Title

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Localized immunomodulation of IL-10 and IL-4 to enhance functional regeneration after spinal cord injury

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Abstract

Trauma to the spinal cord and associated secondary inflammation can lead to permanent loss of sensory and motor function below the injury level, with the resulting environment serving as a barrier that limits regeneration. In this study, we investigate the localized expression of anti-inflammatory cytokines IL-10 and IL-4 via lentiviral transduction ~~at~~ in multichannel bridges. Porous multichannel bridges provide physical guidance for axonal outgrowth with the cytokines hypothesized to modulate the neuroinflammatory microenvironment and enhance sparing ~~and modulate the neuroinflammatory microenvironment~~. IL-10 and IL-4 expression induces a decreased expression of pro-inflammatory genes and increased pro-regenerative genes relative to control. Moreover, these factors led to increased numbers of axons and myelination, with the number of oligodendrocyte myelinated axons significantly increased relative to control. Furthermore, IL-10 and IL-4 expression improves locomotor function after injury. Collectively, these studies highlight the potential for localized immunomodulation to decrease secondary inflammation and enhance regeneration that may have numerous applications.

Trauma to the spinal cord creates an initial injury, with secondary inflammatory responses further exacerbating the damage. Secondary events can include ischemia, anoxia, and excitotoxicity for the first minutes, hours, and days after injury. These events create an inhibitory microenvironment and damage adjacent intact tissue, resulting in permanent loss of function below the level of the injury[1-4]. Activated macrophages and neutrophils release pro-inflammatory cytokines, which induce reactive astrocytes contributing to increase the secretion of inhibitory molecules such as chondroitin sulfate proteoglycans (CSPGs) at the injury site[3, 4]. ~~Inflammatory~~ An inflammatory response at the injured spinal cord leads to swelling and thus compression within the vertebral column[3-6] ~~.~~and secondary damage. Moreover, reactive astrocytes form a glial scar at the lesion over time, which acts as a mechanical and chemical barrier for axonal re-growth.

While inflammation can induce damage, the presence of macrophages is also necessary for regeneration[3, 7-9]. The regenerative function of macrophages may be associated with distinct subsets, which are derived from both resident microglia and hematogenous macrophages. Macrophages have plasticity that depends on the microenvironment, and the phenotypes have been described as ranging from pro-inflammatory M1 (or classically activated) to pro-regenerative M2 (or alternatively activated)[9, 10] although many argue the phenotypes are not binary (refs e.g. Martinez & Gordon 2014) these designations provide a useful frame of reference for analysis. The pro-inflammatory phenotype is induced by prototypical T helper 1 (T_H1) cytokines such as interferon-γ (IFN γ), which induce pro-inflammatory cytokines, reactive oxygen species (ROS), and nitric oxide (NO), all of which contribute to inflammation and tissue damage. By contrast, the pro-regenerative phenotype is derived from T_H2 cytokines including IL-4, IL-13 and IL-10[9, 11-13]. These anti-inflammatory cytokines contribute to wound healing and tissue repair, and enhance re-growth of axons against a myelin inhibitory environment in a spinal cord injury (SCI)[10, 12-15].

Multiple channel bridges, which provide a structural conduit across the injury site, have been employed as a means to promote regeneration after SCI[8, 16-18] (add Pawar 2015 reference). The presence of the bridge reduces the extent of glial scar formation and astrogliosis[18-20], and localized expression of trophic factors have been able to enhance the number of regenerating axons and the extent of myelination[8, 16, 17, 21]. Collectively, the results have indicated the potential to support regeneration through the injury site, which has contributions from a modestly attenuated inflammatory response. Nevertheless, the secondary inflammatory response remains and contributes to cell death and axonal retraction that may limit regeneration, and reducing this inflammation combined with inducing a pro-regenerative response may further enhance regeneration.

