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Yimeng (Lina) Du April 9, 2019

Visual Recovery Following Optic Nerve Crush in Male and Female Wild-type and TRIF Deficient Mice

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Abstract

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Optic Nerve Injury induces neurodegeneration both in the retina and in the visual structures of the brain and some of the major signaling pathways involved in this type of injury have been identified. In addition to the identified pathways, the TIR-domain-containing interferon- β (TRIF) adapting solely to toll-like receptors (TLR)-3, may also play an important role in post-optic nerve crush (ONC) neurodegeneration of the optic nerve and retina, due to its mediation of inflammatory TLR3 signaling when overexpressed. In this study, we hypothesize that mice with the TRIF gene knocked out will demonstrate decreased inflammatory responses and greater recovery from ONC. We found that TRIF knockout mice had reduced inflammation and neurodegeneration, better neuroprotection in the retina and optic nerves and, better recovery of visual function following ONC compared to those of wild type mice. This study allows us to gain more understanding of the role of TRIF pathway in the optic nerve regeneration and retinal neurodegeneration. Since the TRIF pathway is commonly involved in the innate immune response in the brain, our study will also further clarify its role in the central nervous system.

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I. Introduction

Optic nerve injury causes serious disruptions of visual function both in the retina and in the brain (Sabel et al., 2011). Injury to the optic nerve can occur secondary to brain trauma, blast injury or penetrating orbital trauma (Lukas et al., 2009). Other optic neuropathies result from glaucoma, ischemia, tumors, infections, inflammatory processes and demyelinating conditions (Chan et al., 2014). Despite its variety in etiologies, injury to the optic nerve induces substantial morphological degeneration and reorganization in the retina and in the visual areas of the brain; in particular because of the excitotoxicity triggering a cascade of inflammatory responses and thus cytotoxic cell death.

In order to better understand the pathophysiology of optic nerve injury, a well-characterized rodent model of optic nerve injury – optic nerve crush (ONC) – was developed as early as the mid-1980s (Misantone et al., 1984). This model is characterized by an acute insult to the optic nerve that induces retrograde degeneration of most retinal ganglion cells within the first two weeks after injury. The retinal ganglion cells' degeneration is associated with inflammatory responses in both the injured and contralateral eyes (Rovere et al., 2016). Further evidence using the ONC model shows that degeneration of the cells could be prevented, and axons could be regenerated by activation of the intrinsic growth state of retinal ganglion cells, maintenance of their viability, and counteraction of inhibitory signals in the extracellular environment (Benowitz et al., 2010). In terms of signals in the extracellular environment, the alteration of inflammatory pathways in particular leads to some neuronal protection (Laha et al., 2017). For example, amacrine interneurons release zinc after ONC, which limits the regeneration of retinal ganglion cells (Benowitz et al., 2015; Li et al., 2017). Other studies show that the activation of glial cells, such as astrocytes and microglia, may limit RGC regeneration (Davies et al., 1997). Furthermore,

the proteins controlling inflammatory-related cytokines, such as p38 MAP kinase (Dapper et al, 2013), hamper the axonal regeneration while neurotropic factors, such as ciliary neurotropic factor (CNTF), promote axonal regeneration and cell survival (Leibinger et al., 2009; Park et al., 2009).

The TRIF pathway plays a significant role in neuroinflammation following injuries to the brain and retina. After exposure to excitotoxic pathogens, toll like receptors (TLR) activate the innate immune system signaling cascade and stimulate the release of inflammatory cytokines that lead to a series of cytotoxic events. For example, upon activation the toll-like receptor 4 regulates cell death. In fact, the inhibition of the receptor reduces TGF-β2-induced fibrosis thus enhancing RGC survival in the ONC and ischemic injury models (Poyomtip, 2018). Similar to toll like receptor 4, the Toll-like receptor 3 (TLR3) is expressed in neurons, astrocytes, and microglia and mediates the inflammatory processes in these cells (Moraes et al., 2012). The Toll-like receptor (TLR3) has a sole downstream adaptor known as the TIR-domain-containing, adaptor-inducing interferon-β (TRIF). In the optic nerve crush model, acute and chronic neurodegenerative retinal disease may progress if TRIF is overexpressed (Lin et al., 2012).

In order to further identify and better understand the role of the TRIF inflammatory pathway within the processes of RGC death and axon regeneration in the optic nerve crush model, we assessed functional and morphological outcomes in TRIF knock-out mice and wild type mice with induced optic nerve crush. We hypothesize that TRIF deficient mice would demonstrate less RGC degeneration, more axonal regeneration and greater recovery of visual function. Our predictions are as follows:

a. *compared to that of wild type mice, TRIF deficient mice will* ***have less retinal ganglion cell loss, more axonal growth and reduced inflammatory response*** *in both the retina and the optic nerve following ONC;*

b. compared to that of wild type mice, TRIF deficient mice will ***have better recovery of visual function*** *(VEP and visual acuity) following ONC.*

This study broadens our understanding of the interactions between the responses of the innate immune system and subsequent processes of neuronal degeneration and regeneration. Functional testing provides us with evidence for neuronal regeneration as well as potential implications for clinical treatment. Furthermore, both female and male mice are included to address the possible sex differences within the recovery process from optic nerve injury. As we assess the recovery processes from optic nerve crush in mice in relationship to the TRIF-inflammatory pathway, we may better address the possible treatment for diseases such as glaucoma, and other traumatic and ischemic neuropathies resulting in the damage to the optic nerve.

II. Methods

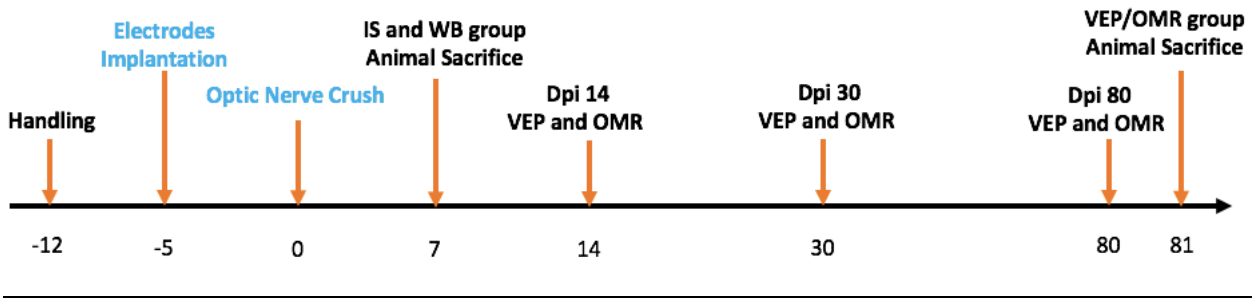


Figure 1. Experimental Timeline

2.1 Animals

All animal related procedures in this study were in accordance with Emory University Division of Animal Resources guidelines for the use of experimental animals and approved by the Institutional Animal Care and Use Committee (Emory University protocol DAR-2003137-063018GN), and conformed to the National Institutes of Health guidelines and the ARVO Statement for the Use of Animals in Ophthalmic and Vision Research.

