Modulation of the Retinal Immune Environment in a Zebrafish System of Rod Photoreceptor-Specific Degeneration

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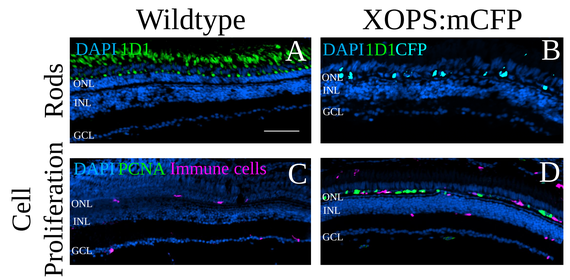
Abstract

Activated and pro-inflammatory microglia, along with accompanying local inflammation, are associated with human retinal degenerative disease. However, it remains unclear if these aspects of the immune response are symptomatic or directly initiate and/or contribute to disease pathology, such as the death of additional retinal neurons. One hypothesis for continued loss of neurons in retinal degenerative disease is that microglia may engulf, or possibly initiate cell death of, otherwise healthy neurons (1,2).

Our project attempts to test this hypothesis using a zebrafish system in which rod photoreceptors die due to a toxic transgene (XOPS:mCFP), but cone photoreceptors survive (3). We first characterized microglial characteristics in XOPS:mCFP retinas compared to wildtype and found that microglia localize to the photoreceptor layer and engulf dying rods, but total numbers of microglia are similar. Next, we successfully induced a pro-inflammatory retinal immune environment by intraocular injection of zymosan (a pro-inflammatory compound) (4), as indicated by our results showing infiltration and accumulation of immune cells in the retina and gene expression of selected transcripts.

Our next goal is to determine if this induction of a pro-inflammatory retinal environment may result in subsequent cone death or disappearance in XOPS:mCFP retinas, thus directly probing contributions of a dysregulated immune environment to retinal degenerative disease.

Figure 1: Perpetual Rod Death and Regeneration in XOPS:mCFP Zebrafish Retinas



Images show a 5µm section of zebrafish retina from wildtype and XOPS:mCFP transgenic fish. Wildtype retinas (A,C) show healthy rods (A, labeled by 1D1) and lack of cell proliferation in the outer nuclear layer (ONL) (C, labeled by PCNA). XOPS:mCFP retinas (B,D) show far fewer rod photoceptors (B, labeled by CFP). 1D1 is not observed in XOPS:mCFP retinas because they do not express the protein rhodopsin (3), which is the 1D1 antibody target. We also observe a constant attempt to regenerate dying rods by proliferative rod precursors (D, labeled by PCNA). In XOPS:mCFP retinas, we also see that microglia localize to the ONL (similar to C) around the PCNA+ cells, but do not express PCNA themselves (D). This tells us that immune cells are not dividing in response to ongoing rod death, but instead the PCNA+ cells likely represent only rod precursors. Scale bar = 50µm and applies to all images. INL = inner nuclear layer, GCL = ganglion cell layer, PCNA = proliferating cell nuclear antigen.

Methods: Modulation of the Retinal Immune Environment

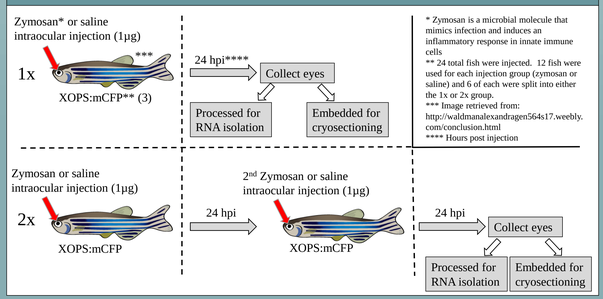
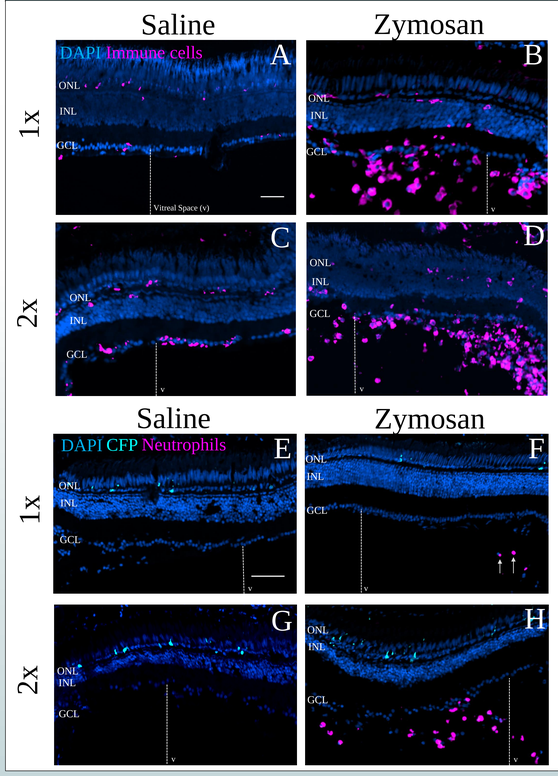


