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Murine *Ccl2/Cx3cr1* Deficiency Results in Retinal Lesions Mimicking Human Age-Related Macular Degeneration

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Abstract

Purpose—Senescent *Ccl2*^{−/−} mice are reported to develop cardinal features of human age-related macular degeneration (AMD). Loss-of-function single-nucleotide polymorphisms within CX3CR1 are also found to be associated with AMD. The authors generated *Ccl2*^{−/−}/*Cx3cr1*^{−/−} mice to establish a more characteristic and reproducible AMD model.

Methods—Single *Ccl2*- and *Cx3cr1*-deficient mice were crossbred to obtain *Ccl2*^{−/−}/*Cx3cr1*^{−/−} mice. Funduscopy, histopathology, retinal A2E quantification, proteomics, RT-PCR gene expression assay, immunochemistry, and Western blotting were used to examine the retina and to evaluate gene expression within the retinal tissue.

Results—By 6 weeks of age, all *Ccl2*^{−/−}/*Cx3cr1*^{−/−} mice developed AMD-like retinal lesions, including drusen, retinal pigment epithelium alteration, and photoreceptor degeneration. Furthermore, choroidal neovascularization occurred in 15% of the mice. These degenerative lesions progressed with age. A2E, a major lipofuscin fluorophore that accumulated during AMD progression, was significantly higher in the *Ccl2*^{−/−}/*Cx3cr1*^{−/−} retina than in the wild-type retina. Complement cofactor was higher in the *Ccl2*^{−/−}/*Cx3cr1*^{−/−} RPE. Proteomics data indicated that four proteins were differentially expressed in *Ccl2*^{−/−}/*Cx3cr1*^{−/−} retina compared with control. One of these proteins, ERp29, an endoplasmic reticulum protein, functions as an escort chaperone and in protein folding.

Conclusions—The authors concluded that *Ccl2*^{−/−}/*Cx3cr1*^{−/−} mice develop a broad spectrum of AMD abnormalities with early onset and high penetrance. These observations implicate certain chemokines and endoplasmic reticulum proteins in AMD pathogenesis. Similar to the mechanism of neurodegeneration caused by dysfunction of endoplasmic reticulum proteins, decreased chaperoning may cause misfolded protein accumulation, leading to drusen formation and retinal degeneration.

Age-related macular degeneration (AMD) is the leading cause of blindness in the elderly in Western countries.¹ Considerable efforts have been made to establish AMD animal models.^{2–9} Even though mice have no macula, existing mouse AMD models have been shown to develop cardinal pathologic features of AMD.^{3,8–10} Strong evidence indicates the

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involvement of immunologic processes in AMD.¹¹ Additionally, several studies have successfully demonstrated associations between AMD and various single-nucleotide polymorphisms (SNPs). Many of these SNPs are within genes encoding immunologic molecules.¹²⁻²³

CX3CR1, the specific receptor for CX3CL1/fractalkine chemokine, is expressed on leukocytes (e.g., lymphocytes, macrophages, NK cells, mast cells), dendritic cells,^{24,25} brain microglia, and astrocytes.²⁶⁻²⁸ CX3CR1 expression is also reported in the eye,²⁹ iris, ciliary body,³⁰ retinal microglia, RPE, and Müller cell.^{31,32} In a functional study, two CX3CR1 SNPs resulted in a decreased number of CX3CL1 binding sites and reduced ligand-binding affinity on peripheral blood mononuclear cells.³³ We have reported that these SNPs are associated with AMD.²² Furthermore, we have demonstrated a decreased number of CX3CR1 transcripts and protein in AMD maculae compared with the maculae of normal eyes.^{22,32} However, no ocular abnormalities have been reported in young adult *Cx3cr1*-deficient mice.

In addition to CX3CR1, CCL2 (MCP-1, a CC chemokine) is thought to play a homeostatic, immunoregulatory role in AMD pathogenesis.³⁴ Aged mice with deficient *Ccl2* or *Ccr2*, the corresponding receptor, develop many cardinal features of AMD including drusen formation, RPE accumulation of lipofuscin and complement factors, and choroidal neovascularization.⁸ In addition, CCL2 may function as an antiapoptotic factor, as reported in in vitro systems.^{35,36}

In the present study, we examined the hypothesis that deficiencies in both *Cx3cr1* and *Ccl2* may induce typical pathologic features of AMD in mice more consistently and at an earlier age of onset than in existing animal models. We found that AMD features in the mice were highly characteristic and reproducible. In addition to inflammatory molecules, we also showed evidence for a potential role of endoplasmic reticulum proteins (ERp) in AMD.