Adult female and male C57BL/6 mice (wild type; WT) and C57BL/6J-*Ticam1*^{Lps2}/J (trif deficient mice; TKO) of age 8-10 weeks were used in the experiment. The C57BL/6J-*Ticam1*^{Lps2}/J mice are bred in the laboratory and are the third generation of the mice originally from the Jackson Laboratory, Bar Harbor, ME, USA. In the C57BL/6J-*Ticam1*^{Lps2}/J mice, tumor necrosis factor (TNF) fails to be produced by macrophages upon challenge from synthetic lipid A, endotoxin lipopolysaccharide (LPS), and dsRNA. Mice were housed on a 12 hours light/dark schedule with water and food available in Emory Hospital Clinic B Division of Animal Resources.

2.2 Experimental Design

Four experimental groups – TKO treatment (10 males and 5 females), WT treatment (10 males and 5 females), TKO sham (5 males and 5 females), and WT sham (5 males and 5 females) were used in the experiment. Mice were acclimated to the laboratory environment by handling for one week. Then in each experimental group, 5 animals were taken for immunohistochemistry, 5 animals for western blot, and 5 animals for western blot. Electrodes were implanted over their primary visual cortex and mice were allowed to recover for five days. ONC surgery or sham operation was then performed on all mice. Visual evoked potentials and optomotor responses (explained below) were assessed on day 14, 30, and 80 after ONC injury. Mice were euthanized and the retinas, optic nerves and brains were collected for immunohistochemistry and western blot of inflammatory markers on day 7 after ONC, and immunohistochemistry of GAP-43 and retinal ganglion cells on day 30 after ONC. The timeline of the experiment is shown in Figure 1.

2.3 Electrode Implantation

Mice were anesthetized with 80 mg/kg ketamine and 16mg/kg xylazine intraperitoneally (i.p.). Animals were fixed on a stereotaxic frame with their body temperature maintained at 37 Celsius with a heating pad. Chlorhexidine solution was used to disinfect the skin after the head was shaved. After the application of the topical anesthetic proparacaine 1% to the scalp, a midline scalp incision was made. The exposed skull was cleaned with saline and dried. Stainless steel screws (Fine Science Tools, Heidelberg, Germany) were inserted over the V1b cortical surface of each hemisphere through small burr holes (V1b: 0.0mm lambda anteroposterior, ±

3.0mm mediolateral) in the skull above the visual cortex. Cyanoacrylate and dental cement were used to stabilize the screws. Animals were allowed to recover for five days prior to any testing.

2.4 Unilateral Optic Nerve Crush

Optic nerve crush was induced in a group of wild type mice and in a group of TKO mice. All mice were anesthetized with 80 mg/kg ketamine and 16mg/kg xylazine i.p.. A lateral incision of the conjunctiva was performed on the conjunctiva, followed by the exposure of the optic nerve by blunt dissection, leaving the dura and blood supply intact. Dumont self-closing forceps N7 (Fine Science Tools) with a calibrating mircrometer screw (Emory University Physics Machine Shop) was used to apply pressure on the optic nerve for 5 seconds at a distance of 1mm from the eye, with the forceps jaws 0.04 apart. All procedures were performed aseptically and on the left eye. Topical antibiotic eye ointment (Bausch and Lomb, USA) was applied to the injured site. No procedures were performed on the right eye. Animals in the sham group underwent the anesthesia as well as the exposure of the optic nerve with nerves left intact.

2.5 Fixation and Sectioning

Seven days after optic nerve crush, mice were deeply anesthetized with 80 mg/kg ketamine and 16 mg/kg xylazine i.p.. They were then perfused intracardially with 0.1 M PBS and then 10% buffered formalin. The retinas were removed from the mice, post-fixed overnight, and 15% sucrose followed by 30% sucrose is used to cryoprotect them. The retinas were cryosectioned into 14 μm cross-sections and the optic nerves into 10 um longitudinal sections.

2.6 Immunohistochemistry

Sections with fixed 14μm cross-sections of the retina were rehydrated in 0.1 M TBS – 0.3% Tween 100 solution. After protein blocking (X0909 Dako, Carpinteria, CA, USA) in the humid chamber for 30 min at room temperature, slides were incubated with primary antibody at 4 Celsius overnight. Primary antibody raised in rabbit against Iba-1 (Wako, WDJ 3047) diluted 1:1000 was used as a marker for microglial cells and primary antibody raised in rat against GFAP (Abcam, ab7260, 1:500) was used to mark astrocytes. Primary antibody raised in Guinea pig against RMPBS (Santa Cruz Biotechnology, sc-2930, 1:400) was used to visualize retinal ganglion cells and primary antibody raised in mouse against GAP43 (Santa Cruz Biotechnology, sc-17790, 1:500) was used to label regenerated axons within the optic nerve. Slides were washed with TBS-T (0.1 M TBS with 0.3% tween) three times, 10 minutes each time, prior to incubation in fluorescent secondary antibodies for 60 minutes at room temperature. Secondary antibody raised in goat against rabbit (SeraCare Life Sciences, 5220-0336, 1:200), goat against rat (Kirkegaard & Perry Laboratories, # 14-16-06, 1:200), goat against guinea pig (Kirkegaard & Perry Laboratories, # 14-17-06, 1:200), and goat against mouse (SeraCare Life Sciences, # 5220-0341, 1:200) were used as secondary antibodies to visualize the respective primary antibodies. Sections incubated with only secondary antibodies served as negative controls. Afterwards, slides were mounted with Vectashield mounting medium with 4', 6-diamidino-2-phenylindole (DAPI, 0.5μg/ ml, Vector Laboratories, Burlingame, CA, USA) coverslipped and then were scanned in a fluorescent microscope(Ziess Axioskop, Zeiss, Jena, Germany).

2.7 Axon Quantification in the Optic Nerve

GAP-43 was used as a marker of re-growing axons. The number of stained axons was counted 0.50 mm from the crush site. Based on the nerve radius and the thickness of the retinal slices, the total number of GAP-43 positive axons was quantified by $\Sigma ad = \pi r2 \times$ [average axons/mm]/t, where total number axons 0.50 mm from the crush site in a nerve having a radius of r and a thickness of t, was estimated.

2.8 VEP Recording and Analysis

Visual evoked potentials were recorded in the left and right primary visual cortices of all mice at 14, 30, and 80 days after optic nerve crush. Prior to testing, mice were anesthetized with 80 mg/kg ketamine and 16 mg/kg xylazine, i.p. and placed on a heating pad in front of a computer monitor. Eyes were lubricated with 0.9% saline drops. A bandpass filter set at 0.3-500 Hz from the UTAS-E3000 system and software (LKC, Gaithersburg, MD, USA) was used to acquire and amplify the signals.

