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Reduced photoreceptor death and improved retinal function during retinal degeneration in mice lacking innate immunity adaptor protein MyD88

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Abstract

The injury inflammatory response mediated by the innate immune system is an important contributor to neurodegeneration in the central nervous system (CNS) and retina. A major branch of the innate immune system is regulated by the Toll-like receptors (TLRs), which are receptors for endogenous damage associated molecules released from injured cells as well as pathogen-derived molecules, and interleukin-1 receptors (IL-1R), which are activated by IL-1α, IL-1β and IL-18 cytokines. TLRs and IL-1R are expressed on immune and non-immune cell types and act as first responders to cell damage, which results in tissue repair, or inflammation and apoptosis. Both TLR and IL-1R require the adaptor protein myeloid differentiation primary response gene 88 (MyD88) for signaling. Although inflammation is implicated in neuronal death in the retina, the role of MyD88-dependent TLR and IL-1R signaling in retinal degeneration is unknown. Therefore, the purpose of this study was to investigate the role of MyD88-mediated signaling in neuronal degeneration in the *retinal degeneration 1* (*rd1*) mouse model, which exhibits a phenotype of rapid photoreceptor death and inflammation. To generate *rd1* mice lacking the *MyD88* gene, *rd1* were bred with MyD88 knockout mice ($MyD88^{-/-}$) for several generations to produce $rd1/MyD88^{+/+}$ and $rd1/MyD88^{-/-}$ genotypes. Chemokine mRNA expression levels were analyzed by qRT-PCR, and recruitment of activated microglia was quantified by immunodetection of the IBA-1 protein. Retinal outer nuclear layer cell counts were performed to quantify photoreceptor degeneration, and retinal function was assessed using electroretinograms (ERG). Our results revealed that retinal expression of Ccl2, Ccl4, Ccl7 and Cxcl10 was reduced by 2 to 8-fold in $rd1/MyD88^{-/-}$ mice compared with $rd1/MyD88^{+/+}$ mice ($p<0.05$), which coincided with attenuated microglial activation, higher numbers of photoreceptors and higher retina responses to photopic and scotopic stimuli. At later ages, $rd1/MyD88^{-/-}$ had reduced chemokine expression and higher photopic responses but no change in microglial recruitment compared with *rd1* mice with functional MyD88. In conclusion, lack of MyD88-mediated signaling increased photoreceptor

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survival and retina function in *rd1* mice, which implicates MyD88-mediated innate immunity pathways as an important pathogenic factor during retinal degeneration.

Keywords

TLR signaling; IL-1R; MyD88; photoreceptor; retinal degeneration; chemokines; microglia; retina; innate immunity

Introduction

Activation of the innate immune system is implicated in the pathogenesis of numerous neurodegenerative conditions in the brain (Glass et al., 2010) and retina (Donoso et al., 2006). Toll-like receptors (TLRs) and interleukin-1 receptor (IL-1R) form a superfamily of innate immunity receptors that require the adaptor protein MyD88 for signaling. TLRs recognize exogenous pathogen-associated proteins, such as lipopolysaccharide, and are also the major receptors for endogenous host-derived danger-associated proteins, such as HMGB1 and heat shock proteins (Mathur et al., 2011). There are 10 TLRs, and all except TLR3 and a minor portion of TLR4 signal through MyD88 (Takeda and Akira, 2004). IL-1R is activated by the IL-1 pro-inflammatory family of molecules, including IL-1β and IL-18.

Upon ligand binding to TLR and IL-1R, MyD88 is recruited to the receptors and in turn recruits the kinases IRAK-1 and IRAK-4, which leads to activation of NF-κB, AP-1, and subsequent induction of cytokines, including IL-6 and TNF-α (Elner et al., 2005; Kumar et al., 2004). During non-pathogen injury such as oxidative stress, TLR and IL-1R are believed to be activated by molecules released from injured cells, and induce MyD88-dependent intracellular signaling cascades that stimulate inflammation and promote further neuronal death (Caso et al., 2007; Kaczorowski et al., 2008; Lehnardt et al., 2003; Marsh et al., 2009; Okun et al., 2009; Tang et al., 2008). Therefore, characterizing how MyD88-mediated signaling influences neuronal viability is important for understanding the role of innate immunity and inflammation in the onset and rate of degeneration.

The retina is a light-sensitive specialized neuroepithelial tissue composed of three layers of functionally distinct neurons and glia that act together to generate vision. Because of its accessibility and low complexity, the retina is often used to study neuroinflammatory events that also occur elsewhere in the CNS. Chemokines and microglia are implicated in photoreceptor death in inherited retinal degeneration (Zeng et al., 2005), but the question of whether the prominent danger signal responders, the TLRs and IL-1R, influence these factors, and more importantly, disease outcome, has not been elucidated. Whereas the contribution of MyD88-independent TLR3 and complement to retinal degeneration have been described (Hageman et al., 2005; Khandhadia et al.; Klein et al., 2008; Yu et al., 2012), the role of MyD88-dependent TLR and IL-1R signaling in inherited photoreceptor degeneration is not known. Therefore, the overall understanding of inflammatory pathways during retinal disease remains incomplete.

Several lines of evidence suggest a pathogenic role for TLR and IL-1R receptors in the retina. TLRs are expressed throughout the retina, and induction of TLR expression was

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demonstrated in retinal degenerations in mouse and age-related macular degeneration (AMD) patients (Kohno et al., 2013; Kokkinopoulos et al., 2005; Maloney et al., 2010; Shiose et al., 2011; Zhu et al., 2013). An active role for TLR signaling via MyD88 in photoreceptor death has been shown in several recent studies using acute injury. TLR4 signaling in microglia contributed to light-induced photoreceptor degeneration (Kohno et al., 2013), and TLR4 activation caused photoreceptor death during oxidative stress in vitro (Yi et al., 2012) and mitochondrial oxidative stress damage in vivo (Ko et al., 2011). Additionally, TLR2 signaling enhanced choroidal neovascularization and promoted the development of diabetic retinopathy (Fujimoto et al., 2010). Inhibiting TLR2/4 increased retinal endothelial cell survival (Tang et al., 2013) and mice lacking TLR4 show improved survival of retinal ganglion cells from axotomy-induced death (Kilic et al., 2008) and have smaller infarct sizes in CNS injury models (Caso et al., 2007; Tang et al., 2007), suggesting that MyD88 functions as a common regulator of cell death. A role for IL-1R in regulating photoreceptor death has also been demonstrated by nature of the involvement of its proinflammatory ligands, cytokines IL-1β and IL-18, in retinal degeneration (Anderson et al., 2013; Tarallo et al., 2012). The lipofuscin component A2E stimulated IL-1β in retinal pigment epithelium (RPE) (Anderson et al., 2013), indicating a potential link between drusen and IL-1R signaling. In AMD retinas, IL-1β and IL-18 secretion was induced by the NLRP3 inflammasome, resulting in detrimental effects in dry AMD (Tarallo et al., 2012) and protective effects in neovascular AMD (Doyle et al., 2012). These studies suggest that TLR and IL-1R are associated with retinal disease, but their precise contribution to degeneration, especially in an inherited model of photoreceptor death, has not been investigated.