Materials and Methods

Animals

Ccl2-deficient and *Cx3cr1*-deficient mice (obtained from Bao Lu and Barrett J. Rollins of Children's Hospital, Harvard Medical School,³⁷ and Philip Murphy of the National Institute of Allergy and Infectious Diseases/National Institutes of Health [NIAID/NIH],³⁸ respectively) were used as the founder generation (F₀) and were crossbred to obtain animals that were heterozygous (F₁) for *Ccl2* and *Cx3cr1* alleles. Heterozygous animals were intercrossed to obtain homozygous *Ccl2*^{-/-}/*Cx3cr1*^{-/-} mice (F₂). Wild-type (WT) mice were of C57BL/6 background. The study was conducted in compliance with ARVO Statement for the Use of Animals in Ophthalmic and Vision Research, and all animal experiments were performed under the protocols approved by the National Eye Institute Institutional Animal Care and Use Committee.

Fundus Photography

Funduscopy examinations were performed every three weeks from 3 to 5 weeks of age until 10 months with the use of a hand-held fundus camera (Kowa Optimed, Torrance, CA) and a specialized lens (90D; Volk Optical, Mentor, OH) after intraperitoneal injection of ketamine (1.4 mg/mouse) and xylazine (0.12 mg/mouse) for systemic anesthesia and topical administration of 1% tropicamide ophthalmic solution (Alcon Inc., Fort Worth, TX) for pupil dilation.

Histopathology

Eyes, brains, livers, spleens, and lungs were harvested after the mice were humanely killed. Tissues were fixed in 10% formalin for at least 24 hours. All tissues were then embedded in methacrylate. Seventy-two eyes (60 of the *Ccl2*^{-/-}/*Cx3cr1*^{-/-} mice and 12 of the WT mice) were subjected to histopathology. The eyes were serially sectioned in the pupillary- optic nerve plane. Each eye was cut into six sections. Other organs were sectioned routinely. All sections were stained with hematoxylin and eosin. If an ocular lesion was found, another 6 to 12 sections were cut through the lesion. These slides were also stained with periodic acid Schiff (PAS) to highlight the Bruch membrane and small neovascular vessels. This study with human ocular tissues was approved by the National Eye Institute institutional review board and was conducted in accordance with the principles expressed in the Declaration of Helsinki.

Transmission Electron Microscopy

Electron microscopy was performed on 4% glutaraldehyde formalin-fixed tissue. The fixed neuroretina-RPE-choroid tissue was embedded in epoxy resin (LX-112; LADD Research Industries, Burlington, VT). Six-micrometer-thick sections stained with toluidine blue were examined under light microscopy. Ultrathin sections were stained with uranyl acetate and lead citrate for examination under a microscope (JEM-100B; JEOL, Tokyo, Japan). Two eyes in each of three age groups (12-20, 21-35, and 36-48 weeks, respectively) of the *Ccl2*^{-/-}/*Cx3cr1*^{-/-} mice and two eyes of the WT mice were used for transmission electron microscopic study.

Immunohistochemistry

After enucleation, mouse eyes were snap-frozen and embedded in OCT compound (Sakura Finetek, Inc., Torrance, CA). Three eyes of the *Ccl2*^{-/-}/*Cx3cr1*^{-/-} mice and two eyes of the control mice were used for immunohistochemistry, as described previously.³⁹ Frozen sections 4-μm-thick were fixed in acetone for 7 to 10 minutes and rinsed with Tris-buffered saline, 0.05 M, pH 7.4. The slides were immersed in 5% normal serum specifically to block potential background from the secondary antibody. Because microglia and complement system activation were suggested to play a role in retina housekeeping and correlated with AMD development,^{12,40} CD11b, a marker for microglia, and CD46, the ligand of complement factors C3b and C4b and a complement regulatory protein,⁴¹ were measured. For the detection of microglia, rat-anti-mouse CD11b antibody (Harlan Sera-Laboratory, Loughborough, UK) was used as the primary antibody; for the detection of CD46, rabbit-anti-mouse CD46 (H-294; sc-9098; Santa Cruz Biotechnology, Inc., Santa Cruz, CA) was used as the primary antibody. Secondary antibodies were biotin-labeled goat anti-rat or anti-rabbit IgG (Vector Laboratories, Burlingame, CA), respectively. For the detection of ERp29, rabbit polyclonal antibody against ERp29 (Abcam Inc., Cambridge, MA) was used as the primary antibody, followed by incubation with secondary antibody (biotin-labeled goat anti-rabbit IgG; Vector Laboratories). Sections were treated with the avidin-biotin-immunoperoxidase system and 3,3'-diaminobenzidine as the substrate and were counterstained with methyl green. Each staining assay for a particular primary antibody was repeated at least once. Exposure times for immunohistochemistry images were matched to ensure appropriate control. Staining was quantified and graded based on the positive number of cells and the color (black) intensity of the stained cells. Cells that were more than 50% blackish were graded as having intense immunoreactivity; in contrast, cells that were less than 20% grayish were graded as having poor immunoreactivity.