Visual stimuli consisting of sinusoidal gratings of 100% contrast (0.05 cycles/deg, reversal cycle 1s) were presented independently to the left and right eyes in random order at 300 stimuli per block and two blocks per eye. To prevent stimulation of the opposite eye, a black plastic monocular patch was used to cover it. Gratings of the vertical orientation were used. Each single evoked response was 500 msec long and averaged for the 300-stimulus block. The peak-to-peak amplitude of the averaged VEP was calculated.

2.9 Measurement of optomotor behavior

To assess the visual spatial frequency threshold a virtual optomotor system was used (OptoMotry system; Cerebral Mechanics, Lethbridge, AB, Canada) (Prusky et al, 2004; Douglas et al., 2005). Stimuli consisted of vertical gratings (12°/s) of 100% contrast with an initial spatial frequency of 0.003 cycles per degree. The ability of mice to detect and react to stimuli was judged by their head movements in each condition. Spatial frequency increased stepwise until the response threshold was crossed three times and the highest spatial frequency to induce head movement in accordance with the stimulus direction was considered the spatial frequency threshold for the specific eye.

2.10 Western Blot

Isoflurane was used to deeply anesthetize the animals before decapitation. Retinas were quickly removed and dissected under a binocular microscope.

Retinas were lysed in homogenizing buffer (tissue protein extraction reagent with protease inhibitor, 99:1). The lysate was separated by centrifugation at 11 rpm at 4 Celsius for 10 minutes. Protein concentration was measured using Bradford protein assay.

20 µg samples were loaded into 20% sodium dodecyl sulfate (SDS) – poly acrylamide gradient gel and transferred onto polyvinylidene fluoride transfer membranes using a 110V for 45 minutes. The blots were washed with TBS-Tween 0.3% and blocked with 5% milk for 2 hours at room temperature. The membranes were incubated overnight at 4 ℃ with primary anti-NF-kB p65 antibody raised in rabbit (1:1000, #8242, Cell Signaling, Danvers, MA, USA), diluted in 5% milk in TBS-Tween 0.3%. Three washes with TBS-Tween 0.3% were followed by incubation with peroxidase-conjugated secondary antibody (species, concentration, info) for 90

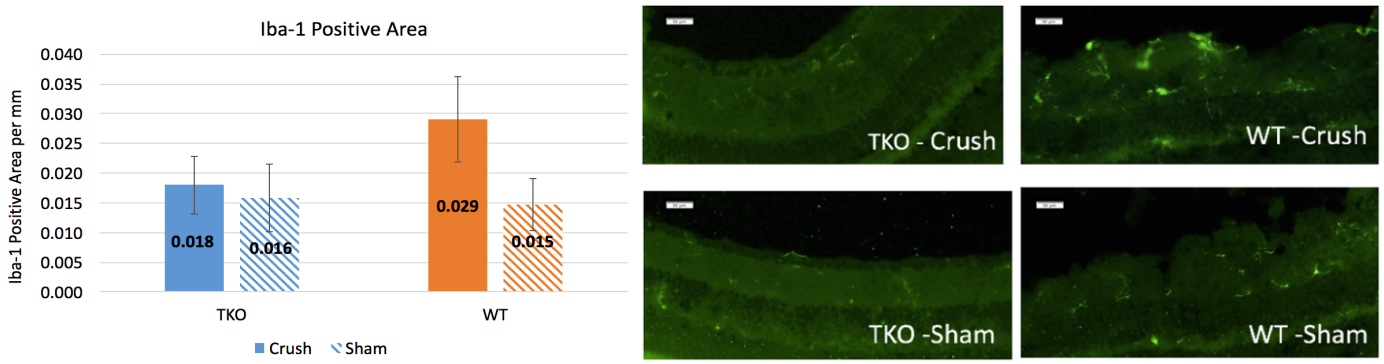
minutes at 25 Celsius. Blots were visualized with ECL reagent and developed with HyBlot CL film in the dark room by an enhanced chemiluminescence system (E2400, Denville Scientific, South Plainfield, NJ, USA). The same membranes were incubated with anti-β-actin for loading control (1:1000, A5316, Sigma). Blots were quantified using Image J software (NIH) and density of NF-kB normalized to density of β-actin in the same gel.

2.11 Statistical Analysis

IBM SPSS Statistics 23.0 for Windows was used to evaluate all data. Repeated and factorial measures ANOVA were used to compare differences in VEP and OMR over time.

Immunofluorescent and western blot densities of each marker were measured in the left and right retina with Image J software. The group differences were evaluated by factorial analyses of variance (ANOVA). A value of $p < 0.05$ was considered significant.

II. Results



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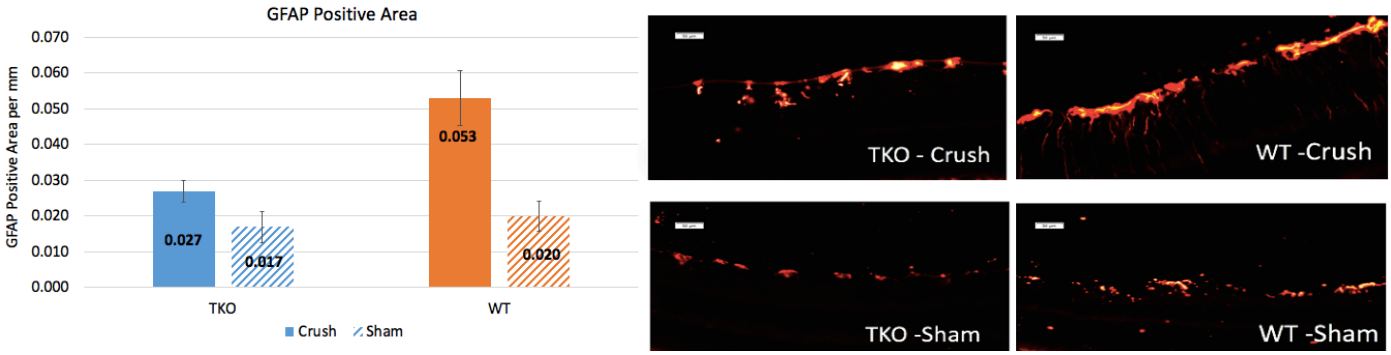