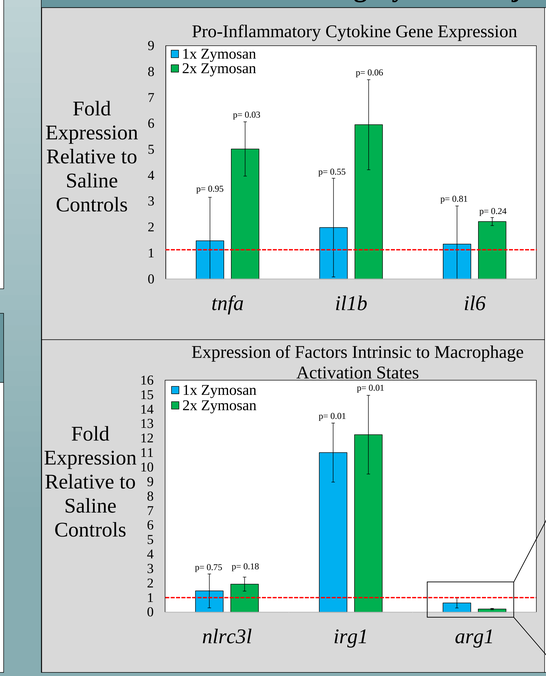
Figure 2: Zymosan Treated Retinas Show Infiltrating Immune Cell Response



Images show 5µm section of XOPS:mCFP retinas with 1x and 2x zymosan or saline (control) injections stained for L-plastin (immune cells, A-D) and MPX (neutrophils, E-H). After 1 injection of zymosan, immune cells infiltrate the retina which is shown by an accumulation of immune cells in the vitreal space (B) and redistribution of immune cells towards the GCL and vitreal interface. After 2 injections of zymosan, the immune cell population continued to increase (D). The immune cells that accumulate following zymosan injection include neutrophils, which is a characteristic of a pro-inflammatory response (arrows, F and H). However, the recruited neutrophils do not account for the entirety of the infiltrating immune cell population.

Scale bar = 50µm, A applies to B,C, and D. Scale bar in E applies to F,G, and H.

Figure 3: Increase in Pro-Inflammatory Gene Expression Following Zymosan Injection

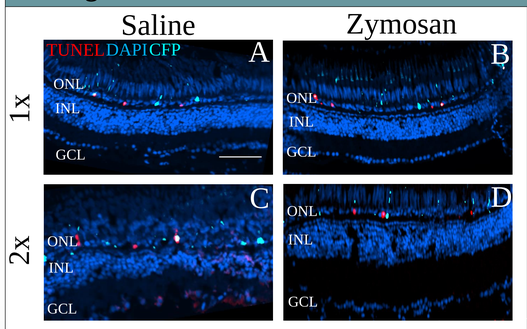


Quantitative PCR analysis of classic pro-inflammatory genes as well as genes that are specific to macrophage activation states. Gene expression was normalized using the housekeeping gene b-actin. The graph shows fold expression of selected genes where a value greater than 1 (designated by a red line on the graph) indicates elevated target gene expression, less than 1 indicates decreased target gene expression, and a fold change of 1 implies no change in gene expression.

tnfa, *il1b*, and *il6* are cytokines that are secreted by innate immune cells (including macrophages) to induce a state of inflammation (5).

nlrc3l, *irg1*, and *arg1* are all genes associated with different macrophage activation states. *nlrc3l* is a gene identified in zebrafish as an anti-inflammatory receptor (6). *irg1* is a gene induced by inflammatory stimuli and macrophage activation (7). And *arg1* is a macrophage gene that promotes wound repair (8).

Figure 4: Cell Death 24 and 48 Hours Following Pro-Inflammatory Induced State



Here, we quantified cell death following injection of zymosan. We used a TUNEL* stain to mark cells that were in late stage apoptosis. TUNEL+ cells were quantified in the ONL exclusively and TUNEL+ cells that were CFP+ rods were accounted for as their death is programmed by the transgenic line. We found that the 1x and 2x zymosan injection groups showed an increase in cell death per 100µm of retina in relation to their corresponding saline control (E, although not statistically significant). Additionally, this increased number of TUNEL+ cells did not represent CFP+ rods (F). This could be evidence for increased cell death in a population other than rods following an inflammatory response.
Scale bar = 50µm and applies to all images.