In this study, we tested the involvement of TLR and IL-1R signaling in retinal degeneration using the *retinal degeneration 1* (*rd1*) mouse model. The *rd1* mouse has a spontaneously-derived mutation in the rod photoreceptor-expressed cGMP phosphodiesterase *β*-subunit (*PDE6β*) gene, which also causes the human retinal degeneration retinitis pigmentosa (Chang et al., 2002; Huang et al., 1995). The *PDE6β* mutation in *rd1* mice leads to aggressive retinal degeneration with photoreceptor apoptosis beginning at post-natal day (P)10, and causes rapidly declining photoreceptor layer thickness (Bowes et al., 1990). The pattern of chemokine expression and microglial recruitment in *rd1* has been well characterized, thus making it a suitable model for this study. We used a genetic approach to block TLR and IL-1R signaling in *rd1* by removing MyD88, and demonstrated that innate immunity mediates the inflammatory response in early stages of retinal degeneration, with reduced chemokine expression and microglial recruitment in mice lacking MyD88. Furthermore, the dampened initial immune response is associated with increased photoreceptor survival and retina function. These findings reveal new insights into the contribution of TLR and IL-1R to retinal degeneration, and identify MyD88 as a potential target for delaying retinal disease progression.

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Materials and Methods

Animals

All procedures involving mice were performed in accordance with the ARVO Statement for the Use of Animals in Ophthalmology and Vision Research and were approved by the Institutional Animal Care and Use Committee at the University of Miami. To block TLR and IL-1R signaling pathways, we bred *rd1* mice with global MyD88 knock-out mice (*MyD88*$^{-/-}$), which have a constitutive deletion of MyD88 exon 3 and show reduced innate immune responses to TLR ligands and IL-1 and IL-18 (Hou et al., 2008). The *rd1* mice (C3H/HeOuJ, The Jackson Laboratory (Bar Harbor, ME, USA), Stock number 000635), are homozygous for the *retinal degeneration 1* mutation ($Pde6\beta^{rd1}$) (Bowes et al., 1990; Gibson et al., 2013) and were bred with *MyD88*$^{-/-}$ mice (B6.129P2(SJL)-MyD88$^{tm1.1Defr}$/J, Stock number 009088, The Jackson Laboratory) (Hou et al., 2008) to produce breeders that were homozygous for the *rd1* mutation and heterozygous for the MyD88 genotype (*MyD88*$^{+/-}$). The breeders were backcrossed for 4 generations and were used to produce litters containing the *rd1/MyD88*$^{+/+}$ and *rd1/MyD88*$^{-/-}$ genotypes. All mice were housed in a 12-hour light/ dark cycle and were kept at equivalent distances from the overhead light.

Electroretinograms (ERGs)

ERGs were performed as previously described (Patel and Hackam, 2014). After 4 hours of dark adaptation, mice were anesthetized by intraperitoneal injection of a solution containing 100 mg/Kg body weight ketamine (Vedco, Inc., St. Joseph, MO) and 10 mg/Kg xylazine solution (AnaSed, Akron, Lake Forest, IL) and the pupils were dilated with 10% phenylepherine hydrochloride (Akorn, Lake Forest, IL). The mice were placed on a small stage and wrapped in a modified heating blanket attached to a constant running water bath to maintain body temperature. A reference electrode was placed subcutaneously at the head and a ground electrode was inserted into the tail. Silver wire electrodes shaped into a large loop were placed on both corneas, and the stage was placed into a Ganzfeld light-emitting chamber (Li et al., 2010). Single flash stimuli were produced using the UTAS system controlled by EM for Windows software (LKC Technologies, Gaithersburg, MD). Scotopic stimuli were flashes of white light ranging from 1 to 100 cds/m^2 on a white background light, and photopic stimuli were flashes of green light on low intensity green background light.

Immunohistochemistry and outer nuclear layer cell counts

Enucleated eyes were fixed with cold 4% paraformaldehyde in PBS and processed through increasing sucrose concentrations from 5% to 20%, then embedded in optimal cutting temperature compound and frozen. Embedded globes were sectioned into 8 μm thick sections and mounted onto Superfrost micro slides (VWR International, Radnor, PA) (Yi et al., 2012). To detect activated microglia, the slides were first blocked with 20% goat serum then incubated with primary antibody rabbit anti-IBA-1 (Wako, Osaka, Japan) overnight at 4°C, washed with PBS and then incubated with secondary anti-rabbit antibody conjugated with Alexa Fluor-488 (Invitrogen, Carlsbad, CA). The retinas were washed and counterstained with 4′,6-diamidino-2-phenylindole (DAPI), and then viewed using a fluorescent microscope (Zeiss Axiovert 200). Control slides omitting the primary antibody

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were viewed using the same microscopy and exposure settings. Microglia counts were performed by counting IBA-1 positive microglia with an ameboid shape from the outer plexiform layer (OPL) to the RPE, and expressed as density of activated microglial cells within a specific area of outer retina. Microglia were counted in the entire section in 3 adjacent retinal sections, in the equivalent regions of the retina in each animal.

For photoreceptor nuclei counts, columns of nuclei were counted at the periphery, near the ora serrata, and centrally, near the optic nerve region, to account for the recognized central-peripheral temporal gradient of degeneration (Carter-Dawson et al., 1978). Ten columns of DAPI-positive nuclei in the outer nuclear layer (ONL) were counted at each site centrally on either side of the optic nerve, and at each side of the cornea peripherally, adjacent to the ora serrata, while viewed at a 20× magnification. Three adjacent retinal sections were counted in each region, and nuclear column counts averaged for central and peripheral regions, for a total of 60 columns per region per eye.

RNA isolation and Quantitative PCR (qPCR)

Total mRNA was isolated from dissected retinas using Trizol reagent (Invitrogen), as described (Silva et al., 2010). One microgram of RNA was reverse-transcribed into cDNA using Thermoscript RT (Invitrogen), and the cDNA samples were used for qPCR in the Mastercycler ep *realplex* (Eppendorf, Biotech, Hamburg, Germany) using SYBRGreen (Biorad, Hercules, CA). Primer sequences specific to the genes of interest were selected to span at least one intron (Table 1). Amplification of each sample was performed in triplicate for each gene, and the expression levels were normalized to the housekeeping gene Acid Ribosomal Phosphoprotein (ARP) using the delta-delta C_t method (Hackam et al., 2004). The expression of each gene was then normalized to expression levels in wild type adult mouse retina, except for TNF-α, which was not detected in wild type retina, and was instead normalized to TNF-α expression levels in the murine microglial cell line BV2.

Statistical Analysis

A two-tailed Student's *t*-test was used where two groups were compared, and ANOVA was used with post-hoc Tukey-Kramer analysis where multiple groups were compared. Null hypothesis was rejected at $p<0.05$.