In the present study, we investigated regeneration after SCI following local immunomodulation using lentiviral vector delivery of anti-inflammatory cytokines IL-10 and IL-4 from a multiple channel PLG bridge. IL-10 and IL-4 were investigated because of their dual neuroprotective and neuroregenerative abilities[22-25]. We hypothesized that the architectural aspects of the bridge ~~will~~ would synergize with the immunomodulation provided by cytokines to modulate neuroinflammation and promote regeneration. A lateral hemisection model ~~is~~ was employed for bridge implantation, and the inflammatory response ~~is~~ was characterized histologically, and through transcriptome analysis for inflammatory and regenerative gene expression. The number of axons and their extent of myelination ~~are~~ were characterized histologically, with locomotor tests performed for functional recovery.

Transgene expression after anti-inflammatory cytokine~~s~~ delivery

Local transgene expression by delivery of IL-10 encoding lentiviral vector (vIL-10) and IL-4 encoding lentiviral vector (vIL-4) from spinal cord tissues was quantified as a function of time (**Figure~~.~~ 1**), with firefly luciferase encoding lentivirus used as a control (vCtrl). By day 7, IL-10 and IL-4 protein expression levels were significantly increased relative to vCtrl, with elevated levels maintained through at least 28 days post-SCI. IL-10 and IL-4 protein levels at day 3 trended greater than control, yet lentiviral expression ~~requires~~ required multiple days to achieve protein levels above background. IL-10 expression increased through day 14, and then declined at day 28, yet was still significantly above control. Conversely, IL-4 levels were maximal at day 7 and persisted through the 28 days of this study.

Anti-inflammatory cytokines modulate microenvironment after SCI

We investigated alteration in the transcriptome caused by anti-inflammatory factors through cDNA microarray at day 7 post-SCI (**Figure 2**). A total of 24,581 genes were measured. A total of 368 transcripts for the vIL-10 condition and 132 transcripts for the vIL-4 condition were ~~two-fold~~ upregulated two-fold or greater. An additional 391 transcripts in the vIL-10 condition and 92 transcripts in the vIL-4 condition were downregulated more than two-fold. We sought to identify patterns of among these genes that differentiated the vIL-10 and vIL-4 conditions from the vCtrl to gain insight into the active biological processes at all conditions. We were primarily interested in ontologies that overlapped between the two cytokine conditions compared with vCtrl; ten representative ontologies that were significant in both conditions are presented in Fig. 2b and 2c. Gene ontology analysis for genes that were two-fold downregulated primarily identified ontologies associated with neuroinflammation responses (e.g. immune response, Fig. 2d). qRT-PCR was used to confirm these results (Fig. 2f). The results agreed broadly with the microarray data at day 7, ~~then~~ when gene~~s~~ expression showed a decrease at later timepoints in response to vIL-10 and vIL-4. Conversely, upregulated genes matched to ontologies related to the functional recovery (e.g. chemical synaptic transmission, Fig. 2e). We confirmed these results via qRT-PCR of genes in these over-represented pathways. Time-course measurements showed an increase in transcript quantity further out from the time of injury (day 14 or 28) along with a modest upregulation at day 7 in response to anti-inflammatory factors, which corresponds with the microarray data (Fig. 2g).

Anti-inflammatory cytokines induce macrophage polarization

We investigated macrophage polarization by cytokine expression, as macrophages are central players in the inflammatory response following SCI (**Figure~~.~~ 3**). Initially, the gene expression profiles associated with M1 and M2 phenotypes were assessed through microarray and qRT-PCR. M1 markers levels were the same or downregulated in vIL-10 and vIL-4 relative to vCtrl~~, i.~~ In contrast, M2 genes levels were upregulated in response to vIL-10 and vIL-4 compared to vCtrl (Fig. 3a). Based on the microarray, subsets of M1 and M2 genes were validated using qRT-PCR over time (Fig. 3b and 3c). The expression levels of inflammation markers CD86, major histocompatibility complex class II (MHC-II), and inducible nitric oxide synthase (iNOS) were significantly decreased in vIL-10 and vIL-4 relative to vCtrl, while anti-inflammatory markers CD206, resistin like alpha (Retnla), and arginase 1 (Arg1) were substantially increased in anti-inflammatory delivery groups over time. Subsequently,

immunofluorescence staining was performed to confirm and quantify the densities of total macrophages (Hoechst$^+$/F4/80$^+$) and macrophages expressing the M2 associated factor arginase 1 (Hoechst$^+$/F4/80$^+$/Arginase1$^+$) (Fig. 3d-3g) at the site of injury (or within the bridge – specify). In agreement with previous study[17], no statistical differences were observed in the density of macrophages within the bridge at day 14 and 28 for all conditions (Fig. 3h); however, the number of macrophages expressing arginase1 was substantially increased in anti-inflammatory cytokine groups relative to bridge only and vCtrl groups (Fig. 3i). These data indicated that overexpression of anti-inflammatory factors influences macrophage polarization at the injury.

