Investigation of the Role of the TLR3 Innate Immunity Pathway in the Pathogenesis of Age-Related Macular Degeneration

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INVESTIGATION OF THE ROLE OF THE TLR3 INNATE IMMUNITY PATHWAY IN THE PATHOGENESIS OF AGE-RELATED MACULAR DEGENERATION

By

Amit K. Patel

A DISSERTATION

Submitted to the Faculty of the University of Miami in partial fulfillment of the requirements for the degree of Doctor of Philosophy

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INVESTIGATION OF THE ROLE OF THE TLR3 INNATE IMMUNITY PATHWAY IN THE PATHOGENESIS OF AGE-RELATED MACULAR DEGENERATION

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Major factors in the pathogenesis of age-related macular degeneration (AMD) include dysregulated innate immunity, inflammation, and elevated oxidative stress. Abnormalities in toll-like receptor (TLR) signaling, mediators of innate immunity, have recently been implicated in the progression of the disease. Several reports show that TLR3 activation leads to retinal cell death, but other studies indicate that TLR3 has cytoprotective activity in different contexts. However, how TLR3 signaling behaves during oxidative stress, or the exact conditions in which TLR3 signaling is protective or pathogenic are still not known. This thesis examines how TLR3 activation during oxidative stress regulates RPE and photoreceptor viability and function. I demonstrated that TLR3 signaling increased RPE and photoreceptor survival, protected against loss of photoreceptor function, and increased overall visual acuity during oxidative injury to the retina, whereas TLR3 activation in the absence of injury was toxic. Furthermore, I showed that the protective effects of TLR3 during injury were mediated by Stat3 pathway activation. Knockdown of Stat3 signaling eliminated the protective effect of TLR3 during oxidative stress and exacerbated retinal degeneration, indicating that Stat3 activation controls whether TLR3 activation
results in protective or pathogenic behavior. Overall, the findings of this dissertation indicate that TLR3 activation in the context of injury is protective via STAT3, indicating that combining inflammatory pathways and oxidative stress triggers protective instead of pathogenic signaling. This study identifies TLR3 as a potential novel therapeutic strategy for AMD, retinal degenerations, and other diseases of the central nervous system in which oxidative stress is a major contributor.
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List of Abbreviations

AMD: Age-related macular degeneration
ERG: Electroretinography
GCL: Ganglion cell layer
GFP: Green fluorescent protein
INL: Inner nuclear layer
IPL: Inner plexiform layer
IS/OS: Inner and outer photoreceptor segments
OCT: Optical coherence tomography
ONL: Outer nuclear layer
OPL: Outer plexiform layer
ROS: Reactive oxygen species
RPE: Retinal pigmented epithelium
PQ: Paraquat
Stat3: Signal transducer and activator of transcription
TLR: Toll-like receptor
Chapter 1: Introduction

1.1. The eye and retina

The eye is a sensory organ that responds to light, ultimately resulting in the sensation of vision. Vision occurs through the transformation of light energy into electrical signals, which are primarily converted by the retina. The retina is part of the central nervous system and is a multilayered tissue that is located in the back of the eye (Figure 1.1). The retina is composed of several layers, including three layers of nerve cell bodies and two layers containing synapses. The three nerve cell body layers are the outer nuclear layer, inner nuclear layer, and the ganglion cell layer, which contain photoreceptors, bipolar cells, and retinal ganglion cells, respectively. Photoreceptor cells are specialized sensory neurons located in the outermost layer of the neural retina and are the initial responders to light (Kolb et al 1995). The primary retinal circuitry is formed by photoreceptors synapsing with bipolar cells which, in turn, synapse with retinal ganglion cells. The axons of the retinal ganglion cells extend all the way to the brain for higher order processing, which results in vision. The inner nuclear layer of the retina also contains horizontal and amacrine cells, which serve as interneurons and act to integrate visual information carried by the primary chain of synapses. In addition to neuronal cells, the retina contains cells that are critical to the neuronal health and maintenance of neuronal signaling. These cells include astrocytes, microglia, Muller glia, which span across the entire retina, and retinal pigmented epithelial cells (RPE), which are in the outermost layer of the retina adjacent to the photoreceptors.
There are two major types of photoreceptors cells: rods and cones. Rod photoreceptor cells are the most numerous (95% of photoreceptor cells) and are primarily active in low-light conditions. In contrast, cone photoreceptor cells (5% of photoreceptor cells) require more photons to achieve the threshold for activation and therefore are generally active in bright light conditions. Cones are also responsible for color vision and high acuity vision. Photoreceptors detect light through opsin molecules located in their outer segments. Rod photoreceptors contain the opsin molecule, rhodopsin, while cone photoreceptors contain one of three different kinds of opsins, each detecting a specific wavelength of light, resulting in color vision (Kolb et al 1995). Light is converted into electrical signals in a process called the visual phototransduction cycle, which starts in the photoreceptor cells. In the absence of light stimulation, cation channels on the photoreceptor membrane are continuously open and create a “dark current”, which leads to depolarized photoreceptors and constant inhibitory neurotransmitter release (Stryer 1991). Opsin molecules undergo a conformational change that triggers the closure of cation channels upon stimulation by photons of light. Loss of cation influx stops the dark current and leads to hyperpolarization of the photoreceptor cell membrane. The hyperpolarization of the photoreceptors stops the release of inhibitory neurotransmitters, leading to activation of bipolar cells, which, in turn, activates retinal ganglion cells, leading to vision.
The relative simplicity of the retinal circuitry allows for easy assessment of retinal cell health and function through the use of electroretinography (ERG), which measures field potentials generated by cells of the retina (Aung et al 2013). Vision can be assessed at the behavioral level using the optokinetic reflex (Tabata et al 2010). Visual information from moving objects is carried to the brain, which forms a reflex arc with muscles surrounding the eye. The reflex leads to involuntary eye movement in pursuit of a moving object. The optokinetic

\[\text{Figure 1.1. Schematic of the human eye and retina.}\]

Light enters the eye through the cornea and is focused onto the retina by the lens. The retina is a multilayered tissue composed of several different populations of neuronal cells. Light travels to the outermost neural layer of the retina and activates opsins molecules in the outer segments of the photoreceptor cells. Visual information is carried down the retinal circuitry, from photoreceptors to bipolar cells to ganglion cells. The axons of the ganglion cells form the optic nerve and carry the visual information to the brain for higher order processing.
reflex exam tests the integrity of the entire reflex arc including photoreceptor function. I used ERGs and optokinetics in my thesis work, as described in the following chapters.

1.2. The retinal pigmented epithelium

The RPE is essential for maintenance of the retina. The RPE is a layer of tightly packed, interconnected epithelial cells that is located between the photoreceptor layer of the retina and the choroidal blood supply. The apical ciliary processes of a single RPE cell ensheath several photoreceptor outer segments (Snodderly et al 2002).

The RPE cells provide crucial support and maintenance functions for the photoreceptors (Strauss 2005). These functions include phagocytosis of photoreceptor outer segments, supply of nutrients to the retina, maintaining pH balance, maintaining the visual cycle, and removal of reactive oxygen species (ROS). Opsin molecules that are converted during phototransduction are shed by the photoreceptors and phagocytized by the RPE. The RPE contains enzymes that recycle opsin molecules back to their original configuration and transport them back to the photoreceptors for reuse, which maintains the visual cycle. The RPE also plays a critical role in forming the blood retinal barrier and is essential for the transport of nutrients and oxygen to the retina and uptake of retinal waste byproducts. Dysfunction or death of the RPE cells leads to photoreceptor cell death (Marmorstein et al 1998). Genetic models of RPE
dysfunction have shown that loss of proper RPE function directly leads to photoreceptor apoptosis (Strauss 2005).

The blood-retinal barrier created by the RPE also results in the retina having immune privilege. The tight junctions of the RPE form a mechanical barrier to block immune cells from the blood stream from entering the inner eye. The RPE also actively inhibits immune cells through surface ligands and secretion of cytokines (Kim et al 2009, Relvas et al 2009). The RPE itself serves as an immune regulatory mechanism for the retina. The RPE expresses receptors for many immune signaling factors, such as MHC receptors, toll-like receptors, and tumor necrosis factor receptors (Jorgensen et al 1998, Kindzelskii et al 2004, Oh et al 1999). Furthermore, the RPE secretes immune modulatory factors that include complement factor H and interleukins (Kim et al 2009, Relvas et al 2009). The immune regulatory properties of the RPE are of special interest due to the association of several retinal diseases with abnormal immune activity.

1.3. Retinal degenerations and age-related macular degeneration

Retinal degenerations are a collection of diseases characterized by progressive loss of photoreceptors, which leads to vision loss and ultimately blindness. One of the leading causes of visual impairment is age-related macular degeneration (AMD). This disease results in the loss of RPE and photoreceptor cells beginning in the macula region of eye and leads to progressive loss of central vision. The incidence of AMD increases with age. About 30% of the population, between 65-85 years of age, will have various degrees of AMD (O’Neill et al
The onset of AMD is characterized by the appearance of drusen, which are deposits of extracellular matrix protein, between Bruch’s membrane and the RPE in the macula and loss of RPE and photoreceptor cells (Kolb et al 1995). AMD progresses in two stages, the “dry” form and the more advanced “wet” form. Dry AMD, also known as geographic atrophy, is characterized by RPE and retina dystrophy and accounts for 90% of AMD cases. The remaining 10% of AMD patients suffer from wet AMD. In wet AMD, also known as exudative AMD, there is also development of choroidal neovascularization and fibrovascular disciform scarring (Kulkarni & Kuppermann 2005, Morris et al 2007).

Although there are therapeutic methods to the alleviate progression of wet AMD, there are currently no treatments for dry AMD. This is most likely due to the lack of understanding about the underlying causes of AMD development and progression. The initial pigmentary changes in the macula of eyes with AMD indicate that initial RPE dysfunction is most likely the first factor in AMD pathogenesis (Ali et al 1996, Barton et al 2004). The close interactions between the RPE and photoreceptors result in concomitant photoreceptor degeneration with RPE death. The exact mechanisms that lead to RPE dysfunction and photoreceptor death during AMD have not yet been ascertained. Several risk factors for AMD have been identified including genetics factors, cigarette smoke, light toxicity, oxidative stress, and, more recently, abnormal immune activity (Anderson et al 2002, Cai et al 2000, Chen et al 2010). However, it is still unknown how these risk factors intersect to induce pathogenesis of disease.
This thesis will examine how two of the most common AMD risk factors, innate immunity and oxidative stress, interact to regulate pathogenesis of AMD.

1.4. Oxidative stress in AMD

Oxidative stress is thought to be one of the key players in the development of AMD because the high oxygen concentration in the retina leaves it susceptible to the formation and propagation of reactive oxygen species (ROS) (Cai et al 2000, Ding et al 2009, Wong et al 2011). ROS are the main sources of oxidative stress and are usually byproducts of normal cellular processes. ROS are mainly generated during cellular respiration in the mitochondria, especially in oxygen heavy areas like the retina (Cai et al 2000). In normal retina tissue, ROS are usually negated through various antioxidant enzymes, such as superoxide dismutase and vitamin C, which are contained within the RPE. The balance between ROS formation and elimination becomes shifts towards ROS formation during the process of aging, which leads to cellular damage (Junqueira et al 2004). Many studies have shown that aging decreases the function of antioxidant systems and increases ROS induced damage to tissues (Cai et al 2000, Junqueira et al 2004, Moreira et al 2005, Muller et al 2007, Shen et al 2007).

Oxidative stress damage is particularly important to the RPE. The RPE is exposed to high levels of oxygen and potential sources of ROS (Kennedy et al 1995). First, the RPE contributes to the blood-retina barrier and is exposed to high levels of oxygen during its transfer from the choroid to the retina (Strauss
In addition, RPE are also constantly phagocytizing photoreceptor outer segment membranes that are potential sources for oxidative stress (Cai et al. 2000, Kennedy et al. 1995). Studies that genetically or biochemically altered oxidative stress showed direct effects on RPE and photoreceptor survival. Exogenous induction of oxidative stress in the retina leads to retinal degeneration of photoreceptors and RPE (Cingolani et al. 2006, Lu et al. 2006). Furthermore, blocking oxidative stress leads to photoreceptor protection and increased RPE integrity (Bailey et al. 2004, Lu et al. 2009, Usui et al. 2009). Although the role of oxidative stress in the pathogenesis of AMD is still not well understood, recent studies suggest that oxidative stress-induced damage may lead to an inflammatory response and further progression of AMD (Hollyfield et al. 2008). However, how innate immunity and oxidative interact within the retina have not been examined together within a single model.

1.5. Innate immunity and AMD

Recently, inflammation and the innate immune system have been implicated as major players in the pathogenesis of AMD and other retinal degenerations (Detrick & Hooks 2010). Acute activation or low levels of inflammation can promote healing, whereas higher levels can lead to tissue damage and autotoxicity (McGeer & McGeer 2004). Chronic inflammation has been associated with a number of neurodegenerative diseases, such as Alzheimer’s disease (Galimberti & Scarpini). Activation of the complement system in AMD patients has been well described. The composition of drusen deposits in AMD eyes contains components of the innate immune complement system (Anderson
et al 2002). Immunolabeling of complement factors is more intense in the macula of the retina than in the peripheral region, indicating that possible complement activation would more strongly affect the macula which is the prime area of AMD pathogenesis (Anderson et al 2002, Prusky & Douglas 2003). Variants of the CFH gene, a key regulator of the complement system, have been associated with increased risk of AMD (Ding et al 2009, Klein et al 2005, Magnusson et al 2006, Postel et al 2006, Shuler et al 2007, Ufret-Vincenty et al 2010). One theory of how innate immunity plays a role in AMD pathogenesis is that the deposition of drusen, oxidative lipid, and cellular debris in the subretinal and sub-RPE space, triggers innate immunity activity (Anderson et al 2002, Kauppinen et al 2012). Inflammation from the initial immune reaction triggers RPE and photoreceptor cell death, which leads to more cellular debris deposition in the subretinal space. The cellular death in the retina escalates the inflammatory response and leads to progressive retinal degeneration and choroidal neovascularization. Evidence of innate immune activity in AMD patients has suggested that inhibition of innate immunity in the retina may be an effective treatment for prevention of AMD (Liu et al 2012, Okun et al 2010).

