

Transneuronal delivery of designer cytokines: perspectives for spinal cord injury

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We recently achieved significant functional recovery after a complete spinal cord injury, allowing previously paralyzed mice to walk again. This was accomplished by a single, unilateral application of an adeno-associated virus (AAV) carrying the cDNA for the designer cytokine hyper-interleukin-6 (hIL-6) into the sensorimotor cortex after spinal cord injury. This treatment resulted in the transneuronal delivery of the designer cytokine to neurons in subcortical locomotor centers, axon regeneration of cortical- and serotonergic neurons across the lesion site, and functional recovery of hindlimb stepping. Here we briefly cover the historical context, current implications, and future perspectives surrounding this new achievement.

Spinal cord injury leads to a permanent and debilitating impairment of motor and sensory functions, the severity of which can vary greatly. In the most severe injury models, such as complete transection or crush, all connections from rostral to caudal of the injury site are severed, resulting in a total loss of voluntary control. These injury types represent a worst-case scenario. Without the redundancy provided by spared axons and their synaptic plasticity, control cannot be regained through rehabilitation therapy alone. The lack of functional regeneration stems partly from a general lack of anatomical regeneration of severed axons in the central nervous system (CNS). Over the years, efforts have been made to remedy this. They can generally be split into two approaches: activation of cell-intrinsic regenerative programs or therapeutic strategies for dealing with the CNS's inhibitory environment.

One spinal cord tract of particular interest in humans and their most popular experimental surrogates, mice, is the corticospinal tract (CST). This tract is responsible for facilitating voluntary fine motor control of skeletal muscles in humans. As such, its lesion severely limits patients' ability to use their extremities voluntarily and actively interact with their environment. Despite its importance, the injured CST has been highly resistant to almost all attempts at stimulating

its regeneration. In 2010, success was announced with the discovery of CST axon regeneration following PTEN knockout in mice (Liu et al., 2010). PTEN functions as a negative regulator of the PI3K/AKT pathway, which gets activated and causes pyramidal neurons to enter a regenerative state, enabling them to cross the lesion site (Liu et al., 2010).

PTEN^{-/-}'s initial regenerative effects were first discovered in a less complex mammalian CNS regeneration model: the mouse optic nerve (Park et al., 2008). In this model, another intrinsic regenerative state-activating pathway came to light: The JAK/STAT3 pathway. We found that lens injury in the eye enabling moderate axon growth beyond the lesion site of the optic nerve (Leibinger et al., 2013b) caused these effects by releasing neurotrophic cytokines, such as ciliary neurotrophic factor (CNTF), leukemia inhibitory factor, and interleukin 6 (IL-6), from the retinal glial cells. These cytokines are the upstream signal transducers of the JAK/STAT3 pathway, as shown by STAT3 knockout in retinal ganglion cells, abolishing lens injury-induced regeneration (Leibinger et al., 2013a). Similarly, knockout of SOCS3, a JAK/STAT3 pathway negative feedback actuator, also allowed moderate regeneration in retinal ganglion cells (Smith et al., 2009). Moreover, the PTEN/SOCS3 double knockout in the optic nerve resulted in significant synergistic stimulation of axon regeneration (Sun et al., 2011).

The previously mentioned JAK/STAT3 pathway-stimulating cytokines transduce their signal into the cell via the gp130 obligate coreceptor, which is widely expressed throughout the nervous system. However, for a neuron to be sensitive to cytokines, it must also express the respective cytokine-specific receptor, which then complexes with gp130 upon ligand binding to initiate signal transduction (Nakashima and Taga, 1998). These cytokine-specific receptors are limited in their expression and thus act as an additional gatekeeper for signal transduction. This natural limitation can be overcome by the designer cytokine hIL-6, a secreted fusion protein made of

the soluble IL-6 receptor covalently linked to its agonist, IL-6, via a flexible peptide linker (Fischer et al., 1997). In the optic nerve, we previously showed hIL-6 to be a highly potent and efficacious stimulator of the cytokine intrinsic regenerative program, enabling more robust optic nerve regeneration than SOCS3^{-/-} or even PTEN^{-/-}. It was also successfully combined with PTEN^{-/-} to synergistically enhance axon growth (Leibinger et al., 2016).