Anti-inflammatory factors promote axonal regrowth and remyelination

We next assessed the impact of anti-inflammatory cytokine expression on axonal regrowth and remyelination in the sub-acute (28 days) and chronic (84 days) phase after SCI (**Figures 4 and 5**). Initially, the microarray results were analyzed for alteration in the expression of neural system development associated-genes. The results demonstrated more than two-fold upregulation of a number of associated-genes ~~for~~ in the vIL-10 and vIL-4 conditions compared to vCtrl. We also confirmed ~~that~~ expression levels for a number of selected genes associated with neural system development, with ~~the~~ qRT-PCR results indicating increased and persistent expression of Lhx5, Hapln4, Trpc5 through the 28 days in vIL-10 and vIL-4 groups, consistent with the microarray studies (Fig. 4a and 4b).

Subsequently, we investigated axonal regrowth and myelination after SCI ~~through~~ via immunofluorescence for myelin basic protein (MBP) and neurofilament 200 (NF200). The data revealed that NF200$^+$ axons were observed throughout the bridges in all conditions in the sub-acute phase (Fig. 4c-4g). No significant differences were observed for the groups, though a trend toward greater densities of NF200$^+$ axons in vIL-10 and vIL-4 groups in comparison to vCtrl were observed (Fig. 4j) at 28-days post bridge implantation. However, the number of Schwann cell~~s~~-derived myelinated axons (NF200$^+$/MBP$^+$/P0$^+$) was significantly increased in anti-inflammatory delivery groups relative to vCtrl. Generally, MPB myelinated (NF200$^+$/MBP$^+$) axons were seen in the center of bridges or midline of spinal cord (channels 2, 5, and 6 in **Supplementary Fig~~.~~ure 1a**), while Schwann cell-derived (NF200$^+$/MBP$^+$/P0$^+$~~–~~) axons were observed around the outer ~~surface~~ edges of the bridge (Fig. 4h and 4i).

At day 84, analysis of immunofluorescence data indicated that regenerating axons were typically found throughout the bridge in bundles of 2 or more axons ~~for~~ in the anti-inflammatory cytokine groups (Fig. 5a and 5b), which was consistent with previous reports[8, 19]. The total number of NF200$^+$ axons was significantly increased in the anti-inflammatory cytokine~~s~~ groups relative to bridge only and vCtrl (Fig. 5c)~~, m~~. Moreover ~~the~~ a greater number of NF200$^+$ axons were observed at day 84 relative to day 28 for all conditions (**Supplementary Fig~~.~~ure 2**). Similar to axon number, myelinated axons (NF200$^+$/MBP$^+$) were also significantly increased in vIL-10 and vIL-4 delivery groups by 3-4 folds compared to bridge only and vCtrl, and about 45% and 44% of which NF200$^+$ axons were myelinated (Fig. 5c and 5d).

In addition to axonal regrowth and myelination, we also evaluated the source of myelination in the chronic phase (Fig. 5e and 5f). The significantly greater number of oligodendrocyte-derived myelinated axons (NF200$^+$/MBP$^+$/P0$^-$) was seen in anti-inflammatory cytokine groups relative to bridge only and vCtrl. About 48% and 52% of myelinated axons were ensheathed by oligodendrocyte-derived myelin in vIL-10 and vIL-4 respectively. Moreover, ~~the~~ a greater number

of oligodendrocyte-derived myelin ~~were~~ was observed at day 84 relative to day 24 for vIL10 and vIL4 (Supplementary Fig. 2c and 2d). However, there were no significantly differences in Schwann-cell mediated myelination (P0^{+}) (Supplementary Fig~~.~~ 2). Collectively, these data demonstrate that the localized expression of anti-inflammatory cytokines promotes axonal regeneration and axonal remyelination by oligodendrocytes.

