin (BSA), 0.5% Triton X-100 in PB for 1 hour to block nonspecific binding sites. The primary antibodies used to assess Müller glia were: rabbit anti-glial fibrillary acidic protein (GFAP; Cat. No# Z0334; Dako, Carpentier, CA, USA) diluted 1:20,000 to label astrocytes and gliotic Müller cells and mouse anti-glutamate synthetase (GS, clone GS-6, Cat. No# MAB302; Merck-Millipore, Frenchs Forest, NSW, Australia) diluted 1:1000 to label Müller cells. The primary antibodies used to assess RPE integrity were: mouse anti-cellular retinaldehyde-binding protein (CRALBP, clone B2, Cat. No# MA1-813; Thermo Scientific) diluted 1:1000 and mouse anti-retinal pigment epithelium-specific 65 kDa protein (RPE65, clone 401.8B11.3D9, Cat. No# AB13826; Abcam, retail by Wako Pure Chemical Industries, VA, USA) dissolved in PB, placed ganglion cell layer (GCL) side up on a slide, coated with a custom glycerol/Mowiol based mounting media and covered with a glass coverslip.

For labeling of retinal and RPE flat mounts, tissues were exposed to three freeze/thaw cycles in 30% sucrose in PB, washed in PB, and blocked as above. Retina/RPE were then incubated for 4 days in primary antibody to label microglia and infiltrating macrophages (rabbit anti-ibA1, 1:1500, Cat. No# 019-19741; Wako Pure Chemical Industries, VA, USA) dissolved in antibody buffer (above). After washing in PB, retina/RPE were incubated overnight with secondary antibodies, goat anti-rabbit conjugated to AlexaFluor 488 or AlexaFluor 594 (Life Technologies, Mulgrave, VIC, Australia) diluted 1:500 as required for 90 minutes. Finally sections were rinsed three times for 10 minutes each in PB. Sections were incubated with secondary antibody: goat anti-mouse or goat anti-rabbit conjugated to AlexaFluor 488 and AlexaFluor 594 (Life Technologies, Mulgrave, VIC, Australia; diluted 1:500) as required for 90 minutes. Far-red (647-nm excitation), green (488-nm excitation), red (594-nm excitation), and far red (647-nm excitation) spectral frequencies. Images were collected using Zeiss LSM or Zen software (Carl Zeiss AG). The tissues were washed in PB, placed ganglion cell layer (GCL) side up on a slide, coated in a custom glycerol/Mowiol based mounting media and covered with a glass coverslip.

A Zeiss Meta or Pascal confocal microscope (air X10, air X20, and oil X40 objective) was used to examine and image immunolabeled tissue samples (Carl Zeiss AG, Oberkochen, Germany). Fluorescence was scanned and imaged with a krypton/argon laser (10% laser power) fitted with appropriate filters for differential visualization of the specific fluorophores. In some instances, autofluorescence was assessed in the absence of immunolabeling by increasing the laser power (30%) and gain of the confocal settings in the blue (405-nm excitation), green (488-nm excitation), red (594-nm excitation), and far red (647-nm excitation) spectral frequencies. Images were collected using Zeiss LSM or Zen software (Carl Zeiss AG). Images were edited using LSM Zeiss software, ImageJ 1.43 freeware (http://imagej.nih.gov/ij/; provided in the public domain by the National Institutes of Health, Bethesda, MD, USA) or Adobe Photoshop CS2 (Adobe Systems Inc.). Image quality was optimized similarly for WT and Cd2−/−.
tissues by adjusting the following parameters: laser power, gain, and black levels.

**Analysis of RPE Cell Morphology in Flat Mount**

Eyecups from 15-month-old WT and Ccl2−/− mice were compared for changes in RPE cell size \((n = 9\) and \(n = 6\), respectively). Six images (three central and three peripheral) from phalloidin-labeled RPE were collected for each animal using an X20 air objective. The image threshold was set to bandpass filter the image to enhance cell membranes for cell size analysis. The image threshold was set to specify cell areas as regions of interest (ROI) and the analyze particle for cell size analysis. Given RPE, hundreds of cell areas were assessed and averaged to generate a single result \((n = 1)\) for both central and peripheral RPE. Final data are presented per animal and \(n \geq 6\) animals in each group were assessed for statistical comparison.