Immunohistochemistry analyzing five human ocular sections (two wet AMD, two dry AMD, and one normal eye) was conducted in conformance with the policies and principles stated in the Federal Policy for the Protection of Human Subjects (US Office of Science and Technology Policy) and in the Declaration of Helsinki. Formalin-fixed human ocular sections were

deparaffinized.^{32,42} Detection of ERp29 on human sections was carried out as described and was repeated at least once in each eye.

A2E Extraction and Quantification

Mice were kept in the dark for more than 12 hours. RPE cells and neural retina were isolated from the eyecups of 15-, 20-, and 24-week-old WT and *Ccl2*^{-/-}/*Cx3cr1*^{-/-} mice. RPE cells were then dissected after removal of the neural retina, in a dark room under dim red light.^{43,44} Six eyes were pooled from each group. Each analysis was repeated. 2-[2,6-Dimethyl-8-(2,6,6-trimethyl-1-cyclohexen-1-yl)-1E,3E,5E,7E-octatetra-enyl]-1-(2-hydroxyethyl)-4-[4-methyl-6(2,6,6-trimethyl-1-cyclohexen-1-yl) 1E,3E,5E,7E-hexatrienyl]-pyridinium (A2E) was extracted as previously described.³ Detection and quantification were performed with the use of HPLC electrospray ionization mass spectrometry (ESI-MS; Thermo Electron, San Jose, CA). A gradient of 75% to 96% acetonitrile was used to separate A2E over a period of 40 minutes at a flow rate of 1 mL/min. A2E was quantified according to external standards, and a standard was used to identify and determine A2E concentrations.

Proteomics

The mouse retina, including the RPE and excluding the choroid, was dissolved into extraction buffer II (Bio-Rad Laboratories, Hercules, CA). Sample preparation—including two-dimensional gel electrophoresis image analysis (Proteomweaver; Definiens, Munich, Germany) according to the manufacture's protocol, in-gel digestion followed by liquid chromatography (LC)-MS/MS on an LC/MS system (ProteomeX; ThermoElectron Corp., San Jose, CA), and protein identification—was conducted according to Okamoto et al.⁴⁵ Protein identification was accepted when MS/MS spectra of at least two peptides from the same protein exhibited at a minimum the default Xcorr versus charge values set by the program (for Z = 1, 1.50; for Z = 2, 2.00; for Z = 3, 2.50). Six eyes in each group were pooled as one sample. Three independent experiments (18 *Ccl2*^{-/-}/*Cx3cr1*^{-/-} and 18 WT mice) were performed. All samples were run in duplicate to guarantee greater than 90% identity before further analysis was performed.

Western Blot Analysis

Mouse retina, including the RPE, was homogenized with an equal volume of 2× lysis buffer (RIPA; Upstate Biotechnology, Lake Placid, NY). The protein was separated by SDS-PAGE under reducing conditions and was transferred to transfer membrane (Immobilon-P; Millipore, Bedford, MA). The membrane was processed with an immuno-detection kit (WesternBreeze; Invitrogen, Carlsbad, CA). Briefly, the membrane was first incubated with 1:500 diluted anti-ERp29 antibody (Abcam, Cambridge, MA), followed by incubation with 1:2000 diluted alkaline phosphatase-labeled secondary antibody (Vector). Signals were visualized by immersing the membrane in chromogenic substrate. Two independent experiments were performed with two eyes pooled for each strain in each run.

