Published in final edited form as:

Exp Eye Res. 2008 April ; 86(4): 675–683. doi:10.1016/j.exer.2008.01.014.

Immunological protein expression profile in *Ccl2/Cx3cr1* deficient mice with lesions similar to age-related macular degeneration

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Abstract

Age-related macular degeneration (AMD) is the leading cause of blindness in the United States. *Ccl2* knock-out (KO) mice sporadically develop the cardinal features of AMD in their senescent stage. Humans bearing a loss of function variant or single nucleotide polymorphism (SNP) in *CX3CR1* are at increased risk of developing AMD. We recently developed *Ccl2*^{-/-}/*Cx3cr1*^{-/-} mice, which consistently develop retinal degeneration with many AMD features. Since there is strong evidence for an immunological role in AMD pathogenesis, we examined ocular immune protein expression levels in *Ccl2*^{-/-}/*Cx3cr1*^{-/-}, *Ccl2*^{-/-}, *Cx3cr1*^{-/-}, and age-matched wild-type (WT) mice. Immunohistochemistry revealed increased complement C3d in Bruch's membrane, retinal pigment epithelium (RPE), choroidal capillaries, and particularly drusen of the *Ccl2*^{-/-}/*Cx3cr1*^{-/-} mice relative to the WT controls. No change was detected in single KO mice. Real-time RT-PCR revealed a 2.5-fold increase in *C3* expression in the *Ccl2*^{-/-}/*Cx3cr1*^{-/-}. While the retinas of four month old WT and *Ccl2*^{-/-} showed minimal immunoreactivity for markers of macrophages and microglia, infiltrates of these mononuclear phagocytic cells were detected in the *Ccl2*^{-/-}/*Cx3cr1*^{-/-} retinal lesions and a few foci in the *Cx3cr1*^{-/-} retina. The *Ccl2*^{-/-}/*Cx3cr1*^{-/-} had reduced toll-like receptor 4 (TLR4) expression in the RPE. Following LPS injection, the *Ccl2*^{-/-}/*Cx3cr1*^{-/-} had significantly reduced endotoxin-induced uveitis scores and showed a diminished increase in *Tlr4* mRNA expression. No changes in TLR4 expression were detected in either single KO. Autoantibodies against the retina and photoreceptors were also detected in the *Ccl2*^{-/-}/*Cx3cr1*^{-/-} serum. Real-time RT-PCR revealed significant increases in *Ccl5* transcript in the *Ccl2*^{-/-}/*Cx3cr1*^{-/-} relative to the WT. These results suggest that innate immunity and possibly adaptive immunity play an important role in *Ccl2*^{-/-}/*Cx3cr1*^{-/-} retinal degeneration. Moreover, since human AMD patients show similar immunopathological profiles, these results support the *Ccl2*^{-/-}/*Cx3cr1*^{-/-} as a suitable model for human AMD.

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Keywords

Age-related macular degeneration; animal model; chemokine; cytokine; complement factor; Toll-like receptor 4; microglia; macrophage

INTRODUCTION

Age-related macular degeneration (AMD) is the leading cause of irreversible severe vision loss in the United States and the third leading cause of blindness internationally (Resnikoff et al., 2004; The Eye Diseases Prevalence Research Group, 2004). The prevalence of this disease is expected to increase as the general population ages (Friedman et al., 2004). While there has been some success in treating neovascular AMD, treatment options for this devastating disease remain limited (Augustin and Offermann, 2006).

Considerable evidence now suggests that immunity plays an important role in AMD pathogenesis. Genetic analysis implicates single nucleotide polymorphisms in the genes encoding Complement Factor H (*CFH*), Complement Factor B (*BF*), Complement Component C2 (*C2*), and Toll-Like Receptor 4 (*TLR4*) (Edwards et al., 2005; Gold et al., 2006; Haines et al., 2005; Klein et al., 2005; Li et al., 2006; Maller et al., 2006; Ross et al., 2007; Zarepari et al., 2005). Investigations into the composition and origin of drusen have reported that drusen contain molecules known to mediate immune processes such as clusterin, vitronectin, complement receptor 1, CFH, and major histocompatibility complex II antigen (Hageman et al., 2001; Johnson, et al. 2007). Moreover, significantly elevated anti-retinal antibodies have been found in human AMD patient serum and in the drusen of a cynomolgus monkey model of AMD (Gu et al., 2003; Patel et al., 2005; Umeda et al., 2005).