Results

Loss of MyD88-mediated signaling reduced chemokine mRNA expression during retinal degeneration

Pro-inflammatory chemokines are upregulated during retinal degeneration and are thought to contribute to photoreceptor apoptosis (Guo et al., 2012; Kohno et al., 2014; Rutar et al., 2012). To investigate whether removing MyD88-mediated innate immunity signaling pathways blocks expression of these potentially destructive molecules in the presence of a mutation, we compared *rd1* mice with a genetic deletion of MyD88 ($rd1/MyD88^{-/-}$) with their littermates that had intact MyD88 ($rd1/MyD88^{+/+}$). The *rd1* mouse is an aggressive model of inherited retinal degeneration, with rod photoreceptor death beginning at P10, reaching a peak of degeneration at P16 (Zeng et al., 2005), and resulting in almost complete

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loss of rods by P17 (Carter-Dawson et al., 1978). A secondary slow progressive cone photoreceptor degeneration follows, in a mechanism that remains poorly understood. The inflammation associated with inherited retinal degeneration occurs prior to and during this period of elevated neuronal death (Gupta et al., 2003; Roque et al., 1996; Yoshida et al., 2013; Zeiss and Johnson, 2004). Therefore, we sought to identify whether MyD88 had a role during the peak of degeneration in the early time-points of degeneration.

QPCR analysis was performed on retinal lysates during the early stage of photoreceptor death at P12, at P14, and during the peak of degeneration at P16. As shown in Fig. 1, expression of Ccl2 (MCP-1), Ccl4 (MIP-1β), Ccl7 (MCP-3) and Cxcl10 mRNA peaked at P12 in the *rd1MyD88*$^{+/+}$ mouse and reduced thereafter, which is consistent with previous studies in *rd1* that showed maximal chemokine expression at P12, reducing subsequently to baseline levels at P18 (Zeng et al., 2005). Notably, at the same time-point, expression of these chemokines was 2-8 fold lower in the *rd1/MyD88*$^{-/-}$ compared with *rd1/MyD88*$^{+/+}$ ($p<0.05$; Fig. 1A-D), suggesting a dampened inflammatory response. In contrast, Ccl3 (MIP-1α) expression increased from P12 to P14 in both *rd1/MyD88*$^{+/+}$ and *rd1/MyD88*$^{-/-}$ ($p<0.05$ for both *rd1/MyD88*$^{+/+}$ and *rd1/MyD88*$^{-/-}$; Fig. 1E), indicating that the delayed and reduced levels of the other chemokine mRNAs were not a global defect in transcription in the *rd1/MyD88*$^{-/-}$ mice. Furthermore, Ccl5 expression remained constant across the ages in both genotypes, but mRNA transcripts in *rd1/MyD88*$^{-/-}$ were consistently 2-fold lower than *rd1/MyD88*$^{+/+}$ at all three time-points ($p<0.05$; Fig. 1F). These results indicate that reduced MyD88-dependent TLR and IL-1R signaling in *rd1/MyD88*$^{-/-}$ leads to reduction in mRNA expression of specific chemokines associated with retinal degeneration.

Loss of MyD88-mediated signaling reduced TNF-α mRNA expression during retinal degeneration

The cytokine TNF-α exacerbates photoreceptor death in retinal degenerations (de Kozak et al., 1997; Nakazawa et al., 2011) and is produced at highest quantities during early degeneration at P12 in the *rd1* mouse, returning to baseline by P18 (Zeng et al., 2005). We previously demonstrated that TNF-α induced photoreceptor death after TLR4-mediated MyD88 signaling in primary retina dissociated cultures (Yi et al., 2012). To determine the contribution of MyD88 to the expression of TNF-α during retinal degeneration, we examined TNF-α mRNA levels in the *rd1/MyD88*$^{-/-}$ and *rd1/MyD88*$^{+/+}$ mice at each time-point. As was the case with chemokines, TNF-α expression was maximal at P12 in the *rd1/MyD88*$^{+/+}$ and reduced thereafter. In contrast, there was a delay in peak expression to P14 in the *rd1/MyD88*$^{-/-}$ (Fig. 1G). Also, TNF-α expression at P12 was 4.4-fold lower in the *rd1/MyD88*$^{-/-}$ compared with *rd1/MyD88*$^{+/+}$ ($p<0.05$).

Loss of MyD88 attenuates activated microglial recruitment only during early degeneration

Microglia are recruited to the outer retina during retinal degeneration where they are thought to exacerbate photoreceptor loss through the release of additional chemokines and toxic proteins (Ng and Streilein, 2001; Zeiss and Johnson, 2004; Zhang et al., 2005). Because TLRs are implicated in microglia activation and subsequent chemokine production (Esen and Kielian, 2006; Jack et al., 2005), and because MyD88-mediated signaling is implicated in microglia recruitment into injured tissue in the CNS (Lim et al., 2011), we next

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investigated whether MyD88-mediated signaling was involved in microglia recruitment to the ONL in the *rd1* retina. Resting microglia reside in the ganglion cell layer (GCL) and inner retina in non-degenerating retina, and are induced to migrate towards the outer retina in conditions of retinal pathology (Zeiss and Johnson, 2004). Therefore, we quantified IBA-1-positive ameboid (activated) microglia in the region from the outer plexiform layer (OPL) to the RPE (Fig. 2A).

The density of microglia in the outer retina increased with disease progression from P12 to P14 in both *rd1/MyD88*$^{+/+}$ and *rd1/MyD88*$^{-/-}$ ($p<0.01$; Fig. 2B), consistent with previous findings (Zeng et al., 2005). However, at P12, mice lacking MyD88 had a 40% reduction in microglial density compared with *rd1/MyD88*$^{+/+}$ mice ($p<0.05$). The difference in microglia density between the two genotypes diminished dramatically at P14. The reduced microglial recruitment at P12 but not at P14 in the *rd1/MyD88*$^{-/-}$ mice suggests that MyD88-mediated signaling may be involved in early microglial recruitment, but as the disease progresses other signaling pathways may play a more prominent role. Loss of MyD88 leads to reduced expression of chemotractant chemokines at P14, therefore one would expect a subsequent attenuation in recruited microglia, which does not occur, suggesting other factors may be involved in the recruitment of these cells.