**Analysis of RPE Cell Protein Expression in Transverse Sections**

Transverse eyecup sections from 15-month-old WT and Ccl2−/− mice were compared for changes in RPE protein expression by quantifying immunofluorescence \((n = 6\) for each). At least four, 16-bit images (two central and two peripheral) from CRALBP- and also RPE65-labeled RPE were collected using a 1.4-μm pin hole, an X40 oil objective at 2X zoom, from at least two sections over 140 μm apart for each animal. Care was taken to ensure WT and Ccl2−/− tissues were matched by placing samples on the same slide, running all the immunohistochemistry at the same time, collecting confocal images on the same day, ensuring confocal laser power settings were 20% below those required to assess autofluorescence, and ensuring confocal settings were not adjusted between samples or during analysis. ImageJ 1.43 freeware (NIH) was used to draw a segmented line across the labeled RPE at the base of the cells. The mean gray level along this line was determined where use of the 16-bit grayscale depth meant a fluorescence unit from 0 to 65,535 could be ascertained as a measure of peptide expression for each image. Final data are presented as fluorescence units, and \(n = 6\) animals for both central and peripheral RPE in each group were assessed for statistical comparison.

**Analysis of Microglia/Macrophage Number and Morphology**

For RPE-microglia/macrophase cell counts, tile scans of IbA1-labeled cells across the entire flat mounted RPE were collected using an X10 air objective. The area of the RPE (mm²) and the number of IbA1-positive cells within that area was assessed using ImageJ 1.43 freeware (NIH) and cell counts were completed using the macro, ITCN. Within the retina, morphological analysis of IbA1-positive microglia was completed. At least three high resolution Z-stack images from central retina containing microglia in the GCL, inner plexiform layer/inner nuclear layer (IPL/INL), and outer plexiform layer (OPL) were captured using an X40 oil objective for each retina. Microglial morphology in each laminae was assessed using the software, MetaMorph Offline (Molecular Devices Corporation, Sunnyvale, CA, USA). Soma area was assessed using the Multiwavelength Cell Scoring Application, and all other morphological parameters were determined using the Neurite Outgrowth application module. For a given retinal laminae (e.g., OPL), from a single animal the morphological properties of at least 15 microglia from that retina layer were assessed. Morphological parameters from \(n \geq 6\) animals in each group were assessed for statistical comparison including: soma area (μm²), number of branches, and total outgrowth of processes, equating to the summed length of all processes (μm), which is a measure of arbor spread.

**Analysis of Müller Cell Gliosis**

Retina from 15-month-old WT and Ccl2−/− mice were compared for signs of retinal stress. Entire transverse retinal sections within 150 μm of the optic nerve head were assessed for Müller cell gliosis. Müller cell gliosis was defined as the proportion of Müller cell GS labeled processes that displayed GFAP immunoreactivity. At least two sections in at least \(n = 5\) animals were assessed and compared for each genotype. For quantification, offset and gain parameters of the confocal system were not altered between photography of WT and Ccl2−/− sections.

**Statistics**

Results are expressed as the mean ± SEM. Retinal pigment epithelium cell size and protein expression across eccentricity (central and peripheral) and genotype (WT versus Ccl2−/−) were analyzed by two-way analysis of variance (two-way ANOVA). Microglial morphology parameters across retinal layer (GCL, IPL, OPL) and genotype (WT versus Ccl2−/−) were analyzed by two-way ANOVA. Electoretinogram responses across time (3, 6, 9, 12, and 15 months) and genotype (WT versus Ccl2−/−) were analyzed by two-way ANOVA. Following two-way ANOVA, a Tukey’s post hoc test was used to make individual comparisons as appropriate (Graphpad Prism version 6; GraphPad Software, Inc.) and statistical significance between genotypes indicated by * for \(P < 0.05\), while for the ERG a statistically significant effect of age is indicated by † for \(P < 0.05\). For immunohistochemical quantification of RPE macrophase numbers and Müller cell gliosis at 15 months, a Student’s \(t\)-test was used to compare results from WT and Ccl2−/− mice. In these figures, statistical significance is expressed as *\(P < 0.05\).