Anti-inflammatory factors improve functional recovery after SCI

cDNA microarray heatmaps ~~indicated~~ revealed a two-fold upregulation of locomotor recovery-associated genes in the anti-inflammatory cytokine groups relative to vCtrl (**Fig~~.~~ ure 6a**). qRT-PCR analysis of selected functional recovery-associated genes indicated a significant increase within vIL-10 and vIL-4 groups relative to vCtrl, which was maintained until 28 days after SCI (Fig. 6b). Subsequently, locomotor function was evaluated using the Basso Mouse Scale (BMS) before injury, at day 3, and then weekly for 84 days post-SCI (Fig. 6c). Before SCI, all mice in all conditions were fully functional. At day 3 post-SCI, no movement was observed in ipsilateral hindlimb in any group, yet BMS scores gradually ~~increased~~ improved with time. The BMS score in vIL-10 and vIL-4 groups indicated a significantly enhanced recovery of motor function compared to vCtrl starting at day 14 after SCI. The vIL-10 group was significantly improved relative to vCtrl through day 84, though the vIL-4 group was not significantly different from vCtrl after day 63, despite the trend towards increased scores.

~~Generally, s~~Specific aspects of locomotor recovery in mice after SCI do not follow a typical recovery pattern, ~~which~~ and are thus ~~are~~ not reflected ~~on~~ in the ~~general~~ overall BMS score[26]. Therefore, BMS subscoring was performed after day 63 to analyze specific components of locomotion[26] (**Supplementary Fig~~.~~ ure 3**). The subscoring indicated that both vIL-10 and vIL-4 delivery substantially improved stepping frequency, paw position, fore-limb and hind-limb coordination, tail position, and trunk stability compared to vCtrl. Furthermore, BMS subscore for the bridge only was greater than that in SCI only, suggesting that the implanted-multichannel bridge itself also has a positive effect on locomotor function recovery after SCI. These data demonstrate that localized expression of the anti-inflammatory factors and multichannel bridge increased motor function following injury.

Discussion

The injured microenvironment cues favor differentiation of macrophage into M1 phenotypes, also, persistent induction of pro-inflammatory cytokines expression was observed[3, 7, 15, 27]. Conversely, anti-inflammatory cytokines necessary for inducing M2 phenotype or attenuating macrophages function were unchanged or decreased[7, 15, 27]. We report that ~~Localized~~ localized delivery of anti-inflammatory cytokines ~~has shown to~~can modulate modulate the local neuroinflammatory microenvironment, ~~which~~ and induce~~d~~ M2-favored conditions at the injury site. The injured microenvironment cues favor differentiation of macrophage into M1 phenotypes, also, persistent induction of pro-inflammatory cytokines expression was observed[3, 7, 15, 27]. Conversely, anti-inflammatory cytokines necessary for inducing M2 phenotype or attenuating macrophages function were unchanged or decreased[7, 15, 27]. cDNA microarray showed that neuroinflammatory-related genes levels were decreased more than two-fold relative to vCtrl, while upregulation of synaptogenesis- and neurogenesis-related gene expression was observed in both vIL-10 and vIL-4 groups (Fig.2 and **Supplementary Figure ~~.~~ 4**). qRT-PCR indicated that anti-inflammatory cytokines downregulated inflammatory response-

