Title: Polycistronic delivery of IL-10 and NT-3 promotes oligodendrocyte myelination and functional recovery

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Abstract

One million estimated cases of spinal cord injury (SCI) have been reported in the United States and repairing an injury has constituted a difficult clinical challenge. The complex, dynamic, inhibitory microenvironment post injury, which is characterized by pro-inflammatory signaling from invading leukocytes and lack of sufficient factors that promote axonal survival and elongation, limits regeneration. Herein, we investigated the delivery of polycistronic vectors, which have the potential to co-express factors that target distinct barriers to regeneration, from a multiple channel PLG bridge to enhance spinal cord regeneration. In the present study, we investigated polycistronic delivery of IL-10, that targets pro-inflammatory signaling, and NT-3 that targets axonal survival and elongation. A significant increase was observed in the density of regenerative macrophages for IL-10+NT-3 condition relative to conditions without IL-10. Furthermore, combined delivery of IL-10+NT-3 produced a significant increase of axonal density and notably myelinated axons compared to all other conditions. A significant increase in functional recovery was observed for IL-10+NT-3 delivery at 12

wpi that was positively correlated to oligodendrocyte myelinated axon density, suggesting oligodendrocyte-mediated myelination as an important target to improve functional recovery. These results further support the use of multiple channel PLG bridges as a growth supportive substrate and platform to deliver bioactive agents to modulate the SCI microenvironment and promote regeneration and functional recovery.

Keywords: spinal cord injury, biomaterial, gene therapy, axon elongation, axon myelination

Impact Statement

Spinal cord injury (SCI) results in a complex microenvironment that contains multiple barriers to regeneration and functional recovery. Multiple factors are necessary to address these barriers to regeneration, and polycistronic lentiviral gene therapy represent a strategy to locally express multiple factors simultaneously. A bi-cistronic vector encoding IL-10 and NT-3 was delivered from a PLG bridge, which provides structural support that guides regeneration, resulting in increased axonal growth, myelination, and subsequent functional recovery. These results demonstrate the opportunity of targeting multiple barriers to SCI regeneration for synergistic effect.

Introduction

Approximately 17,000 estimated new cases of SCI occur in the United States each year and repairing an injury to the spinal cord has constituted one of the most difficult clinical challenges[1]. The CNS does not regenerate largely due to an inhibitory microenvironment post injury. Biomaterial bridges are among strategies to overcome some aspects of the inhibition [2-8]. We have developed multi-channel PLG bridges with an architecture for cellular infiltration and axon elongation. While the bridge provides some support, they alone are insufficient due to complex, dynamic inhibitory microenvironment.

The inhibitory environment consists of multiple barriers to nerve regeneration, most notably pro-inflammatory signaling from invading immune cells and lack of sufficient factors that promote axonal survival and elongation[9]. Analysis of this environment has led to the discovery of the cytokine/neurotrophin axis in axon growth. Cytokines (IL-10, IL-4, IL-6) released by invading macrophages can influence the expression of neurotrophins (NT-3, NT-4, NGF) and their receptors[10]. Macrophages invade the lesion and contribute to both injury and repair [11]. Macrophages exist on a spectrum of activation states ranging from pro-inflammatory to pro-regenerative [12, 13]. Pro-regenerative macrophages present enhanced phagocytosis capabilities [14, 15] and can promote tissue regeneration [11]. The pro-inflammatory/pro-regenerative ratio can expedite or drastically reduce axonal growth into the lesion site[16]. Combinations of interleukins and neurotrophins have been shown to promote or inhibit neurite extension in

vitro, though the ability to locally deliver combinations of factors has limited the in vivo translation [10, 17].

Traditional strategies for localized delivery of factors to augment the microenvironment involve osmotic pumps which can clog and require surgery for removal or direct injection of factors that are rapidly cleared and lead to off-target effects[18-20]. In addition to providing structural support for regenerating tissue, our bridges are also capable of long-term, localized transgene expression of lentivirus. Previously, single viral vectors have been delivered with positive results, but the multiple barriers to regeneration must be addressed simultaneously to create holistic treatments for SCI. Delivering multiple lentiviruses may result in inactivation or increased immunogenicity. These limitations suggest we need an effective way to delivery multiple factors without increased concentrations of lentivirus.

