Hyper-IL-6: a potent and efficacious stimulator of RGC regeneration

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Abstract

Mature retinal ganglion cells (RGCs) normally fail to regenerate injured axons and die soon after optic nerve injury. Research over the last two decades has demonstrated that application of IL-6-like cytokines or activation of respective downstream signaling pathways promote neuroprotection and optic nerve regeneration. However, the overall beneficial effects of natural cytokines remain usually rather moderate, possibly due to intrinsic signaling pathway inhibitors, such as PTEN or SOCS3, or a limited expression of specific cytokine receptors in RGCs. It was recently demonstrated that directly targeting the gp130 receptor, a common signalling receptor of all IL-6-like cytokines, induces stronger RGC axon regeneration *in vitro* and *in vivo* than other known growth-promoting treatments such as inflammatory stimulation or PTEN knockout. Remarkably, continuous expression of hyper-IL-6 (hIL-6) upon intravitreal AAV injection after nerve injury enables long-distance axon regeneration, with some axons growing through the optic chiasm 6 weeks after optic nerve injury. Thus, AAV-mediated hIL-6 delivery is so far one of the strongest single, post-injury treatments for the promotion of optic nerve regeneration and may be suitable for the development of novel, clinically applicable therapeutic treatments for human patients. *Eye* (2017) 31, 173–178; doi:10.1038/eye.2016.234; published online 25 November 2016

Introduction

Mammalian retinal ganglion cells (RGCs) are normally unable to regenerate injured axons.¹ Thus, optic nerve damage caused by either disease or trauma inevitably leads to irreversible and, in severe cases, even complete functional loss of vision.² No treatments are currently

clinically available to reverse this outcome, underlining the high clinical need for effective strategies for optic nerve repair.

Regenerative failure has been attributed to an inhibitory environment for axonal growth cones in the injured optic nerve as well as to an insufficient intrinsic capacity of RGCs for axonal re-growth. As in other parts of the central nervous system (CNS), oligodendrocytes in the optic nerve express inhibitory factors such as myelin-associated glycoprotein, Nogo, and oligodendrocyte-myelin glycoprotein, which negatively impact the stability and motility of axonal growth cones.³ Thus, exposure of transected axonal tips to these inhibitory molecules counteracts the ability of growth cone migration and axonal extension after optic nerve damage.^{4,5} Apart from myelin-derived inhibitors in the optic nerve, glial scar formation at the lesion site represents another barrier for regenerating axons. In this case, proliferating astrocytes produce and release among others specific inhibitory molecules, such as semaphorin 3A, slit-1, tenascin-R, and chondroitin sulfate proteoglycans.^{6–11} Even greater challenges for successful optic nerve regeneration are apoptotic RGC death, which begins a few days after axonal injury, and RGCs' insufficient intrinsic capacity to restart a regenerative, axon growth enabling program, which is markedly reduced in mammals after birth. Hence, mature RGCs barely regenerate axons even in the absence of myelin inhibitors or in the presence of a growth supportive peripheral nerve graft.^{12–14} As a result of this regenerative failure together with a lack of neurotrophic support, >80% of mouse and >90% of rat RGCs undergo apoptotic cell death 21 days after an intraorbital optic nerve injury.^{15–18}

Although no clinical treatments are yet available to substantially stimulate axon regeneration or to functionally repair axonal connections in the visual pathway, experimental

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Figure 1 Schematic cartoon of gp130 signaling by naive cytokines and hyper-IL-6. Inflammatory stimulation (IS) induces the release of different IL-6-like cytokines such as CNTF, LIF and IL-6. These cytokines need to bind to their respective non-signaling α -receptors in order to activate ubiquitously expressed gp130. Activation of JAK/STAT3 and PI3K/AKT/mTOR signaling is restricted by the limited expression of α - and β -receptor subunits (leaving unbound gp130) and by the intrinsic inhibitors PTEN and SOCS3. Removal of the intrinsic brake on PI3K activity via PTEN knockout (PTEN ko, second panel) predominantly increases pAKT levels and promotes considerable axon regeneration. PTEN knockout in combination with increased release of native IL-6-like cytokines upon IS (PTEN ko + IS, third panel) stimulates gp130 signaling and accordingly increases the regenerative response after optic nerve injury in comparison to single PTEN knockout. Application of hyper-IL-6 (hIL-6) is expected to activate most gp130 receptors in neurons (lower panel), thereby causing a stronger stimulus. Despite ongoing presence of the intrinsic brakes PTEN and SOCS3, this strong activation of gp130-dependent signaling leads to optic nerve regeneration comparable to PTEN deletion combined with IS. However, additional PTEN depletion can further enhance axon regeneration (right panel).