Loss of MyD88-mediated signaling increased photoreceptor survival during retinal degeneration

To determine whether MyD88-mediated signaling and associated reduction in pro-inflammatory cytokine mRNA expression and microglia influences photoreceptor viability, we next quantified photoreceptor survival and function. Counting columns of photoreceptor (rod and cone) nuclei in the ONL is an established accurate marker of photoreceptor survival (Michon et al., 1991). As retinal degeneration progressed from P12 to P16, the number of photoreceptor nuclei declined by 5.4-fold in the *rd1/MyD88*$^{+/+}$ ($p<0.0001$; Fig. 3B) and by 3.9-fold in *rd1/MyD88*$^{-/-}$ mice ($p<0.0001$). However, the rate of photoreceptor loss that occurred between P12 to P14 differed from rate of cell loss between P14 to P16, because while the rate of cell loss was 2.26-fold greater in *rd1/MyD88*$^{+/+}$ between P12 to P14, the rate of cell loss in *rd1/MyD88*$^{-/-}$ caught up to *rd1/MyD88*$^{+/+}$ between P14 to P16 and was equivalent. Although photoreceptor nuclei do diminish in the *rd1/MyD88*$^{-/-}$ mouse, the numbers of remaining nuclei are higher in *rd1/MyD88*$^{-/-}$ compared with *rd1/MyD88*$^{+/+}$, with the ONL cell count 1.43-fold higher at P16 ($p<0.05$) in the *rd1/MyD88*$^{-/-}$ mouse compared to *rd1/MyD88*$^{+/+}$. Therefore, eliminating MyD88-mediated signaling in the *rd1* mouse results in a moderate but significant protective effect, and dampens the severity of photoreceptor loss during early retinal degeneration.

Retinal function is higher in the rd1/MyD88$^{-/-}$ mouse during degeneration

Retinal recordings in response to flashes of light, which are measured by ERG, were used to compare retinal function between the *rd1/MyD88*$^{-/-}$ and *rd1/MyD88*$^{+/+}$ mice. In ERGs, light-stimulated a-waves represent photoreceptor hyperpolarization, while the b-waves represent photoreceptor to bipolar cell synaptic connections. The amplitudes of these waves are proportional to the number of surviving photoreceptors and are a reliable measure of retinal function (Dalke et al., 2004). Additionally, the time it takes the waves to reach their peak,

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known as the implicit time, is prolonged in retinal disease (Jae et al., 2013) and therefore is often used as a second measure of disease progression in *rd1* mice. We used b-waves to characterize the retina response in the two genotypes because the photoreceptor a-waves were variable and close to background in the rapidly degenerating *rd1* mouse retina. Analysis of ERG b-waves indicated that loss of MyD88 signaling resulted in improvement of scotopic retina response (rod-driven) at P12, with a maximal 1.46-fold increase in b-wave amplitude, and faster time-to-peak (implicit time) by 18.6 milliseconds compared with *rd1/MyD88*$^{+/+}$ ($p<0.05$) (Fig. 4A). The increased scotopic b-wave amplitudes in *rd1/MyD88*$^{-/-}$ were not maintained at later time points, likely due to the aggressive nature of the rod degeneration in the *rd1* mouse (Fig. 4B, 4C). In contrast, significant improvements of photopic retina responses (cone-driven) b-wave amplitudes were demonstrated in *rd1/MyD88*$^{-/-}$ mice at all time points ($p<0.05$) (Fig. 5A-C). To rule out the possibility that MyD88 loss itself affects the function of the retina, comparisons of adult non-degenerating C57Bl/6J mice with adult *MyD88*$^{-/-}$ mice indicated no difference in ERG responses (Fig. 6A-B). Therefore, loss of MyD88 signaling in *rd1* improved retina responses to both scotopic and photopic stimuli.

Discussion

The purpose of this study was to investigate whether MyD88-mediated signaling contributes to retinal degeneration. The immune response is known to affect disease severity, but it was unknown whether the absence of MyD88-mediated signaling, downstream of TLRs and IL-1R, would change inflammatory markers of degeneration and the extent of photoreceptor loss. This investigation is novel in that by blocking the majority of TLR and IL-1R signaling during degeneration using a genetic knockout of MyD88, we eliminated a crucial component of the innate immune response to neurodegeneration in the retina. We discovered attenuated retinal microglial recruitment during early degeneration and reduced pro-inflammatory chemokine transcript expression. Furthermore, loss of MyD88-mediated signaling resulted in increased photoreceptors, improved early scotopic retina function and improved photopic retina function. Our evidence thus suggests involvement of MyD88-mediated signaling in the inflammation-associated exacerbation of retinal degeneration during early disease.

All TLRs signal through MyD88, with the exception of TLR3 and a portion of TLR4, and are expressed throughout the retina (Kindzelskii et al., 2004). TLR4 has been localized to rod photoreceptors in wild-type and degenerating mice (Yi et al., 2012) and the expression of TLR2-9 confirmed in microglia (Jack et al., 2005), and TLR1-10 in RPE (Kindzelskii et al., 2004; Kumar et al., 2004). During non-pathogen injury such as ischemia and oxidative stress, as exhibited in retinal degeneration, TLRs and IL-1R are believed to be activated by molecules released from injured neurons early in the disease process, and induce intracellular signaling cascades that cause further photoreceptor death. TLRs are activated by molecules collectively known as damage-associated molecular pattern molecules (DAMPs) that are released from injured cells, such as Hsp70 (Vabulas et al., 2002) and HMGB1 (Lotze and Tracey, 2005; Qiu et al., 2010), and signature molecules in degenerating retinas, including oxidized lipids (Chavez-Sanchez et al., 2010; Higgins et al., 2003) and drusen components (Higgins et al., 2003; Kaarniranta and Salminen, 2009). Indeed, photoreceptor outer segments co-incubated with microglia caused their activation

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and subsequent pro-inflammatory chemokine production in vivo (Kohno et al., 2013). IL-1R is activated by cytokines induced during inflammation. Our findings indicate that blocking the TLR and IL-1R response to injury-associated molecules reduces but does not eliminate subsequent retinal degeneration. Our findings of reduced chemokine expression, increased photoreceptor numbers and improved retina function are supported by a recent study by Kohno et al. By knocking out a single upstream component of MyD88 signaling, the TLR4 receptor, in a light-induced retinal degeneration mouse model, Kohno et al. demonstrated reduced chemokine production and microglial activation and increased rod and cone photoreceptor numbers (Kohno et al., 2013).

IL-1R, which also functions through MyD88, is activated by the pro-inflammatory cytokines IL-1β and IL-18 and contributes to retinal degeneration (Anderson et al., 2013; Tarallo et al., 2012). IL-1β and IL-18 are cleaved from their pro-forms by another major class of innate immune receptors, the NLRP3, an important danger signal receptor that responds to signature proteins of macular degeneration as ligands, including lipofuscin component A2E (Anderson et al., 2013), drusen and drusen component complement C1Q (Doyle et al., 2012), and *Alu* RNA (Tarallo et al., 2012). In addition to TLR and IL-1R, MyD88 regulates a viral-response IFN-γR1 pathway independent of TLRs (Sun and Ding, 2006). However, this pathway is unlikely to be relevant in the *rd1* retinal degeneration because viral infection is absent. MyD88-independent inflammatory pathways, including NF-κB and NLRP3 inflammasome activation are associated with the pathogenesis of retinal degenerations. MyD88 regulates complement and the inflammasome and it is possible that there is a compensatory change in these pathways in *rd1/MyD88*$^{-/-}$ mice. Furthermore, blocking MyD88 leaves non-MyD88 TLR pathways active (TLR3/TRIF, some TLR4 signaling), as well as other DAMP receptors (e.g. RAGE), which may contribute to retinal degeneration in the mice, and may account for the fact that we observed only a delay in degeneration instead of halting degeneration entirely.