In the present study, we investigated polycistronic vectors for co-expression of NT-3 and IL-10 using PLG bridges after acute SCI. Polycistronic vectors negate the limitations of single lentiviral vectors by encoding co-expression of multiple genes by adding “self-cleaving” 2A peptide sites between genes. 2A peptides can lead to high levels of downstream protein expression compared to other strategies for multi gene co-expression and are small enough to not negatively interfere with the function of the co-expressed genes[21]. IL-10 can alter the phenotype of invading macrophages towards a pro-regenerative phenotype and lead to improved spinal cord regeneration [22, 23], while NT-3 expression can promote neuron survival and

axonal elongation[19]. Herein, we investigated the potential for co-expression to influence macrophage phenotypes, axonal elongation and myelination, and source of the myelination at 12 weeks post injury, with subsequent studies assessing the functional benefits of co-delivery. This research builds upon the success of the multiple channel poly(lactide-*co*-glycolide) (PLG) bridges by delivering multiple gene factors that target distinct barriers to regeneration to elucidate synergistic relationships.

Methods

Virus Production and validation

Lentivirus was produced as previously described[2]. Briefly, HEK-293FT cells (80-90% confluent, American Type Culture Collection (ATCC), Manassas, VA, USA) were transfected with third generation lentiviral packaging vectors and pLenti-CMV-Luciferase, pLenti-CMV-hNT3, pLenti-CMV-hIL10, or pLenti-CMV-hIL10/NT3. Plasmids were incubated in OptiMEM (Life Technologies, Carlsbad, CA, USA) with Lipofectamine 2000 (Life Technologies) and then added to cells. After 48 hours supernatant was centrifuged to remove and then incubated with PEG-It (System Biosciences, Palo Alto, CA, USA). Virus was centrifuged, supernatant was removed, and the pellet was re-suspended in sterile phosphate buffered saline (PBS; Life Technologies). Virus aliquots were frozen at -80°C until use. Viral titers used throughout the study were 2E9 IU/mL as determined by the Lentivirus qPCR Titer Kit (Applied Biological Materials, Richmond, BC, Canada).

Fabrication of multi-channel bridges

Bridges were fabricated using a sacrificial template variation[24] of the gas foaming/particulate leaching technique, as previously described[19, 25]. Briefly, PLG (75:25 lactide:glycolide; i.v. 0.70 - .90 dL/g; Lactel, Birmingham, AL, USA) was dissolved in dichloromethane (6% w/w) and emulsified in 1% poly (vinyl-alcohol) using a homogenizer (PolyTron 3100; Kinematica AG, Littau, Switzerland) to create microspheres (z-average diameter ~1μm). D-sucrose (Sigma Aldrich), D-glucose (Sigma Aldrich), and dextran MW 100,000 (Sigma Aldrich) were mixed at a ratio of 5.3:2.5:1 respectively by mass. The mixture was caramelized, cooled, and drawn from solution with a Pasteur pipette to make sugar fibers. Fibers were drawn to 150 – 250 μm, coated with a 1:1 mixture of PLG microspheres and salt (63-106 μm) and pressed into a salt-lined aluminum mold. The sugar strands were used to create 9 channels and the salt created a porous structure. The materials were then equilibrated with CO_2 gas at 800 psi in a custom-made pressure vessel. Bridges were cut into 1.15 mm sections and leached to remove salt porogen. The bridges were stored in a desiccator until use.

Virus loading into bridges

Bridges were disinfected in 70% ethanol and washed with sterile water. Bridges were then saturated with 2 μL of virus. After 2 minutes of incubation, sterile filter paper was touched to the surface of the bridge to remove excess moisture. This process was repeated with another 2 μL of virus. The bridges were stored on ice until use. Bridges were used within 3 hours of coating

with lentivirus to preserve viral activity. Lentivirus loading conditions included NT-3, IL-10, and IL-10+NT-3.