research has made significant progress over the last two decades. Significant neuroprotection and even moderate axonal regeneration into the injured optic nerve has been achieved for example by inflammatory stimulation (IS). IS can be induced in rodents by lens injury or by intravitreal application of specific lens proteins, Pam₃Cys, or zymosan.^{12,19–24} Besides several cell types of the innate immune response, IS activates retinal astrocytes and Müller cells to induce retinal expression of IL-6-type cytokines such as CNTF, LIF, and IL-6.^{16,25,26} The essential contributions of each cytokine in mediating the neuroprotective and growth promoting effects of IS were verified by their depletion in respective genetic knockout mice, which compromised or even completely abolished the beneficial effects of IS.^{16,27} Similarly, application of exogenous CNTF, LIF, and IL-6 mediates neuroprotection and stimulates axon regeneration in RGC cultures as well as the injured optic nerve *in vivo*, particularly upon continuous release from virally transduced cells.^{26,28–31}

CNTF, LIF, and IL-6 belong to the family of interleukin-6 (IL-6)-type cytokines activating the signal transducing receptor glycoprotein 130 (gp130) (Figure 1),³² which in the adult retina is mainly expressed in RGCs.³³ LIF requires direct interaction with the LIF-receptor (LIFR) to form a signaling complex with gp130. CNTF needs to bind first to the

CNTF-receptor- α (CNTFR α), which then recruits the signaling subunits LIFR and gp130 to form a functional, ternary receptor complex. Comparably, IL-6 binds to the IL-6 receptor to form a complex with two gp130 receptors.^{27,32} Although all these receptor components are expressed by mature RGCs^{16,26} and cytokines directly interact with RGCs in cell cultures,^{16,27,31,34} expression of the α -receptors (LIFR, CNTFR α , IL6R) is seemingly limited in RGCs. In fact, CNTFR α is reportedly downregulated in RGCs upon axotomy, potentially even decreasing their responsiveness towards this respective cytokine.³⁵ Thus, despite its high receptor-binding affinity,³⁶ CNTF application might not be optimally efficacious for the promotion of optic nerve regeneration.

Upon cytokine stimulation, activated receptor complexes associate with Janus-kinases (JAK1, JAK2 and/or TYK2) and become tyrosine-phosphorylated.³² Phosphorylated receptors serve as docking sites for signal transducer and activator of transcription (STAT; mainly STAT3) and protein tyrosine phosphatase SHP2.^{26,37} In turn, STAT3 monomers become phosphorylated, dimerize and translocate into the nucleus to activate expression of genes with STAT3 responsive promoter elements.^{38,39} The essential role of JAK/STAT3 signaling in optic nerve regeneration has recently been

demonstrated. For example, conditional, RGC-specific knockout of STAT3 prevents IS-mediated transformation of adult RGCs into a regenerative state.¹⁵ As a consequence, STAT3-depleted RGCs showed markedly reduced induction of regeneration-associated genes, decreased neurite growth in culture, impaired axonal regeneration into the optic nerve and compromised neuroprotection upon IS.¹⁵ On the other hand, expression of constitutively active STAT3 promoted RGC survival

and axon re-growth in the injured optic nerve.⁴⁰ Conditional knockout of SOCS3, an intrinsic inhibitor of JAK/STAT3 signaling expressed upon STAT3 activation as a negative-feedback loop, markedly increased the number and length of regenerating axons after CNTF application.⁴¹ Thus, JAK/STAT3 signaling is essential for cytokine-induced neuroprotection and axon regeneration.