Photoreceptor degeneration is preceded by, or accompanied by, pro-inflammatory C-C motif chemokine (Ccl) induction. Increased retinal Ccl2, Ccl3, Ccl4 and Ccl12 were detected in mouse models of AMD and light-induced retinal degeneration (Kohno et al., 2014) and increased Ccl2-Ccl5 and Ccl7 expression were observed prior to and during photoreceptor loss in the *rd1* mouse (Zeng et al., 2005). Additionally, in *retinal degeneration 10* (*rd10*) mice that genetically lacked *CCR2*, there was improved retinal function and structure (Guo et al., 2012), suggesting that pro-inflammatory chemokines are active participants in the pathology of retinal degeneration. Expression of Ccl2, Ccl4, Ccl5, Ccl7 and Cxcl10 and TNF-α transcripts were reduced in *rd1/MyD88*$^{-/-}$ compared with *rd1/MyD88*$^{+/+}$ mice, and maximal expression levels attained in the *rd1/MyD88*$^{-/-}$ mice did not reach those of *rd1/MyD88*$^{+/+}$ at the time-points analyzed. However, the fact that chemokines are induced at all in the *rd1/MyD88*$^{-/-}$ mouse also implicates MyD88-independent pathways. Certainly, administration of TLR4 ligand LPS to MyD88-deficient cells induced several cytokines such as Ccl3, Ccl4, and TNF-α (Covert et al., 2005). Additionally, TLR-mediated activation of transcription factor NF-κB is biphasic and occurs first via the MyD88-dependent followed by the MyD88-independent TRIF pathway (Covert et al., 2005; Yamamoto et al., 2003),

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therefore highlighting a potential mechanism for the delayed peak chemokine expression exhibited in the *rd1/MyD88*$^{-/-}$ mouse.

TNF-α is known to be cytotoxic to neurons (Neniskyte et al., 2014) and exacerbates photoreceptor death in retinal degeneration (de Kozak et al., 1997; Nakazawa et al., 2011). TNF-α produced by primary retinal microglia isolated from the RCS rat retinal degeneration model was toxic to photoreceptors in vitro (de Kozak et al., 1997). In fact, microglia and indeed photoreceptors themselves have been shown to produce TNF-α in response to stimulation by each other in vivo, via microglia stimulated by degenerating photoreceptors (Zeng et al., 2005), and photoreceptors co-incubated with activated microglia (Yang et al., 2007). As in previous studies (Zeiss and Johnson, 2004), TNF-α expression in the *rd1/MyD88*$^{+/+}$ retina peaked at P12 prior to maximal photoreceptor death and reduced thereafter, whereas expression in the *rd1/MyD88*$^{-/-}$ mouse at the same time-point was 4.4-fold lower, and remained 2- to 3-fold lower compared with *rd1/MyD88*$^{+/+}$ at further time-points. Therefore, reduced TNF-α expression as a result of absent MyD88-mediated signaling may contribute to functional and cellular photoreceptor protection in *rd1/MyD88*$^{-/-}$.

Chemokines released secondary to tissue injury are chemotractants for inflammatory cells, most prominently, the microglia. These cells serve not only as phagocytes clearing cellular debris, but also as neurotoxic substance secretors in the CNS (Boje and Arora, 1992) and retina (de Kozak et al., 1997; Roque et al., 1999; Zeng et al., 2005), provoking further neuronal loss in both settings. Maximal expression of the chemotractant chemokines precedes microglial recruitment to the outer retina during retinal degeneration (Zeng et al., 2005), a phenomenon also exhibited in our *rd1/MyD88*$^{+/+}$ mice. However, although reduced chemokine expression and microglial recruitment was observed at P12 in *rd1/MyD88*$^{-/-}$, this reduction did not translate to attenuated microglial recruitment at P14. While MyD88-mediated signaling influences chemokine expression during retinal degeneration, it does not affect the microglial recruitment that follows. An explanation for normal microglial recruitment at P14 lies in the possibility that despite the 2- to 5-fold decline in chemokine expression in *rd1/MyD88*$^{-/-}$ at P12, a minimal cytokine threshold required for microglial recruitment and activation was reached nonetheless. In addition, microglial activation secondary to TLR4 ligand LPS in MyD88-deficient mice was demonstrated by Esen et al. (Esen and Kielian, 2006), raising the possibility that MyD88-independent factors and alternative molecules may also play a role in their recruitment.

Chemokine expression and microglial recruitment precede maximal photoreceptor loss (Zeng et al., 2005), and our trends support this, with the biggest drop of photoreceptor nuclei occurring between P14 and P16. In addition, the rate of photoreceptor nuclei loss between P14 to P16 in *rd1/MyD88*$^{-/-}$ caught up to that of *rd1/MyD88*$^{+/+}$ mice, possibly a reflection of the effect of higher outer retinal microglial recruitment at P14 in *rd1/MyD88*$^{-/-}$. By knocking out MyD88-mediated signaling, we have managed to dampen the initial inflammatory response to dying photoreceptors, preserving photoreceptors and conveying not only a protective effect on scotopic function of the retina during early degeneration, but interestingly, also improved photopic function, which represents the vulnerable bystanders, the cone photoreceptors. The *rd1* mutation affects rod photoreceptors primarily, however cone photoreceptor loss also follows, and although the exact mechanism is unknown, a

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suggested cause is noxious stimuli produced by the inflammatory response mounted against degenerating rod photoreceptors (Zeng et al., 2005). The improved scotopic function in *rd1/MyD88*$^{-/-}$ does not extend past P12 perhaps because the rod photoreceptors that initiate the scotopic response eventually succumb to the ongoing pathology resulting from the aggressive rod *PDE6β* gene mutation. Because cone photoreceptor loss is known to occur after P18 in the *rd1* mouse (Carter-Dawson et al., 1978), the improvement of photopic retina function, which is primarily mediated by cones, in *rd1/MyD88*$^{-/-}$ thus appears to occur prior to photoreceptor loss. Furthermore, photopic b-wave amplitudes do not reduce between P12 to P16 in the *rd1/MyD88*$^{+/+}$ mouse, suggesting no functional deterioration with age. It is also possible that the delayed immune response may preserve the function of the inner retina only, and the outer retina (the photoreceptors) is only preserved at the structural and not functional level.