Mouse spinal cord hemisection

All animal procedures were approved and in accordance with the Institutional Animal Care and Use Committee at the University of Michigan. A hemisection model of SCI was performed on female C57BL/6 mice (6-8 weeks old; Jackson Laboratories, Bar Harbor, ME, USA, N=48) as previously described[5]. After administration of bupivacaine (.8 ml/kg), a laminectomy was performed at C5 to allow for a 1.15 mm lateral hemisection on the left side of the spinal cord for bridge implantation. The injury site was covered using Gelfoam (Pfizer, New York, NY, USA) followed by suturing together of the muscle and stapling of skin. Postoperative care consisted of administration of enrofloxacin (2.5 mg/kg; daily for 2 weeks), buprenorphine (0.1 mg/kg; twice daily for 3 days), and Lactated Ringer's solution (5 mL/100 g; daily for 5 days). Bladders were expressed twice daily until function recovered. No mice were loss using this injury model.

Western blot

Spinal cord tissues were collected at 2 wpi and lysed with RIPA buffer (Thermo Fisher, Waltham, MA, USA) supplemented with Halt Protease Inhibitor Cocktail (Thermo Fisher). The lysate was then sonicated and centrifuged. The supernatant was added with 2x Lammeli Buffer (Biorad, Hercules, CA, USA) and boiled for 5 minutes at 95°C. Samples were ran on a 4-15% gradient SDS-PAGE gel (Biorad) and proteins were transferred to .45

um nitrocellulose membranes. After blocking with BLOK Casein (G-Biosciences, St. Louis, MO, USA), proteins were probed with primary antibodies against rabbit anti-IL10 (Abcam, Cambridge, UK), rabbit anti-NT3 (Abcam, Cambridge, UK), and rabbit anti-β actin (CST, Danvers, MA). The proteins were detected by chemiluminescence (Clarity Substrate, Thermo Fisher, Waltham, MA). Quantification was performed with Image J (NIH, Bethesda, MD).

Immunohistochemistry and quantitative analysis nerve regeneration and myelination

Spinal cords were extracted 12 weeks after SCI and flash frozen in isopentane. For immunofluorescence, spinal cord segments were embedded in Tissue Tek O.C.T. Compound (Sakura Finetek, Torrance, CA, USA) with 30% sucrose. Cords were cryo-sectioned transversely into 12-µm-thick sections. Antibodies against the following antigens were used for immunofluorescence: F4/80 (Abcam, Cambridge, UK), Arginase 1 (Arg1, Santa Cruz Biotech, Dallas, TX, USA), neurofilament 200 (NF-200, Sigma Aldrich), myelin basic protein (MBP, Santa Cruz Biotech, Dallas, TX, USA), and Protein-zero myelin protein (P0, Aves Labs, Tigard, OR, USA). Red fluorescent protein (RFP) was imaged at 488 nm without added antibodies. Tissues were imaged on an Axio Observer Z1 (Zeiss, Oberkochen, Germany) using a 10x/0.45 or 20x/0.75 M27 apochromatic objective and an ORCA-Flash 4.0 V2 Digital CMOS camera (C11440-22CU, Hamamatsu Photonics, Hamamatsu City, Shizuoka, Japan).

For quantification of macrophage phenotypes, nine 12 µm thick transverse tissues were randomly selected from each animal (N=24, 6 per condition). All immunopositive cell events were counterstained with Hoechst 33342 to indicate cell nuclei. F4/80$^+$ cells and F4/80$^+$/arginase1$^+$ (Arg1$^+$) were quantified to determine pro-inflammatory macrophages and non-inflammatory macrophages respectively. Immunopositive cells were counted within the bridge area by 2 blinded researchers independently. Co-staining for multiple markers was assessed by evaluating overlap of different channels in Image J (NIH, Bethesda, MD, USA).

To assess the numbers of regenerated and myelinated axons within the PLG bridge area, NF-200 was used to identify axons, NF-200+/MBP+ to determine the number of myelinated axons, and NF-200+/MBP+/P0+ to determine the amount of myelin derived from infiltrating Schwann cells[20]. Twenty-four tissues distributed between conditions were counted by 2 blinded counters to calibrate software for automated counting as previously described[2, 22, 23, 26]. Briefly, images were imported into MATLAB (Mathworks, Natick, MA, USA) and the area of the tissue section corresponding to PLG bridge was outlined. A Hessian matrix was created by convolution filtering using second derivative of the Gaussian function in the x, y, and xy directions. Following filtering, positive NF-200 events were identified by intensity thresholding, single pixel events were removed, and the number of continuous objects were identified to ensure high branching axons are counted as a single object. For calibration, the software will output a matrix