Progress in understanding the pathogenesis of AMD has been hampered by the lack of a suitable animal model. Mice deficient in *Ccl2* or its receptor develop retinal degeneration with many pathological similarities to AMD. However, this animal model develops the AMD-like phenotype only in its senescent stage (Ambati et al., 2003b). Aged mice knock-outs for *Cx3cr1* also sporadically develop drusen-like lesions, progressive accumulation of subretinal microglia, and photoreceptor degeneration (Combadiere et al., 2007). We recently reported a *Ccl2*^{-/-}/*Cx3cr1*^{-/-} mouse, which consistently develops retinal degeneration with many similarities to human AMD at 6 weeks of age (Tuo et al., 2007). Here we compare the expression of certain inflammation-related molecules in hopes of better understanding molecular basis of AMD-like phenotype in the *Ccl2*^{-/-}/*Cx3cr1*^{-/-} and single knock-out mice.

MATERIALS AND METHODS

Animals

Ccl2 deficient and *Cx3cr1* deficient mice were obtained from Drs. Bao Lu and Barrett J. Rollins of Children's Hospital, Harvard Medical School (Lu et al., 1998) and Dr. Philip Murphy of NIAID/NIH (Combadiere et al., 2003), respectively. Generation of the *Ccl2*^{-/-}/*Cx3cr1*^{-/-} mice has been previously described (Tuo et al., 2007). Briefly, the *Ccl2*^{-/-} and *Cx3cr1*^{-/-} mice were used as the founder generation (F₀) and were crossed to generate mice (F₁) heterozygous for the *Ccl2* and *Cx3cr1* genes. The heterozygous mice were intercrossed to obtain homozygous *Ccl2*^{-/-}/*Cx3cr1*^{-/-} mice. Wild-type (WT) mice were C57BL/6 background. Mice were provided food and water ad libitum and kept on a 12 hr light-dark cycle. All animal experiments were performed under the protocols approved by the NEI Institutional Animal Care and Use Committee. Mice were treated according to the ARVO Statement for the Use of Animals in Ophthalmic and Vision Research.

Immunohistochemistry

Following enucleation, eyes from four month old age-matched WT and *Ccl2*^{-/-} *Cx3cr1*^{-/-} mice were snap-frozen and embedded in O.C.T. compound (Sakura finetek USA Inc, Torrance, USA) or agarose. For immunohistochemistry-confocal microscope detection of complement C3d (C3d), complement receptor C5aR (C5aR), macrophages (F4/80), microglia (CD11b), toll-like receptor 4 (TLR4). Four-μm-thin for O.C. T. embedded eyes and 100-μm-thick for agarose embedded eyes, frozen sections were fixed briefly in 4% formaldehyde and in 0.1 M glycine, and blocked briefly in 5% normal rabbit serum. The sections were immunolabeled with a goat anti-mouse C3d antibody (R & D Systems, Inc. Minneapolis, MN.), rat-anti-mouse F4/80 antibody (Abcam Inc, Cambridge, MA), rat-anti-mouse CD11b antibody (AbD Serotec, Oxford, UK), rabbit-anti-mouse TLR4 (Abcam, Cambridge, UK), or rat-anti-mouse C5aR antibody (a generous gift from P. Gasque, University of Wales College of Medicine, United Kingdom). The primary antibody was followed by incubation with secondary antibodies (Alexa 555 rabbit anti-goat IgG, Alexa 568 goat-anti-rat IgG, Alexa Fluor 568 goat anti-rabbit, or Alex 488 goat anti-mouse IgG; Molecular Probes, Eugene, OR). The DNA-binding dye DAPI was used to fluorescently label nuclei (Blue color). Sections were mounted in an anti-bleaching mounting medium (Gel-Mount). Slides were examined using a confocal microscope (Leica TCS SP-2, Leica Microsystems, Exton, PA). The intensity and size of the positive areas in the entire section of the eye were compared among different mice strains. At least three sections from one eye and three different eyes in each mouse strain were viewed. Representative areas were photographed.