Recent research has found that while dysfunction or uncontrolled activation of innate immunity leads to AMD pathogenesis, low levels of activation of innate immunity is necessary for proper retinal maintenance and may lead to tissue regeneration. Knocking out complement components leads to increased pathogenesis of AMD-like features in animal models. Mice deficient of CCL2, which is a chemokine responsible for recruiting microglia and macrophage
mouse to inflamed sites, had increased retinal degeneration (Luhmann et al 2009, Ross et al 2008). These mice exhibited AMD-like retinal lesions, RPE dystrophy, increased drusen formation, and photoreceptor death, which are all hallmarks of AMD and other retinal degenerations. Direct knockout of complement proteins also resulted in significant retinal cell death. Knocking out C3a and C5a receptors in mice lead to early onset of progressive retinal degeneration (Lu et al 2009). Retinal degeneration occurred in a cell specific manner where retinal cell types that normally exhibited complement receptors had the largest amount of cellular defects, indicating that some level of complement signaling is important for retinal health. Furthermore, complement system activation can lead to retinal regeneration and injury repair mechanisms (Stone et al 2009). Activation of C3a receptors in retinal progenitor cells led to regeneration of the entire chick retina in the absence of exogenous growth factors which are usually necessary to induce regenerative functions. Therefore, inhibition of innate immunity may lead to increased retinal degeneration instead being an effective treatment for AMD (Haynes et al 2013). It is likely that regulated innate immune activity is important for maintaining retinal health and functions during disease and will be explored in this thesis.

1.6. Toll-like receptors

In addition to the complement system, recent studies suggest that toll-like receptor (TLR) signaling pathways, which are another major component of the innate immunity system, play a role in the development of AMD. There are several types of TLRs that respond to a range of different pathogen-associated
molecular patterns (PAMPs) (Zhang et al 2007). TLR pathway activation leads to the induction of NF-kB signaling, a major regulator of inflammatory signaling (Hajishengallis & Lambris 2010). The majority of these pathways act through the MyD88 adaptor molecule; however, a few TLRs, including TLR3, signal through the TRIF adaptor molecule and activate both the NF-kB and IRF3 signaling pathways (Fig 1.2.)(Chen et al 2008, Takeda & Akira 2004).

![Figure 1.2. Toll-like receptor signaling pathways](image)

Membrane bound TLRs recognize external ligands (exogenous and endogenous), while endosomal TLRs (TLR3) recognize intracellular signals. PAMP binding to TLR receptors induces activation of the MyD88 adaptor molecule, which triggers NF-kB signaling and cytokine production. TLR3 signals in a MyD88-independent manner, using a TRIF adaptor protein, and induces the expression of interferons (IFN) and IFN-inducible proteins in addition to NF-kB activation.
Recent studies have implicated TLR signaling in the initiation and progression of non-pathogen (sterile) tissue injury in addition to pathogen mediated injury. TLR activation in non-immune regulatory cell types, such as neurons and glia, was identified in stroke and ischemia models of brain injury (Lu et al 2014, Shichita et al 2012). Following damage to the spinal cord or brain tissue, TLR protein levels in neurons and glia increased in the affected regions (Letiembre et al 2009, Walter et al 2007). Furthermore, general knockdown of TLR2 and TLR4 resulted in increased neuronal survival in the brain and retina following neurodegenerative insults (Kilic et al 2008, Walter et al 2007, Yi et al 2012). However, the role of TLR signaling during disease is not clear, because, paradoxically, TLR signaling is protective in certain instances. TLR2 and TLR4 signaling following peripheral nerve injury also increased axonal regeneration by activating tissue repair and recovery pathways (Boivin et al 2007, Kigerl et al 2007).

Similar reports were found in the retina. Almost all cell types in the retina express TLR receptors, including RPE and photoreceptors (Kumar et al 2004). TLR4 activation in photoreceptors led to mitochondrial damage and cell death (Ko et al 2011, Yi et al 2012). TLR4 mediated microglial activation also resulted in degeneration of RPE and production of inflammatory cytokines, leading to photoreceptor cell death (Tseng et al 2013). Furthermore, TLR4 deficient mice showed increased protection against retinal ischemia reperfusion injury (Dvoriantchikova et al 2010).
Although TLRs play a role in disease progression, low levels of TLR activation are protective. Low level activation of TLR4 signaling within the retina protected retinal neurons against ischemic insult (Fischer et al 2009). Brief stimulation of TLR4 with lipopolysaccharide (LPS) reprogrammed TLR4 to induce tolerance against ischemic damage by preventing microglia activation (Halder et al 2013). Also, TLR4 activation prior to oxidative injury resulted in increased survival of photoreceptors cells, which was mediated by TNFα (Yi et al 2012). Similarly, low levels of TLR2 stimulation greatly attenuated retinal microglial induced inflammatory response, which led to decreased apoptosis of retinal neurons (Aung et al 2013). TLR3 induced IFN-β production in RPE cells is important for protection from excessive retinal inflammation through downregulation of inflammatory chemokines (Hooks et al 2008). Furthermore, proper TLR4 signaling plays a critical role in maintaining the physiological functions of the RPE, including the inhibition of apoptosis signaling pathways (Niu et al 2013). TLR4 also plays an integral role in visual function. It was shown that TLR4 is important in regulating transmembrane signaling of RPE and shed photoreceptor outer segments, which is essential for the recycling of visual proteins (Kindzelskii et al 2004). These contrasting effects of TLR signaling lead to the important question of what conditions regulate TLR signaling to be either pathogenic or protective.

1.7. The role of TLR3 in regulating retinal degeneration

While there are many studies that have examined MyD88-mediated TLR pathways during neuronal degeneration, the role of TLR3 signaling during
neurodegenerative disease remains to be explored. TLR3 serves as an innate immune sentinel for viral infection and is activated by dsRNA binding, leading to dimerization of TLR3 and activation of the adaptor protein TRIF, resulting in interferon and cytokine production (Alexopoulou et al 2001).

TLR3 may play a critical role in regulating cell survival during AMD. TLR3 is expressed in the retina with the RPE having the highest level of expression (Kumar et al 2004). Additionally, RPE have TLR3 receptors on their cell surface, unlike most other cell types that express TLR3 endosomally (Kleinman et al 2012, Kumar et al 2004). Furthermore, TLR3 activation regulates RPE and photoreceptor cell death in the retina (Kleinman et al 2012, Patel & Hackam 2012, Shiose et al 2011). Therefore TLR3 is an excellent candidate to examine during the pathogenesis of AMD.

1.8. Research objective and hypothesis

Recent clinical observations have shown that the innate immune system and inflammatory pathways play a role in AMD and other retinal degenerations. However the exact role of innate immunity in disease progression is not clear. There is evidence for innate immunity inducing both pathogenesis and protection during retinal disease. One possibility for the conflicting roles of innate immunity in retinal disease may lie in the cellular conditions in which innate immunity is stimulated. Interactions between innate immunity and other disease pathways may stimulate a protective response rather than pathogenesis. The overall goal of this thesis is to examine how TLR3 mediated innate immunity regulates retinal
cell survival and function in the presence and absence of AMD-like oxidative injury and to identify a mechanism through which TLR3 regulates retinal survival. The overall hypothesis of this thesis is that TLR3 signaling activates survival pathways leading to increased RPE and photoreceptor activity and viability during the AMD-like oxidative injury. The results of this dissertation show that TLR3 activation is pathogenic in the absence of injury but protective during oxidative damage. Furthermore, I identified the Stat3 signaling pathway as a key mechanism of TLR3 induced regulation of retinal survival and function during AMD conditions. This is the first study to show a protective role for TLR3 signaling in the retina during non-pathogen mediated injury. This thesis provides the first step in identifying the conditions that regulate TLR3 during disease and advance our understanding of the underlying causes in the pathogenesis of AMD.
Chapter 2. Materials and methods

2.1. Animals

Non-degenerating wild-type control mice (strain B6;129SF2/J) and TLR3 knockout mice (strain B6;129S1-Tlr3tm1Flv/J) were used in this study and were purchased from Jackson Laboratories (Bar Harbor, Maine). The TLR3 knockout mice used in this study have a targeted mutation in the gene encoding TLR3, resulting in the production of a truncated non-functional protein (Alexopoulou et al 2001). All procedures that involved mice were conducted following the ARVO Statement for the Use of Animals in Ophthalmic and Vision Research and were approved by the Animal Care and Use Committee at the University of Miami, protocol numbers 10-078 and 13-057.

2.2. Primary mouse RPE cultures

Eyes of wild type and TLR3 knockout mice were enucleated at age postnatal day 21 to generate primary RPE cultures (Patel & Hackam 2012). A total of 10 animals (20 eyes) were used for each experiment. The anterior chamber, lens, and retina were removed, leaving the RPE intact in the eyecup. The eye cup was filled with 0.25% trypsin-EDTA (Cellgro, Manassas, VA) for 1 hour and the RPE was released from Bruch’s membrane of the choroid and eyecup with gentle shaking and aspiration and were harvested for culture. Primary RPE cells were plated at a density of 15,000 cells/ml in 96 well plates and maintained in DMEM/F12 media (Cellgro, Manassas, VA). Media was also supplemented with essential amino acids (Invitrogen, Carlsbad, CA), 10% fetal bovine serum.
(Hyclone, South Logan, UT), 100U/ml of penicillin and 100μg/ml of streptomycin (Cellgro, Manassas, VA), and 1x fungizone (Invitrogen, Carlsbad, CA). Cell cultures were maintained in 5% CO₂ at 37°C.

2.3. ARPE-19 cell line

The ARPE-19 cell line (Dunn et al 1996) was purchased from ATCC (Manassas, VA). Experiments using ARPE-19 cells were conducted at low passage number at sub-confluent density (Patel & Hackam 2012). Cell cultures were maintained using DMEM/F12 media that was supplemented with 10% fetal bovine serum, 100U/ml of penicillin and 100μg/ml of streptomycin. Cell cultures were maintained in 5% CO₂ at 37°C. Cells were passaged every 3 days and were not used after 5 passages.

2.4. TLR3 and oxidative injury stimulation

TLR3 signaling was induced using polyinosinic: polycytidylic acid (Poly (I:C)). Poly (I:C) is a synthetic double stranded RNA used as a prototypic activator of TLR3 (Alexopoulou et al 2001, Kleinman et al 2012, Matsumoto & Seya 2008). Oxidative injury to RPE cultures was induced using 1,1'-Dimethyl-4,4'-bipyridinium dichloride, also known as paraquat (PQ) (Bus & Gibson 1984, Cingolani et al 2006). PQ generates propagating oxygen radicals in the mitochondria, and is commonly used as a model of oxidative injury in the retina and neurons.

TLR3 was activated using 100 μg/ml of Poly (I:C) and oxidative stress was induced using 0.8-1.6 mM PQ in cell cultures; which is within in the concentration
range used by other studies (Fragoso et al 2012, Shiose et al 2011). TLR3 and oxidative stress was induced in the retina using 1µg of Poly (I:C) and 1 mM PQ respectively in the in vivo studies (Cingolani et al 2006, Shiose et al 2011). Both drugs were dissolved into 2 µl of PBS and subretinally co-injected into the retina.

2.5. Viability assays

Cell viability was assessed 24 hours following treatment to determine RPE survival following TLR3 stimulation and/or oxidative injury (Patel & Hackam 2012). RPE survival was quantitated using the Cell Titer Blue assay (Promega, Madison, WI). The cell titer blue assay is a modified MTT assay in which survival can be quantified two hours following addition of cell titer blue reagent to cells through fluorescence measured by an ELISA plate reader (excitation 530 nm, emission 590 nm). Untreated cells grown in normal media served as a control for normalization. Cells were grown in 96-well plates and treated for twenty four hours. Cells were washed with fresh media and incubated in a 1:5 mixture of Cell titer blue reagent to normal growth media for 2 hours before fluorescence was measured.

2.6. Immunohistochemistry

ARPE-19 cells were grown on precoated multi-well chamber slides (Nalge Nunc, Penfield, NY) and treated with a TLR3 activator and oxidative stress inducer. Twenty-four hours following treatment, cells were washed in 1x in PBS, then fixed in 4% paraformaldehyde. The slides were washed in 1x in PBS for 5 minutes following fixation and then permeabilized in 50% methanol/50% acetone
solution at -20°C and then washed again in 1x PBS for 5 minutes following permeabilization. The slides were incubated in primary mouse anti-p65 antibody (1:100 dilution, Cell Signaling Technology, Beverly, MA) or rabbit anti-phosphorylated Stat3 (1:100 dilution, Cell Signaling Technology, Beverly, MA) diluted in 0.5% Triton-X100 in PBS. Primary antibody incubation was overnight at 4°C and then washed three times in 1x PBS for 5 minutes each. The slides were then incubated in secondary goat anti-mouse Alexa 488 and goat anti-rabbit Alexa 546 antibody (1:600 dilution, Molecular Probes, Carlsbad, CA) at room temperature for 45 minutes and then washed three times in 1x PBS prior to mounting. The coverslips were mounted using 10% glycerol in PBS containing 4',6-diamidino-2-phenylindole (DAPI, 1:1000 dilution) to counterstain for cell nuclei and were imaged using a fluorescent microscope (Zeiss Axiovert 200). Similarly, retinal sections were stained using rabbit anti-TLR3 antibody (1:100 dilution, Abcam, Cambridge, MA).