of predicted axon counts based on filtering parameters inputted by the user. These values are directly compared to manual counts for the twenty-four tissues used for calibrating the software. The appropriate filter size and threshold sensitivity are selected based on the lowest mean percentage error between the manual and automated counts. For these studies, the mean percentage error was 3%. Once calibration was complete, nine tissues per animal (N=24, 6 per condition) were quantified. To obtain axon densities, total NF-200 counts were divided by the area of the PLG bridge. MBP and P0 events were identified similarly as described above. NF200 objects containing pixel locations overlapping with positive MBP or P0 staining were counted and compared to total NF200 counts to determine percentages of axons populations.

Behavioral analysis

The ladder beam walking task was used to evaluate locomoter recovery over a period of 12 weeks post-SCI as previously described for all conditions (N=48, 12 per condition) [27]. Briefly, animals were trained to walk across a ladder beam of 50 rungs into an enclosure over the course of 2 weeks before injury. Baseline scores were determined to separate animals in equal groups prior to SCI. The mice were tested at 2, 4, 8, and 12 weeks. Observations and ladder beam scoring were performed by two blinded observers for 3 trials per animal. Animals were scored by average left forepaw full placements on the ladder beam during the task.

Thermal hyperalgesia

Cold sensitivity was assessed by acetone evaporative cooling over a period of 12 weeks post-SCI as previously described [28]. Through a mesh floor, 5 applications of acetone were applied to the bottom of the left and right forepaw. Each application was separated by at least 5 minutes. Individual responses were scored based on lifting, licking, or shaking of the forepaw that continued past the initial application. Scores were averaged over the ten applications between left and right forepaws to yield a percentage of positive responses.

Statistical analysis

For multiple comparisons, statistical significance between groups was determined by two-way ANOVA with Tukey's post-hoc. For single comparisons, the statistical significance between pairs was determined by unpaired t-test. All statistics test significance using an α value of 0.05. For all graphs: a, b, c denotes $p<0.05$ compared to Blank control, d and e denotes $p<0.05$ compared NT-3 condition, and f denotes $p<0.05$ compared to IL-10 condition. Error bars represent standard error in all figures. Prism 7 (GraphPad Software, La Jolla, CA, USA) software was used for all data analysis.

Data Availability

The datasets generated during and/or analyzed during the current study are available from the corresponding author on reasonable request.

Results

Bi-cistronic vectors conserve IL-10 and NT-3 production in vivo

We initially assessed protein levels following expression from the lentivirus with a single construct and also the bi-cistronic construct (**Error: Reference source not found**). Bridges were implanted into mice and explanted at 2 weeks post injury (wpi). This time point was selected based on previous studies using bioluminescence imaging that demonstrates robust transgene expression [19]. The proteins used in these studies are human which enabled differentiation of the IL-10 and NT-3 produced by our constructs from the mouse proteins. No significant difference in protein expression was observed between single constructs or the bi-cistronic construct. Furthermore, for the bi-cistronic construct, no significant difference in protein expression was observed between the first gene, IL-10, and the second gene, NT-3.

IL-10 delivery increases anti-inflammatory macrophages

We next investigated the macrophages populations in the bridge at 12 wpi (**Error: Reference source not found**), as this population could be influenced by the cytokine and neurotrophin expression. This analysis was performed at 12 wpi to analyze the chronic infiltration and phenotype of macrophages. We observed most of the macrophages at the injury were localized to the bridge and not the surrounding tissue (**Error: Reference source not found****A**). The macrophages in the bridge were evenly distributed and were observed in all conditions at similar densities (**Error: Reference source not found****F**). For these studies, pro-regenerative macrophages were assessed by co-localization of F4/80 and arginase (Arg)

(**Error: Reference source not found**G). A significant 3-fold increase in the density of regenerative macrophages was observed for conditions with expression of IL-10 compared to the Blank and NT-3 conditions. Differences among these conditions were also significant for the percentage of pro-regenerative macrophages. Notably 31% and 26% of F4/80^{+} macrophages were regenerative for IL-10 and IL-10+NT-3 conditions respectively, a 2-fold increase over Blank and NT-3 conditions (12% and 14%) (**Error: Reference source not found**H).