associated expression relative to vCtrl throughout day 28 post-SCI (Fig. 2). These factors also resulted in upregulation of M2 markers at the injury site, while M1 markers were downregulated compared to vCtrl (Fig. 3a-3c). The transgene expression herein, and as also reported in a previous study, showed that delivered-lentivirus started producing transgene at day 3 and was substantially increased at day 7 compared to control[21, 28]. This may lead to significant alteration of gene expression profiles in vIL-10 and vIL-4 groups relative to vCtrl as a function of time. Both IL-10 and IL-4 downregulate the secretion of pro-inflammatory factors after injury, but ~~using~~ via different pathways. Nuclear factor-κB (NF- κB) plays a critical role in the ~~developing~~ development of inflammation by regulating gene encoding pro-inflammatory cytokines[29]. IL-10 has been shown to suppress NF-κB activation and its DNA binding through a STAT3-mediated pathway[29, 30]. Furthermore, glia cells express the IL-10 receptor in the CNS, where it acts as an antagonist to inhibit secretion of pro-inflammatory factors from glia cells after injuty[22, 23, 25]. IL-4 utilizes ~~the~~ a STAT6-mediated cascade, leading to expression of anti-inflammatory genes via suppression of IFNγ signaling[24, 31]. In addition, IL-4 prevents production of inducible enzymes, which inhibits inflammatory factor-mediated NO synthesis, resulting in expression of anti-inflammatory factors[32]. In agreement with previous ~~study~~studies, arginase 1 gene level increases in all conditions and then return to baseline at day 14, since microglia/macrophages have both M1 and M2 phenotypes early in the SCI[14, 15]

The local microenvironment modulation by anti-inflammatory cytokines controls macrophage activation, differentiation, and polarization (Fig. 3). In the injured spinal cord, an analogous M1-to-M2 macrophage polarization as in ~~the~~ normal tissue~~s~~ does not occur. As a function of time, M2 macrophages are decreased, while pathological M1 macrophages remain elevated at the injury site. Therefore, a transient and low-number of pro-regenerative M2 macrophages in the damaged microenvironment may ~~cause~~ allow ~~the~~ chronic inflammation and secondary damages after primary injury[15, 27]. Although immunofluorescence showed a trend toward greater number of infiltrated F4/80$^+$ macrophages at day 14 post-SCI compared to empty bridge, there were no significant changes between conditions. These observations are supported ~~as~~ by prior reports that tha ~~infiltrated~~ infiltrating number of macrophages is known to peak around 7-10 days at the injured site and plateau for several days before decreasing[7, 17]. More importantly, both vIL-10 and vIL-4 delivery significantly increased the number of M2 (F4/80$^+$/Arginase1$^+$) macrophages at day 14 and 28 relative to empty bridges (Fig. 3i). Increased number of F4/80$^+$/Arginase1$^+$ macrophages may be correlated with ~~Increased~~ increased arginase1 levels and induction of an M2-favored microenvironment by IL-10 and IL-4 expression, since there is a linear trend between IL-10 and IL-4 expression and M2 numbers with a positive slope (**Supplementary Fig. ure 5**). Moreover, IL-10 has shown to induce expression of IL-4 receptor alpha (IL4Ra), and ~~an~~ M2 macrophages produce~~s~~ both IL-10 and IL-4, which may ~~lead to~~ creat~~ing~~e a feed forward process when IL-10 and IL-4 are overexpressed at the injured site[10, 17].

Immunomodulation by anti-inflammatory cytokines creates positive effects on axonal regrowth after SCI. In the neuroinflammatory microenvironment, the M1 phenotype produces pro-inflammatory cytokines that are cytotoxic to neurons and cause them to extend short neurite sprouts after SCI[9, 15, 27]. IL-10 has been shown to upregulate anti-apoptotic factors such as B-cell lymphoma 2 (Bcl-2) and to provide a direct trophic influence on neurons to overcome the neurotoxic microenvironment [22, 23, 25]. IL-4 controls macrophage activation in the acute SCI phase, and reduces the release of pro-inflammatory factors at the injury[24, 25, 31]. In the experiments reported here, IL-10 and IL-4 had a similar impact on ~~the~~ inflammation-related

gene expression dynamics, though a few differences in the dynamics were observed (e.g., Retnla). Moreover, the M2 phenotype forms in response to IL-10 and IL-4, and upregulates arginase1 activity, which in turn promotes overexpression of polyamines. Polyamines activate downstream of cAMP to enhance axonal regrowth against a myelin inhibitory environment[10, 14, 15]. In addition, an M2 macrophages are thought to promotes removal of apoptotic and necrotic cells via CD206 without any toxic byproducts[12, 15]. Through these pathways, M2 macrophages may facilitate phagocytosis and nerve tissue remodeling to promote axonal regeneration after SCI.