**Terminal deoxynucleotidyl transferase dUTP nick end labeling*

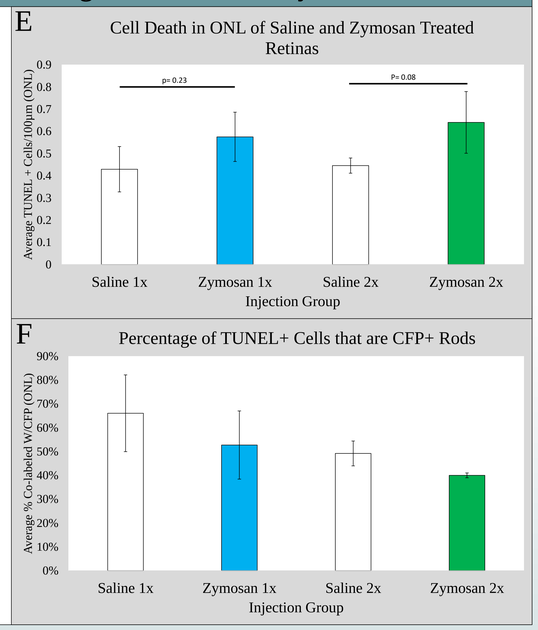
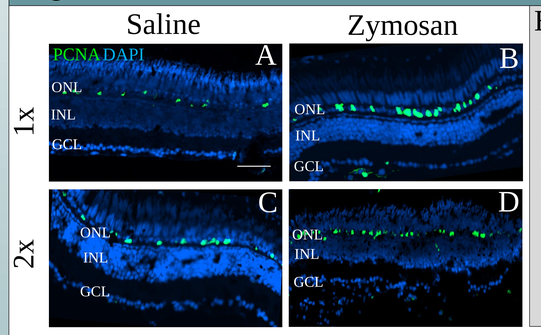
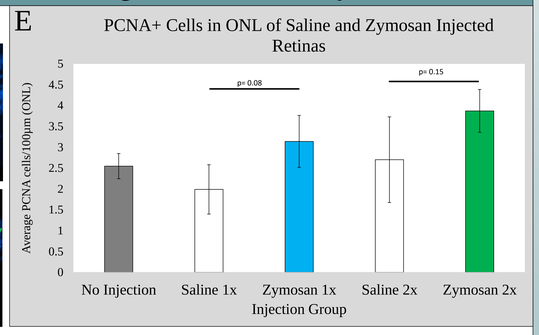


Figure 5: Cell Proliferation 24 and 48 Hours Following Pro-Inflammatory Induced State





Images show 5µm section of XOPS:mCFP retinas with 1x and 2x zymosan or saline (control) injections stained for PCNA to indicate cell proliferation. PCNA+ cells were quantified in the ONL exclusively. We found that the1x and 2x zymosan injection groups had more PCNA+ cells per 100µm of retina when compared to their corresponding saline control (E). XOPS:mCFP eyes that did not receive an injection were also quantified as a control. We found that the zymosan treated eyes had an increased number of PCNA+ cells when compared to eyes receiving no injection, but was not statistically significant (p= 0.70, 1x) (p= 0.18, 2x). Both groups of saline injected controls showed no significant change when compared to eyes receiving no injection (p= 0.03, 1x) (p= 0.38, 2x). We observed that only rarely were L-plastin+ cells also PCNA+ (not shown) indicating that immune cells in the ONL did not represent significant numbers of the PCNA+ cells quantified. Scale bar = 50µm and applies to all images.

Conclusions and Future Directions

- We have shown that we can induce an inflammatory response in the zebrafish retina by using the microbial compound zymosan to mimic infection. Resident and infiltrating immune cells mount an inflammatory response as early as 24 hpi and continue to infiltrate up to 48 hpi. Along with infiltrating immune cells characteristic of an inflammatory response, we observed an increase in gene expression for target genes that are indicative of a pro-inflammatory state of macrophage activation.
- In 1x and 2x zymosan injected XOPS:mCFP retinas, TUNEL+ and PCNA+ cells were slightly more numerous than in saline injected controls (although differences were not statistically significant). Also, less cell death appeared to be attributed to CFP+ rods in the zymosan injected retinas.
- The future of this project will be to determine if sustained inflammation (for longer periods) in the retinal microenvironment may result in subsequent cone death, other other signs of pathology, in XOPS:mCFP retinas, more akin to simulating a chronic degenerative disease. Moving forward we hope to directly probe the contributions of a dysregulated immune environment to retinal degenerative disease.

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