Delivery of IL-10+NT3 enhances axonal growth into bridges

The extent of axonal elongation into the bridge was subsequently analyzed, which reflects the impact of expressing the factors on the capacity of the environment to promote regeneration (**Error: Reference source not found**). Axons were observed throughout the bridge in all conditions. Axons in treatment groups appeared longer and more diffuse while axons in the control condition appeared bundled and shorter in length (**Error: Reference source not found**A-D). NT-3 and IL-10 delivery did not significantly increase axonal density compared to control. However, combined delivery of IL-10+NT-3 produced a significant increase of axonal density compared to all other conditions (**Error: Reference source not found**E).

IL-10+NT-3 delivery promotes myelination of regenerating axons

Myelination of axons is necessary for signal propagation in the spinal cord, and myelinated axons (NF200^{+}/MBP^{+}) were observed throughout the bridges for all conditions (**Error: Reference source not found**).

Significantly more myelinated axons were observed in the IL-10 condition compared to the Blank bridge. IL-10+NT-3 delivery resulted in significantly more myelinated axons compared to Blank and NT-3 conditions (**Error: Reference source not found**E). No significant difference was observed in the percentage of myelinated axons across the conditions, with levels at approximately 27% of total axons (**Error: Reference source not found**F).

Myelinated axons were sub-divided into oligodendrocyte-derived myelin ($NF200^+/MBP^+/P0^-$) and Schwann cell-derived myelin ($NF200^+/MBP^+/P0^+$) (**Error: Reference source not found**) to determine their relative contribution to total myelination. A significant 2-fold increase in oligodendrocyte-derived myelin density was observed for IL-10+NT-3 compared to Blank and NT-3 conditions (**Error: Reference source not found**E). IL-10 delivery resulted in a significant increase of oligodendrocyte myelin compared to control. However, no significant difference in density of Schwann cell-derived myelin was observed across all conditions (**Error: Reference source not found**F).

IL-10+NT-3 improves forelimb locomotor recovery

The ladder beam task was used to evaluate functional motor recovery of the left forelimb to determine if the observed regeneration correlated with an increase in function (**Error: Reference source not found**A). A significant functional improvement was observed for all conditions compared to Blank. Furthermore, a significant increase in functional recovery was obtained with IL-10+NT-3 delivery compared to all other conditions at 12 wpi. Interestingly,

a greater prolonged improvement over time was observed for IL-10+NT-3 condition compared to other conditions. IL-10 and NT-3 delivery were similar in functional recovery, however co-delivery produced a significant additive effect in locomotor recovery.

We also assessed cold hypersensitivity using the acetone test to identify any potential negative effects that may be associated with combined delivery (**Error: Reference source not found**B). IL-10 delivery alone reduced cold hypersensitivity compared to all other conditions. NT-3 delivery alone exacerbated hypersensitivity compared to all conditions. The combined expression of IL-10+NT-3 led to increased hypersensitivity compared to IL-10 alone, yet decreased hypersensitivity relative to Blank and NT-3 conditions.

As a final analysis, we investigated the association of tissue recovery characteristics with forelimb locomotor function recovery (**Error: Reference source not found**). The values for axon density (**Error: Reference source not found**A), myelinated axon density (**Error: Reference source not found**B), oligodendrocyte-derived myelinated axon density (**Error: Reference source not found**C), and Schwann cell-derived myelinated axon density (**Error: Reference source not found**D) were plotted against the ladder beam score for each animal. The oligodendrocyte myelinated axon density was positively correlated with functional recovery (r=0.59, p<0.01). This relationship also segments animals by condition along the

interpolated linear fit line. This relationship was the only significant connection between tissue characteristics and functional recovery.

