PERSPECTIVE

Neuroinflammation in glaucoma: soluble tumor necrosis factor alpha and the connection with excitotoxic damage

Inflammation is a complex and highly regulated response that occurs early after infection or injury. This process is initiated by cells of the immune system to re-establish tissue homeostasis. When the injury is persistent, however, chronic inflammation leads to overproduction of noxious mediators that contribute to cell dysfunction and death. The inflammatory response in the central nervous system (CNS), known as neuroinflammation, is achieved by activation of resident glia and monocyte-derived cells. Accumulating evidence indicates that this cellular response occurs in the early stages of numerous neurodegenerative diseases, triggering a cascade of events that converge to promote neuronal damage. Indeed, neuroinflammation has been reported in a host of CNS disorders including Alzheimer's disease, Parkinson's disease, amyotrophic lateral sclerosis, Huntington's disease, multiple sclerosis, stroke, and glaucoma.

Glaucoma is a prevalent neurodegenerative disease and the leading cause of irreversible blindness worldwide affecting over 60 million people. Glaucoma is characterized by the progressive degeneration of retinal ganglion cells (RGC) and their axons in the optic nerve resulting in gradual vision loss. High intraocular pressure is the most significant known risk factor for developing the disease, but the mechanism by which elevated pressure promotes RGC damage is currently unknown. Current therapies are aimed at lowering intraocular pressure, but many patients continue to experience visual field loss even when pressure lowering treatments are implemented. A better understanding of the mechanisms causing glaucomatous neurodegeneration triggered by ocular hypertension injury is, therefore, essential to develop effective therapies.

Accumulating evidence indicates that neuroinflammation plays a key role in RGC damage in glaucoma. A number of studies have confirmed the presence of hallmark features of neuroinflammation in glaucoma animal models and human specimens including glial cell activation, upregulation of proinflammatory cytokines, induction of the complement cascade, and trans-endothelial cell migration of leukocytes (Soto and Howell, 2014). A critical modulator of the neuroinflammatory response in glaucoma is tumor necrosis factor alpha (TNFα). RGCs express the TNFα receptors 1 and 2 (TNFR1/2) and TNFα signaling has been linked to RGC death. For example, exogenous administration of TNFα promotes RGC loss and optic nerve degeneration, and genetic or pharmacological depletion of TNFα or its receptors stimulates RGC survival (Tezel et al., 2008). High-throughput characterization of the retinal proteome revealed significant upregulation of TNFα signaling in human glaucoma (Yang et al., 2011). TNFα levels have been shown to be elevated in aqueous humor samples from glaucoma patients (Sawada et al., 2010; Balaiya et al., 2011; Xin et al., 2013). Notably, TNFα gene polymorphisms are associated with primary open angle glaucoma (Fan et al., 2010; Bozkurt et al., 2012; Xin et al., 2013). A recent meta-analysis study (> 3,000 cases) showed that the TNFα 308G/A polymorphism is significantly linked with higher risk of developing primary open angle glaucoma, predominantly in the Asian population, but not with low tension or exfoliation glaucoma (Xin et al., 2013).

What is the source of TNFα in glaucoma? Chronically reactive glial cells are thought to become a sustained source of proinflammatory cytokines in the CNS. Traditionally, microglia are thought to be the primary source of TNFα after injury or in disease. Using a well-characterized rat model of ocular hypertension glaucoma (Morrison et al., 2015), our team recently demonstrated that high intraocular pressure stimulates production of TNFα by retinal glia (Cueva Vargas et al., 2015). Intriguingly, our results show that Müller cells, the most abundant glial cell type in the retina, rapidly upregulate TNFα in response to increased eye pressure. Müller cells are specialized radial glia that play critical structural, metabolic and support roles for retinal neurons. Consistent with their role as a source of TNFα, Müller cells exposed to selective blockers of the neurotrophin receptor p75NTR, an upstream activator of TNFα production in these cells, promoted RGC survival in models of traumatic axonal injury and excitotoxic damage (Lebrun-Julien et al., 2009a, b). In addition, we observed increased TNFα expression in retinal microglia with amoeboid shape, characteristic of a reactive state, rather than in quiescent cells with ramified morphology (Cueva Vargas et al., 2015). This finding is consistent with previous reports showing TNFα expression in microglia from human glaucomatous optic nerve head and rat retinas subjected to ocular hypertension (Roh et al., 2012). Of interest, high-dose irradiation leading to reduced microglial activation, and presumably decreased levels of proinflammatory mediators, attenuated RGC degeneration in a mouse model of inherited pigmentary glaucoma (Howell et al., 2012). Collectively, these data suggest that both Müller cells and microglia respond rapidly to ocular hypertension by increasing TNFα production.