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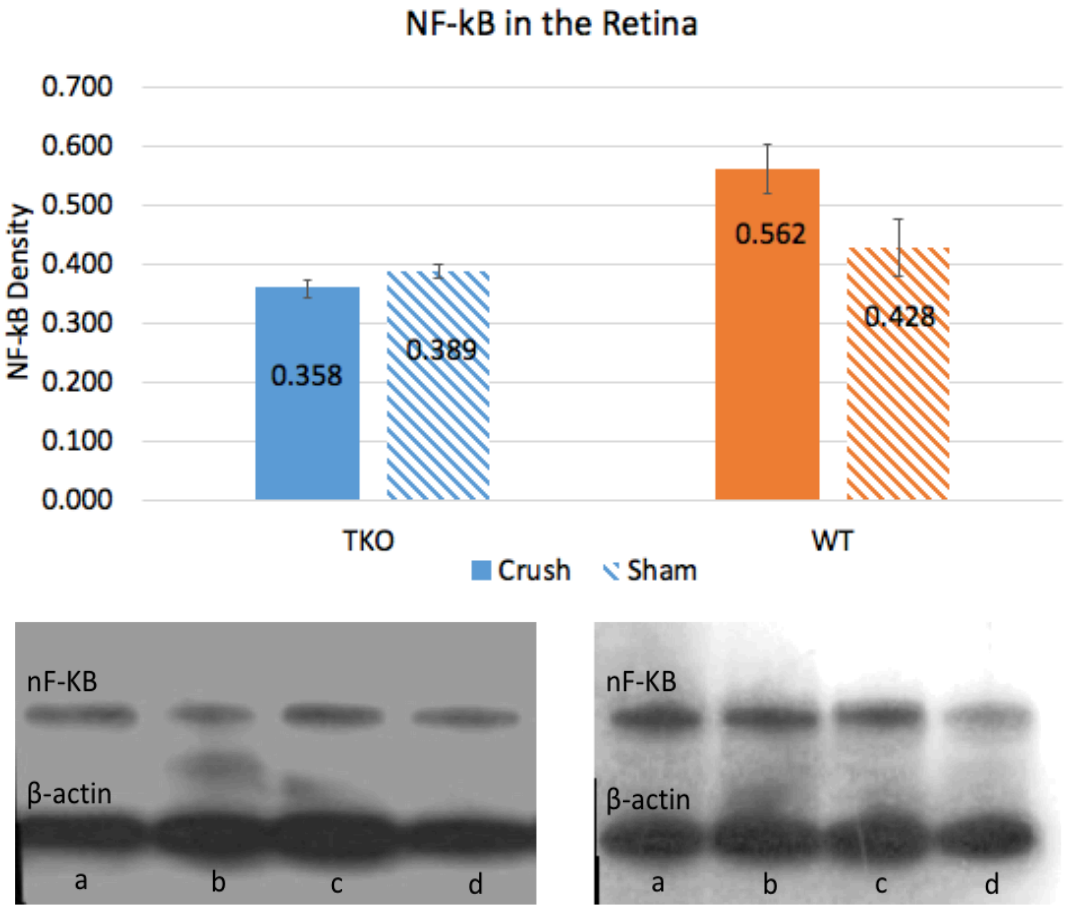


C)

Figure 2. TKO mice had significantly less inflammatory response compared to WT mice 7 days after Optic Nerve Crush. (A) Fewer microglia were present (indicated by reduced IBa-1 positive area, $p = 0.03$). (B) Fewer astrocytes were present (indicated by reduced GFAP positive area, $p < 0.01$). (C) Level of the inflammatory cytokine NF-kB was lower ($p = 0.01$). Left panels show results from 9 mice per crush conditions and 6 mice for sham conditions in both TKO and WT groups; significance tested by one-way analysis of variance; error bars indicate standard errors. Right panels of (A) and (B) show immunofluorescent images of the relevant marker; scale bar is 50 µm. The bottom panel of (C) shows a sample of Western blot. The left picture shows samples of a)WT-M Crush, b)TKO-M Crush, c)WT-M Sham, and d)TKO-M Sham; the right picture shows samples of a)WT-M Crush, b) TKO-F Crush, c) TKO-M Crush, and d)TKO-M Sham.

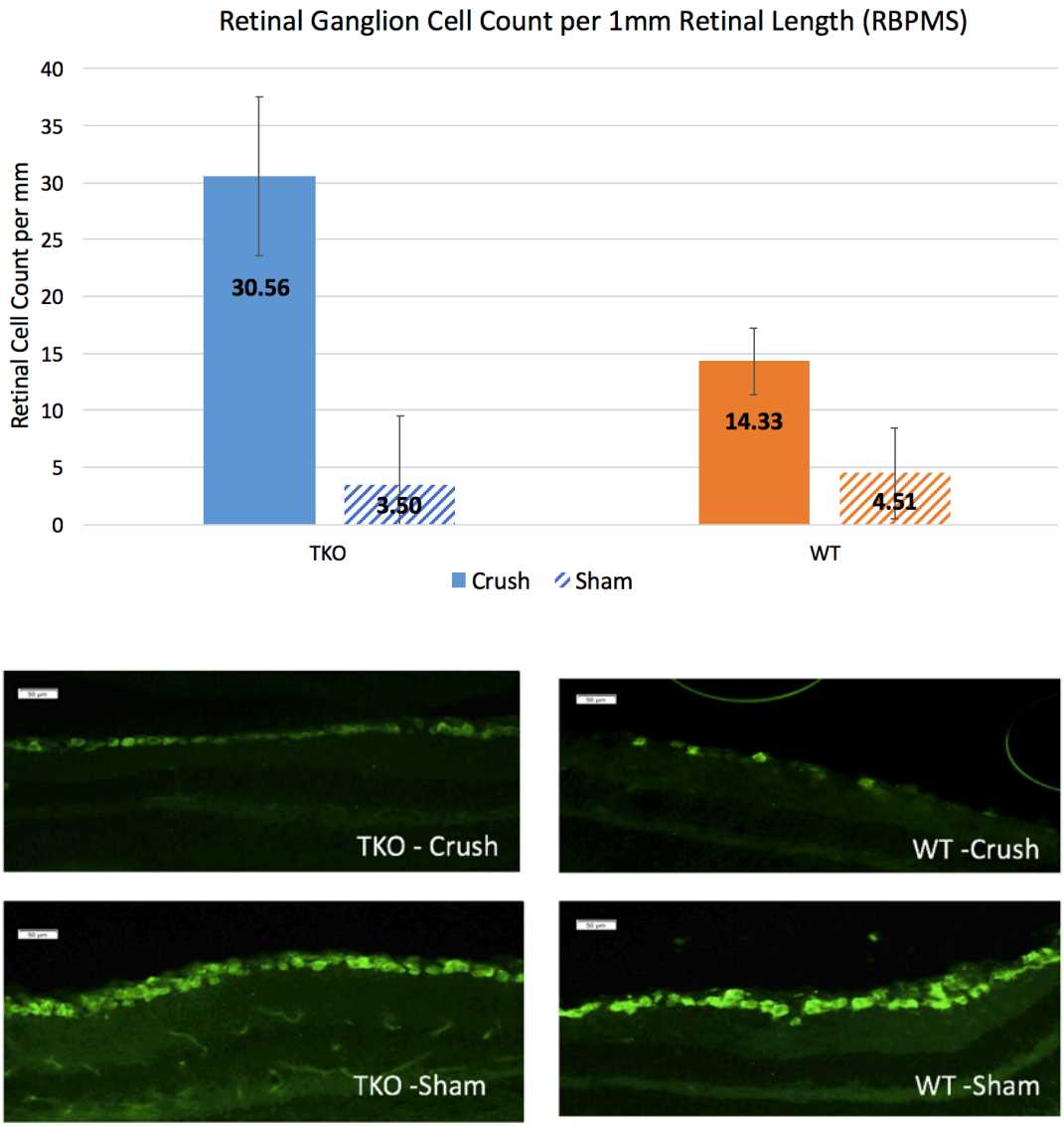
3.1 Reduced inflammatory response in the retina of TRIF deficient mice 7 days after optic nerve crush