Detection of *Ccl2*, *Cx3cr1*, and *ERp29* Transcripts by RT-PCR

Five micrograms RNA from mouse retina/RPE was used for cDNA synthesis (Superscript II RNase H⁻ Reverse Transcriptase; Invitrogen, Grand Island, NY). Real-time PCR was performed (Stratagene Mx3000 Real-Time PCR System and Brilliant SYBR Green QPCR Master Mix; Stratagene, La Jolla, CA). Primers for *Ccl2* and *Cx3cr1* were synthesized by and supplied as a gene expression assay kit (RT² Real-Time; Super-Array Bioscience Corp., Frederick, MD). For the internal control, β -actin was amplified using primers 5'-CCCAGCACAAATGAAGATCAA-3' and 5'-ACATCTGCTGGAAGGTGGAC-3'. To determine *ERp29* transcript in mouse ocular tissue, validated and inventoried *ERp29* and *GAPDH* gene expression kits (TaqMan; Applied Biosystems, Foster City, CA) were used

according to the manufacturer's instruction. The comparative C_t method was used to establish relative quantification of the fold change in gene expression (User Bulletin 2; ABI Prism 7700 Sequence Detection System, PE Applied Biosystems, Foster City, CA; 1997). Fold changes were normalized first by the level of *GAPDH*. The average fold change resulting from gene manipulation was again normalized to the transcript level of WT mouse and presented graphically. Four biological samples were analyzed from each group. Each sample was analyzed at least twice.

Results

Systemic Manifestation of *Ccl2*^{-/-}/*Cx3cr1*^{-/-} Mice

Among the 400 F2 offspring genotyped, 12 animals were *Ccl2*^{-/-}/*Cx3cr1*^{-/-}, indicating an abnormal Mendelian segregation (1 in 16 expected). *Ccl2*^{-/-}/*Cx3cr1*^{-/-} mice had normal body weight compared with the controls. *Ccl2*^{-/-}/*Cx3cr1*^{-/-} mice were less prolific when maintained as a separate lineage, with an average of four pups per litter compared with eight per litter in the WT controls. Twenty percent of the *Ccl2*^{-/-}/*Cx3cr1*^{-/-} mice had progressive patchy skin depigmentation, primarily on the face and upper extremities (data not shown).

Ocular Features in *Ccl2*^{-/-}/*Cx3cr1*^{-/-} Mice

Ophthalmic examination findings on 103 *Ccl2*^{-/-}/*Cx3cr1*^{-/-} mice appeared normal except the retina and choroid. Sequential funduscopic examinations were performed on 76 *Ccl2*^{-/-}/*Cx3cr1*^{-/-} mice and 27 age-matched WT mice every 3 weeks beginning at 3 weeks of age. Unlike WT mice that were normal at all ages (Fig. 1A), all 6- to 9-week-old *Ccl2*^{-/-}/*Cx3cr1*^{-/-} mice spontaneously developed drusenlike lesions characterized by heterogenous, round or domed-shaped, soft-bordered, yellowish deposits within the subretina (Fig. 1B). With aging, these lesions enlarged or flattened and became confluent (Fig. 1C). Some of the lesions progressed to form chorioretinal scars and depigmented atrophic areas (Fig. 1D). In comparison, none of the single knockout *Ccl2*^{-/-} or *Cx3cr1*^{-/-} mice developed retinal lesions at such young ages (Ross RJ, et al. *IOVS* 2007;48:ARVO E-Abstract 2355).⁸