**RESULTS**

**Fifteen-Month-Old Ccl2−/− Mice Display Fundus Lesions, Subretinal Macrophages, and Central RPE Cell Change**

The retinal fundi of 9- and 15-month-old WT and Ccl2−/− mice were imaged using a Micron III fundus camera (Figs. 1A–D). At 9 months, there were no abnormalities observed in the fundus of either WT (Fig. 1A) or Ccl2−/− mice (Fig. 1B). By 15 months of age, in five out of six Ccl2−/− mice investigated, multiple yellow fundus lesions were observed in all quadrants of the retina (Fig. 1D). In contrast, in WT mice, four out of five had no lesions, while one had a small number of spots in the fundus (Fig. 1C). The fundus lesions are thought to represent bloated subretinal IbA1 positive microglia/macrophages containing lipofuscin.7,8 In order to confirm this previous work, we labeled the RPE of 15-month-old WT and Ccl2−/− mice for microglia/macrophages using an antibody against IbA1 (Figs. 2A–D). In general, the RPE of Ccl2−/− mice had many adherent subretinal IbA1-positive cells (Figs. 2B, 2D) when compared with WT RPE (Figs. 2A, 2C), and this was found to be significant when quantified (Fig. 2E; WT, \(n = 9\) versus Ccl2−/−, \(n = 6\), Student’s \(t\)-test \(P = 0.0286\)). In addition, the RPE of Ccl2−/− mice appeared to have higher levels of autofluorescence, indicative of lipofuscin buildup, across the RPE surface.
in all spectrums assessed (far red and green, Figs. 2I, 2J, respectively; red and blue, data not shown). The autofluorescence detected in the far red spectrum was the most condensed, in particular within adherent microglia/macrophages (Figs. 2I–K). The level of autofluorescence across the RPE was generally lower in WT mice, even in areas where subretinal IbA-1 positive microglia/macrophages were present (Figs. 2F–2H; images taken with same confocal settings as in panels I–K). Interestingly, in the only 15-month-old Ccl2−/− mouse in which there were no obvious lesions observed on funduscoppy, there were still large numbers of microglia/macrophages adhered to the RPE (data not shown). The converse was true for WT mice in that mice without fundus lesions did have microglia/macrophages adhered to the RPE. This suggests that the presence of subretinal microglia/macrophages may not consistently correlate with fundus lesions in WT and Ccl2−/− mice.

Previously, fundus lesions have also been shown to develop in mice with retinal degeneration due to loss of retinal neurons and/or the RPE.22,30 Thus the morphologies of the neural retina and RPE in the aged Ccl2−/− mouse were assessed (Fig. 3). The retinal morphologies of 15-month-old WT and Ccl2−/− mice were compared using toluidine blue-stained, resin sections of neural retina of Ccl2−/− segments, loss of cell bodies, or loss of synaptic layers in the retina. I, Inferior; N, Nasal; ON, optic nerve head; S, Superior; T, Temporal.}

FIGURE 1. Ccl2−/− mice have an increase in fundus lesions by 15 months of age. The retina of 9- and 15-month-old mice were imaged using a Micron III fundus camera. At 9 months of age, no abnormalities were observed in (A) WT or (B) Ccl2−/− mice fundi. (C) By 15 months of age, 20% of WT mice had a small number of lesions apparent in the fundus. (D) In contrast, 83% of 15-month-old Ccl2−/− mice investigated had multiple yellow fundus lesions in all quadrants of the retina. I, Inferior; N, Nasal; ON, optic nerve head; S, Superior; T, Temporal.

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FIGURE 1. Ccl2−/− mice have an increase in fundus lesions by 15 months of age. The retina of 9- and 15-month-old mice were imaged using a Micron III fundus camera. At 9 months of age, no abnormalities were observed in (A) WT or (B) Ccl2−/− mice fundi. (C) By 15 months of age, 20% of WT mice had a small number of lesions apparent in the fundus. (D) In contrast, 83% of 15-month-old Ccl2−/− mice investigated had multiple yellow fundus lesions in all quadrants of the retina. I, Inferior; N, Nasal; ON, optic nerve head; S, Superior; T, Temporal.

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Figure 2. Ccl2−/− mice have an increase in macrophages/microglia on the RPE and an increase in RPE autofluorescence. The RPE of 15-month-old mice were labeled with the microglial/macrophage marker, anti-Iba1 (green) and imaged in flat mount on a confocal microscope. (A) Entire tile scans of RPE from a WT mouse labeled for Iba1-positive cells and (C) magnified inset from (A) near the ON. (B) Retinal pigment epithelium from a Ccl2−/− mouse, labeled for Iba1-positive cells and (D) magnified inset showing increased macrophages adhered to the RPE from (B) near the ON. (E) Quantification of Iba1-positive macrophages per area of RPE in WT (black, n = 9) and Ccl2−/− (gray, n = 6), showing a significant increase in Iba1-positive cells in Ccl2−/− mice (Student’s t test, *P < 0.05). (F–K) Confocal laser power was increased to detect autofluorescence, and areas of
in RPE cell morphology toward the periphery. Together these data indicate that aged Ccl2−/− mice have normal retinal morphology but many signs of RPE distress, including RPE cell loss and autofluorescence/lipofuscin buildup, coupled with increased numbers of subretinal microglia/macrophages containing RPE-specific debris.