In addition to microglia and photoreceptors, Muller glia and RPE also express TLRs and signal via MyD88 (Jack et al., 2005; Kindzelskii et al., 2004; Kumar et al., 2004; Yi et al., 2012). Future avenues for exploration would investigate how MyD88 inhibition in each cell type, particularly RPE, would affect their role in the recognition of endogenous danger signals and the subsequent initiation of inflammation in retinal degeneration. In addition, TLR-independent MyD88-mediated signaling in the RPE plays a role in pathogenesis of retinal degeneration (Kerur et al., 2013; Tarallo et al., 2012). *Alu* RNA accumulation in RPE is implicated in dry AMD and stimulated TLR-independent NF-κB activation and subsequent NLRP3 inflammasome activation, which lead to IL-18 maturation and subsequent release. IL-18-induced IL-1R MyD88-mediated signaling in the RPE resulted in RPE loss and subsequent photoreceptor death (Kerur et al., 2013). Interestingly, TLR4 signaling regulates complement (Song, 2012), and activates the NLRP3 inflammasome in RGCs after injury (Qi et al., 2014), indicating interactions among MyD88-dependent and -independent pathways. Additional studies would indicate whether MyD88-dependent and -independent pathways cross-talk in the RPE to regulate retinal degeneration.

In this study, we used the *rd1* retinal degeneration mouse model because the inflammatory response has been well characterized in the literature. *Rd1* is an aggressive degeneration model with rapid photoreceptor loss that begins prior to retinal maturation (Gibson et al., 2013), and was chosen because its inflammatory profile and rate of degeneration is well established. Future avenues will investigate whether protection from lack of MyD88 would also be observed in models of less severe inherited retinal degeneration. Because pathways that regulate photoreceptor death in inherited retinal degenerations are often also implicated in the pathogenesis of AMD, these findings provide insight into potential therapeutic strategies for dry AMD.

Conclusion

In summary, knocking out innate immunity MyD88-mediated signaling in an aggressive inherited retinal degeneration mouse model dampens the initial immune response, which results in a protective delay in loss of the scotopic retina response, and improvement in photopic retina function. This study highlights the destructive role of TLR/IL-1R MyD88 mediated inflammatory pathways in neurodegenerative tissue injury.

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List of Abbreviations

AMD	age-related macular degeneration
Ccl	chemokine (C-C motif) ligand
Cxcl10	chemokine (C-X-C motif) ligand 10
HMGB1	High-mobility group protein B1
Hsp70	70 kilodalton heat shock protein
IFN-γ	interferon- γ
IL-1R	interleukin-1 receptor
IRAK	Interleukin-1 receptor-associated kinase
MAPK	mitogen-activated protein kinase
MyD88	myeloid differentiation primary response gene 88
NF-κB	nuclear factor kappa-light-chain-enhancer of activated B cells
NLRP3	NOD-like receptor family, pyrin domain containing 3
PDE6β	phosphodiesterase β-subunit
RPE	retinal pigment epithelium
TLR	Toll-like receptor
TNF-α	tumor necrosis factor-α
rd1	*retinal degeneration 1*.

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Highlights

- Knocking out MyD88-dependent TLR/IL-1R signaling in retinal degeneration reduces retinal proinflammatory chemokine and TNF-α mRNA expression
- Absence of MyD88-mediated signaling leads to attenuated microglial recruitment during early degeneration but not at later time-points.
- Dampened immune response in the absence of MyD88 leads to an improvement in retina function.
- Therefore, TLR/IL-1R MyD88 mediated inflammatory pathways play a destructive role in neurodegenerative tissue injury in the retina.

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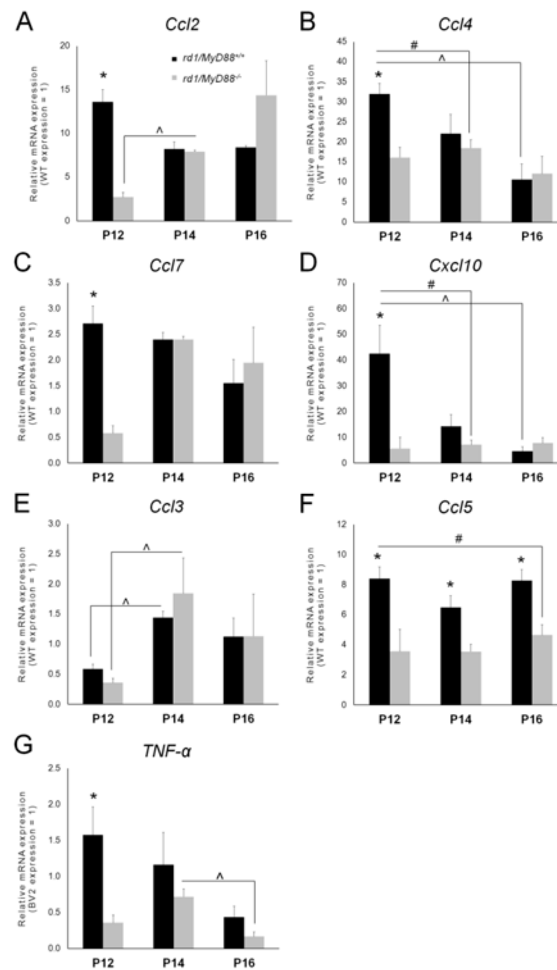

Fig. 1.

Reduced pro-Inflammatory chemokine and TNF-α transcript expression in the *rd1* mouse in the absence of MyD88-mediated signaling. (A-F) Expression of Ccl2 (MCP-1), Ccl4 (MIP-1β), Ccl7 (MCP-3), Cxcl10, Ccl3 (MIP-1α), and Ccl5 (RANTES) in *rd1/MyD88*$^{+/+}$ and *rd1/MyD88*$^{-/-}$ mice at postnatal day 12 (P12), P14 and P16, as measured by qPCR. Reduced transcript levels and a shift in the peak expression from P12 to P14 was observed in *MyD88*$^{-/-}$ compared with *MyD88*$^{+/+}$, for Ccl4, Ccl5, Ccl7, and Cxcl10 (n=3-5, $p<0.05$). In contrast, Ccl5 expression level remains relatively constant as retinal degeneration progresses with a consistent 2-fold reduction in the *rd1/MyD88*$^{-/-}$ mouse (n=3-5, $p<0.05$), whereas Ccl3 expression was not different between the two genotypes. **(G)** Retinal expression of TNF-α is reduced by 4.4 fold in the *rd1/MyD88*$^{-/-}$ compared with *rd1/MyD88*$^{+/+}$ at P12 (n=3 each, $p<0.05$). TNF-α expression was normalized to ARP and was expressed relative to expression in the BV2 microglial cell line due to lack of expression in WT retina. Mean ± SEM are shown. * $p<0.05$, comparison between genotypes within a given time-point $p<0.05$; (x0005E) $p<0.05$, comparison among ages of the same genotype; # $p<0.05$, comparison of peak expression between *rd1/MyD88*$^{+/+}$ and *rd1/MyD88*$^{-/-}$across all time-points. Samples were compared with the house-keeping gene acid ribosomal phosphoprotein (ARP). All samples were normalized to that of a wild type adult mouse retina, except for

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TNF-α, which was not detected in wild type retina, and was instead compared with the murine microglial cell line BV2.