Histologic Features in *Ccl2*^{-/-}/*Cx3cr1*^{-/-} Retina

Histopathologic examination was conducted on the eyes of 60 *Ccl2*^{-/-}/*Cx3cr1*^{-/-} mice (20 mice 8-15 weeks of age, 20 mice 15-21 weeks of age, and 20 mice 21-60 weeks of age) and 12 WT mice (four younger than 12 weeks of age, four 12-24 weeks of age, and four older than 60 weeks of age). Eyes of the age-matched WT mice were entirely normal and lacked drusen formation, neovascularization, photoreceptor degeneration, and RPE atrophy (Fig. 2A is a representative image). All *Ccl2*^{-/-}/*Cx3cr1*^{-/-} eyes showed focal thickening of the Bruch membrane. Drusen (dome-shaped areas of hyaline excrescences within Bruch membrane) was found in some eyes (Fig. 2B); most druse were small (5-15 μ m). Local RPE hypopigmentation and vacuolation (Fig. 2C), photoreceptor outer segment disorganization, and photoreceptor atrophy were commonly observed. These local changes could be present at various severities in the same eye or in different eyes. With careful examination of the series of consecutive sections, choroidal neovascularization was found in 15% of *Ccl2*^{-/-}/*Cx3cr1*^{-/-} mouse eyes; earliest onset was at 12 weeks of age. A few of the fragile, small choroidal neovascular vessels that penetrated Bruch membrane and entered the outer retinal layers were surrounded by hyperplastic RPE cells or atrophic RPE areas (Fig. 2D).

Ultrastructure of *Ccl2*^{-/-}/*Cx3cr1*^{-/-} Retina

Transmission electron microscopic examinations were performed on the mice at different ages. Retinas of the *Ccl2*^{-/-}/*Cx3cr1*^{-/-} mice showed a decrease in melanosomes and an increase in lipofuscin within the RPE (hypopigmentation; Figs. 3A, 3B), thickened Bruch membrane

(500-1000 nm compared with 240-350 nm in the normal areas of the *Ccl2*^{-/-}/*Cx3cr1*^{-/-} and WT mice; Fig. 3E) with amorphous granular and heterogeneous material deposits (Fig. 3C), and disorganization or atrophy of the photoreceptors (Fig. 3D). In addition, loss of tight junctions and cellular membrane folding were noted in some RPE cells. These ultrastructural findings indicated degeneration of the RPE and photoreceptors and are reminiscent of the ultrastructural changes observed in human AMD cases.^{46,47} Moreover, these abnormalities were more severe in the older mice, indicating a progressive degenerative process during aging. In contrast, none of the abnormalities were found in the eyes of the age-matched WT mice.

Accumulation of A2E in *Ccl2*^{-/-}/*Cx3cr1*^{-/-} RPE

Accumulation of lipofuscin and the fluorophore A2E is an early pathologic feature observed in human AMD.⁴⁸ The stable A2E, a pyridinium bis-retinoid derived from all-*trans* retinal and phosphatidyl-ethanolamine, is toxic to RPE.⁴⁹ A2E levels were measured within the RPE with the use of HPLC/ESI-MS, and a significant increase of more than threefold was found in 15-week-old and older *Ccl2*^{-/-}/*Cx3cr1*^{-/-} mice (3.4 pmol of A2E per eye) compared with the age-matched WT (approximately 1 pmol; Fig. 4).

Complement Cofactor and Microglia in *Ccl2*^{-/-}/*Cx3cr1*^{-/-} Retina

CD46 (membrane cofactor protein [MCP]) immunoreactivity was detected on the entire RPE of the *Ccl2*^{-/-}/*Cx3cr1*^{-/-} but not the apical RPE of the WT mice (Figs. 5A, 5C), indicating possible enhanced complement (C3b and C4b) activation. No difference in CD46 expression pattern was noted in the choroids of the *Ccl2*^{-/-}/*Cx3cr1*^{-/-} and WT mice, an expected finding because CD46 is widely distributed on vascular endothelial cells and fibroblasts in the choroid. The infiltration of microglia (CD11b⁺ cells) was detected in the retinal lesions of the *Ccl2*^{-/-}/*Cx3cr1*^{-/-} mice (Figs. 5B, 5D) but not of the WT mice. Immunostaining results for CD46 and CD11b were consistent within the strain.

Lower Expression of ERp29 in *Ccl2*^{-/-}/*Cx3cr1*^{-/-} Retina

Proteomic analysis of retinal lysate using two-dimensional gels uncovered differential expression of four proteins between the *Ccl2*^{-/-}/*Cx3cr1*^{-/-} and WT control mice (Fig. 6). Three independent experiments resulted in a consistent pattern of protein distribution. Three of the four proteins successfully identified by LC/MS/MS were ERp29 precursor, calcium-binding 140k protein, and RIKEN cDNA 2210010C04. ERp29, an endoplasmic reticulum protein, functions in protein folding and is associated with various degenerative diseases.^{50,51} We therefore investigated the expression of ERp29 in the retinas of WT and *Ccl2*^{-/-}/*Cx3cr1*^{-/-} mice. Immunostaining, Western blotting, and RT-PCR data demonstrated significantly reduced expression of ERp29 in the retinas of *Ccl2*^{-/-}/*Cx3cr1*^{-/-} mice compared with those in WT controls (Fig. 7).