All four strains of mice were also examined using the avidin-biotin-complex immunoperoxidase (ABC) system. Frozen sections 4 μm thick were fixed in acetone for 7–10 minutes and rinsed with Tris-buffer saline, 0.05 M, pH 7.4. The slides were immersed in 5% normal serum specifically to block potential background from the secondary antibody. The primary antibody (rat-anti-mouse F4/80, rat-anti-mouse CD11b, goat anti-mouse C3d, rabbit-anti-mouse TLR4; manufacturers listed above) was then applied. Biotin labeled goat-anti-rat IgG (VECTOR laboratory, Burlingame, CA) was used as the secondary antibody for the detection of F4/80 and CD11b. Biotin labeled horse-anti-goat IgG (VECTOR laboratory, Burlingame, CA) was used as the secondary antibody for the detection of C3d. For the detection of TLR4, biotin labeled goat-anti-rabbit IgG (VECTOR laboratory, Burlingame, CA) was used as the secondary antibody. The sections were treated using the avidin biotin complex immunoperoxidase system with 3,3'-diaminobenzidine as the substrate and counterstained with methyl green as previously described (Tuo et al., 2006, 2007).

Detection of retinal auto-antibodies

Indirect immunohistochemical technique was applied as previously described (Chan, et al. 1985). The 4 μm thick, frozen sections of fresh normal mouse retina tissue were fixed and blocked in 5% normal goat serum. Diluted mouse serum (1:100) was placed on the section at room temperature for one hour. After washing the slide was then layered with Alex 488 goat anti-mouse IgG for another hour. The DNA-binding dye DAPI was used to fluorescently label nuclei (Blue color). The slide was then visualized using a confocal microscope (TCS SP-2; Leica Microsystems, Exton, PA).

Detection of *Cx3cl1*, *Ccl5*, and *C3* transcripts by real-time RT-PCR

Ten micrograms of RNA taken from the eyes of age-matched, four-month old mice were used for cDNA synthesis (Superscript II RNase H- Reverse Transcriptase, Invitrogen, Grand Island, NY). Real-time RT-PCR was performed using an Applied Biosystems 7500 Real-Time PCR System and validated and inventoried Taqman gene, expression kits (Applied Biosystems, Foster City, CA). The gene expression kits *Cx3cl1* (Mm00436454_m1), *Ccl5* (Mm01302428) *C3* (Mm00437858_m1), and *Gapdh* (Mm99999915_g1) were used according to the

manufacture's instruction. The comparative C_t method was used to establish relative quantification of the fold changes in gene expression according to User Bulletin #2: ABI Prism 7700 Sequence Detection System, PE Applied Biosystems, 1997. Fold changes were normalized first by the level of *Gapdh*. The average fold change due to the gene manipulation was again normalized to the transcript level of WT mouse and presented graphically. Each sample was analyzed at least three times.

For detection of *Tlr4* mRNA, real-time RT-PCR was performed using a Stratagene Mx3000™ Real-Time PCR System and Brilliant SYBR Green QPCR Master Mix (Stratagene, CA). The primers for *Tlr4* were synthesized by SuperArray and supplied as the RT² Real-Time™ Gene Expression Assay Kit. Reactions were performed in a final volume of 50 μ l with 2 μ l of single-strand cDNA. The real-time PCR cycling conditions were: 95°C for 10 min followed by 45 cycles of 30 s at 95°C, 60 s at 55°C and 60 s at 72°C and finally fluorescence measurement. For the internal control, beta-actin was amplified using primers 5'-CCCAGCACAAATGAAGATCAA-3' and 5'-ACATCTGCTGGAAGGTGGAC-3'. For the internal control, all PCR conditions were the same as for *Tlr4* except that the annealing temperature was 58°C. Following PCR, a thermal melt profile was performed for amplicon identification. To determine the C_t , the threshold level of fluorescence was set manually in the early phase of PCR amplification. Each sample was analyzed at least twice. ABI SDS 1.3.1 software and the $2^{-\Delta\Delta C_t}$ analysis method were used to determine relative amounts of product using beta-actin as an endogenous control. The average fold change was presented graphically.