Seven days after optic nerve crush, TKO mice showed reduced inflammatory responses in the retinas than those of WT mice (Figure 2). This was evident as reduced proportions of microglial cells (as shown by less Iba-1 staining, $p = 0.03$) and reduced astrocyte generation (as shown by less GFAP staining, $p < 0.01$) is shown in retinas of TKO mice. In addition, the inflammatory protein NF-kB was significantly lesser in that of TKO mice seven days after optic nerve crush. The sham surgery groups of TKO and WT mice were comparable in all inflammatory measures.

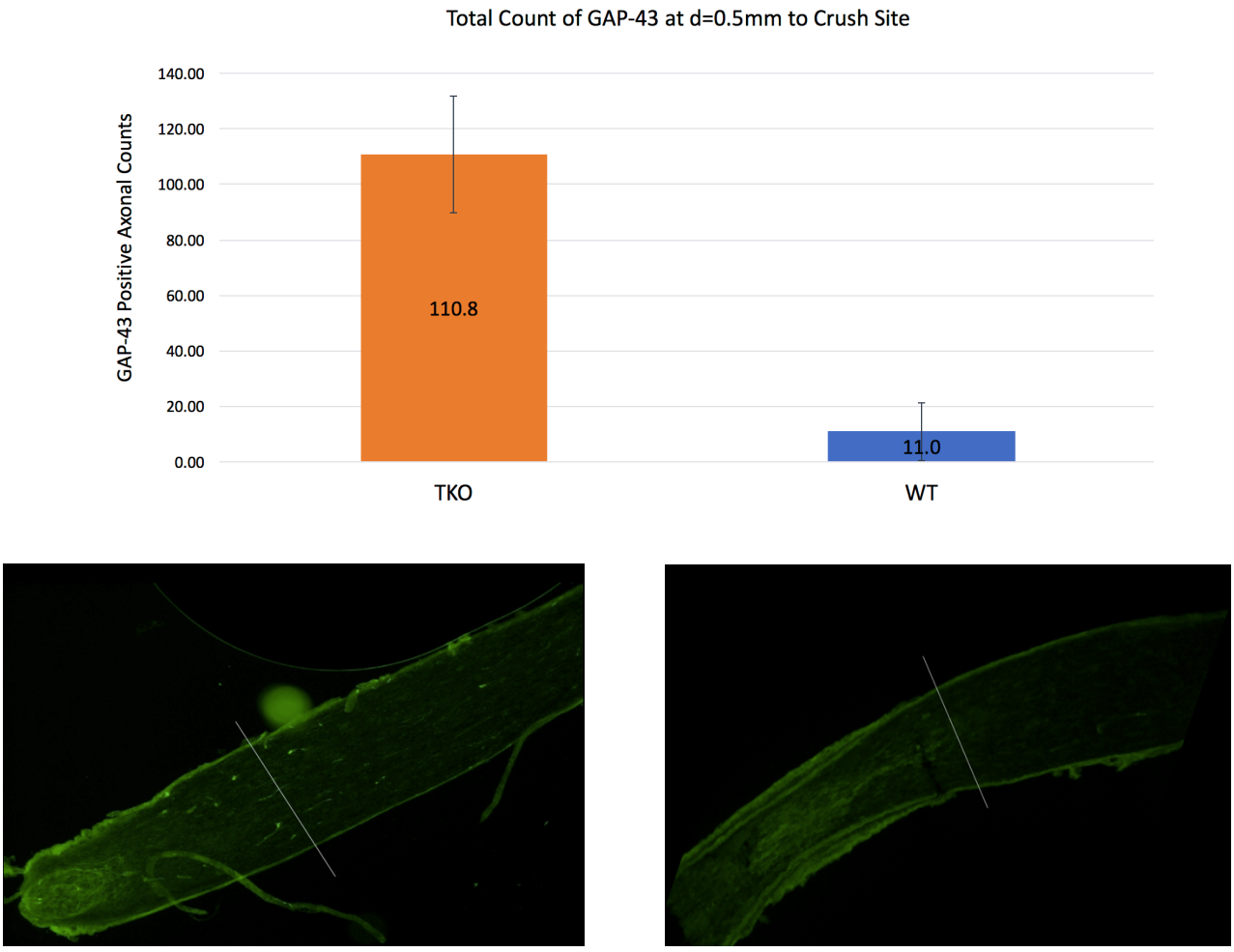
3.2 Less retinal ganglion cell loss and greater axonal regeneration in TRIF deficient mice 30 days after optic nerve crush

In addition to the reduced inflammatory responses, retinal ganglion cells in the retina were visualized seven days after ONC (bottom panel of figure 3A). It is shown that TKO mice demonstrated significantly less retinal ganglion cell loss than those of wild type mice (shown by less RBPMS staining, $p = 0.027$).

Furthermore, compared to those of WT mice, TKO mice exhibited considerably greater GAP-43 levels 30 days after ONC, indicating greater axonal growth after nerve injury ($p = 0.01$). The bottom panel of figure 3B shows the site of GAP-43 measurement at a distance of 0.50 mm from the crush site in the optic nerve of TKO and WT mice.

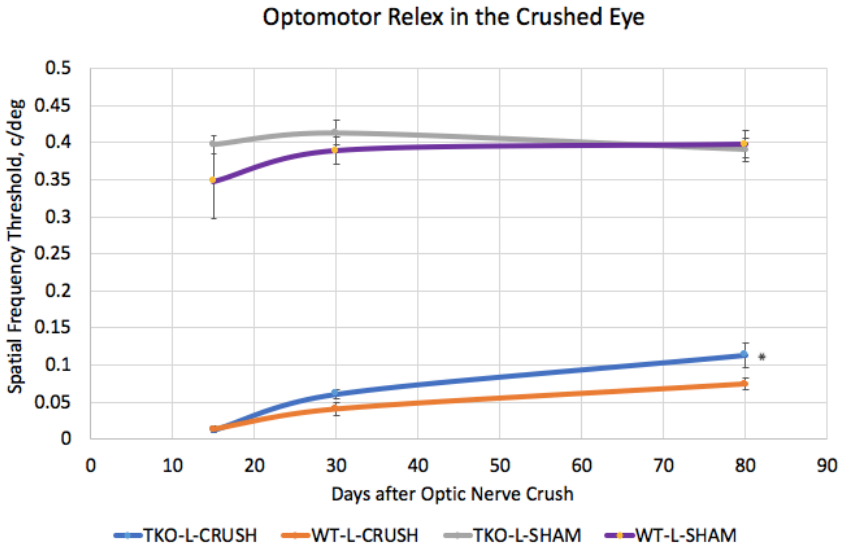

Retinal Ganglion Cell Count per 1mm Retinal Length (RBPMS)
Retinal Cell Count per mm
40
35
30
25
20
15
10
5
0
30.56
3.50
14.33
4.51
TKO
WT
Crush
Sham
TKO - Crush
WT -Crush
TKO -Sham
WT -Sham