Lower Expression of ERp29 in Human Archived AMD Slides

Decreased expression of ERp29 protein was found by immunohistochemical staining in the neuroretina and RPE cells of human maculae with AMD compared with normal human maculae. A representative image is shown in Figure 8.

Discussion

This study presents a mouse strain, created by knocking out a chemokine (*Ccl2*) and a chemokine receptor (*Cx3cr1*), that exhibits pathologic features of human AMD. *Ccl2*^{-/-}/*Cx3cr1*^{-/-} mice are shown to spontaneously develop retinal degenerative lesions, including choroidal neovascularization. In addition, elevations of A2E in the RPE and enhanced expression of complement regulatory protein (CD46) and microglia are also observed in the

Ccl2^{-/-}/*Cx3cr1*^{-/-} mice. These findings are compatible with human AMD eyes in which A2E accumulates in RPE cells⁴⁹ and CD46 localizes on RPE cells adjacent to and overlying drusen.⁵² These ocular manifestations observed in the *Ccl2*^{-/-}/*Cx3cr1*^{-/-} mice implicate important roles of the immune system and specific chemokines and ligands in the pathogenesis of resultant lesions. Because of the high penetrance and early presentation of many AMD-like features these mice display, this phenotype makes the model an alternative for studying the genetics and pathologic mechanisms of AMD and may aptly aid in the evaluation of various therapies for this blinding disease.

Researchers have linked the immune system and inflammatory processes to the pathophysiology of human AMD.^{53,54} Moreover, under normal conditions, a dynamic balance is struck between the generation of macular deposition stimulated by various internal and external factors and the elimination of these deposits by inflammatory cells attracted to the site by chemokines. It is hypothesized that the absence of adequate macrophage recruitment is involved in AMD development.^{8,22} We have found lower expression of *CX3CR1* transcripts in the maculae than in the perimacular retina within AMD eyes. In contrast, similar levels of *CX3CR1* transcript expression were detected in the maculae and perimaculae of subjects with normal eyes.²² We have also reported exacerbated retinal degeneration and choroidal neovascularization after the injection of subretinal basement membrane preparation (Matrigel; BD Biosciences, San Jose, CA) in *Ccl2*-deficient mice.⁵⁵ Many chemokines, including CCL2, exhibit protective effects against neuronal apoptosis.^{36,56,57} In a toxic model of Parkinson disease and a model of genetic motor neuron disease, *Cx3cr1*^{-/-} mice showed more extensive neuronal cell loss than did *Cx3cr1*^{+/+} littermate controls.⁵⁸

Activated microglia are associated with AMD.^{40,59} We observed microglia in the retinal lesions of *Ccl2/Cx3cr1*-deficient mice but not of WT mice, suggesting the activation of retinal microglia that may cause adjacent photoreceptor death.^{60,61} CD46 is a membrane-bound complement regulator that facilitates inactivation of the activated complement component C3b and C4b.⁶² Similar to the finding of CD46 in the RPE of the senescent *Ccl2*, *Ccr2*, or *Sod1* (*Cu*, *Zn-superoxide dismutase*) knockout mice,^{8,9} our *Ccl2/Cx3cr1* deficient mice also demonstrated the existence of this complement regulatory protein in the eye. The data are parallel to those for human eyes with AMD.⁵²

Significantly lower ERp29 protein and transcript expression was detected in the ocular tissue of *Ccl2*^{-/-}/*Cx3cr1*^{-/-} mice than of WT controls. Immunohistochemistry also illustrated decreased ERp29 in human AMD maculae compared with controls. Expression of ERp29 is controlled primarily through the XBP-1/IRE-1 pathway.⁶³ IRE-1 is differentially expressed in the inflammatory state,⁶⁴ which might account for the lower ERp29 expression in this chemokine-deficient mouse. Neurodegenerative diseases are known to involve cell death initiated by endoplasmic reticulum (ER) stress and are thus regarded as ER stress-associated diseases or conformational diseases.⁶⁵⁻⁶⁷