The Rod and Cone Pathway ERG Responses Reduce With Age but Are Not Altered in Ccl2−/− Mice

The general function of the retina was assessed in WT and Ccl2−/− mice at 3, 6, 9, 12, and 15 months of age using a twin flash ERG paradigm to isolate the rod and cone pathway responses. Representative rod ERG waveforms are presented.

Figure 3. Gross retinal morphology appears normal, but Ccl2−/− mice have an increase in central RPE cell size by 15 months of age. Eyecups were fixed, embedded in epon-resin, cut at 1 μm, and stained with toluidine blue to inspect the gross structural morphology of retina and RPE from WT (A retina; D higher magnification of RPE), and two separate regions of retina from Ccl2−/− mice (B, C retina; E, F higher magnification of RPE). The gross morphology of (A) WT and (B, C) Ccl2−/− retina was qualitatively similar. Generally the RPE of (D) WT and (E) Ccl2−/− mice was similar; however, (F) some regions of Ccl2−/− RPE showed signs of distress, including vacuoles and subretinal nuclei containing RPE melanin (arrowhead). (G-I) Transverse cryostat sections of (G) WT and (H) Ccl2−/− eyecups were labeled with an antibody against CRALBP (green) and imaged for autofluorescence in the far red spectrum (red). (I) Relative expression of CRALBP was quantified by careful analysis of fluorescence intensity across the RPE and was found to be similar between WT and Ccl2−/− mice (WT, n = 6 and Ccl2−/−, n = 6; two-way ANOVA for genotype, P = 0.25, for eccentricity, P = 0.0014). (J, K) Transverse cryostat sections of (J) WT and (K) Ccl2−/− eyecups were labeled with an antibody against RPE-65 (green). (L, M) The RPE were labeled with the RPE marker, phalloidin (red), imaged in flat mount, and representative images of central RPE from (L) WT and (M) Ccl2−/− mice are presented. (N) Quantification of RPE cell size in central and peripheral eccentricities showed a significant increase in central RPE cell size in Ccl2−/− (gray, n = 6) compared to WT mice (black, n = 9; two-way ANOVA, Tukey’s posttest for central RPE size from WT versus Ccl2−/− (*P < 0.05). Scale bars: 50 μm (A–C); 20 μm (D–K); 50 μm (L, M). Ch, choroid; Os, outer segments; ONL, outer nuclear layer.
for WT (black) and Ccl2−/− mice (gray, Fig. 4A) and show that the overall amplitude of the ERG response reduces with age but is not qualitatively different between the two strains, at any age. When quantified, the amplitude of the rod photoreceptor response (Rod PIII, a-wave) was reduced significantly by 9 months of age but was not different between WT and Ccl2−/− mice at any age (two-way ANOVA, age \( P < 0.0001 \), genotype \( P = 0.154 \)). (C) There was no significant change in the rod photoreceptor (rod PIII) sensitivity with age or between strains (two-way ANOVA, age \( P = 0.06 \), genotype \( P = 0.340 \)). (D) The amplitude of the rod postphotoreceptor response (rod PII, b-wave) amplitude was reduced significantly by 9 months of age but was not different between strains (two-way ANOVA, age \( P < 0.0001 \), genotype \( P = 0.85 \)). (E) There was an increase in time to peak of the rod postphotoreceptor response with age but no effect of genotype (rod PII implicit time, two-way ANOVA, age \( P = 0.0012 \), genotype \( P = 0.79 \)). (F) The OP response was reduced with age, but there was no difference between strains (rod summed OPs[2-4] amplitude; two-way ANOVA, age \( P < 0.0001 \), genotype \( P = 0.67 \)). Tukey’s post hoc for age; for both genotypes the significant effects of age compared with the 3-month response was apparent from 9 months onwards as indicated by \( ^{+}P < 0.05 \).