Control slides that omitted the primary antibody incubation step were used to verify the specificity of antibodies. Microscopic and photographic settings, including fluorescence exposure times, were kept constant between antibody and control staining for comparison.

2.7. siRNA and shRNA knockdown

TLR3, RIG-1, and STAT3 were knocked down in ARPE-19 cell cultures using the Silencer siRNA AM1640 Kit (Ambion, Carlsbad, CA). siRNA was delivered to cells using Lipofectamine 2000 reagent (Invitrogen, Carlsbad, CA) at a
concentration of 2 µM. Cells were incubated in transfection reagent for 5 hours using serum free OptiMEM media (Cellgro, Manassas, VA) and were then washed in normal growth media twice and allowed to grow for another 20 hours following transfection before being treated by the various drugs used in this study. The sequences for the siRNAs are listed in Table 1 (Table 2.1.). A scrambled siRNA was used as a control for any effects that siRNA introduction into the cells may have on cell viability. TLR3, Rig-1, and Stat3 knockdown efficiency was verified by Western blotting and QPCR of transfected cells (see methods below).

In vivo knockdown of Stat3 was conducting using Stat3 specific shRNA in lentivirus and was delivered to the retina using subretinal injections (see below). Lentivirus was used at a titer of 2 x 10^8 IFU/ml. shRNA constructs were provided by Dr. Denise Hilfiker-Kleiner and Michaela Scherr from Hannover Medical School (Haghikia et al 2011) and were packaged into the virus at the Miami Project to Cure Paralysis Viral Vector Core. Scrambled shRNA in lentivirus was used as a control for any effects of viral infection or shRNA. All lentivirus used in the experiments in this thesis also co-express green fluorescent protein (GFP).
2.8. Western Blot analysis

Cells and retinas were incubated in lysis buffer (50 mM Tris, pH7.4, 150 mM NaCl, 1% NP40, 0.05% SDS) and were homogenized by vigorous pipetting (Fragoso et al 2012, Patel & Hackam 2012). Proteinase and phosphatase inhibitor cocktails were added to prevent protein degradation. Twenty microliters
of cell or retina lysate were loaded in a 4-12% gradient sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) gel. Resolved proteins were transferred to polyvinylidene fluoride (PVDF) membranes at 15 V for 90 minutes using a semi-dry transfer system (Biorad, Hercules, CA). The membranes were blocked using Rapid Block blocking solution (Amresco, Solon, OH) for 10 minutes and were probed using primary antibodies against TLR3 (1:100 dilution, Abcam), phosphorylated STAT3 (1:200 dilution, Cell Signaling), total STAT3 (1:200 dilution, Cell Signaling), and β-actin (1:8000 dilution, Sigma Aldrich), each diluted in Rapid Block buffer. The membranes were incubated in primary antibody overnight at 4°C and were then washed three times in tris-buffered saline with 0.1% tween-20 followed by incubation in anti-rabbit and anti-mouse secondary antibodies conjugated with horse radish peroxidase (HRP) enzyme (1:1000 dilution, Santa Cruz, Dallas, TX) diluted in Rapid Block solution. Secondary antibody incubation lasted for 1 hour at room temperature. The protein bands were visualized through chemiluminescence using either SuperSignal West Femto maximum sensitivity substrate kit (Thermo Scientific, Waltham, MA) or LumiGLO chemiluminescent substrate system (KPL, Gaithersburg, MD) and imaged using a Fujifilm LAS 4000 imaging system. The density of the protein bands were quantified using NIH Image J (Abramoff et al 2004). Band densities were normalized using β-actin density to correct for loading differences.
2.9. Subretinal injection

Subretinal injections were performed in adult wild type and TLR3 knockout mice (age 8 weeks, both sexes). Mice were anaesthetized using a ketamine (1.5 mg/0.1 ml) and xylazine (0.3 mg/0.1 ml) cocktail delivered through intraperitoneal injection (0.2 ml/20 g of body weight). The eyes were locally anesthetized using 1 drop of Proparacaine Hydrochloride Ophthalmic Solution (0.5%, Akorn, Lake Forest, IL). A small incision was made in the conjunctiva and sclera of the eye exposing the subretinal space and a 1.5 cm 33-gauge Hamilton needle (Hamilton Company, Reno, NV) was inserted between the RPE and retina. Poly (I:C) was used to induced TLR3 signaling in the retina and oxidative stress was induced using paraquat (PQ). The mice were injected in one eye with 2 µl of PBS, Poly (I:C) (1 µg), and/or paraquat (1mM), all dissolved in 2 µl PBS. The fellow eye was used as an uninjected control. Injections of drugs led to temporary bleb formation in the subretinal space and retinal detachment that rapidly resolved, which is typical of this type of injection technique. The distribution of injected compounds was verified by subretinal injection of lentivirus expressing GFP (2 x 10^8 IFU/ml). GFP expression across the retina was visualized using a confocal laser scanning ophthalmoscope (Heidelberg Engineering, Carlsbad, CA).

2.10. Optical coherence tomography

In vivo imaging of the mouse retina was conducted using a spectral domain optical coherence tomography (SD-OCT) system (Bioptigen, Research Triangle
Mice were anesthetized using a ketamine/xylazine cocktail delivered through intraperitoneal injection and were placed on a stage with the body of the animal wrapped in a heating blanket. Eyes were dilated with topical phenylephrine and kept moist using artificial tears (Systane, Alcon, TX). Scans were centered on the optic disk and consisted of 100 × 100 (horizontal × vertical) depth scans. A total volume of $1.3 \times 1.3 \times 1.56 \text{ mm}^3$ of the mouse retina was imaged. The average photoreceptor layer thicknesses across retinas were quantified by measuring the thickness of the outer nuclear layer and inner and outer segments of the photoreceptors. The measurements of retinal layer thickness were obtained through segmentation of the OCT images using
MATLAB software and programs developed by the Ophthalmic Biophysics Center at the University of Miami.

2.11. Electroretinography

Mice were dark adapted for 4 hours prior to electroretinography (ERG) and were then anaesthetized using a ketamine/xylazine cocktail and placed on a stage, with the body of the animal wrapped in a heating blanket at a continuous temperature of 37 °C. Eyes were dilated with topical phenylephrine (10%, Akorn, Lake Forest, IL) and kept moist with application of artificial tears (Systane, Alcon, TX) to prevent drying of the cornea. A grounding electrode was placed in the tail of the mouse and a reference electrode was placed under the skin of the forehead between the eyes to normalize recordings (Frantz et al 2001). Silver wire electrodes were placed on the corneas of mice. Following electrode connections, the stage was inserted into a Ganzfeld light emitting chamber modified for use in small animals (Figure 2.2).
Flash intensity and timing and response recording was conducted using the UTAS system controlled by EM for Windows software (LKC Technologies, Gaithersburg, MD). Mice were exposed to flashes of white light ranging from 0.01 to 10 cd·s/m² in the scotopic and photopic range. For photopic recordings, green flashes were used in the presence of a green background light intensity of 1 cd/m². Both eyes were recorded simultaneously. Ten 250 µs flashes per intensity were averaged and recorded with an interstimulus time of 5 sec between each flash.

ERG a-wave and b-wave amplitudes were calculated using maximum and minimum points from the ERG waveform. The a-wave amplitude was obtained...
from the lowest voltage to point to the baseline voltage between 0-50 ms of time following the flash. The b-wave is calculated as the total voltage difference between the minimum point between 0-50 ms and the maximum peak between 30-100 ms.

2.12. Optokinetic reflex eye exam

Mice were placed on a raised platform in the center of a chamber surrounded by four monitors displaying an optokinetic stimulus under photopic conditions (Pearson et al 2012). The mice were given time to adjust to the platform before beginning the optokinetic exam. The optokinetic exam was performed by using an optokinetics apparatus that rotating sinusoidal gratings with alternating white and black color, as described in Prusky et al (Prusky & Douglas 2003). The optokinetic stimulus was made using four computer monitors bordering the chamber (Figure 2.3). The monitors displayed continuous optokinetic sine wave gratings of decreasing thickness with alternating black and white stripes that rotated in either a clockwise or counterclockwise direction. The stripe rotation direction was changed every 30 seconds for a total of 6 changes per stripe thickness. Stripe thickness was decreased stepwise by a factor of 2 until the animal could no longer track the movement of stripes. Mice were scored on vision based on whether the mice tracked the direction of stripe movement with their head and upper body. Each stripe thickness was converted to relative spatial frequency (Equation 2.1). Visual acuity was defined as the highest spatial frequency yielding a head turning behavioral response from the mouse. Two
observers monitored the movement of the animals, and the observers were masked to the identity of the treatment given to each animal.

2.13. Statistical Analysis

Analysis of variance (ANOVA) with appropriate post-hoc analysis and Student’s t-test were used for statistical analysis. P values less than 0.05 were regarded as significant. Regression and correlation analysis was conducted and reported as a Pearson correlation coefficient.

**Figure 2.3. Setup of the optokinetic exam**

The optokinetic reflex eye exam chamber was built using four computer monitors arranged to display a continuously rotating sinusoidal grating that alternates between white and black color. The mouse is place on the stage in the center of the chamber and is given time to adjust before stripes are displayed. Animals will track the rotation of the stripes with movement of their head and body in the direction of the stripe rotation. Stripe thickness is sequentially decreased until mice can no longer track the rotation of the stripes, which indicates their maximum visual acuity.
Spatial Frequency =

\[
\frac{2 \times \pi \times \text{distance of mouse from screen (cm)}}{360 \text{ deg}} \times \frac{\text{cycles}}{\text{cm}}
\]

**Equation 2.1. Equation for assessment of visual acuity by optokinetic examination**

The visual acuity of mice was measured as spatial frequency, which was calculated by the number of sinusoidal grating cycles present in each degree of vision. One cycle is equivalent to the width of the black and white stripe together. Visual acuity is equivalent to the highest spatial frequency that can be seen by the animal (Pearson et al 2012).
Chapter 3: The role of TLR3 signaling during oxidative injury in the RPE

There have been many studies that separately examine the roles of innate immunity and oxidative stress in cellular death. However there is no study that has examined how innate immune signaling and oxidative stress interact to regulate cellular survival. The research objective of this chapter is to identify how the TLR3 innate immune receptor regulates survival of cells that affected in AMD, focusing on the RPE. This objective is important because the pathogenesis of several retinal diseases, including AMD, begins with RPE dysfunction. Several studies have shown that TLR3 activation is detrimental to RPE survival (Kleinman et al 2012, Shiose et al 2011). This chapter will examine how TLR3 signaling regulates RPE survival during oxidative injury, which is another major risk factor for the development of AMD.

3.1. Verification of primary mouse RPE cultures and ARPE-19 cell line

I first used mouse primary RPE cultures and the ARPE-19 cell line as model systems to test whether TLR3 plays a role in regulating RPE cellular survival during oxidative injury. The use of in vitro cell cultures allows a reductionist approach for discovering how TLR3 influences cellular survival in a relatively simple environment, free from possible confounding interactions from other retinal cell types. Mouse primary RPE cells were derived from non-degenerating wild-type control mice and from TLR3 knockout mice. The purity of primary RPE cultures was verified by morphology and by the presence of RPE gene expression and absence of neuronal and glial cell marker genes, which was
measured by QPCR (Figure 3.1). Primary RPE cells displayed visible melanin pigment in the cytoplasm and had similar morphology to primary RPE cultures generated by other groups (Ramo et al 2008). Furthermore, the primary cultures were enriched in RPE65, an RPE specific protein responsible for recycling opsin molecules used during visual phototransduction, and tyrosinase, an enzyme controlling melanin production.

![Figure 3.1. Verification of primary mouse RPE cultures.](image)

A representative image of wild type mouse RPE primary cells is shown. After 5 days in culture the primary cells display pigmentation and typical RPE preconfluent morphology (left, 20× magnification, scale bar represents 100 μm). Verification of culture purity was confirmed by PCR amplification of RPE cell markers RPE65 (112 bp) and Tyrosinase (276 bp) and the photoreceptor marker rhodopsin (315 bp). RPE primary cultures are enriched for RPE markers and do not express other retinal cell markers.

One limitation of primary RPE cells is that a large number of animals are required to generate enough RPE cells for a single experiment. Therefore, I also utilized the ARPE-19 cell line. The ARPE-19 cell line is a non-transformed cell
line derived from adult human RPE (Dunn et al 1996). ARPE-19 cells share many properties with RPE cells in vivo, including phagocytic activity, tight junction formation, polarization, immunologic responses.