In addition to JAK/STAT3, IL-6-like cytokines also stimulate the PI3K/AKT/mTOR pathway in RGCs.^{31,36}

Figure 2 Hyper-IL-6 effectively promotes optic nerve regeneration. (a) Representative pictures of longitudinal sections of optic nerves with regenerating axons visualized by fluorescent CTB 3 weeks after optic nerve crush (ONC). Adult floxed PTEN mice were intravitreally injected either with AAV-Cre to induce RGC-specific PTEN deletion (PTEN^{-/-}) or with AAV-GFP (-) as control treatment 14 days prior to ONC. ONC was combined either with inflammatory stimulation (IS) or intravitreal injection of either AAV-hIL-6 or AAV-GFP. Asterisks indicate the crush site. Scale bar = 200 μm. (b-e) Magnification of the respective optic nerve sections depicted in (a). Scale bar = 200 μm. (f) Quantification of regenerating axons at indicated distance beyond the crush site in adult mice treated as described in a. Treatment effects: *** $P \leq 0.001$, NS, non-significant. (g) Representative longitudinal optic nerve section with optic chiasm 6 weeks after ONC and simultaneous AAV-hIL-6 administration. Several regenerating axons reach the optic chiasm and the contralateral optic nerve (arrows). The asterisk indicates the crush site. Scale bar = 200 μm.

Normally, mTOR activity declines in mature RGCs upon optic nerve injury, but is sustained upon IS, application of IL-6-type cytokines or, independently of cytokines, by genetic depletion of the intrinsic PI3K inhibitor PTEN.^{17,17,42} Inhibition of mTOR by rapamycin markedly compromised optic nerve regeneration after PTEN knockout,¹⁷ but only affected long-distance regeneration induced by IS.⁴² Therefore, sustained mTOR activity upon cytokine treatment is seemingly not necessarily required for switching RGCs into, but rather for maintaining the regenerative state.^{17,42} Overall, the effects of PTEN knockout on PI3K/AKT activation, neuroprotection and optic nerve regeneration are much stronger compared with IS or direct cytokine application,^{36,43,44} indicating a contribution of further targets beyond mTOR in these processes. Despite the remarkable effects of intrinsic PTEN or SOCS3 depletion on RGCs survival and regeneration, such approaches are, however, unfavorable from a clinical point of view due to their high cancerogenic potential and the requirement for pre-injury neuronal transduction to achieve optimal results.

Owing to the described intracellular negative-feedback loops (PTEN, SOCS3) and the limited expression of α -receptor subunits, activation of JAK/STAT3 and PI3K/AKT signaling seems only suboptimally activated by application of naive IL-6-type cytokines. In this case, it should be possible to boost axon regeneration by directly binding to and activating the signaling receptor gp130. The designer cytokine hyper-IL-6 (hIL-6) consists of the covalently linked bioactive parts of IL-6 and soluble IL-6R α . This molecule directly binds to gp130 without the need of additional co-receptors and activates cytokine-specific signaling pathways in neurons and other cells^{36,45} (Figure 1). Indeed, application of hIL-6 also stimulated neurite growth of adult cultured RGCs with significantly higher efficacy than CNTF or IL-6.³⁶ Unlike CNTF, hIL-6 also abrogated axon growth inhibition by CNS myelin, which is highly relevant for optic nerve regeneration.⁵ Most remarkably, continuous hIL-6 expression upon RGC-specific AAV transduction lead to stronger axon regeneration than other known growth promoting treatments such as IS or PTEN knockout, with some axons even growing through the optic chiasm 6 weeks after injury (Figure 2). As hIL-6-treatment alone was only moderately neuroprotective combinatorial, neuroprotective approaches will likely further increase the regenerative outcome. In fact, application of hIL-6 together with neuroprotective PTEN knockout yielded stronger regeneration than each treatment alone (Figure 2).³⁶ Alternatively to a gene-therapeutic approach, hIL-6 could also be delivered intraocularly for example by encapsulated and therefore removable cells,⁴⁶ which might be a promising therapeutic concept to promote axon regeneration in human patients. This possibility

needs to be addressed in the future. In addition to its potential therapeutic use, hIL-6 also offers advantages for forthcoming experimental research. For one, viral transduction after nerve injury significantly shortens the length of experimental studies. Moreover, the lens and vitreous body remain translucent upon AAV-hIL-6 transduction as opposed to zymosan injection or lens injury.

In summary, hIL-6 is so far the strongest single, post-injury treatment for the promotion of optic nerve regeneration, which on its own is even more effective than prophylactic PTEN knockout or IS. In the future, this novel approach could not only enable broadened experimental regeneration studies, but also hold exciting promise for the development of clinically applicable therapeutic treatments in human patients.

Conflict of interest

The author declares no conflict of interest.

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