Endotoxin-induced uveitis (EIU)

EIU was induced by a single intraperitoneal injection of 0.1 mg *Salmonella typhimurium* lipopolysaccharide (LPS) endotoxin (Difco Laboratories, Detroit, MI) in 0.1 ml PBS (Li et al., 1995; Shen et al., 2000; Tuaillon et al., 2002). Mice were sacrificed at 24 hours after injection. Eyes were enucleated for histopathology and RNA isolation for RT-PCR of β -Actin, *Il6*, *Tnfa*, *Ifn β* , and *Tlr4*. Real-Time PCR and $\Delta\Delta C_t$ methods were used to quantitatively measure the increase of gene expression following LPS injection as described previously (Tuaillon et al., 2002). Three independent experiments using 20 DKO and 18 control mice were performed.

RESULTS

Increased complement expression in the *Ccl2*^{-/-}/*Cx3cr1*^{-/-}

Confocal microscopy showed increased immunoreactivity against complement C3d in Bruch's membrane, drusen-like lesions, RPE cells, photoreceptors, and choroidal capillaries of the *Ccl2*^{-/-}/*Cx3cr1*^{-/-} relative to the WT controls. The most pronounced immunoreactivity against C3d was found overlying drusen-like lesions (Figure 1, A and B). Similar findings have been reported for human AMD (Baudouin et al., 1992; Hageman et al., 2005). Immunoreactivity against C5aR appeared increased in the photoreceptor inner segments of the *Ccl2*^{-/-}/*Cx3cr1*^{-/-} relative to the WT control (Figure 1, C and D). A three-fold increase in C3 mRNA transcript was found in the *Ccl2*^{-/-}/*Cx3cr1*^{-/-} in comparison to the WT (Figure 2, A).

Mononuclear phagocytic cell infiltrates in the *Ccl2*^{-/-}/*Cx3cr1*^{-/-}

Confocal microscopy revealed increased immunoreactivity for CD11b, a marker for microglia, in the subretina and retinal lesions of the *Ccl2*^{-/-}/*Cx3cr1*^{-/-} mice as well (Figure 3). Immunoreactivity against F4/80, a marker for macrophages, was also increased in the outer nuclear layer and in the lesions of the four-month old *Ccl2*^{-/-}/*Cx3cr1*^{-/-} mice relative to the WT controls. Since CX3CR1 and CCL2 ordinarily play crucial roles in the recruitment of macrophages and microglia, we measured the expression of other cytokines and chemokines (*Cx3cl1*, and *Ccl5*), assuming they would be upregulated to recruit mononuclear phagocytic

cells to the pathologic tissue. The mRNA transcript for *Ccl5* showed a five-fold increase in the *Ccl2^{-/-}/Cx3cr1^{-/-}* while the *Cx3cl1* transcript was unchanged (Figure 2, B and C).

Detection of significantly lower TLR4 expression and inhibition of EIU in *Ccl2^{-/-}/Cx3cr1^{-/-}*

Immunostaining demonstrated significantly reduced TLR4 protein levels in the *Ccl2^{-/-}/Cx3cr1^{-/-}* as compared to the control (Figure 4, A and B). Although similar levels of mRNA were found in the eyes of both *Ccl2^{-/-}/Cx3cr1^{-/-}* and wild-type mice, *Tlr4* transcript was significantly increased in wild-type mice (12.69) but not in *Ccl2^{-/-}/Cx3cr1^{-/-}* (1.00) after LPS treatment that induces EIU. EIU, an acute ocular inflammation, was less severe in *Ccl2^{-/-}/Cx3cr1^{-/-}* mice than the wild type controls (Figure 5, A–D). The number of ocular inflammatory cells was 6.7 ± 1.3 (mean $\pm$ SE) in DKO and 11.3 ± 3.5 in WT. Elevation of ocular inflammatory cytokine transcripts such as IL-6, TNF α and IFN γ , from EIU were also much less in DKO than WT (Figure 5 E).