TNFα plays both homeostatic and pathophysiological roles in the CNS. TNFα is generated as a membrane-bound precursor that is cleaved by the cell surface protease TNFα-converting enzyme (TACE/ADAM17) to release the soluble 17-kDa protein. Both the transmembrane and secreted forms of TNFα are biologically active and play distinct roles *in vivo*. Soluble TNFα binds primarily to TNFR1 and regulates apoptosis and chronic inflammation, whereas membrane-bound TNFα displays a higher affinity for TNFR2 and mediates immunity against pathogens, resolution of inflammation and promotes myelination. Consistent with this, mice expressing only transmembrane TNFα suppress the onset and progression of autoimmune demyelination while maintaining host defenses against bacterial infection, septic shock and pulmonary fibrosis. Therefore, modulation of soluble *versus* transmembrane TNFα signaling might be a powerful strategy to achieve homeostasis in diseases with a neuroinflammatory component.

Which form of TNFα, soluble or transmembrane, is responsible for RGC death in glaucoma? To investigate this, we used an engineered dominant negative peptide, called XPro1595, that selectively inhibits soluble TNFα without interfering with transmembrane TNFα signalling (Zalevsky et al., 2007). XPro1595 binds only to soluble TNFα monomers and forms

inactive heterotrimers that are unable to interact with TNFα receptors. Our data demonstrate that intraocular administration of XPro1595 effectively promoted RGC survival in a rat model of ocular hypertension glaucoma, without altering intraocular pressure (Cueva Vargas et al., 2015). Consistent with the idea that the primary site of degeneration in glaucoma is at the level of RGC axons, we found that glaucomatous eyes had more pronounced axon loss than cell body loss. XPro1595 effectively protected a similar proportion of RGC soma and axons suggesting a dynamic crosstalk between these compartments.

Both TNFR1 and TNFR2 are upregulated by RGCs during ocular hypertension (Cueva Vargas et al., 2015), thus it is likely that blockade of soluble TNFα with XPro1595 minimizes the detrimental effect of TNFR1 activation while preserving beneficial TNFR2-mediated signaling. Recently, other studies have also reported a beneficial effect of XPro1595 in models of Parkinson's and Huntington's disease, spinal cord injury, and experimental autoimmune encephalomyelitis, confirming that soluble TNFα plays a harmful role in the context of multiple neurodegenerative conditions. Etanercept, a drug that blocks both soluble and transmembrane TNFα, also protects RGCs in a rat glaucoma model (Roh et al., 2012). However, non-selective TNFα inhibitors such as etanercept, infliximab and adalimumab have been associated with serious adverse effects including impaired host defense, autoimmunity, lupus, demyelination syndromes and congestive heart failure. Collectively, these findings highlight the benefits of inhibiting soluble TNFα while preserving transmembrane TNFα function during neurodegeneration.