3.2. TLR3 signaling protects RPE cells from oxidative injury

TLR3 was activated by Poly (I:C) during oxidative stress conditions that were simulated using PQ in both primary mouse RPE and ARPE-19. The cells were treated with the drugs for 24 hours followed by measurements of cell viability using the Cell Titer Blue viability assay. Poly (I:C) treatment combined with PQ significantly increased RPE viability in both the primary culture (Figure 3.2) and the cell line (Figure 3.3). PQ treatment alone in primary RPE cells lead to a 40% reduction in primary RPE cell viability and a 60% reduction in ARPE-19 cell viability, compared with viability of untreated cells. The differences in the amount of cell death due to PQ treatment between the primary RPE cells and the cell line is most likely due to inherent differences in tolerance of oxidative stress. The combination of Poly (I:C) and PQ increased cell viability by 50% in both the RPE primary culture and cell line, indicating that TLR3 signaling induces robust protection during oxidative stress injury in the RPE. Interestingly, Poly (I:C) treatment on its own decreased ARPE-19 viability by 25% compared with untreated cells, similar to other studies (Kleinman et al 2012, Shiose et al 2011).
Figure 3.2. Poly (I:C) protected wild type mouse primary RPE cultures from oxidative stress

Poly (I:C) significantly increased cell survival of primary RPE cultures obtained from wild type mice in the presence of oxidative stress compared with oxidative stress alone (n=3, *p<0.05). Cell viability was measured 24 hours after treatment began using Cell Titer Blue assay and was normalized to untreated cells. UT, untreated (growth media only); PQ, paraquat.
3.3. TLR3 signaling is required for Poly (I:C) induced protection of RPE during oxidative injury

Poly (I:C) is a double stranded RNA and, therefore, could activate any of the several dsRNA sensing pathways. Primary RPE cultures derived from TLR3 knockout mice, described in section 2.1.1., were used to verify that the protection induced by Poly (I:C) treatment during oxidative injury occurs through a TLR3 dependent pathway. Furthermore, TLR3 signaling was knocked down in the
ARPE-19 cell line using TLR3 specific siRNA. Loss of TLR3 signaling resulted in the abolishment of the protective effects of Poly (I:C) during oxidative injury, in both the primary RPE cultures and the ARPE-19 cell line (Figures 3.4 and 3.5). Furthermore, loss of TLR3 resulted in no change to cell viability when cells were treated by Poly (I:C) alone compared with untreated cells. Together, these results show the necessity of TLR3 signaling in Poly (I:C) induced RPE protection during oxidative injury. All further studies of RPE cells in culture will be conducted in the APRE-19 cell line due to similar effects in cell viability by Poly (I:C) treatment and oxidative stress between the cell culture models and the difficulty of generating primary mouse RPE cells.

ARPE-19 cells were transfected with scrambled siRNA as a control for the effects that siRNA transfection may have on cell viability. Control siRNA transfected cells showed similar changes in cell viability with respective treatments as the untransfected cells (Figure 3.5). Knockdown efficiency of TLR3 siRNA was verified using QPCR, which showed an 84% decrease in TLR3 RNA expression and a 46% decrease in TLR3 protein (Figure 3.6). RNA expression of TLR4 in TLR3 siRNA transfected cells was also examined to verify that siRNA was specific to TLR3. QPCR analysis of cDNA obtained from cells transfected with control scrambled siRNA or TLR3 specific siRNA showed no difference in TLR4 RNA expression, indicating that siRNA knockdown was specific to TLR3.
Figure 3.4. TLR3 signaling is required for Poly (I:C) induced protection during oxidative stress conditions

Poly (I:C) did not increase cell survival of primary mouse RPE cultures obtained from TLR3 KO mice in the presence of oxidative stress (n=3, *p<0.05). Cell viability was measured 24 hours after treatment began using Cell Titer Blue assay and was normalized to untreated cells. UT, untreated (growth media only); PQ, paraquat.
Poly (I:C) protection of APRE-19 cells during oxidative stress is TLR3-dependent. Poly (I:C) did not rescue cells from paraquat treatment when TLR3 was knocked down by siRNA. Control siRNA transfection still resulted in rescue of cell viability when treated with poly (I:C) and oxidative stress (*p<0.05, n=5). Cell viability was measured 24 hours after treatment began using Cell Titer Blue assay and was normalized to untreated cells. UT, untreated (growth media only); PQ, paraquat.
Figure 3.6. TLR3 siRNA significantly reduced TLR3 protein compared with control siRNA in ARPE-19 cells

Protein expression of TLR3 was reduced by 46%, measured by Western blotting using an anti-TLR3 antibody (p<0.05, n=3). Detection of β-actin was used as a loading control for normalization. Representative protein bands are shown (top).
I knocked down retinoic acid-inducible gene 1 (RIG-1) to further verify the specificity of Poly (I:C) induced protection to a TLR3 dependent mechanism. The RIG-1/MDA5 pathway is another dsRNA recognizing pathway that could potentially mediate Poly (I:C) induced changes in cell viability. However, knockdown of RIG-1 using siRNA showed no difference in viability between control siRNA transfected cells in each of the respective treatments, indicating that RIG-1 is not necessary for Poly (I:C) induced protection during oxidative stress (Figure 3.8) and that the effects of Poly (I:C) on RPE viability are mediated by TLR3 signaling.
Figure 3.8. Poly (I: C) did not regulate viability through RIG-1 signaling in ARPE-19 cells

RIG-1 siRNA transfected cells show no difference in cell viability compared with control siRNA transfected cells in each treatment. Knockdown of RIG-1 resulted in a modest increase in cell death from Poly (I: C) by 20% compared with untreated cells, similar to control transfected cells. Both control siRNA and RIG-1 transfections resulted in rescue of cell viability when treated with Poly (I: C) and oxidative stress, similar to untreated cells in figure 3.3 (*p < 0.05, n = 5) indicating that RIG-1 does not play a role in Poly (I: C) induced protection. ARPE-19 cell cultures were treated with Poly (I: C) and/or 0.8 mM paraquat for 24 h and viability was measured using Cell Titer Blue assay. UT, untreated (growth media only); PQ, paraquat.
3.4. Conclusions and significance

TLR3 has long been recognized as an innate immune sentinel for viral infections. However, this is the first work to present TLR3 as a mediator of cellular protection during non-pathogen mediated injury. The results reported in this chapter are also the first to look at how innate immunity and oxidative stress regulate cellular survival in a single model. While previous studies have shown the detrimental effects of TLR3 in the context of retinal degeneration, this is first study to demonstrate that TLR3 can be protective during oxidative injury conditions. The results shown in this aim offer new knowledge of how TLR3 has context specific activity and raise the interesting question of what factors promote TLR3 towards a cytoprotective function rather than pathogenic, which will be explored in chapters 5 and 6.
Chapter 4. The role of TLR3 in photoreceptor survival and function during AMD-like conditions

In the previous chapter, I explored the role of TLR3 in regulating RPE cellular survival during oxidative injury \emph{in vitro}. \emph{In vitro} studies are an excellent method to identify interactions between pathways in a simple controlled system. However, a major question that has yet to be examined is whether TLR3 regulates survival of neuronal cells, and how TLR3 behaves in a complex environment during injury. The research goals of this chapter are to identify how TLR3 activation in the retina regulates neuronal photoreceptor cell survival in the presence and absence of AMD-like oxidative injury \emph{in vivo}, and to determine its molecular mechanism of action.

4.1. Subretinal injection as a technique for delivering drugs to the retina

I used non-degenerating wild-type control mice and TLR3 knockout mice to identify the role of TLR3 in regulating photoreceptor survival during oxidative injury \emph{in vivo}. For details on the TLR3 knockout mice, refer to chapter 2.1.1.

I utilized the subretinal injection technique to induce TLR3 activation and oxidative stress in the retina. Subretinal injections directly apply compounds to subretinal space of the eye, the space between the RPE and photoreceptors (Figure 4.1) (Ali et al 1996, Martin et al 2002). Therefore, any compounds injected into this space will interact with photoreceptors and RPE. Furthermore, the subretinal space has immunoprivilege and typically has no inflammation.

**Figure 4.1. Schematic of the subretinal injection technique**

A small incision is made in the sclera of the eye and a needle is inserted into the subretinal space. Injection typically results in a mild temporary retinal detachment. Subretinally injected compounds primarily interact with the RPE and photoreceptor cells due to proximately of cells to injection site.

4.2. Verification of TLR3 expression and activation in the retina

TLR3 expression in the retina of mice was verified by the cellular localization of TLR3 in the retinal cells using immunohistochemistry. Immunodetection of TLR3 was observed throughout most retinal cell layers. In particular, there was significant localization in the photoreceptors, retinal ganglion cells, and RPE indicating a potential role for TLR3 signaling in these cell types (Figure 4.2). Immunohistochemistry on retinal sections obtained from TLR3 knockout mice or wild type retinal sections incubated in secondary antibody only showed no staining for TLR3, which confirms the specificity of the antibody used for detecting TLR3.
Figure 4.2. TLR3 is expressed throughout the mouse retina

TLR3 (green) showed prominent localization in photoreceptors and retinal pigmented epithelium in wild type mice (top). The specificity of the antibody was verified using TLR3 knockout mouse retinal sections (middle) and wild-type sections with only 2° antibody incubation (bottom). DAPI staining (blue) was used as a marker for cell nuclei. Scale Bar: 50 µm.
Lentivirus expressing GFP was subretinally injected into wild type mouse eyes to verify the distribution of compounds though the subretinal injection technique. The GFP fluorescence generated by the lentivirus was used as a marker for distribution and is assumed to have similar delivery kinetics as the other compounds injected in this study. GFP fluorescence was expressed in about one third of the retina one week following the subretinal injection, as imaged using a Heidelberg scanning laser ophthalmoscope (Figure 4.3), which indicates that subretinally injected compounds will likely have a large area of distribution across the retina as well.

Gene expression of typical markers of TLR3 signaling were examined to determine whether subretinal injection of Poly (I:C) is sufficient to induce TLR3 signaling in the retina. Injection of Poly (I:C) in the retina increased interferon regulatory factor 3 (IRF3) and interleukin 6 (IL-6) expression compared with vehicle control PBS injected retinas, at two weeks following injections (Figure 4.4). The expression of IRF3 and IL6 are lower than reported in other studies, which may be due to the timing of IL-6 and IRF3 measurement and suggest that TLR3 activation is sustained up to two weeks following injection. In contrast, subretinal injections of Poly (I:C) did not induce significant changes to IRF3 or IL-6 expression in TLR3 knockout mice. Together, these results demonstrate that Poly (I:C) induces TLR3 signaling in the adult mouse retina.
4.3. Distribution of compounds across the retina following subretinal injection

Lentivirus expressing GFP was subretinally injected into the retina. Fundus image shows a relatively normal looking retina following subretinal injection (left). GFP expression in the retina was measured by fluorescence using a confocal scanning laser ophthalmoscope (right). GFP expression in the retina was distributed across roughly 1/3 of the retina.
Figure 4.4. Subretinal injection of Poly (I:C) increased expression of downstream markers of TLR3 signaling

Confirmation of TLR3 activation in wild type retinas following subretinal injection of Poly (I:C) was verified using QPCR analysis of downstream TLR3 pathway molecules IL6 and IRF3. Poly (I:C) significantly increased IRF3 RNA expression (n=3-4, *p<0.05) while increases of IL6 expression approached significance. Injections of Poly (I:C) in TLR3 KO mice did not alter either IRF3 or IL6 expression. Expression of housekeeping gene ARP was used as a control for internal normalization.
4.3. Optical coherence tomography as a method of assessing photoreceptor survival

As described in chapter 1.1., the neural retina is stratified into several layers, each consisting primarily of only a few different types of cells. Furthermore, degeneration of the retina often occurs in a predictable pattern, which usually results in progressive thinning of the retinal layers. Therefore, quantifying the thickness of the different retinal layers is an accurate method of assessing survival of specific cell types of the retina. Measuring the thickness of the outer nuclear layer of the retina, which is the layer comprised of the cell bodies of the rod and cone photoreceptors (Figure 4.5), is a commonly used method of assessing photoreceptor cell survival in vivo (Ferguson et al 2012, Fischer et al 2009, Knott et al 2011).

Optical coherence tomography (OCT) is a technique used to noninvasively image the retina using the light scattering properties of the retinal layers. Layers containing nerve cell bodies exhibit less backscattering of light and appear as dark layers in the OCT image. Layers containing synapses, axons and dendrites of the retinal neurons have higher backscattering properties and appear white in the image (Figure 4.5). OCT is commonly used in clinical practices to track the progression of cellular degeneration, including photoreceptor death in AMD patients, where decreased retinal layer thickness closely correlates with reduced cellular survival (Witkin et al 2009). Recent advances in OCT technology has allowed the application of this technique to small rodents, including mice (Fischer et al 2009, Ruggeri et al 2007).
Figure 4.5. Retinal layers imaged using OCT correlate with retinal layers seen by histology

Images of retinal structure taken by OCT and histology show similar retinal layer thicknesses. Photoreceptor layer thickness is quantified by determining the outer nuclear layer (ONL) and inner and outer nuclear layer thicknesses (IS/OS). NFL: Nerve fiber/ganglion cell layer, RGC: retinal ganglion cell layer, IPL: Inner plexiform layer, INL: Inner nuclear layer, OPL: Outer plexiform layer, ONL: Outer nuclear layer, IS/OS: Inner and outer photoreceptor segments.
4.4. TLR3 activation protects photoreceptors from oxidative injury

In this study, the effect of TLR3 activation on photoreceptor survival during oxidative stress was assessed using OCT. TLR3 activation by Poly (I:C) in the presence of PQ-induced oxidative injury significantly increased photoreceptor layer thickness by 21% compared with eyes that were exposed to oxidative stress alone (Figure 4.6). Oxidative stress alone caused significant death to photoreceptor cells which was indicated by a 28% decrease in photoreceptor layer thickness. In contrast, TLR3 activation in the absence of injury leads to photoreceptor death, which was shown by a 14% loss in photoreceptor thickness and is consisted with reports from other studies (Kleinman et al 2012, Shiose et al 2011). These results indicate a context specific role of TLR3 signaling in regulating relating neuronal survival: TLR3 acts a pro-survival regulator during oxidative injury in the retina whereas it has a pathogenic role in the absence of injury.