Recently, we implemented hIL-6 in the context of severe spinal cord injury (Leibinger et al., 2021), in which all axons are severed. As with previous spinal cord regeneration studies using PTEN^{-/-}, hIL-6 was applied to the pyramidal neurons in the layer V sensorimotor cortex. An AAV serotype 2 was used as a vector for hIL-6 expression due to its neuronal tropism. The efficacy of the hIL-6 could be seen by staining for activated STAT3 (pSTAT3) in the nuclei of transduced and surrounding cells, demonstrating its paracrine effects (Leibinger et al., 2021). Unlike gene knockout approaches, this strategy does not require genetically modified mouse lines. Moreover, it is applicable after injury rather than before, greatly enhancing its transferability.

Strikingly, not only did hIL-6 treatment manage to stimulate the CST axons to regenerate across the lesion site, but the distance they regenerated (6 mm) was considerably further than that of the PTEN^{-/-} group (1.5 mm). The combined treatment of hIL-6 and PTEN^{-/-} resulted in an increased number of axons regenerating over a short distance and slightly increased the longest axons' length to about 7 mm (Leibinger et al., 2021). In this regard, PTEN^{-/-} resulted in robust AKT and S6 phosphorylation indicating mTOR pathway activation without affecting STAT3, whereas hIL-6 showed the opposite effect (Leibinger et al., 2021).

While both PTEN^{-/-} and hIL-6 display significant anatomical regeneration of the CST, what sets hIL-6 apart in the complete spinal cord crush context is its unique ability to facilitate functional recovery. In the 8 weeks before examining the anatomical regeneration, motor function was tested weekly using the open field Basso Mouse Scale (BMS, uninjured animals score 9) test and finally confirmed by automated catwalk gait analysis. While control mice or PTEN^{-/-} mice could maximally only restore basic ankle movement (BMS 2), hIL-6-treated animals could be seen to take steps with liftoff, followed by the swing phase and the reestablishment of body weight support (BMS 4–5) (Leibinger et al., 2021). The

catwalk gait analysis measured, among other parameters, the regularity index of the steps. It revealed, on average, a 40% recovery of interlimb coordination compared to uninjured animals (Leibinger et al., 2021). Interestingly, the observed functional regeneration was equally evident in both hindlimbs, even though AAV2-hIL-6 was only injected into one of the cortical hemispheres, with bilateral AAV2-hIL-6 application showing no additional benefit. This fact, combined with the poor synergistic effects of *PTEN*^{-/-} in functional regeneration, put into doubt whether the CST's anatomical regeneration could be responsible for the observed hindlimb recovery. In fact, despite the CST's well-characterized role in mediating skilled movements, stereotyped motor behaviors such as overground locomotion are known to be induced and modulated by extrapyramidal tracts originating from subcortical nuclei in the midbrain, pons and medulla (Guertin, 2012). Therefore, we investigated whether the cortical hIL-6 application could possibly affect these brain regions.

The pyramidal neurons of the CST first project through the cortex's internal capsule and then coalesce into the pyramidal tracts that run under the brainstem. While passing the midbrain and the brainstem, the pyramidal tracts extend collaterals to various regions responsible for locomotion, including the red-, vestibular-, and reticular nuclei. Moreover, we discovered that the raphe nuclei are an additional target of such collaterals (Leibinger et al., 2021). It turns out that AAV2-hIL-6 application to the sensorimotor cortex not only causes the secretion of hIL-6 at the somata of transduced neurons but also at their axon terminals (Figure 1). This had the effect of transneuronally stimulating the cytokine pathway in these secondary nuclei, as could be seen by the presence of pSTAT3⁺ neurons (Leibinger et al., 2021). Of the brainstem nuclei mentioned above, the raphe nuclei are named so due to being located along the brainstem's midline instead of being separately duplicated in both halves. Their stimulation would be a possible explanation for the bilateral recovery of the hindlimbs from a unilateral cortical treatment. In fact, the pyramidal tract from one hemisphere projects equally into both raphe hemispheres resulting in equal amounts of pSTAT3 activation (Leibinger et al., 2021). This represents an additional advantage of hIL-6 over other cortical treatment methods in that its full effect requires only unilateral application, making the procedure just