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**Figure 4.** The rod-derived ERG reduces with age but is not different between WT and Ccl2−/− mice. (A) Representative, rod ERG waveforms are presented for WT (black) and Ccl2−/− mice (gray) collected in response to a 2.1-log cd.s/m² full field flash. The various waveforms of the rod ERG were isolated and modeled. (B) The amplitude of the rod photoreceptor response (Rod PIII, a-wave) was reduced significantly by 9 months of age but was not different between WT and Ccl2−/− mice at any age (two-way ANOVA, age \( P < 0.0001 \), genotype \( P = 0.154 \)). (C) There was no significant change in the rod photoreceptor (rod PIII) sensitivity with age or between strains (two-way ANOVA, age \( P = 0.06 \), genotype \( P = 0.340 \)). (D) The amplitude of the rod postphotoreceptor response (rod PII, b-wave) amplitude was reduced significantly by 9 months of age but was not different between strains (two-way ANOVA, age \( P < 0.0001 \), genotype \( P = 0.85 \)). (E) There was an increase in time to peak of the rod postphotoreceptor response with age but no effect of genotype (rod PII implicit time, two-way ANOVA, age \( P = 0.0012 \), genotype \( P = 0.79 \)). (F) The OP response was reduced with age, but there was no difference between strains (rod summed OPs[2-4] amplitude; two-way ANOVA, age \( P < 0.0001 \), genotype \( P = 0.67 \)). Tukey’s post hoc for age; for both genotypes the significant effects of age compared with the 3-month response was apparent from 9 months onwards as indicated by \( ^{+}P < 0.05 \).
The cone pathway responses were also isolated, and although the cone photoreceptor response could not be assessed due to the small number of cones in mice, cone postphotoreceptor ERG responses were obtained for WT (black) and Ccl2+/− mice (gray, Fig. 5A). As was seen for the rod pathway response, by 9 months of age there was a significant age-related decrease in the cone postphotoreceptor (cone PII, b-wave) amplitude (Fig. 5B; two-way ANOVA, age \( P < 0.0001 \), genotype \( P = 0.92 \)). (C) There was an increase in time to peak of the cone postphotoreceptor response with age but no effect of genotype (cone PII implicit time, two-way ANOVA, age \( P = 0.0002 \), genotype \( P = 0.88 \)). Tukey’s post hoc for age; for both genotypes the significant effects of age compared with the 3-month response was apparent from 9 months onwards as indicated by \( ^{+}P < 0.05 \).

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**Dark Adaptation Is Unaffected by Age in WT and Ccl2+/− Mice**

The time course of dark adaptation provides valuable insights into function at the level of the RPE and photoreceptor layers, which is where the biochemical cycle operates to recycle bleached photopigment. To determine the effect of age and lack of Ccl2 on the function of the photoreceptor/RPE photopigment regeneration cycle, we compared the dark adaptation responses of mice over 45 minutes, following a bleaching stimulus. The dark adaptation recovery profile for Ccl2+/− (gray) and WT mice (black) at 3 and 15 months are shown in Figures 6A and 6B, respectively, with the prebleach rod photoreceptor responses shown to the right of the dark adaptation curves. An exponential equation was used to model the recovery of the a-wave, and these data were used to assess the amplitude of the recovered response and the kinetics, specifically the time to reach 50% of the maximum amplitude. The postbleach responses indicated that the rod photoreceptors recovered and reached a plateau with time; however, the plateau did not reach the initial amplitudes obtained before retinal bleaching. The average plateau amplitudes of the modeled responses at all ages for Ccl2+/− and WT mice are shown in Figure 6C. The average plateau of the rod photoreceptor response (a-wave) increased significantly with age, and this was not different between the genotypes (plateau amplitude; two-way ANOVA, age \( P = 0.015 \), genotype \( P = 0.41 \); post hoc for age, \( ^{+}P < 0.05 \) for both genotypes). This is consistent with the changes observed in the rod PIII amplitude with age described previously (Fig. 4B). In order to assess the kinetics of the dark adaptation response, the time to reach half the maximum response was also measured (Fig. 6D). There was no difference in the rate of recovery of dark adaptation as a function of age or genotype (half time; two-way ANOVA, age \( P = 0.78 \), genotype \( P = 0.62 \)). Overall, these results demonstrate that the rate of dark adaptation following a 14% bleach is not affected by lack of Ccl2 or advancing age up to 15 months in the mouse.
Müller Cells and Microglia Become Gliotic in Aged Ccl2−/− Mice

Müller cells are responsible for many homeostatic processes and are also believed to be the source of Ccl2 within the retina, so the morphology of Müller cells was investigated. The retina of control and Ccl2−/− mice at 15 months were labeled with antibodies against GS (red), which stains all Müller glia, and GFAP, which selectively labels gliotic Müller cells and astrocytes. (green, Fig. 7). There was an increase in GFAP expression in the Müller cells of the Ccl2−/− animals (Fig. 7B) compared to controls (Fig. 7A) at 15 months of age, indicative of Müller cell gliosis. This gliosis was quantified and found to be significantly higher in Ccl2−/− mice as compared to WT mice (Fig. 7C, Student’s t-test, P < 0.01).