The ER is a central organelle in lipid synthesis, protein folding, and protein maturation. All newly synthesized membranes and secretory proteins are folded and processed in the ER. Cells need correctly folded and processed membrane proteins for function, and when proteins are unfolded or misfolded, they tend to form toxic aggregates (e.g., lipofuscin in the RPE) that are harmful to the cells. Conditions of ER malfunction are called ER stress. ER stress is induced by the accumulation of unfolded protein aggregates, called the unfolded protein response. In ER stress, transcription factors are activated to induce the expression of ER-resident chaperones to deal with accumulated protein aggregates. ERp29 is one such ER-resident chaperone that prevents protein aggregation by keeping the unfolded proteins in a folding-competent state and that functions as a component of the ER-specific protein-degrading apparatus to eliminate denatured proteins.^{50,63,68,69} Decreased chaperoning may cause

misfolded protein accumulation. Moreover, ERp29 acts as an escort chaperone that brings proteins to different locations in the cell.⁶³ AMD is a deposit accumulation disease. In our model, substantial accumulation of lipofuscin could result from the inability of ERp29 to escort. The involvement of ER distress and ERp29 protein in AMD pathogenesis has been reported.⁷⁰⁻⁷³ Furthermore, decreases of retinal ERp29 level have been reported with age.⁵¹ In a recent proteomic analysis of human AMD eyes, Ethen et al.⁷³ report a 33% decrease of ERp29 in the macula with the onset stage of AMD. We are investigating a possible mechanistic role of ERp29 in the phenotypic development in this mouse strain.

In another recent study, Azfer et al.⁷⁴ reports activation of a cluster of ER stress-related genes, including *ERp29*, during the development of myocardial deterioration and dysfunction in the heart of *Ccl2* transgenic mice, which protected the cardiomyocytes from the adverse effect of stress in the early stage. However, with chronic inflammation, these efforts failed, and the cells died to the death-inducing processes. In our model, *CCL2* and *CX3CR1* levels were low, which might have resulted in inadequate ER stress protein production and ER dysfunction. Under conditions of ER impairment, unfolded proteins accumulated in the ER lumen, a signal responsible for activation of the unfolded protein response.

In summary, *Ccl2*^{-/-}/*Cx3cr1*^{-/-} mice developed early-onset and progressive retinal degenerative disease with broad-spectrum pathologic features mimicking human AMD. The phenotype is highly penetrant, reliable, and reproducible. Data from proteomics, immunohistochemistry, Western blot, and RT-PCR indicate that the ERp29 protein is involved in this model, a finding that provides new insight into AMD pathogenesis. The observations made in this study implicate certain chemokines and ER proteins as having important roles in the development of AMD.

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Figure 1. Fundus photographs. **(A)** A normal retina is illustrated in a 21-week-old WT mouse. **(B)** Multiple subretinal lesions mimic drusen formation (*arrow*) in a 9-week-old *Ccl2*^{-/-}/*Cx3cr1*^{-/-} mouse. **(C)** Enlarged and confluent subretinal drusenlike lesions (*arrow*) in the same eye of the *Ccl2*^{-/-}/*Cx3cr1*^{-/-} mouse at 18 weeks. **(D)** Scarring and atrophic retinal lesions (*arrow*) indicate disease progression in the same eye of this mouse at 33 weeks.

Figure 2.

Ocular photomicrographs. (A) Retina, RPE, Bruch membrane, and choroidal capillaries are normal in a 6-month-old WT mouse. (B) Drusen deposits (*arrows*) are shown as small dome-shaped hyaline material within the Bruch membrane of two *Ccl2*^{-/-}/*Cx3cr1*^{-/-} mice. (C) Hypopigmentation and lacy degeneration (*arrow*) of the RPE are observed in a *Ccl2*^{-/-}/*Cx3cr1*^{-/-} mouse. (D) Choroidal neovascularization is identified by small patent vessels (*arrows*) from the choroid piercing Bruch membrane and RPE into the retina of two *Ccl2*^{-/-}/*Cx3cr1*^{-/-} mice. GCL, ganglion cell layer; INL, inner nuclear layer; PR, photoreceptor outer and inner segments; CH, choroids. Hematoxylin and eosin staining. Scale bar, 100 μ m.

Figure 3.