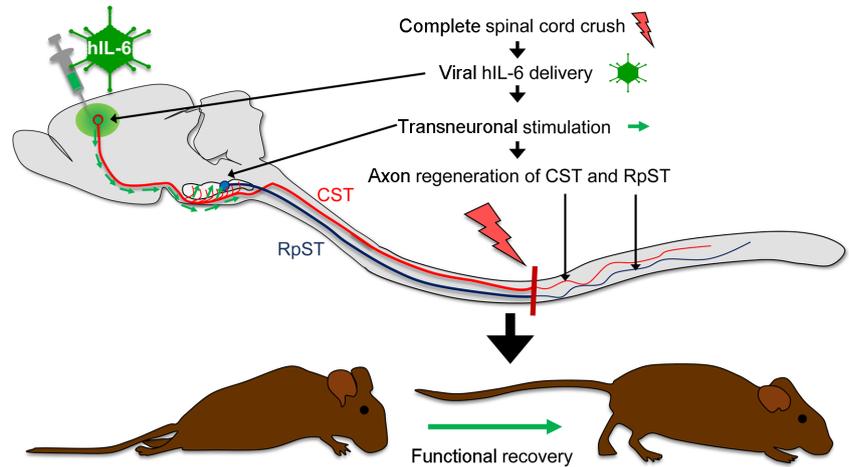


Figure 1 | Schematic depiction of hIL-6 treatment after spinal cord injury in mice.

The transduction of AAV2-hIL6 into cortical cells results in the expression of hIL-6, which is then transneuronally transported along the CST to raphespinal neurons via collaterals in the medulla. This stimulates them to enter a regenerative state, causing the injured RpST and CST to regenerate across the lesion site, resulting in functional recovery of hindlimb locomotion. CST: Corticospinal tract; hIL-6: hyper-interleukin-6; RpST: raphespinal tract.

half as invasive as bilateral injections. The CST transneuronal delivery approach also minimized possible immune system side effects of the designer cytokine. The protein was axonally transported and extracellularly released at axonal endings to bind to gp130 of serotonergic neurons in the raphe nuclei. Thus, AAV-hIL-6 treatment did not increase the number of macrophages or activated microglia in the spinal cord (Leibinger et al., 2021).

As serotonergic neurons (5HT⁺) from the raphe nuclei are responsible for certain locomotor recovery types, 5HT⁺ fiber regeneration was analyzed caudal to the lesion site. Whereas 5HT⁺ fibers of control, or *PTEN*^{-/-} mice showed only very short (< 1 mm) sprouting caudal to the lesion 8 weeks after injury, axons up to 7 mm in length could be found in hIL-6-treated mice (Leibinger et al., 2021). To test their functional impact, they were eliminated using 5,7-dihydroxytryptamine (DHT), a toxin specific to serotonergic neurons. This had the immediate effect of bringing these mice's BMS scores down almost to control levels. In contrast, control mice were not affected, and even uninjured mice showed practically no impairment after DHT treatment (Leibinger et al., 2021). Taken together, transneuronal delivery of hIL-6 to brain stem nuclei elicits functional regeneration by stimulating 5HT⁺ fibers to regenerate across the lesion site and somehow appeared to reinnervate the hindlimbs intraspinal central pattern generators (CPG). However, the exact mechanism how serotonergic axons contributed to the functional recovery, needs to be further elucidated.