A)



B)

Figure 3. TKO mice had significantly better neuronal recovery 30 days after ONC. (A) More retinal ganglion cells were present in the retina (measured as RBPMS immunoreactive cells, p = 0.027). (B) More axons were present in the optic nerve (measured as GAP-43 immunoreactive cells, p = 0.01). No GAP-43 is manifested under the sham condition. Left panels show results from 9 mice per crush condition and 6 mice per sham condition in both TKO and WT groups; significance tested by one-way analysis of variance. Bottom panel shows sample axon GAP-43 count of TKO (left) and WT (right) mice at 0.5mm from the crush site (white line) ; scale bar is 50 µm; error bars indicate standard errors.



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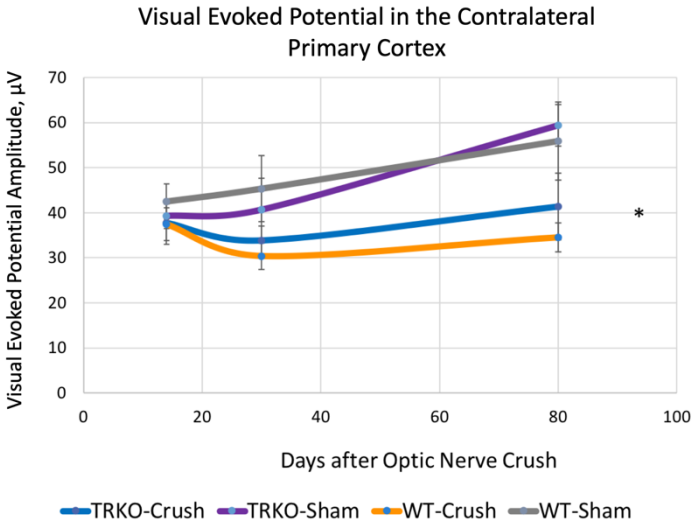

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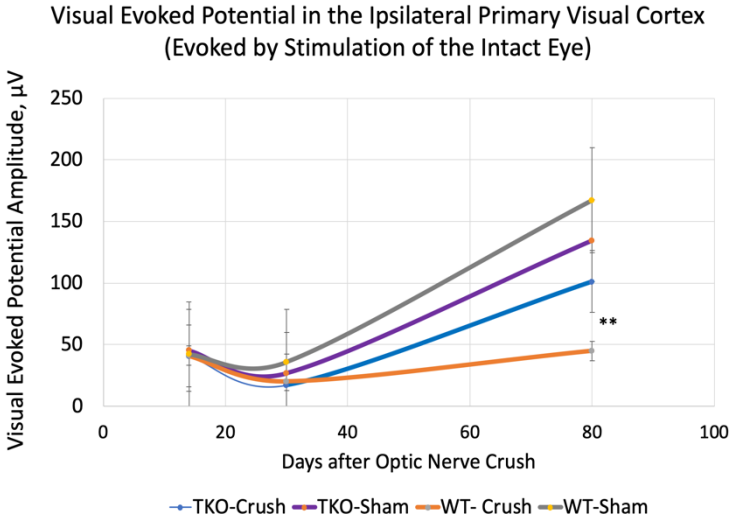


C)

Figure 4. TKO mice had slightly improved visual function 80 days after ONC. No significant differences were found at 14 or 30 days after ONC. (A) Oculomotor reflex spatial frequency threshold was slightly higher ($p < 0.05$). Visual evoked potentials of TKO mice were greater in the primary visual cortices both contralateral (B) and ipsilateral (C) to the crushed nerve compared to WT controls ($p = 0.047$ and $p = 0.006$, respectively). Data came from 9 mice per crush and 6 mice per sham condition in both TKO and WT groups; significance tested by one-way analysis of variance; error bars indicate standard errors.

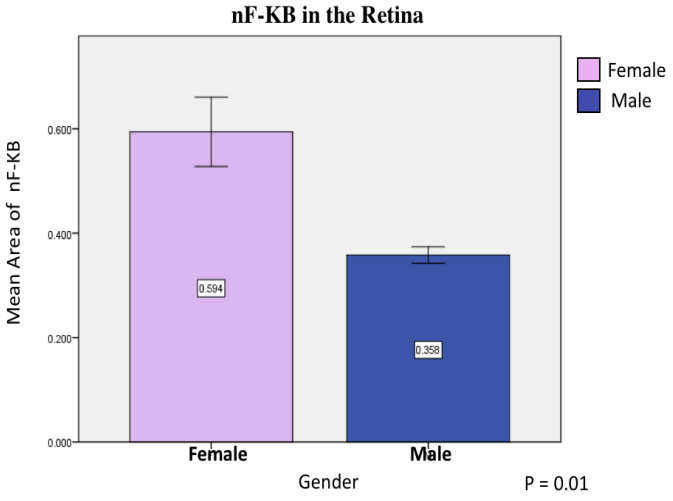
3.3 Better recovery of visual function in TRIF deficient mice 80 days after optic nerve crush

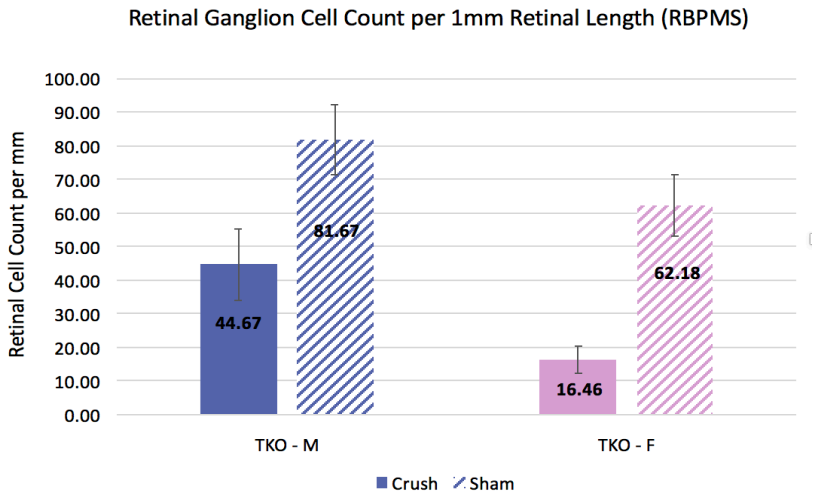
Visual function in mice was measured by the spatial frequency threshold in the optomotor reflex test and the visual evoked potential in the primary visual cortex at 14, 30, and 80 days after ONC. For the optomotor reflex test, no difference between TKO and WT mice was seen 14 and 30 days after the injury. The higher spatial frequency threshold in TKO mice compared to WT mice only appears 80 days after ONC (figure 4A; $p < 0.05$).