Ccl2^{-/-}/Cx3cr1^{-/-} serum contain anti-retinal autoantibody

Confocal microscopy showed strong staining of the outer layers in the retina as well as weak staining of the inner retina of the *Ccl2^{-/-}/Cx3cr1^{-/-}* mice (Figure 6B). In contrast, minimal immunoreactivity was detected for the WT controls (Figure 6 A). A similar result was reported in human AMD patients (Patel et al., 2005).

Immunostaining in the single knock-out mice

Four month old *Ccl2^{-/-}* mice did not appear to have any change in F4/80, CD11b, C3d, or TLR4 immunoreactivity relative to age-matched wild-type mice. *Cx3cr1^{-/-}* mice at four months of age showed no change in immunoreactivity for C3d or TLR4 but appeared to have increased mild to moderate immunoreactivity for F4/80 and CD11b in focal areas of the retina (Table 1).

DISCUSSION

We have profiled the relative expression of protein molecules, which influence and mediate the immune process in the *Ccl2^{-/-}/Cx3cr1^{-/-}*, *Ccl2^{-/-}*, *Cx3cr1^{-/-}* and WT mice. We detected F4/80 positive macrophages and CD11b positive microglia in the retina and subretina highlighting the lesions of *Ccl2^{-/-}/Cx3cr1^{-/-}* mice and in few foci in the retina of the *Cx3cr1^{-/-}*. CX3CR1 mediates monocyte migration to noninflamed tissues, where they differentiate into resident macrophages and dendritic cells (Geissmann et al., 2003). CCL2 is essential to monocyte recruitment in several murine inflammatory models (Lu et al., 1998). Thus, knocking out *Ccl2* and *Cx3cr1* influences two distinct monocyte recruitment pathways. The mononuclear phagocytic cells (MPCs) detected in the double knock-out must be recruited by an alternative chemoattractant, such as CCL5, for which we have demonstrated increased expression in the *Ccl2^{-/-}/Cx3cr1^{-/-}*.

Since the MPCs localize to the lesions in the *Ccl2^{-/-}/Cx3cr1^{-/-}* and foci in the retina of the *Cx3cr1^{-/-}*, they appear to be recruited following the development of retinal degeneration. Interestingly, the absence of CX3CR1 increases the neurotoxicity of microglia in the central nervous system, which suggests that the recruited microglia could contribute to the pathology (Cardona et al., 2006; Combadiere et al., 2007). Microglia and macrophages have been documented in various AMD lesions (Gupta et al., 2003; Grossniklaus et al., 2005; Penfold et al., 1985).

Our results also suggest that activation of complement may mediate the retinal degeneration of the *Ccl2^{-/-}/Cx3cr1^{-/-}* mice. In mammals, C3 is cleaved to C3a and C3b by the C3-convertase; C3b is further fragmented to iC3b, C3dg and then C3d. The presence of detectable

C3d indicates that complement C3 is undergoing rapid and complete fragmentation (Negi et al., 2000; Soames and Sim, 1997). Differential expression of C3d was likely not observed in the *Ccl2*^{-/-} or *Cx3cr1*^{-/-} because these four month old mice were not at the age at which they typically develop AMD-like pathology (> 9 months of age) (Ambati et al., 2003b; Raoul et al., 2007). Several studies have shown the presence of activated complement fragments in drusen of human patients, a finding duplicated in the *Ccl2*^{-/-}/*Cx3cr1*^{-/-} animal in this study (Baudouin et al., 1992; Hageman et al., 2005). This similarity provides further evidence that the *Ccl2*^{-/-}/*Cx3cr1*^{-/-} may serve as an adequate model of AMD. We also show increased immunoreactivity for C5aR but in the photoreceptor inner segments rather than Bruch's membrane. Since C5aR is not universally expressed throughout the retina and Bruch's membrane has not been shown to express C5aR in the healthy eye, it is possible that Bruch's membrane does not express C5aR even when exposed to concentrated complement activation fragments (Vogt et al., 2006). Photoreceptor inner segment immunoreactivity for C5aR without detectable C3d immunoreactivity may be the result of an especially robust response to complement activation. We interpret the increased immunoreactivity for C5aR similarly to the previously reported findings (Ambati et al., 2003a). C5a has been shown to increase the expression of C5aR. Activation of C5aR leads to production of CCL2 in the healthy mouse (Riedemann et al., 2002). CCL2 in turn recruits macrophages, which phagocytose C5 and targets opsonized by it (Ambati et al., 2003a). In our *Ccl2*^{-/-}/*Cx3cr1*^{-/-} deficient mouse, macrophages cannot be recruited leading to the accumulation of C5a and other complement products. Notably, C3d also acts as a powerful adjuvant and has been shown to decrease the antigen concentration needed to elicit an antibody response by 10,000 fold (Nielsen and Leslie, 2002; Wang et al., 2004). In the present study, we demonstrate intense immunoreactivity for anti-retinal autoantibodies in the *Ccl2*^{-/-}/*Cx3cr1*^{-/-}. While these results agree with papers implicating anti-retinal autoantibodies in human AMD (Gu et al., 2003; Patel et al., 2005), at this time we cannot determine whether the presence of autoantibodies is a cause or consequence of retinal degeneration in the *Ccl2*^{-/-}/*Cx3cr1*^{-/-}.