Discussion

The central nervous system has the innate capacity to repair itself post-SCI, yet the spinal cord environment lacks sufficient factors that promote regeneration and has an abundance of factors that inhibit regeneration[9]. We have developed multichannel PLG bridges that can promote nerve regeneration by both acting as a physical guide and serve as a platform for gene therapy vector delivery. The bridges are acellular at implantation, indicating that any cells, extracellular matrix, or proteins present at the time of extraction must have originated from the host tissue. Similarly, axons observed inside the implant are either regenerating injured axons or sprouting of new axons from spared or contralateral tissue. This bridge provides a defined space for histological analysis, analysis of cell populations at and near the lesion site, and treatment outcomes. The bridge has an architecture that supports regeneration by combining micro-porosity for cellular infiltration with channels that direct axonal elongation along the major axis of the cord. These bridges can also be seeded with recombinant lentiviral particles containing genes of interest for subsequent cellular transduction. Unlike other viral vectors, lentivirus does not influence the phenotype of progenitors[29] or cause significant inflammation[30]. Lentiviral vectors physical properties are also independent of the gene of interest, which enables delivery of multiple vectors encoding distinct inductive factors

without modification to the base biomaterial. Poly-cistronic lentiviral vectors can deliver multiple genes by the inclusion of self-cleaving 2A peptide sites between genes. This technology is useful and beneficial to target multiple barriers to spinal cord regeneration by inducing the expression of multiple proteins simultaneously. The genes being used in this study were chosen because each addresses a distinct aspect of the inhibitory microenvironment around the injury site. NT-3 enhances axon elongation and neuroprotection of regenerating and spared axons[19, 31, 32]. IL-10 is largely responsible for dampening and resolving the immune response toward restoring homeostasis [13, 33, 34]. This research builds upon the success of the multiple channel poly(lactide-*co*-glycolide) (PLG) bridges by delivering 2 distinct transgenes alone and in combination. The goal of this investigation was to activate growth promoting cues while attenuating growth inhibitory cues to observe additive effects on spinal cord regeneration.

Similar macrophage infiltration into bridges was observed across experimental conditions delivering, yet transgene expression could alter the macrophage phenotype. Lentiviral constructs are minimally immunogenic, yet the transgene can influence the microenvironment[30, 35-37]. Macrophage phenotypes exist on a spectrum of inflammatory to regenerative and we have previously shown that $Arg1^+$ macrophages have a regenerative phenotype in SCI [11, 23]. For the purposes of this paper, regenerative macrophages were assessed by co-localization of $F4/80^+$ and $Arg1^+$. The early phase of the regenerative response following SCI relies on pro-

inflammatory macrophages, which participate in recruitment of immune cells and clearance of cell debris. Subsequently, these macrophages shift to a pro-regenerative phenotype to coordinate cell differentiation and tissue reconstruction. Dysregulation of this transition from pro-inflammatory to pro-regenerative hampers regenerative success and tissue recovery. In the CNS, microglia and macrophages express several neurotrophins and their receptors, allowing them to act both as sources and targets creating a feedback loop that can modulate proliferation and morphology of axons [38]. Neurotrophins impact immune cell function in varying ways. BDNF stimulates microglial proliferation while NT-3 can stimulate phagocytic activity of microglial cells in vitro and upregulation of nitric oxide production [39-41]. NGF can also act directly on microglial cells by promoting chemotactic migratory activity, potentially contributing to recruitment of additional immune cells at injury sites[42]. Neurotrophins, including NT-3, have been suggested as modulating monocyte chemotaxis without altering their production of inflammatory cytokines [43]. NGF can act directly on microglia and shift them toward a neuroprotective phenotype [42]. In these studies, we did not observe an anti-inflammatory effect of NT-3 on macrophage activity. IL-10 delivery alone produced ~31% of regenerative macrophages, but when combined with NT-3, this percentage decreased to 26%. This difference was not significant. IL-10 has been extensively revered as an anti-inflammatory cytokine capable of shifting the phenotype of macrophages[22, 44-48] and these studies support the previous findings.

A trend of increased axons for NT-3 and IL-10 compared to blank, yet the combination of IL-10+NT-3 produced an additive effect for axonal outgrowth. Axonal outgrowth can be impacted by delivery of NT-3 and IL-10 individually [19, 22, 23], however in our studies, these individual factors did not substantially impact axon density relative to control, which likely results from the relative types of neurons in the C5 lateral hemisection model compared with the previous reports. A larger number of propriospinal neurons are present in the cervical spinal cord than the thoracic spinal cord, with intrinsic differences also reported for growth factors, cell surface receptors, apoptosis, axonal regeneration, neuroprotection, and cell survival [49-52]. Our results indicate that the combined expression of IL-10 and NT-3 does significantly enhance axon density at the cervical hemisection. This synergy is likely related to the sparing of axons by IL-10 and the neurotrophic effects of NT-3. Effectively, the greater survival of neurons by IL-10 leads to an increase number of axons that can regenerate into the injury.