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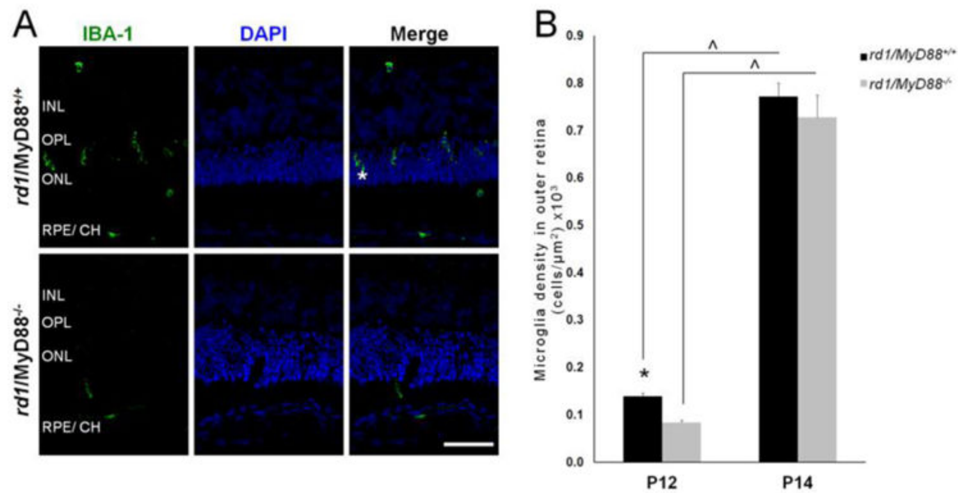

Fig. 2.

Activated microglia recruitment to the outer retina is reduced at P12 but not at P14 in the *rd1/MyD88*$^{-/-}$ mouse. (A) Retinal sections from P12 *rd1/MyD88*$^{+/+}$ and *rd1/MyD88*$^{-/-}$ mice immunostained for the activated microglia marker IBA-1 (asterisk), alongside DAPI and merge images. INL= Inner Nuclear Layer, OPL= Outer Plexiform Layer, ONL= Outer Nuclear Layer, RPE/ CH= Retinal Pigment Epithelium/Choroid. Scale bar: 50µm. (B) Microglia cell density in outer retina (from OPL to RPE). Numbers of activated microglia were counted and expressed as number of cells per area (µm^2) of outer retina $\times 10^3$. At P12, there is a 40% reduction in activated microglia in the outer retina of *rd1/MyD88*$^{-/-}$compared with *rd1/MyD88*$^{+/+}$ mice (n=3 each, p<0.05). Microglia density increases in both mice from P12 to P14, at which point outer retinal microglia numbers are similar in both *rd1/MyD88*$^{+/+}$ and *rd1/MyD88*$^{-/-}$ (n=4, 3 respectively). Mean ± SEM are shown. * p<0.05, comparison among genotypes within a given time-point; (x0005E) p<0.05, comparison between ages of the same genotype.

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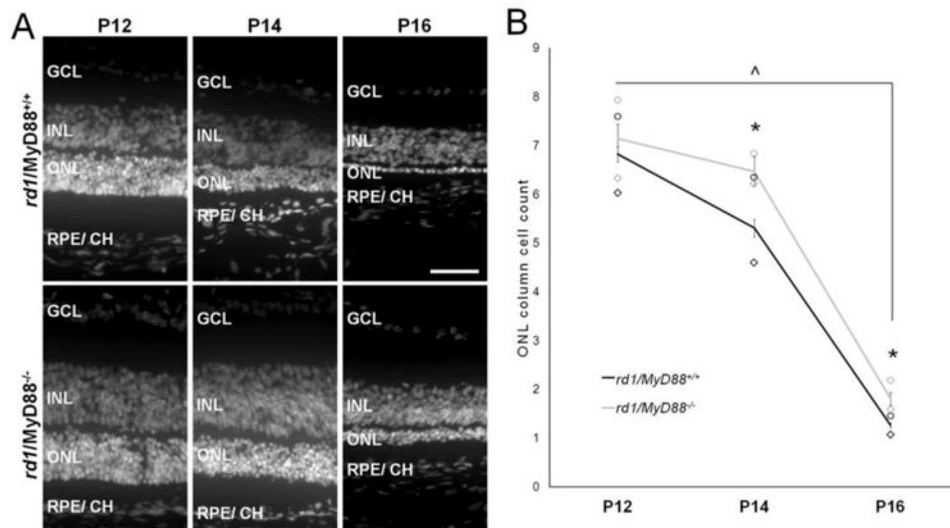

Fig. 3.

Fewer photoreceptors are lost during retinal degeneration in the *rd1/MyD88*$^{-/-}$ mouse. (A) Representative IHC sections with nuclear DAPI stain of P12 to P16 *rd1/MyD88*$^{+/+}$ and *rd1/MyD88*$^{-/-}$retinas revealing increased cells in the outer nuclear layer in the *rd1/MyD88*$^{-/-}$ across all ages. GCL= Ganglion Cell layer, INL= Inner Nuclear Layer, ONL= Outer Nuclear Layer, RPE/ CH= Retinal Pigment Epithelium/ Choroid. Scale bar: 50μm. (B) Average ONL column counts at P12, P14 and P16 in *rd1/MyD88*$^{+/+}$ and *rd1/MyD88*$^{-/-}$ including averages for central ( ) and peripheral (o) counts. ONL cell number decreases in both genotypes with age, with a 4.9-fold decline from P12 to P16 in the *rd1/MyD88*$^{+/+}$ (P12 n=6, P16 n=3, $p<0.0001$) and a 3.3-fold decline in *rd1/MyD88*$^{-/-}$ (P12, P16 n=3, $p<0.0001$). The amount of photoreceptor death was lower at P14 and P16 in the *rd1/MyD88*$^{-/-}$ mice compared with *rd1/MyD88*$^{+/+}$, with 22% more nuclei remaining at P14 (*rd1/MyD88*$^{+/+}$ n=4, *rd1/MyD88*$^{-/-}$n= 3, $p<0.05$) and 43% more nuclei at P16 (*rd1/MyD88*$^{+/+}$ n=3, *rd1/MyD88*$^{-/-}$ n= 3, $p<0.05$) in the *rd1/MyD88*$^{-/-}$ mice compared to *rd1/MyD88*$^{+/+}$. Mean ± SEM are shown. * $p<0.05$, comparison between genotypes within a given time-point; ^ $p<0.05$, comparison among ages of the same genotype.