The pattern of visual evoked potential in the contralateral primary cortex of the injured left eye was also different for WT and TKO mice 80 days after ONC, with TKO mice having a greater response to the stimulus than WT mice (figure 4B; $p = 0.047$). The pattern of visual evoked potential in the ipsilateral primary visual cortex evoked by the stimulation of the intact eye in WT and TKO mice also exhibited differences at 80 days, as WT mice showed a reduced response in the right visual cortex to visual stimuli in the right/intact eye compared to that of TKO mice (figure 4C; $p = 0.006$).

3.4 Sex differences in the recovery pattern of optic nerve crush

Interestingly, TKO female and male mice showed different levels of NF-kB after nerve injury. Particularly, TKO males demonstrated lesser NF-kB levels compared to TKO females seven days after optic nerve crush ($p = 0.01$). Sex differences in recovery from ONC were also manifested in the differences in retinal ganglion cell losses between TKO females and males. Shown in figure 5B, TKO male had less retinal ganglion cell loss compared to that of TKO female mice seven days after optic nerve crush (shown by RBPMS staining, $p = 0.048$).





A) *B)*

Figure 5. Female TKO mice had a greater inflammatory response and greater retinal ganglion cell loss than male TKO mice seven days after ONC. (A) Female TKO mice had significantly more NF-kB than males (p = 0.01). (B) Female TKO mice had significantly fewer retinal ganglion cells than males (p = 0.048). Data came from 6 males and 3 females in the crush condition and 4 males and 2 females in the sham condition; significance is tested by one-way analysis of variance; error bars indicate standard errors.

III. Discussion

4.1 The TRIF adaptor in Inflammatory Pathways of the CNS

So far, there are only a few studies concerning the involvement of the TRIF pathway in optic nerve crush model and only the current study has looked specifically at its role in the recovery of visual function. Here, visual function is directly assessed using optomotor tests and visual evoked potentials. We also measured injury-associated biomarkers in TRIF deficient mice with optic nerve damage. These included markers of inflammatory responses (GFAP, Iba-1), retinal ganglion cells (RBPMS), and regenerating axons (GAP-43). The functional visual tests were conducted for 80 days, allowing significant recovery of the optic nerve. The longer evaluation of recovery time led to greater observable differences between TRIF deficient and wild type mice in restitution of visual function. Seen in RGC survival and visual acuity, the recovery pattern from optic nerve crush of females and males also differ from each other. The sex differences were observed in the inflammatory processes, neuronal cell losses, as well as visual acuity. These differences will be more fully discussed in section 4.5.

As seen in our study, the recovery of visual function is linked to the reduced inflammatory response in TRIF deficiency. Other studies have demonstrated the significance of the TRIF adaptor in inflammatory responses. For example, it has been shown that TRIF signaling is required for the caspase 11 dependent immune response and thus is involved in the lethality in endotoxemia and sepsis (Shimizu et al., 2010). Furthermore, TRIF deficiency reduced the number of natural killer (NK), NK-T-lymphocytes, and CD8-T cells infiltrating into the spinal cord of mice with Amyotrophic lateral sclerosis (Komine et al., 2017). Other studies also show that TRIF protein is essential in activating the common MyD88-dependent signaling pathway as

well as a MyD88-independent pathway. It is worth noting that the dual signaling of MyD88 and TRIF is crucial for dendritic maturation (Yamamoto, 2003). Here, we studied the role of the TRIF-mediated-inflammatory pathway in a mouse model of optic nerve injury.

In line with the work of Lin et al. (2012), our study showed that NF-kB is reduced as a result of TRIF deficiency after injury in the RGCs. In addition, expression of the downstream proteins TBK1 and IKKε in the TRIF pathway was also decreased in TRIF deficient mice (Lin et al. 2012). Lin et al., further showed that inflammatory factors such as nitric oxide synthase, tumor necrosis factor-a, interferon-β, and interleukins-1β, 6 and 17 were found to decrease in the retinal ganglion cells of TRIF deficient animals. This suggests that the deletion of the TRIF gene results in decreased activation of the inflammatory NF-kB pathway and in the decreased recruitment of neurotoxic factors in the injured retina.

In addition, we found that expression of both GFAP and Iba-1 markers was reduced in TRIF deficient mice 7 days after optic nerve crush, suggesting reduced astrocyte and microglial activation. This may be the result of attenuated activation of NF-kB in both types of cells. TRIF is only expressed in the microglial cells of the retina (Lin et al. 2012). Since astrocytes also compose the microenvironment of the unmyelinated part of the RGC axon, TRIF deficiency may lead to interactions that indirectly result in the reduction of astrocytes activation (Laha et al., 2017). One possible mechanism for this change could be the reduction of miRNA-21 activation in astrocytes resulting from decreased cytokines in the microenvironment, thus leading to decreased astrocytosis (Ma et al., 2011).

Since the focus of our experiment is the direct comparison of post-ONC inflammatory responses between TRIF deficient mice and wild type mice, the measure of inflammatory responses was only conducted at 7 days after the injury. Further studies could analyze the

progression of astrocytes and that of the microglial cells over the time course of the inflammatory response to illustrate the association between post-injury developments of these two types of glial cells. In addition, the shape of the microglial cells after optic nerve crush could have been examined to evaluate cellular activation since the form of retinal microglial cells change from a resting dendritic-like shape to an ameboid form upon activation after injury (Wang et al, 2016).

4.2 The TRIF Inflammatory Pathway and Retinal Ganglion Cell Death

In this study, we found that 7 days after optic nerve crush, retinal ganglion cell death as well as inflammatory markers in the retina of TKO mice were significantly less present than those of WT mice. Reactive gliosis, or the reaction of glia in the optic nerve and retina in response to optic nerve crush, is generally considered as pro-inflammatory. Usually, inflammation is associated with nerve degeneration and considered detrimental to regeneration. Simply stated, inflammation facilitates the progression of nerve degradation and hampers nerve regeneration. For example, anti-inflammatory p38 kinase inhibitors prevent the degradation of axons in the optic nerve (Dapper et al, 2013). Moreover, removal of components of astrocyte gliosis, such as GFAP and vimentin, is associated with increased post-trauma adult neurogenesis and greater survival of neuronal grafts (Pekny and Pekna, 2014). In this study, the retinal ganglion cell survival and the GAP-43 protein, a marker of re-growing axons, were significantly higher in male and female mice with reduction in TRIF-induced inflammation. Therefore, down-regulation of TRIF-induced-inflammatory pathways as well as reduced glia cell proliferation are also seen to correlate with better RGC survival and axonal regeneration in male TRIF deficient mice. Likewise, RGC survival and axonal regeneration are up-regulated by the inhibition of

miRNA-21, which reduces muller cell gliosis (Li et al., 2019). Both down-regulation of glial cells and inflammatory pathways are regarded as extrinsic factors in the optic nerve environment; in contrast to the intrinsic factors; such as the intracellular transcription factors in the RGC axons (Laha et al., 2017).