4.5. Poly (I:C) induced photoreceptor survival during oxidative stress is TLR3 dependent

There are several receptors for double stranded RNA in mammalian cells in addition to TLR3. Therefore, TLR3 knockout mice were also injected with either PBS, Poly (I:C), PQ, or a combination of Poly (I:C) and PQ to verify that effects of Poly (I:C) occur through TLR3 dependent signaling. The changes in photoreceptor thickness shown in Figure 4.7 are consistent with the idea that Poly (I:C) induced photoreceptor survival during oxidative stress is TLR3 dependent.
dependent (Figure 4.7). The photoreceptor layer thickness of mice injected with Poly (I:C) during oxidative stress were no different than in mice injected with PQ alone, in contrast to the results seen in wild type mice. Injections of PQ resulted in similar loss of photoreceptor thickness in wild type and TLR3 knockout mice, which indicates that oxidative stress induced death does not operate through a TLR3 dependent mechanism (Figure 4.7). Furthermore, Poly (I:C) alone did not induce photoreceptor death in TLR3 knockout mice compared with PBS injected mice in contrast to the effects observed in wild type mice. Together, these results are most consistent with Poly (I:C) induced regulation of photoreceptor survival during oxidative stress being TLR3 dependent.

4.6. Electroretinography as a measurement of retinal function

It has been established that cells often undergo functional loss well before actual cell death occurs (Porciatti & Ventura 2012). Photoreceptor cells, like all neuronal cells, have electrical signaling functions. Photoreceptors are sensory neurons of the retina that begin the signaling cascade that converts light into electrical activity, ultimately resulting in vision.

Electroretinography (ERG) is a technique that measures the electrical responses of retinal cell types. ERGs are recorded as field potentials taken near the eye, generally by placing electrodes on the cornea. The relative simplicity of the retinal circuitry causes the ERG waveform to be stratified similar to the layers of the retina, with each peak pertaining to specific cell types (Figure 4.8).
Figure 4.6. TLR3 signaling during oxidative stress significantly increased photoreceptor layer thickness in wild type mice

*In vivo* imaging of retinal cell layers using optical coherence tomography (OCT) shows that TLR3 activation protects photoreceptors from oxidative stress damage in wild type mice. Heat maps showing the thickness across retina are shown (top). Colors that approach red indicate thicker areas of the retina while blue indicates thinning of the retina. Photoreceptor cell layer thickness was quantified by measuring the outer nuclear layer (ONL) and inner/outer segment layer (IS/OS) thickness using MATLAB software (bottom). OCT segmentation was conducted by outlining 70-80 cross-sectional images approximately 0.5-0.6 mm from optic disc. A statistical significance of $p<0.05$ is marked as * PBS vs Poly (I:C), # PBS vs PQ, and ## PQ vs Poly (I:C)+PQ . (n=7)
Figure 4.7. TLR3 signaling is required for Poly (I:C) induced protection of photoreceptors during oxidative stress

In vivo imaging of retinal cell layers using optical coherence tomography (OCT) shows that subretinal injection of Poly (I:C) in TLR3 KO mice did not protect photoreceptors from oxidative stress damage. Heat maps indicating thicknesses across retina are shown (top). Colors that approach red indicate thicker areas of the retina while blue indicates thinning of the retina. Photoreceptor cell layer thickness was quantified by measuring the outer nuclear layer (ONL) and inner/outer segment layer (IS/OS) using MATLAB software (bottom). OCT segmentation was conducted by outlining 70-80 cross-sectional images approximately 0.5-0.6 mm from optic disc. A statistical significance of p<0.05 is marked as # PBS vs PQ, ^ PBS vs Poly (I:C)+PQ, ** Poly (I:C) vs PQ, and ^^ Poly (I:C) vs Poly (I:C)+PQ. (n=5)
The first peak of the ERG waveform (a-wave) represents photoreceptor activity. The negative peak of the a-wave is indicative of a special property of photoreceptors, in which they become negatively polarized respective to the cellular environment in response to light stimulation. The second peak (b-wave) is primarily representative of the bipolar cells responding to photoreceptor synapses and, thus, is also an indirect measurement of photoreceptor activity.

The retina has two main types of photoreceptors, rods and cones. Rods are primarily active during dim light (scotopic) conditions. Cones are stimulated under well lit (photopic) conditions where rods have already saturated and no

**Figure 4.8. Schematic of a standard flash ERG waveform**

This figure shows a representative mouse scotopic (dark adapted) ERG waveform. The amplitude of the a-wave represents the rod photoreceptor response. The b-wave amplitude is a measurement of the bipolar cell response to the synapses generated by the photoreceptor cells. The b-wave is often used as a measurement of photoreceptor function as well.
longer detect light. Rods and cones can be measured independently of each other using ERG based on the conditions in which the eye is stimulated. Under dark adapted conditions, in which ERG recordings are conducted in the absence of light, light flashes will stimulate rod photoreceptors. In contrast, ERG recordings conducted in light adapted animals will elicit cone photoreceptor responses (Frantz et al 2001).

4.7. TLR3 activation protects neuronal function of the retina during oxidative injury

Retinal electrical responses from mice injected with either PBS, Poly (I:C), PQ, or a combination of Poly (I:C) and PQ were examined using ERG in both scotopic and photopic conditions to ascertain the role of TLR3 in regulating neuronal function during injury. My main finding is that Poly (I:C) increased electrical signaling in the retina during oxidative stress injury, which is consistent with the improvement in photoreceptor layer structure measured by OCT in the previous chapter (Figure 4.9).

Mice injected with PQ, to induce oxidative stress, had significant loss in electrical activity across all scotopic flash intensities with a maximum loss of 56% in a-wave amplitude and 58% in b-wave amplitude compared with control animals injected with PBS. In contrast, mice co-injected with Poly (I:C) and PQ had scotopic ERG responses that approached the amplitudes of the PBS injected control animals. Poly (I:C)+PQ injections resulted in an increase of 230% and 210% in a-wave and b-wave amplitudes compared with animals
injected with PQ alone. Interestingly, Poly (I:C) injection on its own led to a loss of scotopic ERG signal compared with control PBS injected animals, with a maximum loss of 36% in a-wave amplitude and 22% in b-wave amplitude. ERG recordings from injected animals under photopic conditions showed a similar trend as the scotopic ERGs (Figure 4.10). However, the differences in maximum ERG amplitudes in both the a-wave and b-wave between the different treatments were not as large as the differences seen in the scotopic ERG. This difference is most likely due to the low number of cone photoreceptors in the mouse retina.

4.8. Poly (I:C) induced protection of photoreceptor electrical function during oxidative stress is TLR3 dependent

ERG responses from TLR3 knockout mice injected with Poly (I:C) and PQ were analyzed to verify that Poly (I:C) induced photoreceptor functional protection during oxidative stress occurs through a TLR3 mediated pathway. The results from the ERG recordings from TLR3 knockout mice were consistent with the hypothesis that Poly (I:C) regulates photoreceptor functions through a TLR3 dependent mechanism. Scotopic and photopic ERG amplitudes of mice injected with Poly (I:C) were no different from control PBS injected mice (Figure 4.11). Furthermore, eyes injected with Poly (I:C) and PQ had the same decrease in a-wave and b-wave amplitudes as mouse eyes injected with PQ alone, indicating that TLR3 is required for Poly (I:C) induced functional protection.
Figure 4.9. TLR3 activation protected against the loss of scotopic photoreceptor function during oxidative stress in wild type mice

Representative scotopic ERG waves of WT mice injected with PBS, Poly (I:C), PQ, and Poly (I:C)+PQ stimulated at 1 cd·s/m² are shown (top). Poly (I:C)+PQ co-injected eyes had significantly higher a-wave (right) and b-wave (left) amplitudes compared with eyes injected with PQ alone. Injections of Poly (I:C) alone decreased maximum a-wave and b-wave amplitudes compared with PBS injected animals. A statistical significance of p<0.05 is marked as * PBS vs Poly (I:C), # PBS vs PQ, ** Poly (I:C) vs PQ, ^^ Poly (I:C) vs Poly (I:C)+PQ, and ###PQ vs Poly (I:C)+PQ. (n=6-7)
Figure 4.10. TLR3 activation protected against the loss of photopic photoreceptor function during oxidative stress in wild type mice

Poly (I:C) injected in the presence increases photopic ERG a-wave (right) and b-wave (left) amplitudes during PQ induced oxidative stress compared with animals injected with PQ alone. Poly (I:C) alone decreased Poly (I:C) alone decreased maximum a-wave and b-wave amplitudes compared with PBS injected animals. (n=6-7)
Figure 4.11. TLR3 signaling is required for Poly (I:C) induced protection of photoreceptor function during oxidative stress

Scotopic ERG recordings from TLR3 KO mice injected with Poly (I:C)+PQ were no different in a-wave (top right) and b-wave (top left) amplitudes compared with eyes injected with PQ alone. Injections of Poly (I:C) alone did not decrease a-wave and b-wave amplitudes compared with PBS injected animals. A similar trend in amplitudes was seen in ERG recordings of photopic a-wave (bottom right) and b-wave (bottom left) amplitudes, indicating that regulation of photoreceptor function by Poly (I:C) is TLR3 signaling dependent. A statistical significance of p<0.05 is marked as # PBS vs PQ, ^ PBS vs Poly (I:C)+PQ, ** Poly (I:C) vs PQ, and ^^ Poly (I:C) vs Poly (I:C)+PQ. (n=5-6)
4.9. The optokinetic reflex as a measurement of visual behavior

The overall vision of the injected mice was examined to assess whether TLR3 induced structural and functional protection during oxidative stress translated to improved visual behavior. The optokinetic eye movement reflex exam is a thoroughly characterized technique to detect the level of visual behavior (Pearson et al 2012, Tabata et al 2010). This visual exam utilizes the optokinetic reflex, which is an involuntary visual reaction to a moving stimulus. The optokinetic eye movement reflex exam has been adapted for use in small rodents. To assess visual acuity in mice, animals are placed in a chamber with a rotating optokinetic drum displaying vertical stripes of alternating black and white color (Figure 4.12). Vision is assessed based on whether or not the mouse can track the direction of rotation of the stripes. Stripe thickness is continually decreased until mice can no longer track the direction of stripe rotation, indicating the threshold of visual acuity. For more detailed methods, refer to Chapter 2.

Figure 4.12. Setup of the optokinetic exam

Mice were placed in center of the optokinetic chamber and were surrounded by rotating stripes of alternating contrast. Stripe thickness was sequentially decreased until the mouse could no longer track the direction of line rotation by head movement.
4.10. TLR3 signaling preserves mouse visual behavior during oxidative injury

TLR3 signaling resulted in significantly improved mouse visual acuity during oxidative injury (Figure 4.13). Control PBS injected wild type mice had a normal visual acuity of 0.49 cycles/deg (Pearson et al 2012). PQ injected animals had significantly reduced visual acuity, averaging at 0.10 cycles/deg. Poly (I:C) and PQ co-injected animals had an average visual acuity of 0.38 cycles/deg, significantly higher than animals injected with PQ alone. Interestingly, visual acuity of animals injected with Poly (I:C) alone also averaged at 0.38 cycles/deg, indicating that TLR3 signaling alone induces degeneration.

TLR3 specificity of Poly (I:C) induced changes to visual acuity in the presence and absence of oxidative injury was verified using injected TLR3 knockout mice (Figure 4.14). Visual acuity of TLR3 knockout mice injected with Poly (I:C) were similar to PBS injected animals, averaging at 0.48 and 0.49 cycles/deg respectively, indicating that degradation of vision by Poly (I:C) is TLR3 dependent. Furthermore, visual acuity of mice injected with Poly (I:C) and PQ was no different from mice injected with PQ, averaging at 0.12 and 0.15 cycles/deg, respectively. These results indicate that the structural and functional protection induced by TLR3 signaling during oxidative stress translate to improved visual behavior.
Figure 4.13. TLR3 activation protected visual behavior during oxidative injury in wild type mice

Poly (I:C)+PQ subretinally co-injected into wild type mouse eyes significantly increased visual acuity compared with mice injected with PQ alone. Visual acuity is quantified in terms of spatial frequency, a measurement of how well an animal can detect objects of different size (stripe thickness in the case of the optokinetic exam). A statistical significance of $p<0.05$ is marked as # PBS vs PQ and ## PQ vs Poly (I:C)+PQ. (n=5)
Figure 4.14. TLR3 signaling is required for Poly (I:C) induced protection of visual behavior during oxidative injury

Poly (I:C)+PQ subretinally co-injected into TLR3 KO mouse eyes did not have different average visual acuity compared with mice injected with PQ alone. Furthermore, eyes injected with Poly (I:C) did not have significantly different visual acuity compared with PBS injected animals. Visual acuity is quantified in terms of spatial frequency, a measurement of how well an animal can detect objects of different size (stripe thickness in the case of the optokinetic exam). A statistical significance of p<0.05 is marked as # PBS vs PQ, ^ PBS vs Poly (I:C)+PQ, ** Poly (I:C) vs PQ, and ^^ Poly (I:C) vs Poly (I:C)+PQ. (n=5)
4.11. Visual acuity correlates with photoreceptor thickness and function

The optokinetic reflex exam tests the integrity of the entire reflex arc, which includes photoreceptors, bipolar cells, retinal ganglion cells and muscles of the eyes and head (Pearson et al 2012). Correlation analyses between visual acuity of mice, assessed by the optokinetic reflex exam, and photoreceptor layer thickness, and function, was conducted to confirm that visual acuity can be used as a measurement to assess photoreceptor health.