Drusen are considered to be comparable to atherosclerotic plaque (Sivaprasad et al., 2005), and TLR4 is found to be associated with atherosclerotic plaques (Edfeldt et al., 2002; Xu et al., 2001). Because of this relationship and the association between a *TLR4* polymorphism and AMD, we investigated the expression of *Tlr4* in the eyes of both *Ccl2*^{-/-}/*Cx3cr1*^{-/-} and wild-type mice. Here, we also demonstrate that decreased TLR4 expression on the RPE and decreased TLR4 function may contribute to the retinal degeneration in the *Ccl2*^{-/-}/*Cx3cr1*^{-/-} mice. TLR4 has been reported to be essential in RPE scavenging of the photoreceptor outer segments possibly by clustering and interacting with the receptor CD36 (Kindzelskii et al., 2004). With decreased TLR4 expression and function, the *Ccl2*^{-/-}/*Cx3cr1*^{-/-} has impaired ability to scavenge the photoreceptor outer segments, potentially leading to the accumulation of waste products at the interface between the RPE and photoreceptors. This observation correlates well with the *TLR4* variants associated with increased risk of AMD since the AMD associated allele results in reduced TLR4 mediated LPS signaling (Arbour et al., 2000).

Tlr4, a gene product that regulate LPS response and EIU development, showed significantly reduced mRNA transcript following LPS injection. DKO mice had a significant hyporeactive ocular response to LPS. The lack of CCL2 and CX3CR1 causes inefficient leukocyte recruitment in response to inflammation and may result in suppressed *Il6* and *Tnfa*. The mechanism behind EIU in DKO mice is closely related to TLR4 signaling and monocyte attractants. Increased expression of *Il6*, *Tnfa*, and *Inf γ* is characteristic of a normal TLR4 mediated LPS response (Fan et al., 2006). By demonstrating that the *Ccl2*^{-/-}/*Cx3cr1*^{-/-} mice have a hyporeactive response to LPS as measured by proinflammatory cytokine expression and EIU score, we provide functional evidence that TLR4 signalling is decreased in the *Ccl2*^{-/-}/*Cx3cr1*^{-/-} mice.

In summary, we have shown that several inflammatory proteins, which mediate or are involved in innate immune responses, are differentially expressed in the *Ccl2*^{-/-}/*Cx3cr1*^{-/-} relative to the WT controls, and we have demonstrated the presence of anti-retinal autoantibodies in the *Ccl2*^{-/-}/*Cx3cr1*^{-/-} serum. These results support an immunological role in the retinal degeneration of the *Ccl2*^{-/-}/*Cx3cr1*^{-/-} mice. Moreover, the immune characteristics similar to human AMD such as complement deposition, MPC recruitment, and anti-retinal autoantibody detection further support the *Ccl2*^{-/-}/*Cx3cr1*^{-/-} mouse as a good model for human AMD.

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Figure 1.