In addition to phagocytosis and axonal regeneration, anti-inflammatory cytokines may promotes axonal remyelination. As a major component of the regenerative process, remyelination is mediated by multipotent oligodendrocyte progenitor cells (OPCs) after CNS injury. Previous study studies demonstrates that skewing the M1:M2 ratios towards M2 activation is required for the differentiation of OPCs after SCI[33, 34]. *In vitro* study shows that M2-conditioned media enhances oligodendrocyte differentiation (ref?). Moreover, activin-A, an M2 derived-regenerative factor, directly binds to OPCs and contributes to M2-mediated oligodendrocyte differentiation in remyelinating lesions[35, 36]. Therefore, a switch to M2 from M1 is an essential part of the regenerative process for enhancing differentiation of oligodendrocytes for remyelination[33, 34]. In this study, IL-10 and IL-4 had similar effects on gene expression associated with regeneration, with a few differences in dynamics (e.g., Lhx5, Scn1a). Collectively, vIL-10 and vIL-4 delivery decreased inflammation, which ultimately increased the number of oligodendrocyte-derived myelinated axons at the injury site.

Ultimately, providing spatial organization for axonal regeneration using multichannel bridges with anti-inflammatory cytokines creates synergistic effects on functional recovery after SCI. Previously, we have demonstrated that the a naked PLG bridge altered the chemical balance and physical cues toward a more permissive environment[8, 17-20]. In addition, the bridge porosity enables endogenous supportive cells such as Schwann cells and fibroblasts to infiltrate into injured area, then release growth factors and deposit growth-stimulating extracellular matrix (ECM), leading to long-term axonal growth[8, 16, 19, 20]. Furthermore, our gene expression data herein showed that the bridge itself upregulated axonal guidance- and synaptogenesis-associated genes expression compared to SCI only (Fig. 2g and 6b).

While myelinated axons are observed in the middle of the bridge, Schwann cell derived-myelinated axons are observed particularly around and outside of bridge, and are significantly increased in both vIL-10 and vIL-4 relative to vCtrl. Both oligodendrocytes- and Schwann cell-derived remyelination of spared and regenerating axons have been shown to recover saltatory conduction and carry signals across the injury site which are considered as key factors for functional recovery[34-38]. We have shown that naked PLG bridges can support descending axonal regeneration, and an increase in GAP43 above and below the implantation site, followed by the emergence of functional recovery on a forepaw cylinder reaching task (Pawar et al 2015). Furthermore, previous studies demonstrate that spontaneous axonal sprouting after injury results in rewiring new synaptic connections and/or strengthening of existing synapses with other spinal neurons, enhancing locomotor recovery in hemisection SCI[39, 40]. Taken together, although axonal numbers are not correlated with locomotor recovery in the sub-acute phase, multichannel bridge itselfs provides a permissive microenvironment for improved axonal sparing resulting in greater locomotor recovery compared to SCI only group. Furthermore, locomotor recovery in the chronic phase may be associated with both improved axonal regeneration and sparing, and remyelination at the injury site, which may result from synergistic effects of both

immunomodulation by anti-inflammatory cytokines and topographical guidance via multichannel bridge as a function of time. While most of studies show improved functional recovery by anti-inflammatory factors, others have indicated that they may have negative effects on locomotor recovery after SCI[41, 42]. This discrepancy may be due to differences in the rodent stains, SCI models, and/or delivery methods.

Localized delivery of therapeutics to the injured spinal cord has been unable to achieve long-term expression of therapeutics without additional surgery procedures. Although the osmotic pumps and catheters have been extensively used for long-term sustained delivery of therapeutics, implantation inside the intrathecal space have been shown to generate scar tissue and additional damages at the implanted site[43-45]. Recently, as alternative methods to reduce tissue damage, therapeutics have been delivered via intramuscularly or intravenously[46, 47]. While these methods reduce the additional damage to spinal cord, they do not localize the therapies and increased dosages can initiate an immune response[47, 48]. In this study, we report that delivery of anti-inflammatory cytokines encoding lentivirus from a multichannel bridge provides a tool for long-term and sustained localized expression of potential therapeutics for tissue regeneration without additional damage. These observations are supported by a previous study, which that demonstrated that local lentiviral vector delivery of sonic hedgehog could leading lead to sustained and localized transgene expression over 12 weeks at the injured site[28].