Comparisons of photoreceptor thickness measured by OCT vs visual acuity in the mice showed high correlation between degeneration and vision loss (Figure 4.15). Animals injected with PBS clustered at the far right of the graph indicating that they had both high visual function and normal photoreceptor thickness, while animals injected with PQ clustered at the far left of the graph due to poor vision and a degenerated photoreceptor layer. The linear correlation between all animals had a correlation coefficient of 0.8193, indicating that decreased visual acuity predicts decreased photoreceptor thickness (n=20). Similar results were seen in correlations of photoreceptor function measured by ERG and visual acuity (Figure 4.16). Scotopic b-wave amplitudes and visual acuity had a correlation coefficient of 0.8263 and scotopic a-wave amplitudes and visual acuity had a correlation coefficient of 0.9101, indicating that high ERG amplitudes are predictive of higher visual acuity. Together, these results validate the use of the optokinetic reflex exam as a measure of visual function.
Figure 4.15. Correlation between OCT and optokinetics as measurements of retinal health

Mice subretinally injected with PBS, Poly (I:C), PQ, or a combination of Poly (I:C) and PQ, were examined using OCT and the optokinetic exam two weeks following injections. Individual points represent each mouse used in this analysis. Correlation between all the mice from all injections resulted in a correlation coefficient of 0.8193 and a linear equation of $y = 0.066x + 0.0664$. ($y =$photoreceptor layer thickness and $x =$ spatial frequency, $n=20$)
Mice subretinally injected with PBS, Poly (I:C), PQ, or a combination of Poly (I:C) and PQ, were examined using ERG and the optokinetic exam two weeks following injections. Individual points represent each mouse used in this analysis. Correlation between scotopic ERG b-wave obtained at 1 cd·s/m² and spatial frequency resulted in a correlation coefficient of 0.8263 and a linear equation of $y = 548.82x + 131.27$ (top). Correlation between scotopic ERG a-wave obtained at 1 cd·s/m² and spatial frequency resulted in a correlation coefficient of 0.9101 and a linear equation of $y = 257.42x + 31.514$ (bottom). ($y =$photoreceptor layer thickness and $x=$ spatial frequency, $n=20$)
4.12. Conclusions and significance

Here, I demonstrate that TLR3 signaling is neuroprotective to photoreceptors in the context of oxidative injury \textit{in vivo}. This is the first study to show that TLR3 has contrasting roles in regulating neuronal survival in the context of non-pathogen mediated injury.

This finding has several implications. First, TLR3 signaling may have several roles in addition to its classically defined role as mediator of innate immunity. TLRs were first identified in the context of immunity; however recent studies are beginning to show that TLRs are associated with non-pathogen mediated activity. Second, I show that TLR3 signaling protects neuronal function as well as retinal structure. Furthermore, TLR3 signaling improved visual acuity of mice during oxidative stress. The presence of TLR receptors on neurons and glia have led to questions, such as, whether TLRs play a role in maintenance of homeostatic cell functions, including electrical signaling. This question will be further discussed in the final chapter.
Chapter 5. Stat3 is an essential mediator of TLR3 induced cellular protection in the retina during oxidative stress

In the previous chapters, I established TLR3 as a cell survival/death regulatory pathway. TLR3 is pathogenic in the absence of injury, whereas TLR3 signaling behaves as a protective mediator during oxidative injury. However, the exact conditions and pathways that regulate TLR3 toward a pathogenic or protective role remain to be discovered. The Stat3 pathway was examined because it is a known promoter of cellular survival and interacts with inflammatory pathways. The research goal of this chapter is to identify whether Stat3 mediates of TLR3 induced regulation of retinal survival.

5.1. The Stat3 pathway

Signal transducer and activator of transcription 3 (Stat3) is a transcription factor that regulates many cellular processes including proliferation, inflammation, and survival. Stat3 signaling is activated by specific ligands that bind to JAK receptors, including growth factors and cytokines released by TLR signaling (Aaronson & Horvath 2002). Ligand binding to Stat3 receptor complexes on the cell surface induces Stat3 phosphorylation, leading to dimerization and translocation to the nucleus where Stat3 binds to specific consensus sites within the promoters of Stat3 target genes and activates transcription (Figure 5.1). STAT3 activity is critical for a wide range of functions in numerous cell types, including embryonic development and cellular differentiation, cell-cycle progression, and cellular proliferation and survival. In particular, Stat3 is involved
in the regulation of several anti-apoptotic pathways (Battle & Frank 2002, Liu et al 2010) and has been associated with retinal protection during oxidative stress and other injury paradigms (Barry et al 2009, Fragoso et al 2012, Ozawa et al 2008, Peterson et al 2000, Sarafian et al 2010). Furthermore, Stat3 also plays a role in regulating immune responses and inflammation (Aggarwal et al 2009). Therefore, Stat3 is a compelling candidate for mediating TLR3 induced cellular protection in the retina.

Figure 5.1. Stat3 signaling pathway.

Ligand-receptor binding recruits Stat3 to the plasma membrane leading to Stat3 activation via phosphorylation of a tyrosine residue by receptor tyrosine kinases. Stat3 dimerizes and then translocates into the cell nucleus to bind to consensus sequences on the promoters of Stat3 target genes that regulate cellular functions, such as survival and immune regulation.
5.2. TLR3 signaling activates Stat3 signaling

To investigate the role of Stat3 in TLR3 mediated regulation of retinal cell survival, I first examined whether TLR3 activation induces Stat3 signaling. A commonly used marker of Stat3 activation is nuclear localization of phosphorylated Stat3 (Fragoso et al 2012). I found that TLR3 activation using Poly (I:C) led to a significant increase in STAT3 activation, which was measured using immunocytochemistry on ARPE-19 cells (Figure 5.2). Immunodetection of nuclear phosphorylated STAT3 in ARPE-19 cells increased by 78% when treated with Poly (I:C) compared with untreated cells. TLR3 activation was measured by nuclear localization of p65, a marker of nuclear factor kappa beta (NF-κβ) signaling. The co-immunolabeling of phospho-Stat and p65 implies that TLR3 signaling induces Stat3 activation in RPE cells.

Protein analysis of Stat3 in ARPE-19 cell lysates demonstrated that treatment with Poly (I:C) led to a 141% increase in phosphorylated STAT3 and a 131% increase in total Stat3 compared with untreated cells, confirming that TLR3 activation induces Stat3 signaling (Figure 5.3). Furthermore, when cells were treated with Poly (I:C)+PQ, there was a 2.5 fold increase in total Stat3, suggesting that total available Stat3 is important for TLR3 induced protection.

A time-course analysis of Stat3 activation following TLR3 stimulation in ARPE-19 cells was conducted to further characterize the kinetics of TLR3 induction of Stat3. I found that Poly (I:C) increased both total STAT3 and phosphorylated STAT3 protein in a time-dependent manner, measured by
Western blotting (Figure 5.4). Overall, these results show that TLR3 activation induces Stat3 signaling.

**Figure 5.2. TLR3 signaling increased STAT3 activation in ARPE-19 cells**

Activation of STAT3 in ARPE-19 cells was performed by immunodetection using an anti-phospho STAT3 antibody. TLR3 activation was confirmed using an anti-p65 antibody. Poly(I:C) induces STAT3 phosphorylation in approximately 70% of cells and p65 in approximately 50% of cells after 24 h of treatment indicating that TLR3 activation leads to increased STAT3 signaling. Arrows show nuclear localization; inset shows higher magnification; 20× magnification, scale bar represents 50 μm.
Figure 5.3. TLR3 signaling increased both phosphorylated and total Stat3 protein in ARPE-19 cells

Poly(I:C) treatment increased both total and phospho-specific STAT3 compared with untreated cells after 24 h. Paraquat treatment decreased both total and phospho-STAT3 levels compared with untreated cells. Treatment of cells with both Poly (I:C) and paraquat increased both total STAT3 and phospho-STAT3 expression compared with untreated cells. Detection of β-actin was used as a loading control for normalization (n = 3, *p < 0.05).
Total and phosphorylated STAT3 increased with Poly (I:C) stimulation in a time dependent manner. Protein expression of phosphorylated and total STAT3 levels 0–8 h after TLR3 activation by Poly (I:C), measured by Western blotting. Both phosphorylated (middle) and total STAT3 (bottom) were significantly increased at 5 h and 8 h post stimulation compared with 0 h treatment (n = 3, *p < 0.05). β-actin levels were measured as a loading control.

Figure 5.4. Poly (I:C) increased both total and phosphorylated Stat3 in a time-dependent manner
*In vivo* analysis of the mouse retina following injections of Poly (I:C)+PQ revealed a 23-fold increase in Stat3 mRNA and a 175% increase in STAT3 activation compared with PBS injected animals, measured by QPCR and Western blotting (Figure 5.5). Injections of Poly (I:C)+PQ in TLR3 knockout mice showed no difference in Stat3 activation compared with PBS injected retinas, indicating that Poly (I:C) induced Stat3 activation is TLR3 dependent. Interestingly, injections of Poly (I:C) or PQ alone only increased Stat3 mRNA expression by 4-fold and did not significantly alter Stat3 protein levels. This suggests the possibility that Stat3 signaling may be synergistically activated by TLR3 activation and oxidative stress to promote neuroprotection. Another possibility is that the combination of TLR3 activation and oxidative stress regulate endogenous Stat3 signaling inhibitors, which will be further discussed in the following chapter.

5.3. Stat3 signaling is required for TLR3 induced retinal protection during oxidative injury

Stat3 signaling was knocked down in the ARPE-19 cells using siRNA to ascertain whether Stat3 signaling is necessary for TLR3 induced protection during oxidative stress. Cell viability was measured in transfected cells following treatment with Poly (I:C), PQ, or Poly (I:C)+PQ with untreated cells serving as a control. Cells transfected with scrambled siRNA also served as a control for any changes in viability due to transfection. Efficiency of Stat3 knockdown was measured by QPCR and western blotting, which showed a decrease of Stat3 expression by 79% and 34% respectively, compared with controls (Figure 5.6).
Figure 5.5. TLR3 activation during oxidative stress increased Stat3 signaling in vivo

QPCR of cDNA from mice injected with Poly (I:C) revealed a 23 fold increase in Stat3 mRNA expression compared with PBS injected mice (top left, n=3). Western blotting of retinal lysates obtained from injected mice showed increased phospho-Stat3 to total Stat3 ratio in Poly (I:C)+PQ injected animals compared with PBS, Poly (I:C), or PQ injected animals. In contrast, TLR3 KO animals showed no differences in phospho-Stat3 to total Stat3 ratio among different injected treatments. Representative western blots (right, n=4) and quantification of Stat3 signaling in wild type retinas (middle left, n=4) and TLR3 knockout retinas (bottom left) are shown. Both phospho-stat3 and total stat3 blots were normalized to β-actin as a loading control. A statistical significance of p<0.05 is marked as ^ PBS vs Poly (I:C)+PQ, ^^ Poly (I:C) vs Poly (I:C)+PQ and ## PQ vs Poly (I:C)+PQ.
Cell viability following drug treatment were consistent with the model that TLR3 induced protection is Stat3 dependent. Knockdown of Stat3 abolished the protective effects of Poly (I:C) during oxidative stress while control scrambled siRNA transfected cells behaved similarly to untransfected cells when treated with the various drugs (refer to Chapter 3) (Figure 5.7).