How does TNFα promote RGC death in glaucoma? In physiological conditions, TNFα exerts homeostatic control of synaptic strength by regulating α-amino-3-hydroxy-5-methyl-isoxazolepropionic acid receptor (AMPAR) trafficking in the CNS (Pribiag and Stellwagen, 2014). AMPAR are tetramers assembled from GluA1-4 subunits, and lack of GluA2 confers calcium (Ca^{2+}) permeability through the AMPAR pore. TNFα strengthens synapses in hippocampal pyramidal neurons by inducing rapid exocytosis of AMPAR that lack or have low stoichiometric amounts of the GluA2 subunit thus enhancing intracellular Ca^{2+} levels. Moreover, TNFα was shown to induce expression of Ca^{2+}-permeable-AMPAR (CP-AMPAR) exacerbating neuronal death during acute ischemia and excitotoxicity (Lebrun-Julien et al., 2009b; Pribiag and Stellwagen, 2014). Our team recently reported that ocular hypertension triggered robust upregulation of CP-AMPAR in RGCs in a TNFα-dependent manner. Using a cobalt (Co^{2+}) permeability assay based on the selective transport of Co^{2+} through CP-AMPAR, but not Ca^{2+} channels or N-methyl-D-aspartate (NMDA) receptors, we demonstrated that RGCs accumulate Co^{2+} soon after induction of ocular hypertension (Cueva Vargas et al., 2015). Co^{2+} uptake was blocked by XPro1595 demonstrating TNFα-dependent CP-AMPAR upregulation in these neurons. Furthermore, intraocular delivery of a non-competitive AMPAR antagonist (GYKI 52466) or a polyamine-derived compound that selectively antagonizes CP-AMPAR (philantotoxin 343), blocked Co^{2+} uptake and promoted striking survival of RGC soma and axons in hypertensive eyes (Cueva Vargas et al., 2015), confirming the role of CP-AMPAR in TNFα-induced RGC death.

How do AMPAR become Ca^{2+} permeable in glaucoma? The vast majority of AMPAR in the CNS (> 90%) are not Ca^{2+} permeable, but can become so after injury or in disease. The Ca^{2+} permeability of AMPAR varies depending on whether the GluA2 subunit is present and, if so, whether it has undergone mRNA editing. A possible mechanism for AMPAR to become Ca^{2+} permeable is defective GluA2 mRNA editing. Typically, the change from an uncharged amino acid glutamine (Q) to a positively charged arginine (R) in GluA2 is sufficient to confer Ca^{2+} impermeability due to electrostatic repulsion by the arginine residues lining the AMPAR pore. Accordingly, abnormal mRNA processing can result in a Ca^{2+}-permeable AMPAR pore. Using a molecular approach, we recently found that retinal GluA2 is fully edited in glaucoma, ruling out a post-transcriptional editing defect as a mechanism by which AMPARs become permeable to divalent cations in this disease (Cueva Vargas et al., 2015). A second mechanism that would allow Ca^{2+} influx through AMPAR is low stoichiometric amounts of the GluA2 subunit. Using biochemical and immunohistochemical analyses, we showed that GluA2 expression in RGCs is selectively downregulated by ocular hypertension thus setting the stage for increased Ca^{2+} permeability and excitotoxic injury (Cueva Vargas et al., 2015).

Several factors may contribute to the susceptibility of RGCs to excitotoxic damage *via* TNFα-induced CP-AMPAR upregulation, including poor cytosolic Ca^{2+} buffering leading to mitochondrial Ca^{2+} overload and generation of reactive oxygen species (Crish and Calkins, 2011). A rise in cytosolic Ca^{2+} *via* CP-AMPAR is likely to stimulate signaling cascades that exacerbate RGC degeneration. Excessive intracellular Ca^{2+} can activate Ca^{2+}-dependent calpains that degrade components of the RGC axon cytoskeleton impairing axonal transport (Crish and Calkins, 2011). Ca^{2+} overload can also promote oxidative stress compromising the ability of mitochondria to buffer Ca^{2+}, and might disable Na^+/K^+ ion pumps causing electrical failure of RGC axons. CP-AMPAR are also permeable to zinc (Zn^{2+}), which can be particularly toxic for neurons. Zn^{2+} is known to rapidly accumulate in hippocampal neurons following ischemia, and was recently shown to play a role in oxidative stress and age-related neurodegeneration (McCord and Aizenman, 2014). The future elucidation of the precise role of Ca^{2+} and Zn^{2+} excitotoxicity in RGC death is of great interest to understand their potential contribution to CP-AMPAR-mediated damage in glaucoma.