A currently unknown aspect of hIL-6-

stimulated spinal cord regeneration is if also other neurons apart from the raphespinal tract project across the lesion site. While 5HT⁺ axons have been seen to regenerate, and DHT used to eliminate them, this does not rule out the contribution of other secondary tracts. DHT treatment has little effect in uninjured animals and suggests that redundant systems do exist for coordinating hindlimb locomotion. These open questions could be answered through a range of axonal tracing experiments. The simplest of these would be the injection of a dye, such as fast blue, caudal to the lesion site to stain regenerating axons and retrogradely label their cell bodies. Co-staining against 5HT would reveal whether serotonergic neurons are the only population of regenerating axons and show where they are located in the CNS. Alternatively, co-staining synaptic markers and green fluorescent protein (from the AAV2-hIL-6 construct) in the raphe nuclei would likely visualize collaterals from transduced cortical neurons forming synapses with regenerated 5HT⁺ neurons, themselves retrogradely labeled with fast blue. A more complex tracing strategy would involve the retrograde transsynaptic tracing of the direct connection chain from the hindlimb leg muscles all the way up to the motor cortex. This would ideally be accomplished by retrograde tracing using, for example, pseudorabies virus. Once the locations of all relevant regenerating neuronal populations have been identified, their contribution to functional recovery could be tested experimentally.

After transneuronal hIL-6 treatment, functional regeneration seems to be

achieved with a moderate number of regenerated serotonergic axons, suggesting an active mechanism forms specific synapses with crucial neurons of the lumbar CPG. Furthermore, in addition to regenerating neurons requiring the capability to form synapses, the postsynaptic neurons must also be receptive to this process. Very little is currently known about the process of synapse initiation. However, the spinal cord is capable of independently learning movements (Hodgson et al., 1994). Thus, it could also be capable of a certain amount of synaptic plasticity to facilitate new connections. It is conceivable that strategies that enhance synaptic plasticity, such as BDNF treatment, could increase the number of receptive postsynaptic neurons or perhaps better integrate the few connections made into the lumbar CPG (Garraway and Huie, 2016). Alternatively, instead of just supporting the few existing regenerated axons, future strategies that induce the fasciculation of follower axons with the successfully regenerated pioneering axons could be developed to boost the overall number of axons crossing the lesion site. This positions the hIL-6 spinal cord injury as an ideal model for combinatorial studies, as it allows treatment strategies and compounds that alone achieve no functional recovery to possibly demonstrate an improvement when used concomitantly. The designer cytokine strategy pioneered by hIL-6 could theoretically be used in other signaling systems involving secreted ligands that are limited by the presence or absence of a non-transmembrane signal transducing receptor. However, the efficacy and potency of other fusionproteins, such as hyper-CNTF (a fusionprotein of the CNTFR and CNTF) may be limited as they would still require the heterodimerization of gp130 and the transmembranous leukemia inhibitory factor-receptor. With gp130 expression being widespread in neurons, hIL-6, fortunately, induces regenerative signaling in neurons efficaciously and potently (Leibinger et al., 2016).

As for judging hIL-6's therapeutic potential in larger animals, including humans, knowing the location of the critical bridging synapses will be highly relevant. In the mouse, regenerating 5HT⁺ axons of up to 7 mm in length reach the lumbar enlargement after a thoracic crush (Leibinger et al., 2021). However, this same lesion location is hundreds of millimeters away from the lumbar enlargement in humans. It is currently unknown where over the 7 mm the functional connections are formed and, if they are towards the

longer end, whether hIL-6-mediated regeneration can overcome the equivalent distances in humans. However, if the key synapses can be shown to form shortly after crossing the lesion site with propriospinal interneurons, then testing hIL-6 in larger animals such as pigs and dogs would be warranted.

Hindlimb stepping in quadrupedal mice is more straightforward than that of bipedal humans with our need for balance. It is currently unknown whether reinnervating the lumbar CPG would even provide sufficient control over human locomotion, mainly since there is no undisputable direct proof for the existence of such intraspinal CPGs in humans. This is primarily due to the lack of experimental possibilities to investigate isolated spinal pattern generators *in vivo*. However, studies on complete SCI patients revealed convincing evidence, such as observing rhythmic stepping-like movements in lying patients evoked by epidural electrical stimulation of the lumbar spinal cord distal to the injury site (Guertin, 2012). Notwithstanding, any new motor connections formed after cortical hIL-6 treatment in humans could become usefully integrated when combined with rehabilitation training, thus providing tangible quality of life improvements. Finally, it remains to be seen whether hIL-6 treatment can also induce functional regeneration in a chronic spinal cord injury model when applied several weeks after injury. Should this be the case, and key synapses are also formed after short distances, then a therapeutic application in existing spinal cord injured patients could one day become feasible.

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