Stat3 was knocked down in vivo in the mouse retina using lentivirally delivered Stat3 specific shRNA, using scrambled shRNA as a control. Lentivirus has been used to target retinal cells for gene delivery in numerous studies and is known to infect both RPE and photoreceptors (Bemelmans et al 2006, Binley et al 2013). Furthermore, lentiviral infection results in limited inflammation. Lentiviral shRNA expression in the retina was verified by expression of GFP throughout the retina in various cell types (Figure 5.8). GFP expression was distributed throughout the whole retina. Importantly, significant GFP expression is localized in the RPE and photoreceptor cells. The efficiency of Stat3 knockdown was verified by Western blotting of retinal lysates from virus injected animals. Total stat3 protein was reduced by 90% in retinas injected with Stat3 shRNA compared with PBS injected control eyes (Figure 5.9). Interestingly, injections of lentiviral control scrambled shRNA increased total Stat3 in the retina by 4-fold. This is most likely due to virus mediated Stat3 signaling.
Figure 5.6. Verification of efficiency of siRNA knockdown of Stat3 in ARPE-19 cells

STAT3 specific siRNA decreased STAT3 RNA expression by 79% measured by QPCR compared with control siRNA transfected cells 24 h after transfection (top, n = 3, *p < 0.05). Protein expression of STAT3 was reduced in Stat3 siRNA transfected cells by 34%, as measured by Western blotting (n=3, *p<0.05). Quantification of western blots (bottom left) and representative blots (bottom right) are shown. β-Actin levels were measured as a loading control.
Figure 5.7. Knockdown of Stat3 abolished the protective effects of TLR3 signaling in ARPE-19 cells during oxidative stress

STAT3 signaling mediates TLR3 induced protection of RPE from oxidative stress. Knocking down STAT3 using siRNA eliminated rescue by Poly (I:C) during oxidative stress conditions. Poly (I:C) and PQ treated cells had a similar decrease in viability compared with PQ only treated cells. Control siRNA resulted in 50% rescue of cells when treated with poly (I:C) and oxidative stress (n=5, *p<0.05). UT, untreated (growth media only); PQ, paraquat.
Figure 5.8. Lentiviral Stat3 shRNA expression is localized in photoreceptors and RPE

Wild type mouse eyes were injected with lentivirus co-expressing Stat3 shRNA and GFP. There is GFP expression throughout the retina with significant localization in photoreceptors and RPE (in and above the outer nuclear layer (ONL)), indicating that lentiviral shRNA is expressed in these cell types. No GFP expression is seen in the retinas injected with PBS as a vehicle control.
TLR3 activation induced neuroprotection of the retina during oxidative stress in mice injected with control scrambled shRNA, measured by photoreceptor layer thickness, photoreceptor function, and visual behavior, similar to results from mice that were not injected with lentivirus (refer to Chapter 4). Photoreceptor layer thickness was 128% thicker in eyes injected with Poly(I:C) + PQ than in eyes injected with PQ alone (Figure 5.10), matching the results from the previous chapter. Lentivirus injection slightly decreased photoreceptor thickness by 9% compared with eyes not injected with virus, seen by a
comparison of PBS injected eyes in both groups. The small decrease in photoreceptor thickness is most likely due to the fact that virus injection activates TLR3 signaling, which was shown to be toxic to retinal cell health on its own. Photoreceptor function was significantly increased in eyes injected with Poly (I:C) + PQ by 200% in scotopic a-wave amplitude and 150% in scotopic b-wave amplitude, compared with eyes injected with PQ alone (Figure 5.11). A similar, but lesser, effect is seen in the photopic ERG response from these mice, most likely due to the small number of cone photoreceptors in the mouse retina. Optokinetic analysis of control shRNA virus injected mice revealed increased visual behavior of mice when Poly (I:C) was injected with PQ, with visual acuity increasing by 210% compared with mice injected with only PQ (Figure 5.12). One concern using lentivirus as delivery method for shRNA is that, as a virus, it has the potential to activate TLR3. Injections of control shRNA revealed that possible effects of viral activation of TLR3 did not confound the effects of Poly (I:C) activated TLR3 in terms of cellular survival.

Knockdown of Stat3 using lentivirally delivered shRNA abolished the neuroprotective effects of TLR3 signaling during oxidative injury compared with control shRNA injected animals (Figures 5.10-5.12). Interestingly, knockdown of Stat3 led to increased degeneration when TLR3 was activated during oxidative stress compared with eyes with only oxidative injury. Photoreceptor layer thickness, function, and overall visual acuity decreased by up to 64% in eyes injected with Poly (I:C) + PQ compared with eyes injected with PQ alone. This result implies that Stat3 is necessary for TLR3 mediated protection during
oxidative stress. Furthermore, these results can be extrapolated to show that Stat3 activation is a key signaling pathway that controls whether TLR3 signaling is pathogenic or protective. Together, these results show that Stat3 signaling is necessary for TLR3 mediated protection during oxidative stress.

5.4. Conclusions and significance

The mechanisms of TLR3 signaling during immunogenic responses have been well characterized. However, recent evidence suggests that TLR3 may be active in non-pathogen mediated responses that regulate cellular survival. The mechanisms that control TLR3 signaling to be either pathogenic or protective are not well understood. The role of Stat3 as a protective mechanism during neurodegeneration has been well documented. Here, I present the first study to link Stat3 to neuroprotection induced through an immune receptor. This chapter shows the necessity of Stat3 signaling in TLR3 mediated protection. Knockdown of Stat3 resulted in abolishment of TLR3 induced functional and structural protection during oxidative injury. Furthermore, TLR3 activation increased photoreceptor degeneration during oxidative injury following knockdown of Stat3 in vivo. Together, these results point to Stat3 as a mechanism for controlling how TLR3 regulates cellular signaling during non-pathogen mediated responses.
Figure 5.10. Stat3 is required for TLR3 mediated protection of photoreceptor cells during oxidative injury

Control shRNA injected mice also injected with Poly (I:C)+ PQ had increased photoreceptor layer thickness compared with mice injected with shRNA and PQ, indicating increased photoreceptor survival (top). Photoreceptor thickness was quantified by measurement of outer nuclear layer + inner and outer segment thickness of retinal imaging generated by OCT. However, mice injected with Stat3 shRNA and Poly (I:C) + PQ had noticeably decreased photoreceptor thickness compared with mice injected with shRNA and PQ, indicating that Stat3 is required for TLR3 induced protection (bottom). A statistical significance of p<0.05 is marked as * PBS vs Poly (I:C), # PBS vs PQ, ^ PBS vs Poly (I:C)+PQ, ** Poly (I:C) vs PQ, ^^ Poly (I:C) vs Poly (I:C)+PQ, and ## PQ vs Poly (I:C)+PQ. (n=6)
Figure 5.11. Stat3 is required for TLR3-induced protection of photoreceptor function during oxidative stress

Wild type mice injected with control scrambled shRNA and different treatments had similar scotopic ERG responses compared with mice injected in the absence of lentiviral shRNA (Figure 4.9.). Importantly, control shRNA injected mice injected with Poly (I:C)+ PQ had increased photoreceptor a-wave and b-wave amplitudes compared with mice injected with shRNA and PQ, indicating increased photoreceptor function. Mice injected with Stat3 shRNA and Poly (I:C) + PQ had noticeably decreased scotopic amplitudes compared with mice injected with shRNA and PQ, indicating that Stat3 is required for TLR3 induced protection. Average a-wave and b-wave amplitudes at scotopic and photopic flash intensities ranging from 0.01 to 10 cd·s/m² are shown. (n=6)
Wild type mice injected with control scrambled shRNA and different treatments had similar visual acuity, measured by spatial frequency, as mice injected with treatments alone. Importantly, control shRNA injected mice also injected with Poly (I:C) + PQ had significantly increased visual acuity compared with mice injected with shRNA and PQ, indicating improved visual behavior (top). Mice injected with Stat3 shRNA and Poly (I:C) + PQ had noticeably decreased visual acuity compared with mice injected with shRNA and PQ, indicating that Stat3 is required for TLR3 induced protection (bottom). A statistical significance of p<0.05 is marked as # PBS vs PQ, ^ PBS vs Poly (I:C)+PQ, ** Poly (I:C) vs PQ, ^^ Poly (I:C) vs Poly (I:C)+PQ, and ###PQ vs Poly (I:C)+PQ. (n=6)
Chapter 6: Discussion

This thesis dissertation examined the role of TLR3 during elevated oxidative stress, which is a model of toxicity during age-related macular degeneration. I identified an important dual role of TLR3 in regulating RPE and photoreceptor survival. TLR3 activation protects against oxidative stress induced damage in RPE and photoreceptor cells, while TLR3 signaling on its own leads to retinal cell death. I also showed that TLR3 signaling not only protected against oxidative stress induced cellular damage, but it also protected against photoreceptor neuronal functional loss and improves overall vision. Furthermore, I identified the Stat3 pathway as a critical regulator of TLR3 induced protection of the retina during oxidative stress. The results from this study identify the TLR3 pathway and the Stat3 pathway as novel targets for further investigation and will help to develop new therapeutic approaches to age-related macular degeneration, retinal degenerations, and diseases of the central nervous system.

6.1. Limitations of this study

One limitation of this study is that the cell-specific contributions of TLR3 activation in the retina are not known. OCT is a good tool for measuring the retinal layers of the neural retina; however the use of OCT is limited in defining thin layers such as the RPE and nerve fiber layer. It is difficult to visualize changes in RPE cell survival in vivo using OCT imaging because the RPE is a monolayer of cells. Furthermore, the localization of TLR3 within the retina indicates that protection of photoreceptors in vivo could be mediated by TLR3
signaling in RPE, acting in a paracrine manner, or within photoreceptors themselves, by autocrine signaling. In addition to TLR3 activation directly leading to increased survival in both photoreceptors and RPE, there may be interactions between RPE and photoreceptors that are mediated by TLR3 signaling, which may also regulate neural protection. The use of Stat3 as a marker for TLR3 activation to distinguish exact cell types and mechanisms inducing survival proves difficult due to expression of Stat3 throughout the retina during injury. Furthermore, with the exception of the RPE, TLR3 is expressed endosomally in the retina. TLR3 activation through the cell surface may signal through a different pathway than endosomal activation of TLR3, resulting in different downstream effects. One way to distinguish the how TLR3 regulates survival between photoreceptors and RPE would be to use cell-specific conditional knockouts for TLR3 and should be examined in future studies.

Another limitation of this study is that the experimental design did not permit examination of the contribution of microglial regulation. TLR signaling is known to regulate microglial activity (Gonzalez-Scarano & Baltuch 1999, Haynes et al 2013). Buildup of photoreceptor outer segment proteins in the subretinal space activates TLR4 signaling, which leads to retinal degeneration mediated by microglial activation (Kohno et al 2013). A brief stimulation of TLR4 led to downregulation of microglia activity, which decreased retinal degeneration induced by ischemia (Halder et al 2013). TLR3 also plays a role in regulation of microglia activity in the retina. TLR3-deficient mice have fewer invasive microglia and macrophages in the retina during degeneration compared with wild type
mice (Shiose et al 2011). Therefore it is highly possible that microglial activity plays a role in the TLR3 mediated cellular protection during oxidative stress. Further studies are necessary to identify microglial regulation in this context.

6.2. TLR3 as a pathogenic pathway in the retina

There are several studies that link TLR3 signaling to cellular apoptosis and retinal degeneration. Activation of TLR3 in wild type retinas using intravitreally injected dsRNA led to degradation of the RPE monolayer of the retina, leading to photoreceptor death (Kleinman et al 2012). Furthermore, TLR3 ablation led to decreased disease phenotype expression in a mouse model of cone-rod dystrophy (Shiose et al 2011). TLR3 activation was also shown to be a negative regulator of adult central and peripheral nervous system axonal regrowth mechanisms (Cameron et al 2007). My results paralleled the findings of previous studies that showed that TLR3 activation in the absence of injury did indeed induce RPE and photoreceptor death in vitro and in vivo (Kleinman et al 2012, Shiose et al 2011). However, the difference between my work and others is that I found that when an injury paradigm is present within the retina, TLR3 switches to a protective role.

6.3. TLR3 as a neuroprotective pathway

Although much of the current published literature shows a negative role for TLR3 in regards to cellular survival, there is some precedence for innate immunity having a protective role during disease. It was recently shown that inflammatory cytokines, such as those released during innate immune activation, can stimulate
a protective response against oxidative stress induced degeneration in RPE (Juel et al 2013). Furthermore, TLR3 activation in astrocytes triggers secretion of neuroprotective mediators leading to increased protection of brain cells following traumatic injury (Bsibsi et al 2010). Similarly, my findings showed that TLR3 activation during oxidative stress was neuroprotective to the retina.

6.4. TLR3 signaling has a dual role in regulation of cell survival

The idea that TLR3 has dual roles in regulating cell survival has been looked at in the context of pathogen-mediated disease. A context sensitive dual role for TLR3 during Theiler’s virus mediated demyelination disease was examined by Jin et al. (Jin et al 2011). They found that TLR3 activation during viral infection protected axons of the brain and spinal cord from demyelination. In contrast, TLR3 activation prior to viral infection led to increased pathogenesis of the disease. Other TLR signaling pathways also show similar patterns. TLR4 activation leads to photoreceptor death or protection during oxidative stress injury, depending on timing of TLR4 signaling (Yi et al 2012). Furthermore TLR9 also has a similar dual role in the development of lupus, in which TLR9 can either stimulate or induce tolerance to autoimmune responses leading to increased or decreased pathogenesis respectively (Ehlers & Ravetch 2007).

6.5. TLR3 regulation of cellular survival is dependent on Stat3

Stat3 activation in the retina has been previously reported to mediate cellular survival during oxidative stress. This dissertation shows that TLR3 induced protection during oxidative stress is Stat3 dependent. Interestingly, when Stat3
is knocked down in the retina, TLR3 activation increases degeneration of retina during oxidative stress. This finding implies that TLR3 behavior in regard to cellular survival is controlled by Stat3 activation. Studies have been conducted that support this theory. Stat3 activation induced protection from oxidative stress while deletion of Stat3 lead to increased oxidative stress and decreased mitochondrial function in astrocytes (Barry et al 2009). I found that deletion of Stat3 did not increase oxidative stress injury \textit{in vitro} or \textit{in vivo} in my study. In addition, TLR3 activation did not induce increased cell death during oxidative injury when STAT3 was knocked down in RPE cells \textit{in vitro}, unlike the \textit{in vivo} results. This may be due to varying levels of Stat3 expression and oxidative stress tolerance among the different adult retinal cell types.