As Ccl2 recruits microglia/macrophages to inflamed tissues, the lack of Ccl2 may affect the total number of microglia and their morphology within the neural retina. To test this hypothesis, we used immunohistochemistry to fluorescently stain microglia in flat mounted retina and collected entire z-stacks so as to analyze microglia from the GCL, IPL, and OPL. Figure 8 shows representative images of Iba1-positive cells in the IPL of retina from WT (Figs. 8A, 8B, B inset from A) and Ccl2−/− mice (Figs. 8C, 8D, D inset from C). There were no significant differences in the number of microglia within any of the retinal layers between Ccl2−/− and WT mice at 15 months of age (data not shown). However, there were changes in morphology consistent with microglial activation. While there was no change in soma area in any of the retinal layers (Fig. 8E), microglia in Ccl2−/− mice had significantly reduced process area when compared with WT mice (Fig. 8F; two-way ANOVA, genotype P = 0.0001 and layer P < 0.0001; WT versus Ccl2−/− in GCL P = 0.038, IPL P < 0.0001, OPL P = 0.028). This reduction in process area correlated with a reduction in the number of branches in the microglial processes of aged Ccl2−/− mice (Fig. 8G; two-way ANOVA, genotype P < 0.0001 and layer P < 0.0001; WT versus Ccl2−/− in GCL P = 0.014, IPL P < 0.0001, OPL P = 0.26).

DISCUSSION

This study investigated retinal function and retinal morphology in Ccl2−/− mice compared with WT controls during aging. Ccl2 is expressed in the retina by Müller cells and the RPE, and is thought to be involved in neuroprotection via its roles in modulating the microglial/macrophage response. Fifteen-month-old Ccl2−/− mice had apparent fundus lesions, coupled with increased RPE autofluorescence/lipofuscin deposition and increased numbers of subretinal microglia/macrophages containing RPE specific debris when compared with WT mice. There was also an increase in RPE cell size in the aged Ccl2−/− mice, indicative of a loss of RPE cells. Within the retina, gross neuronal structure was maintained; however, there was an increase in Müller cell gliosis, indicative of retinal stress, and changes in microglial morphology consistent with microglial activation. These morphological changes at the retinal/RPE interface and within the retina itself did not correlate with a change in either rod or cone ERG pathway...
function nor with the rate of dark adaptation. These data show that Ccl2 is important for maintaining normal RPE, Müller cell, and microglial morphology with age but that the RPE and retina compensate to maintain functional vision such that at 15 months of age there is no functional deficit in these mice.

Changes in Retinal and RPE Morphology in Mice Lacking Ccl2

In the retina, Ccl2 is likely released from glia and RPE when the retina is under stress to attract microglia/macrophages expressing the Ccr2 receptor to sites of damage. At 15 months of age, mice lacking Ccl2 had signs of both RPE and Müller glia dysfunction. At the level of the RPE, aged Ccl2−/− mice had increased fundus lesions, subretinal microglia/macrophages, and a significant increase in central RPE cell size compared with WT controls. The fundus lesions seen on ocular fundoscopy in mice have been suggested to be bloated macrophages/microglia stuck at the subretinal space. However, our data indicate that the presence of subretinal microglia/macrophages may not consistently correlate with fundus lesions in these mice. The exact morphological basis underlying the fundus lesions may instead result from a combination of factors. Our data indicate that changes in gross retinal morphology such as loss of neuronal layers and/or presence of photoreceptor rosettes do not cause the fundus lesions observed in the Ccl2−/− mice as has been seen in other

Figure 7. There was an increase in Müller cell gliosis in aged Ccl2−/− mice. Sections of transverse retina of (A) WT and (B) Ccl2−/− mice at 15 months were labeled with antibodies against GS, which stains all Müller glia (red), and GFAP, which selectively labels gliotic Müller cells and astrocytes (green). (C) There was an increase in GFAP expression in the Müller cells of the Ccl2−/− animals compared to WT controls indicative of Müller cell gliosis (n ≥ 5; Student’s t test, **P < 0.01). Scale bar: 20 μm.

Figure 8. Intraretinal microglia show signs of activation in aged Ccl2−/− mice. Entire flat mounted retina of 15-month-old animals were labeled for Iba1-positive microglia, and Z-stacks through from the GCL to the ONL were captured. Flattened Z-stacks of Iba-positive cells in the IPL are presented: (A) WT at ×20 objective magnification and (B) magnification inset from (A); and (C) Ccl2−/− at ×20 objective magnification and (D) magnification inset from (C). Scale bars: 50 μm. Microglial morphology was assessed. (E) There was no significant change in the soma area of Iba1-positive cells in any layer between WT and Ccl2−/− mice. (F) There was a reduction in microglial total process outgrowth in Ccl2−/− mice (two-way ANOVA, genotype P < 0.0001 and layer P < 0.0001; asterisk indicates P < 0.05 in Tukey’s post hoc between WT versus Ccl2−/−). (G) There was a reduction in the number of microglial process branches in the GCL and IPL of aged Ccl2−/− mice (Fig. 7G; two-way ANOVA, genotype P < 0.0001 and layer P < 0.0001; WT versus Ccl2−/− in GCL *P = 0.014, IPL *P < 0.0001, OPL P = 0.26).
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mouse strains. Rather, our data point to RPE dysfunction as there were higher levels of autofluorescence in all spectra across the RPE of Ccl2−/− mice, suggestive of increased lipofuscin deposition, A2E accumulation, and oxidized melanin within the RPE similar to that reported in the Abca4−/− mouse. In addition, autofluorescence was densely associated within subretinal microglial/macrophages in the Ccl2−/− mouse, and to a much lesser extent in the WT mouse. It is likely that a combination of these autofluorescent macrophages/microglia and other independent regions of RPE autofluorescence converge to produce the lesions observed upon fundoscopy.