An increase in myelinated axons for IL-10+NT-3 delivery, notably with oligodendrocyte myelinated axons was observed. Oligodendrocyte myelin is necessary to support saltatory conduction and prevent axon degeneration [53]. Oligodendrocyte proliferation is not altered by NT-3, NT-3 exposure *in vitro* lead to significantly more MBP production by oligodendrocytes through an unknown posttranscriptional mechanism [54, 55]. Some evidence suggests that NT-3 weakly induces the maturation of neural precursor cells into myelinating oligodendrocytes [56], yet other reports have shown NT-3

promotes quiescence or even Schwann cell differentiation of neural precursor cells[20, 57-59]. These differences could indicate concentration dependent effects or vary with surrounding interactions with other factors. We observed that IL-10 delivery had significantly more oligodendrocyte-derived myelin relative to control. IL-10 polarization of immune cells enhances the ability of neural precursor cells to promote oligodendrocyte differentiation and supports mature oligodendrocyte survival by promoting an anti-inflammatory environment rather than acting on neural precursor cells directly [60]. Our results are consistent with this observation that IL-10 promoted the survival of myelinating oligodendrocytes, which can synergize with the expression of NT-3 to potentiate their myelin production to ensheath more axons. This mechanism has been suggested in other work combining IL-10 and NT-3 delivery for treatment of multiple sclerosis [61]. We did not observe an increase in oligodendrocyte myelination for NT-3 delivery alone, possibly due to lack of surviving oligodendrocytes and weak effects of NT-3 on their proliferation and differentiation [56].

Oligodendrocyte myelination significantly correlated increased functional recovery. We assessed locomotor recovery on the ladder beam walking task over the course of 12 weeks. All treatment conditions had significant improvements in functional recovery relative to control, yet IL-10+NT-3 delivery was substantially improved relative to all other conditions. A significant correlation between ladder beam score and oligodendrocyte myelinated axon density was observed, suggesting that oligodendrocyte

myelination of axons is a limiting step in functional recovery for our model. The functional benefit of oligodendrocyte myelin versus Schwann cell myelin have been debated in the literature, yet any conclusion remains nebulous. Some reports suggest oligodendrocyte myelin is not necessary in spontaneous functional recovery [62]; however, prolonged recovery as seen in these studies may require oligodendrocyte myelin for restoration of function. The various injury models may differentially impact oligodendrocytes as oligodendrocyte have been seen as less important in contusion models [62]. Our hemisection model severs and removes existing axons and cells in the lesion, while contusion models retain the damaged tissue.

A decrease in hypersensitivity was observed with IL-10 delivery while NT-3 delivery alone increased hypersensitivity. IL-10 has been shown to ameliorate neuropathic pain but the role played by neurotrophins and NT-3 has been nebulous [63]. NT-3 can be involved in a long-term change of neuronal excitability[64]. NT-3 also promotes an extensive growth of lesioned axons in the dorsal columns which contain mostly sensory projections. Furthermore, the effects of NT-3 on neuropathic pain may be concentration or receptor dependent[65-67]. Our results suggest NT-3 exacerbates neuropathic pain. However, there exists a tradeoff of positive and negative effects. We observed the highest degree of functional recovery when combining IL-10+NT-3, yet we also observed increased neuropathic pain compared to IL-10 alone.

Conclusion

Overall our results show that the combination of IL-10+NT-3 enhanced axonal growth and oligodendrocyte myelinated axon density, and with increased locomotor functional recovery compared to IL-10 or NT-3 alone. Hypersensitivity with the combination was increased compared to IL-10 alone yet decreased relative to NT-3 alone. Furthermore, a positive correlation was observed between oligodendrocyte myelinated axon density and functional recovery, suggesting oligodendrocyte myelination as a target to further improve functional recovery. Poly-cistronic vectors provide a mechanism for expression of multiple transgenes that can simultaneously address multiple aspects that limit regeneration. Multichannel PLG bridges provide a growth supportive substrate and a platform to deliver bioactive agents as well as a defined space to investigate the SCI microenvironment and to assess the biological impact of treatments.

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Disclosure Statement

No competing financial interests exist.

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