Retinal transmission electron micrographs. (A) Melanosomes are markedly decreased and lipofuscin is present in the RPE cells (arrow) of a *Ccl2*^{-/-}/*Cx3cr1*^{-/-} mouse. (B) Lipofuscin (arrow) and lipid droplets (open arrow) deposits are shown as the homogeneous electron dense-cytoplasmic inclusion in the RPE of another *Ccl2*^{-/-}/*Cx3cr1*^{-/-}. (C) Thickened Bruch membrane and amorphous deposits (arrow) are depicted in a *Ccl2*^{-/-}/*Cx3cr1*^{-/-} mouse. (D) Disorganized and atrophic photoreceptors (asterisk) and lipofuscin (arrow) in the RPE are observed. (E) Normal RPE, Bruch membrane, and choroid are illustrated in a WT mouse. Scale bars, 500 nm (A, B, D); 2 μm (C, E).

Figure 4.

Quantification of A2E in *Ccl2*^{-/-}/*Cx3cr1*^{-/-} mice compared with normal controls.

Chromatograms represent absorbance at 440 nm. A2E peaks elute at 25.5 minutes. *Ccl2*^{-/-}/*Cx3cr1*^{-/-} RPE at 4 months: A2E approximately 3.4 pmol (*black*). Normal RPE at 4 months: A2E approximately 1.1 pmol (*gray*). Each represents a 200-μL injection of six RPE extracts.

Figure 5. Ocular photomicrographs of complement cofactor and microglia. (A, C) CD46 (arrows, black) staining in entire RPE and small drusen of a *Ccl2*^{-/-}/*Cx3cr1*^{-/-} mouse compared with none in the retina of a WT mouse (open arrow, RPE with brown pigment). (B, D) Microglia (CD11b⁺ cells, arrows) are found in the retinal lesions of a *Ccl2*^{-/-}/*Cx3cr1*^{-/-} mouse; none are found in a WT mouse (open arrow, RPE). Insets: higher magnifications of the RPE cells in two strains. (A, B: WT mice; C, D: *Ccl2*^{-/-}/*Cx3cr1*^{-/-} mice). INL, inner nuclear layer; ONL, outer nuclear layer. Scale bar, 100 μm.

Figure 6.

Proteomics of retinal lysate from WT and *Ccl2*^{-/-}/*Cx3cr1*^{-/-} mice by two-dimensional PAGE analysis. (A) Representative two-dimensional PAGE image from one WT retina. (B) Representative two-dimensional PAGE image from one *Ccl2*^{-/-}/*Cx3cr1*^{-/-} retina. Four silver-stained protein spots are identified as differentially expressed between the two groups, as indicated by circles. The three proteins from those four spots were successfully identified by LC/MS/MS. Repetition of the analysis reveals consistent protein patterns for each group. The three identified proteins were (1) calcium binding 140k protein, (2) ERp29 precursor, and (3) RIKEN cDNA 2210010C04.

Figure 7.

ERp29 expression in the retina. **(A)** Photomicrographs of mice retinal sections show less ERp29 reactivity (*black, arrows*) and presence of retinal lesions (*asterisks*) in a *Ccl2*^{-/-}/*Cx3cr1*^{-/-} mouse than in a WT mouse (avidin-biotin-immunoperoxidase staining). Scale bar, 100 μm. **(B)** Western blotting of retina lysate shows less density of ERp29 in a *Ccl2*^{-/-}/*Cx3cr1*^{-/-} mouse than in a WT mouse. **(C)** Lower ocular ERp29 mRNA detected by quantitative RT-PCR in the ocular tissue of *Ccl2*^{-/-}/*Cx3cr1*^{-/-} mice than in WT mice.

Figure 8.

Photomicrographs of ERp29 in human eyes. **(A)** Positive ERp29 reactivity (*arrows, black*) is detected in the inner segments of photoreceptor layer, the junction of the outer plexiform and inner nuclear layers, the ganglion cell layer, and the RPE cells of a normal retina. **(B)** Immunoreactivity (*arrows*) was lower in an AMD eye, particularly in the diseased retinal outer layers and RPE cells, than in the normal eye. NFL, nerve fiber layer; GCL, ganglion cell layer; IPL, inner plexiform layer; INL, inner nuclear layer; OPL, outer plexiform layer; ONL, outer nuclear layer; Pho, inner and outer segments of photoreceptor cells; C, choroids; S, sclera. Avidin-biotin-immunoperoxidase staining. Scale bar, 100 μ m.