Our data support a model in which glia-derived soluble TNFα contributes to neurodegeneration in glaucoma by increasing cell membrane expression of CP-AMPAR (**Figure 1**), an excitatory ionotropic glutamate receptor involved in fast synaptic transmission, thus promoting Ca^{2+} overload and RGC death. These findings identify TNFα as an important molecular link between reactive glia and RGC excitotoxicity mediated by TNFα-induced cell surface CP-AMPAR expression. The connection between *de novo* TNFα production by glial cells and neuronal excitotoxicity is increasingly being recognized as an important mechanism in neurodegenerative diseases (Olmos and Lladó, 2014). In addition to regulating AMPAR trafficking, TNFα increases NMDA receptor expression and reduces inhibitory GABA receptor levels, thus altering the balance of excitatory to inhibitory synapses. In this scenario, TNFα enhances the synaptic excitation/inhibition ratio hence potentiating neuronal excitotoxicity. TNFα can also inhibit glutamate uptake by astrocytes further increasing extracellular glutamate levels. In microglia, TNFα induces release of glutamate which, in addition to contributing

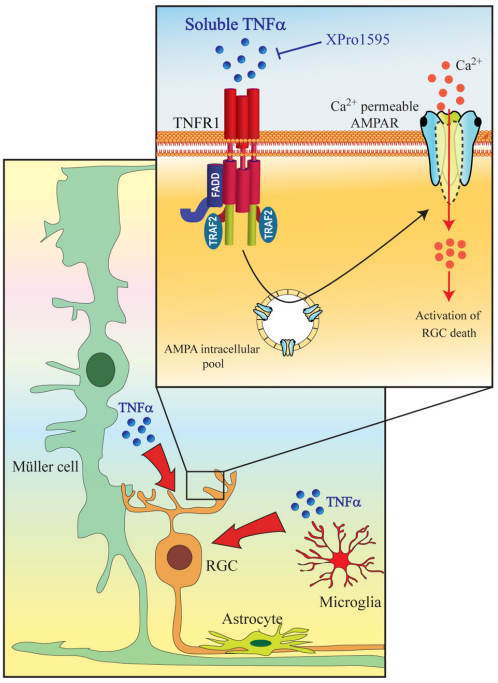

Figure 1 Glia-derived tumor necrosis factor alpha (TNFα): a molecular link between neuroinfammation and retinal ganglion cell (RGC) excitotoxic death in glaucoma.
Working model in which soluble TNFα secreted by Müller cells and microglia contributes to neurodegeneration by increasing cell membrane expression of Ca^{2+}-permeable AMPA receptors (CP-AMPAR), an excitatory ionotropic glutamate receptor involved in fast synaptic transmission, thus promoting Ca^{2+} overload and RGC death in glaucoma. XPro1595, an engineered dominant negative peptide that selectively inhibits soluble TNFα, blocks CP-AMPAR trafficking to the membrane and promotes robust RGC soma and axon survival.

to excitotoxic damage, can act on microglial metabotropic glutamate receptors through an autocrine loop to stimulate more TNFα synthesis. Collectively, these findings reveal a complex mode of action of TNFα: a direct effect on neurons to shift the balance between excitatory and inhibitory synaptic receptors, and an indirect effect on glial cells to regulate their ability to buffer glutamate and produce TNFα.

In conclusion, while endogenous TNFα plays critical physiological roles in retinal homeostasis and neurotransmission, excess soluble TNFα results in CP-AMPAR upregulation, Ca^{2+} overload and neuronal death in glaucoma. These findings suggest that modulation of soluble TNFα signaling might be beneficial to counter the harmful effect of neuroinflammation and synaptic alterations in glaucomatous optic neuropathies.

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