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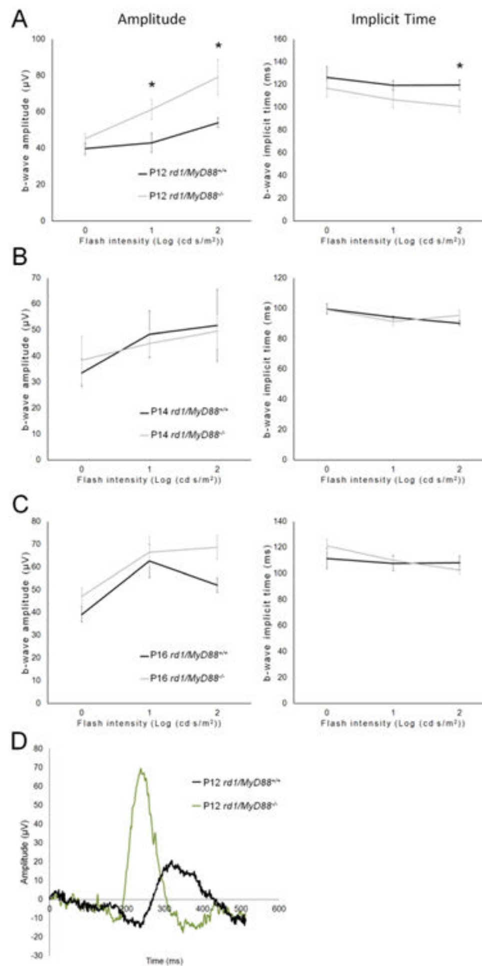

Fig. 4.

The scotopic retina response is improved at P12 in *rd1* mice lacking MyD88. Graphs (A-C) show the retina response to scotopic (rod-driven) light stimuli at different flash intensities. Absence of MyD88-mediated signaling in *rd1* resulted in improved retina function compared with *rd1*/$MyD88^{+/+}$ at P12 only (*rd1*/$MyD88^{+/+}$ n=6-7, *rd1*/$MyD88^{-/-}$ n=5). Representative ERG waveforms for each genotype at P12 are shown in panel D. Mean ± SEM are shown. * $p<0.05$

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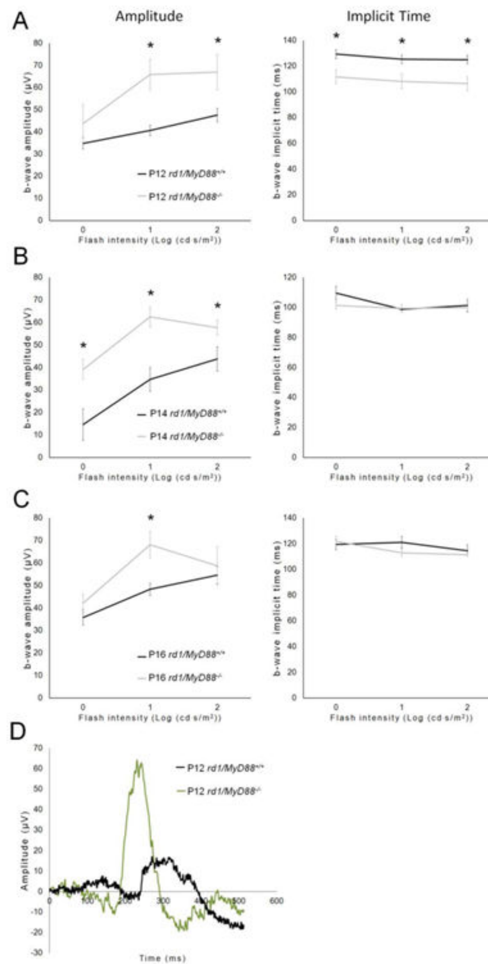

Fig. 5.

The photopic retina response is improved during retinal degeneration in *rd1/MyD88*$^{-/-}$ mice. Graphs (A-C) show the retina response to photopic (cone-driven) stimuli at different flash intensities. An improvement in retina function was demonstrated in *rd1/MyD88*$^{-/-}$ at all three time-points compared with *rd1/MyD88*$^{+/+}$ (*rd1/MyD88*$^{+/+}$ n=4-7, *rd1/MyD88*$^{-/-}$ n=4-10). Representative ERG waveforms for each genotype at P12 are shown in panel D. Mean ± SEM are shown. * $p<0.05$

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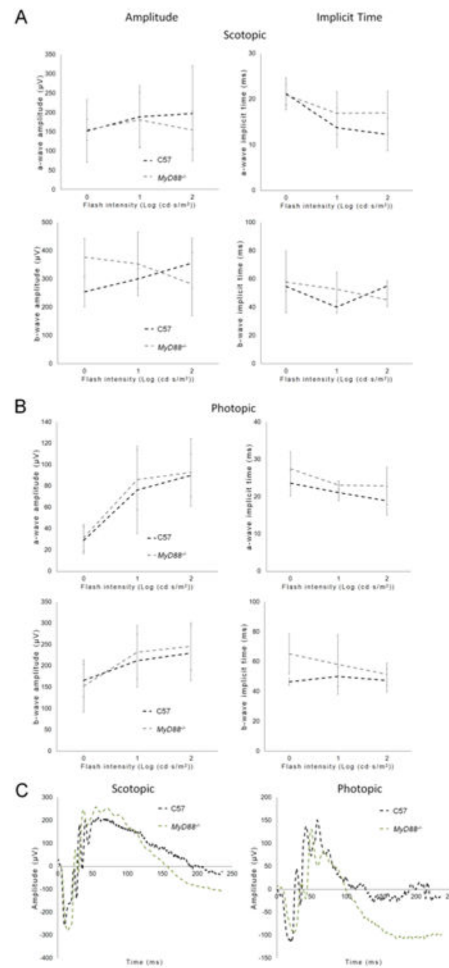

Fig. 6.

MyD88 loss in the absence of the *rd1* mutation does not affect retina function. Average ERG retinal response (amplitude) to (A) scotopic and (B) photopic stimuli in adult non-degenerating C57Bl/6J mice and adult *MyD88*$^{-/-}$ mice show equivalent retina responses between the genotypes (C57 n=5, *MyD88*$^{-/-}$ n=4). (C) Representative ERG waveforms are shown. Mean ± SD are shown.

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Table 1

Primers used for real-time PCR

Gene	Primers: Oligonucleotide sequence 5′-3′	
ARP	Forward	CGACCTGGAAGTCCAACTAC
	Reverse	ATCTGCTGCATCTGCTTG
Ccl2	Forward	AGGTCCCTGTCATGCTTCTG
	Reverse	ATTTGGTTCCGATCCAGGTT
Ccl3	Forward	GCAACCAAGTCTTCTCAGCG
	Reverse	TGGAATCTTCCGGCTGTAGG
Ccl4	Forward	TGTGCAAACCTAACCCCGAG
	Reverse	ATACCACAGCTGGCTTGGAG
Ccl5	Forward	AGCAGCAAGTGCTCCAATCT
	Reverse	ATTTCTTGGGTTTGCTGTGC
Ccl7	Forward	CCATGAGGATCTCTGCCACG
	Reverse	ACCCACTTCTGATGGGCTTC
Cxcl10	Forward	GCTGCAACTGCATCCATATC
	Reverse	CACTGGGTAAAGGGGAGTGA
TNF-α	Forward	TCTTCTCATTCCTGCTTGTGG
	Reverse	CACTTGGTGGTTTGCTACGA