Aged Ccl2−/− mice had an increased number of subretinal microglia/macrophages compared with WT controls. It is possible that the accumulation of immune cells at the subretinal space is a normal aspect of aging, as to a lesser extent Iba1-positive cells were also observed on the RPE in the WT controls. It may be that normally microglia aid the aging RPE in the clearance of photoreceptor debris. As the retina ages, oxidized unsaturated fatty acids and other lipoproteins in the photoreceptor outer-segments accumulate. The oxidized materials stimulate RPE cells to produce various cytokines/chemokines, including granulocyte-macrophage colony-stimulating factor, RANTES, Ccl2, IL-4, IL-6, and IL-8, some of which may induce microglial/macrophage migration and phagocytosis of this material. It is possible that redundancy in the chemokine signaling pathways allows these other signaling mechanisms to enhance immune cell migration to a dysfunctional RPE in the Ccl2−/− mouse.

Others have suggested that the presence of macrophages at the subretinal space may actually lead to photoreceptor degeneration, and drusen formation. Our data do not support this, as there was no photoreceptor loss despite the increased presence of subretinal microglia/macrophages in the 15-month-old Ccl2−/− mice. There was, however, evidence of RPE cell loss in the central retinal regions, with larger RPE cells found centrally. In line with this, the subretinal microglia/macrophages were found to contain RPE melanin granules, consistent with a role for these cells in phagocytosis of dying RPE cells. It is possible that the increased presence of macrophages in the subretinal space of Ccl2−/− mice may negatively impact the RPE, as has been reported. Conversely, the larger number of immune cells in the subretinal space may be acting as a positive support for a dysfunctional RPE caused by age-related RPE failure.

Within the retina also, lack of Ccl2 had detrimental effects on macro- and microglial morphology. While there were no gross changes to retinal structure or changes to the number of intraretinal microglia apparent at 15 months of age, the existing microglia were morphologically different, with less process spread and reduced branching but no change in soma size, indicative of a mildly activated phenotype. Aspects of this type of microglial activation have been shown in a variety of retinal pathologies including diabetes, models of retinopathy of prematurity, foreign body contact and heat, and also in mice lacking both Ccl2 and Cx3cr1. In addition, the macroglia were also relatively gliotic as there were more Müller cells expressing GFAP in Ccl2−/− retina at 15 months of age relative to WT controls. Müller cells become gliotic in response to generalized retinal stress, and previous studies have found Müller glia become more gliotic with normal aging. It is likely that an upregulation of Ccl2 is required during the aging process as Ccl2 levels were found to be higher in the retina of 20-month-old mice compared to 3-month-old mice. Therefore, changes in the retinal molecular environment with age may cause an earlier RPE, macro- and microglial stress response in mice lacking Ccl2.

Retinal Function Is Not Altered in Aged Mice Lacking Ccl2

While our data show no evidence of photoreceptor loss, given the changes in RPE, microglia, and Müller glia, we expected that general visual function and particularly dark adaption would be reduced in Ccl2−/− mice earlier than in controls. This was not the case. While we found significant visual deterioration in the rod and cone pathways by 15 months of age, evident from 9 months, there was no difference between Ccl2−/− and WT mice. This suggests that there is a global decline in retinal function with age in both strains and that there are no losses in specific neuronal subtypes. Reduced retinal function with age has been shown previously for pigmented mice. The number of photoreceptors declines with age and this may be linked to the age-related declines in retinal function. Indeed, specific losses in rod function correlate with a marked loss of 40% of rod photoreceptors in pigmented mice by 2.5 years of age. Our previous studies have also shown that the cone ERG is reduced with age in WT mice, but to our knowledge this is the first assessment in mice lacking Ccl2.