Poly (I:C) increased Stat3 activation in the presence and absence of oxidative stress in RPE cells \textit{in vitro}. However in the mouse retina, Stat3 activation was only increased when TLR3 was stimulated in the presence of oxidative stress. The discrepancy between \textit{in vitro} and \textit{in vivo} TLR3 induced Stat3 activation may be due to high levels of endogenous Stat3 inhibitory molecules in the retina. Photoreceptors in particular have high expression of Soc53, a Stat3 inhibitory molecule (Ozawa et al 2008). It is possible that Soc53 may inhibit any changes to Stat3 activation induced by TLR3 activation. The combination of oxidative stress and TLR3 activation may regulate both Soc53 and Stat3 to synergistically induce Stat3 activation and ultimately retinal protection. Further studies are required to identify how endogenous mechanisms of Stat3 signaling are regulated during TLR3 activation and oxidative stress.
6.6. Preconditioning paradigm for TLR3 induced protection during oxidative stress

The TLR family of innate immunity has the ability to induce tolerance to future injuries or insults following an initial activation event, in a phenomenon known as preconditioning. TLR preconditioning promotes neuroprotection of the brain after ischemic injury through TLR3 or TLR7 activation prior to injury (Leung et al 2012, Stevens et al 2011). In the retina, preconditioning of Muller glia-photoreceptor cultures with TLR4 activation protected against oxidative stress damage (Yi et al 2012). In the studies detailed in this dissertation, oxidative stress was induced using paraquat. Paraquat is a compound that induces free radical propagation, leading to oxidative stress buildup over time. In contrast, TLR3 is most likely rapidly activated by Poly (I:C), leading to a possible preconditioning paradigm. TLR3 activation may have promoted cellular tolerance to oxidative stress, leading to cellular protection. Future studies will be necessary to determine the exact kinetics of these drugs in vivo and identify if a preconditioning effect is occurring in the retina.

6.7. Acute vs chronic TLR3 activation

In this dissertation, I examined the effects of acute TLR3 activation on retinal health. While acute TLR3 activation is protective during injury, chronic TLR3 activation may not be (Galimberti & Scarpini 2011). It has been shown that acute or low levels of inflammation can have beneficial effects following injury, such as during optic nerve regeneration (Benowitz & Popovich 2011). However, chronic
inflammation has been shown to exacerbate neurodegenerative diseases, through increased secondary degeneration. There is evidence of over-activated innate immunity in AMD, including polymorphisms in innate immune regulators, leading to chronic inflammation (Edwards et al 2008, Edwards et al 2005, Yang et al 2008). Therefore, it is likely that precise control of innate immune activity is essential for controlling progression of disease. I have shown that activation of TLR3 during the AMD-like condition of oxidative stress leads to increased retinal cell survival, neuronal function, and overall better vision. My results show that TLR3 innate immune signal can protect against development of some AMD phenotypes, such as photoreceptor death and vision loss, and implies that some level of innate immune activity is important for cellular survival during injury.

Another possibility is that TLR3 may not be acting in a typical innate immune function. TLR3 was first discovered as an innate immune sentinel and therefore has been well characterized in terms of innate immunity. However the role of TLR3 in non-immune regulated functions is only just beginning to be explored. Other TLRs have been shown to play a role in non-immune related functions, including phototransduction and neuronal synaptic regulation (Freria et al 2012, Kindzelskii et al 2004). Therefore, it is possible that TLR3 also has functions other than innate immunity.
6.8. The interaction between inflammation, TLR signaling, and oxidative stress pathways

Studies have shown that the innate immune system and oxidative stress pathways can reciprocally regulate each other (Hollyfield et al 2008, Juel et al 2013). Inflammasomes, a group of multimeric protein complexes that regulate inflammation, have been identified in neuronal and glial cells and inflammasome activity has been attributed to neurodegenerative diseases (Halle et al 2008). NLRP3 inflammasome activity was upregulated during AMD pathogenesis (Tseng et al 2013) and inhibition of the NLRP3 inflammasome alleviated neovascularization during wet AMD (Marneros 2013). However, the exact role of inflammasome activity in retinal disease is not well understood. Oxidative stress was shown to increase NLRP3 inflammasome activity in RPE cells (Kauppinen et al 2012). NLRP3 activation by oxidative stress related proteins was shown to protect against formation of retinal lesions seen in AMD (Doyle et al 2012). The NLRP3 inflammasome activity is also regulated by TLR dependent NF-kB signaling (Franchi et al 2012), indicating a potential point of cross talk between oxidative stress and TLR signaling.

TLR signaling has also been shown to directly mediate oxidative damage in the context of pathogen-mediated damage (Gill et al 2010). Cytokine production by lung macrophages induced oxidative stress via TLR4 signaling in lung tissue (Imai et al 2008). Furthermore, TLR4 signaling can trigger the formation of NADPH oxidase derived ROS, leading to oxidative stress (Lee et al 2012). However, TLR2 signaling was shown to have an anti-apoptotic effect on
cardiac monocytes exposed to elevated levels of ROS through NF-kB signaling (Frantz et al 2001).

However the interaction between TLR3 activity and oxidative stress is unknown during non-pathogen mediated injury. It would be interesting to see whether TLR3 activation during oxidative stress regulates the levels of ROS present within retinal cells in the model discussed in this thesis. It is possible that TLR3 signaling increases antioxidant mechanisms, such as superoxide dismutase (Perez de Diego et al 2013), through the Stat3 pathway, which has been shown to attenuate oxidative stress damage (Barry et al 2009, Patel & Hackam 2012, Zhang et al 2008). Further studies are necessary to identify how TLR3 signaling regulates ROS formation and oxidative stress in the model presented in this thesis.

6.9. Endogenous activators of TLR3 signaling

Recently, TLRs are beginning to be examined in the context of non-immune regulated functions. This has led to identification of novel TLR activators that are not the typical PAMP ligands. TLR4 was shown to be activated by proteins expressed on rod photoreceptor outer segments, contrary to the typical ligands generated from bacterial sources (Kindzelskii et al 2004, Tseng et al 2013).

Typically, TLR3 is activated by double stranded RNA released into cells during viral infection. However, there is increasing evidence of endogenous sources of TLR3 activation. In the retina, mRNA or double stranded RNA released from dying necrotic cells may be a potential source of endogenous
activators for TLR3 (Cavassani et al 2008). Analysis of drusen found in AMD affected eyes revealed the presence of double stranded RNA as a component (Murakami et al 2013). Furthermore, buildup of endogenous Alu repeats, double stranded RNA, triggers TLR mediated death in the retina (Kaneko et al 2011).

There are endogenous small molecule activators of TLR3, in addition to RNA sources. Shiose et al. showed that secreted proteins from dying cells were sufficient to induce TLR3 signaling (Shiose et al 2011). Bsibsi et al. identified stathmin, a protein that regulates microtubule formation, as a ligand for TLR3 (Bsibsi et al 2010). Furthermore, stathmin activation of TLR3 in astrocytes resulted in secretion of neuroprotective mediators. It is likely more endogenous ligands for TLR3 exist, which may differentially activate TLR3 to behave in either a pathogenic or protective role. Therefore identifying endogenous activators of TLR3 are important for determining the conditions that regulate the TLR3 pathway as a prosurvival pathway \textit{in vivo} and will lead to better characterization of the behavior of TLR3 signaling during disease.

6.10. TLR3 may have a potential role in regulating synaptic function

In chapter 4, I showed that TLR3 signaling during oxidative stress protected against loss of photoreceptor function, in terms of electrical signaling. One reason for the increase in electrical activity in the retina may be due to the increased number of surviving neurons. While this may be true, there is also evidence that suggests that TLR3 may play an active role in synaptic transmission. TLR expression has been identified at the site of the synapse in
neurons and glia (Freria et al 2012). TLR3 may have a dual role in synaptic function, similar to what studies have shown in regard to cellular survival. TLR3 knockout mice had improved responses from the hippocampus, shown by increased memory and learning abilities, compared with wild-type mice, however responses from the amygdala of the brain were significantly decreased (Okun et al 2010). Differences in molecular mediators of the neuronal activity between brain regions may account for why TLR3 reduces neuronal activity in the hippocampus while improving neural activity in the amygdala, which is similar to the context driven role of TLR3 signaling during cellular survival.

In terms of synaptic function, TLR3 deficient mice have impaired synaptic transmission in the spinal cord and decreased sensory neuron responses during induced pain (Liu et al 2012). Therefore, TLR3 may play a critical role in sensory neuron synapses during injury. While TLR3 knockout mice injected with PBS did not have significantly different electrical responses in the retina compared with their wild type counterparts, activation of TLR3 signaling during oxidative stress fully preserved photoreceptor function in wild-type mice. While increased photoreceptor function should be expected with increased cellular survival, there was still a modest amount of death in wild type mice injected with Poly (I:C) and PQ compared with mice injected with PBS. The fact that photoreceptor response was almost fully preserved in spite of some cellular death may indicate TLR3 also regulates synaptic function during injury, by enhancing surviving photoreceptor synaptic activity. There is precedence for other TLRs being involved in synaptic activity. TLR4 was shown to be necessary in the
preservation of presynaptic terminals and increased synaptic activity following injury, while TLR2 stimulation had the opposite effect (Freria et al 2012). Further investigation is needed to identify the role of TLR3 in synaptic activity.

6.11. A model of how TLR3 regulates AMD pathogenesis

By extrapolation from the data presented in this dissertation, I propose the following model for how TLR3 regulates retinal cell survival during the AMD-like condition of oxidative stress. In the absence of injury, TLR3 promotes cellular death through a non-Stat3 dependent mechanism. One possibility of how TLR3 induces death is through caspase-3 mediated signaling (Kleinman et al 2012). Kleinman et al. showed that non-internalized siRNAs induced retinal degeneration in mice by activating TLR3 on the surface of the RPE. Activation of TLR3 increased nuclear translocation of IRF3, leading to caspase-3 mediated apoptotic death of RPE and subsequently photoreceptors. In the presence of oxidative injury, TLR3 activation is protective to the retina in a Stat3 dependent manner. Interestingly, knockdown of Stat3 leads to increased retinal degeneration when TLR3 is activated during oxidative stress. Therefore, activation of Stat3 is likely a controller of TLR3 signaling behavior in regulation cellular survival (Figure 6.1). Stat3 and caspase-3 are reciprocal regulators of each other (Barton et al 2004, Darnowski et al 2006). While Stat3 activates anti-apoptotic pathways that regulate caspase-3 activity, such as BCL-2 (Barton et al 2004), high expression of caspase-3 is also known to cleave Stat3 (Darnowski et al 2006). The effects of TLR3 are, most likely, dependent on a balance between caspase-3 and Stat3. While TLR3 activation alone shifts the balance in favor of
caspase-3, the combination of oxidative injury and TLR3 activation shifts the balance towards increased Stat3 activation.

A second possible theory of why TLR3 has both pathogenic and protective behaviors may be mediated by NF-kB signaling, which has been shown to be both pro-apoptotic and anti-apoptotic depending on the level of activation (Fan et al 2008). Furthermore, NF-kB signaling can be reciprocally regulated by the Stat3 pathway and cross-talk between the two pathways has been shown to protect against oxidative stress cardiovascular tissue (Brasier 2010). Additionally, regulation of cellular survival may be mediated by NF-kB mediated inflammasome activity, described in section 6.8.

6.12. Implications of TLR3 in diseases of the central nervous system

Although this thesis focused on the role of TLR3 in the pathogenesis of AMD; these results can be extrapolated to many diseases of the central nervous system. The retina is part of the central nervous system and therefore has much of the same properties (Kolb et al 1995). There are several diseases of the central nervous system in which innate immunity, inflammation, and oxidative stress are critical factors, including Alzheimer’s disease and Parkinson’s disease (Ridolfi et al 2013, Stone et al 2009). While TLR3 activation often induced death in cell types affected by these diseases, there is a lack of knowledge about how TLR3 behaves in the presence of other contributors of neurodegenerative diseases. The study described in this dissertation is the first step in
understanding the complex behavior of TLR3 in non-pathogen mediated disease conditions.

Figure 6.1. Proposed model of TLR3 regulation of retinal cell survival during AMD pathogenesis

TLR3 signaling is protective to retinal cells during oxidative stress via Stat3 activation. One theory is that there is a balance between caspase-3 and Stat3. The balance between caspase-3 and Stat3 is shifted in favor of caspase-3 mediated cell death when TLR3 is activated in the absence of injury, while TLR3 signaling induces Stat3 pathway activation leading to robust neuroprotection in the presence of oxidative injury. Speculatively, the combination of oxidative stress and TLR3 pathways may lead to Stat3 activation, possibly through synergistic downregulation of endogenous Stat3 inhibitors. An alternate protective pathway may be through TLR3 mediated NF-kB induced protection during oxidative stress, regulated by Stat3.
6.13. Conclusion

In summary, this dissertation presents a novel neuroprotective role for TLR3 and Stat3 signaling during AMD disease conditions. I found that TLR3 activation during AMD-like oxidative injury to the retina is neuroprotective through the Stat3 signaling pathway. This study establishes a context driven role for TLR3 in regulation of non-immunoregulatory cells during non-pathogen mediated disease. The results from this study also suggest that TLR3 may have regulatory functions that play an important role in maintaining homeostatic cellular conditions during disease progression. Understanding exactly how TLR3 signaling regulates cellular processes is critical to uncover the underlying mechanisms of AMD pathogenesis. Future directions of this project include examining the kinetics of TLR3 induced neuroprotection during oxidative injury and investigating how TLR3 differentially behaves in the individual cell types of the retina. The results presented in this thesis will lead to the development of novel therapeutic approaches to AMD, retinal degenerations, and other diseases of the central nervous system in which oxidative stress plays a major role.
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