Photomicrographs of complement C3d and C5aR in the retina detected by confocal microscopy. (A) Complement C3d in a wild-type mouse. (B) Complement C3d in a *Ccl2*^{-/-}/*Cx3cr1*^{-/-} mouse. Enhanced C3d intensity is illustrated in Bruch's membrane and drusen (arrows). (C) C5aR in a wild-type control mouse. (D) C5aR in a *Ccl2*^{-/-}/*Cx3cr1*^{-/-} mouse. Enhanced C5aR intensity (arrow) is illustrated in the photoreceptor inner segments. (ONL, outer nuclear layer; RPE, RPE cells; bar = 20 μ m, A & B; 40 μ m, C & D)

Figure 2.

Relative expression of *C3*, *Ccl5*, and *Cx3cl1* mRNA. (A) *C3* mRNA expression is increased 2.5-fold in the *Ccl2^{-/-}/Cx3cr1^{-/-}* mouse relative to the wild-type control. (B) *Ccl5* mRNA expression is increased 5-fold relative to the wild-type. (C) *Cx3cl1* mRNA expression is not significantly different in the *Ccl2^{-/-}/Cx3cr1^{-/-}* relative to the wild-type controls.

Figure 3.

Photomicrographs of microglia and macrophages in the eye. (A) Microglia (CD11b positive cells, arrow) in a wild-type mouse. (B) *Ccl2*^{-/-}/*Cx3cr1*^{-/-} retina demonstrating enhanced microglia staining (arrows). (C) Macrophages (F4/80 positive cells) in a wild-type mouse. (D) *Ccl2*^{-/-}/*Cx3cr1*^{-/-} retina showing enhanced macrophage staining (arrow). (ONL, outer nuclear layer; RPE, RPE cells; bar = 30 μm)

Figure 4.

TLR4 expression in wild-type and *Ccl2*^{-/-}/*Cx3cr1*^{-/-} mouse. (A) wild type mouse (red emission, arrow). (B) *Ccl2*^{-/-}/*Cx3cr1*^{-/-} mouse; decreased staining for TLR4 (arrow) in the RPE as compared to that of a wild-type mouse. (ONL, outer nuclear layer; RPE, RPE cells; bar = 20 μ m)

Figure 5.

Ccl2^{-/-}/*Cx3cr1*^{-/-} mice have a hyporeactive response to lipopolysaccharide. (A) Anterior chamber of a wild-type mouse with EIU showing infiltration of inflammatory cells (arrow). (B) Anterior chamber of a *Ccl2*^{-/-}/*Cx3cr1*^{-/-} mouse with EIU showing fewer inflammatory cells as compared to WT (arrow). (C) Wild-type mouse posterior vitreous with infiltration of inflammatory cells (arrow). (D) Posterior vitreous of an EIU *Ccl2*^{-/-}/*Cx3cr1*^{-/-} with no inflammatory cells. (E) Real-time PCR results showing attenuated increase in cytokine and *Tlr4* expression in response to LPS stimulation of the *Ccl2*^{-/-}/*Cx3cr1*^{-/-}. Lower EIU scores in response to LPS in *Ccl2*^{-/-}/*Cx3cr1*^{-/-} mice as compared to that of wild-type mice are found. (bar = 150 μm, A & C; 80 μm, B & D)

Figure 6. *Ccl2*^{-/-}/*Cx3cr1*^{-/-} serum contains increased anti-retinal autoantibodies. (A) Wild-type control mouse with minimal anti-retinal autoantibody staining. (B) *Ccl2*^{-/-}/*Cx3cr1*^{-/-} mouse with anti-retinal autoantibody staining (green color) in the retina and photoreceptors (arrows). (ONL, outer nuclear layer; RPE, RPE cells; bar = 20 μ m)

Table 1

Immunoreactivity results for four mouse strains at 4 months of age

Mouse Strain	F4/80 Macrophage	CD11b Microglia	C3d Complement	TLR4 Toll-like receptor 4
WT	–	–	–	–
<i>Cx3cr1</i> ^{–/–}	+	+	–	–
<i>Ccl2</i> ^{–/–}	–	–	–	–
<i>Ccl2</i> ^{–/–} / <i>Cx3cr1</i> ^{–/–}	+	+	+	–

Symbols: + positive immunoreactivity using the ABC system, – undetected immunoreactivity using the ABC system