It is important to note that recent studies on other extrinsic factors, have shown that reactive gliosis and inflammation can mediate processes that promote the regeneration of axons (Calkins et al., 2017; Yin, 2009). In a study by Anderson et al. (2016), the prevention of astrocyte scar formation resulted in failed regrowth of transected corticospinal axons after severe spinal cord injury lesions in mice. Other papers have reported that additional recruitment of microglia as a result of inflammation caused more significant neural tissue regeneration in the central nervous system as well as peripheral nervous system (Shechter and Schwartz, 2013; Niemi et al, 2013). Therefore, it appears likely that some degree of inflammation could be necessary to initiate neuronal regeneration. Nonetheless, persistent activation of the inflammatory responses typically leads to damage in the optic nerve. Studies show that reduced microglial activation is associated with reduced retinal and optic nerve degeneration in mice with glaucoma (Bosco 2012). Interestingly, an increase in TLR4 and other TLRs in retinal glaucoma leads to greater microglial activation and exacerbation of glaucoma (Luo et al., 2010). The over-expression of TLR receptors and the consequent increase in TRIF protein is associated with greater inflammatory responses leading to detrimental effects to the optic nerve; including the apoptosis of retinal ganglion cells and interruption of nerve regeneration.

4.3 Retinal Ganglion Cell Survival and Axonal Regeneration

Although more GAP 43 is observed with more RGC survival 30 days after injury in TRIF deficient mice, retinal ganglion cell survival and regeneration appear to be governed by different mechanisms (Calkins, 2017). Factors such as brain-derived neurotrophic factor (BDNF) enhance the effect of intraocular inflammation, leading to increased survival of the RGC, yet it eliminates the effect of intraocular inflammation on axonal regeneration (Goldberg et al., 2002). Similarly, deletion of dual leucine zipper kinase (DLK) in RGCs is shown to increase survival of RGCs but hampers regeneration. However, in our experiment we observed linked pro-survival and pro-regeneration effects of molecular factors and pathways; as demonstrated in our analyses of the TRIF protein. Similar linkages were also observed in the effects of mTOR downregulation on recovery from ONC as has been associated with both the survival of RGC as well as the regeneration of the optic nerve (Kurimoto, 2010). Further studies are needed to demonstrate the mechanisms underlying TRIF's involvement in prevention of cell apoptosis and up-regulation of GAP-43 to help understand the connection between its pro-survival and pro-regeneration effects.

4.4 Optic Nerve Regeneration and Recovery of Visual Function

The increased number of GAP-43 positive axons is indicative of better nerve regeneration in TRIF deficient mice at 30 days after injury. Further evidence of axonal regrowth is demonstrated by better recovery of visual function in TKO mice. Although recovery of visual function is indicative of axon regeneration and protection against neuronal degeneration, no studies have looked at the recovery of visual function in TRIF deficient mice. Damaged optic nerve results in significant decrease in visual acuity, contrast sensitivity, and electrical responses

in the visual cortex after optic nerve crush. Mice with TRIF deficiency demonstrate a higher spatial frequency threshold in the optomotor test, thus reflecting the recovery of retinofugal conduction (Prusky, 2004). Moreover, using VEP as a direct measurement of electrical responses in the visual cortex and as a useful measurement in mice with severe visual impairment (Tokashiki, 2018), we noticed that the amplitudes of the visual evoked potentials are higher in TRIF deficient mice in both the affected and unaffected eye. The recovery of visual function suggests the target specificity of axon regeneration (Calkins, 2017); in other words, the regenerating axons are projecting to the right area of the visual cortex and gradually forming an accurate map of the visual space. Further studies using anterograde labeling technique (i.e. intraocular injection of cholera toxin B) will verify the projection areas of the regenerating axons.

Many studies regarding the functional recovery from optic nerve crush measured function only to 21 days or 38 days after the injury (Sabel et al, 2001). However, as axons extend progressively overtime, no axonal regeneration is seen two weeks after optic nerve crush and no functional recovery is seen until ten weeks after optic nerve crush (Lima, 2014). The differences in improvement of visual function between wild type and TRIF deficient mice is seen 80 days after optic nerve crush. In our hands, a longer duration of recovery time increases the chances for better visual function by allowing the damaged optic nerve to recover more extensively.

4.5 Sex Differences in Recovery from Optic Nerve Crush

The sex differences in traumatic brain injury, including optic nerve injury, have often been overlooked (Wright, 2014). As a result, another highlight of our study is the measured differences, both physiological and functional, in the recovery pattern of male and female mice. Of the few studies that investigated the effect of sex on recovery from injury, many signs and symptoms and complications associated with traumatic brain injury are found to be expressed differently in males and females (Kraus, 2009). Sex differences in brain injury outcomes are controversial and depend on a number of factors that demand more attention in pre-clinical studies (Arambula et al., 2019; Gupte et al., 2019). For example, females on average experience a prolonged recovery time from TBI compared to that of males (Styrke, 2013). This similar effect is also seen in our work as TKO male mice show better recovery of visual optomotor reflex compared to female mice 80 days after optic nerve injury. Although sex differences are manifested in the morphological changes in the brain post-TBI (Geddes, 2016) and other cortical functions, including visual discrimination (Clark, 1989), we may be the first one to look at the sex differences in functional recoveries from optic nerve crush. The underlying mechanisms associated with sex differences remain to be investigated. Interestingly, in addition to sex differences in recovery of visual function, greater protection against retinal ganglion cell loss and less inflammation is also shown in TKO male mice. This finding strengthens the correlation between inflammation and neuronal degeneration. Also, it directs our attention to possible gonadal hormonal effects on neuronal degeneration and inflammation, which leads to sex differences in recovery of vision.

4.6 Clinical Significance of the TRIF Pathway

Optic nerve regeneration has been associated with many transcription factors, neurotrophic factors, cell-intrinsic suppressors, and intraocular inflammation (Benowitz, 2015). The inhibition of the TRIF pathway, a possible way to suppress the immune response following neural injury, was rarely investigated in the wound area of the optic nerve injury. Our findings offer a potential novel clinical target for visual recovery after ONC. The elimination of the TRIF gene suppresses the inflammatory response in the retina leading to greater protection against retinal ganglion cell death and more significant axonal regeneration. The regenerated projections of the axons are also refined and targeted in that they restore visual function and visual evoked responses to a certain extent. As a result, the target of the TRIF gene may not only be a treatment for damage to the optic nerve itself, but also could be relevant to the treatment of glaucoma and other neuropathies involving toll-like receptors.

IV. References

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