To investigate whether subtle visual loss had occurred, we investigated the dark adaptation response of WT and Ccl2−/− mice as a function of age. Dark adaptation measures the restoration of the photo-activated (bleached) rhodopsin to its previous dark state, which is achieved by a complex chain of biochemical reactions in the visual cycle involving both the photoreceptors and the RPE. Dark adaptation has been shown to be effective in the detection of early rod loss. Using a similar ERG paradigm to that used in the current study, the rate of dark adaptation has been shown to be altered in diabetic rodents. However, neither Ccl2−/− nor WT mice showed any significant loss in dark adaptation sensitivity with age, indicating that following a 14% bleach the RPE-photoreceptor driven visual cycle operates normally in aged mice with and without Ccl2. This finding runs counter to the dark adaptation losses found in studies of aged humans; however, our results are consistent with dark adaptation studies in mice. Kolesnikov et al. compared WT mice at two time points, 3 months and 30 months, and found no change in the rate of dark adaptation in these mice. It is possible that the different dark adaptation mechanisms between mouse and human could account for this difference. Factors within the dark current processing that could be affected in aged humans as opposed to mice include the primary phototransduction cascade where pigment meta-products HII decay to free opsin, rates of rhodopsin phosphorylation and/or arrestin binding, changing lipid composition and disc membranes, and the activity of photoreceptor retinol dehydrogenase toward releasing chromophore (all-trans-retinal) could also affect rods following a bleaching stimulus.

It is interesting to note that dark adaptation occurred normally in mice lacking Ccl2 despite changes in RPE morphology and increased numbers of subretinal macrophages/microglia. However, it is possible that under the bleaching conditions used in the current experiment, the RPE may not be participating in the visual cycle. Further experiments, such as a greater than 50% bleach of rhodopsin might provide further insight into the RPE/retina recycling of the visual chromophore. However, in the 15-month-old WT and Ccl2−/− mice, there were no gross changes in retinal morphology such as loss of photoreceptor outer segments, which might predispose to altered dark adaptation. In
addition, despite signs of RPE cell loss, assessment of CRALBP and RPE65 showed that there were no changes in expression of peptides, which are critical for chromophore recycling between WT and Ccl2 $^{-/-}$ mice. This indicates that RPE and retinal function is preserved even when homeostatic mechanisms are stressed by alterations in immunoregulatory pathways.

Recent Developments Regarding the Genetic Background of Ccl2 $^{-/-}$ Mice

Recent evidence has shown that the naturally occurring retinal degeneration mutation, $Crb1^{Rd8/Rd8}$, a single base pair deletion of a cytosine in exon 9 of the $Crb1$ gene, is present in the background strain of several major lines of commercially available genetic knockout mice (specifically, the C57BL/6N lines). 18–20 In the retina, $Crb1$ is produced by Müller cells and is crucially important in the formation of the outer limiting membrane at the posterior site of the outer nuclear layer of the retina. 20 This mutation results in retinal degeneration, and there is a possibility that the Ccl2 $^{-/-}$ line used in previous studies may have been confounded by this mutation. 18–20 These findings also shed light on why studies on Ccl2 $^{-/-}$ mice have produced considerably mixed results varying from geographic atrophy and neovascularization versus accumulation of swollen autofluorescent subretinal macrophages/microglia, RPE vacuolization, and little else. 7, 17 In the present study, the mice used did not harbor the Rd8 mutation, thus the current results aid in the clear interpretation of the role that Ccl2 plays in the retina with age.

In conclusion, this study provides insight into the role of Ccl2 within the retina during the aging process. Lack of Ccl2 did lead to morphological changes in the retina and RPE indicative of RPE cell loss and retinal stress. Therefore, upregulation of Ccl2 may be specifically required to modulate macrophage/microglial movements at the subretinal space and also RPE cell and Müller cell integrity. However, we found that lack of Ccl2 did not cause retinal dysfunction as measured by the ERG. This may mean that, (1) Ccl2/Ccr2 has no significant role to play in retinal function, (2) upregulation of compensatory mechanisms masks any effects, (3) a later time point is needed to detect functional change between the cohorts, or (4) that a stressor is needed to observe a response to the lack of this chemokine pathway. These data show that while Ccl2 is important for maintaining normal RPE and glial morphology with age, compensation occurs to maintain gross retinal structure and vision such that at 15 months of age there is no functional deficit consistent with retinal degeneration in these mice. However, changes in Ccl2 signaling could be a significant contributor to long-term chronic stress whereby a vulnerable RPE and retina may have an increased susceptibility to disease with age. These results highlight the importance of the innate immune response in maintaining the integrity of the retina and RPE with age.

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References