While the results herein demonstrate that anti-inflammatory cytokines effectively improve functional recovery, transgene expression requires a week to achieve significant levels. Therefore, earlier or alternative immune interventions may be required to restrict acute immune response-mediated tissue damages. Nanoparticles (NPs) have received increasing attention in recent years as a tool for immune modulation in various diseases[49-51]. NPs can be utilized to target immune cells to attenuate their potential for causing immune pathology because they are generally considered rapidly as invasive pathogens[50, 51]. Furthermore, NPs can be employed to deliver alone or in any combination, thus delivery of NPs in combination with anti-inflammatory cytokines could act synergistically to promote further regeneration.

Collectively, the current study demonstrates that multichannel bridges and localized expression of anti-inflammatory cytokines can modulate the neuroinflammatory microenvironment after SCI. Localized IL-10 and IL-4 expression enhances macrophage polarization from a pro-inflammatory M1 to a pro-regenerative M2 phenotype compared to control. Under these conditions, the number of axons and oligodendrocyte-derived myelinated axons are significantly increased relative to control as a function of time. Moreover, expression of anti-inflammatory cytokines promotes locomotor recovery after SCI. Our results suggest that providing a permissive environment to regeneration by a multichannel bridge, combined with localized anti-inflammatory cytokines gene delivery, which may provide a novel therapeutic strategy to overcome neuroinflammatory microenvironment for nerve regeneration after SCI.

Methods

Virus production

Lentivirus was produced from HEK293T cells based on ~~the~~ establishe~~d~~ds ~~method~~s[17]. Cells were co-transfected using third generation lentiviral packaging vectors (pMDL-GagPol, pRSV-Rev, pIVS-VSV-G, and the gene of interest (pLenti-CMV-Luciferase or pLenti-CMV-Human IL10 or pLenti-CMV-Human IL4) using Lipofectamine 2000 (Life Technologies, Grand Island, NY, USA). After 48 hours, supernatant was collected and concentrated in PEG-it (System Biosciences, Mountain View, CA) for 24 hours. Then ~~it,~~ the supernatant was precipitated using ultracentrifugation, resuspended in PBS and stored at −80 °C until use. Virus titers were determined by ~~the~~ a Lentivirus qPCR Titer Kit (Applied Biological Materials, Richmond, BC, Canada) and 2E9 IU/mL were used throughout the study.

Multichannel bridges fabrication

Multichannel bridges were fabricated using a gas foaming/particulate leaching method as previously described[8, 16]. Briefly, PLG (75:25 lactide:glycolide; i.v. 0.76 dL/g; Lakeshore Biomaterials, Birmingham, AL, USA) was dissolved in dichloromethane (6% w/w) and emulsified in 1% poly(ethylene-alt-maleic anhydride) 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. To make a sugar fiber, the mixture was caramelized, cooled, and drawn from solution using a Pasteur pipette. These fibers were coated with a 1:1 mixture of PLG microspheres and salt, then pressed into a salt-lined aluminum mold. Next, ~~it~~ the bridge was equilibrated and gas formed with high pressure CO_2 gas (800 psi) for 16 h in a custom-made pressure vessel. The pressure was released over a period of 40 min, which fused adjacent microspheres to produce a continuous polymer structure. The bridges were cut into 2.25mm sections, and the porogen was leached in water for 2 hours. The bridges were dried over-night and stored in a desiccator.

Virus loading into the multichannel bridges

Bridges were disinfected in 70% of ethanol and washed with water, then dried ~~in~~ at room temperature. In order to maximize lentivirus loading into bridges, repeated addition of lentiviruses was performed. Initially, 2µl of virus was added onto the bridge ~~and,~~ waiting until it was completely adsorbed (approximately X minutes). We performed these procedures 3 additional times; then, bridges were stored at -80 ºC ~~deep freezer~~ until ~~in~~ use.

Spinal cord hemisection injury model and animal care

All animal surgery procedures and animal care were performed according to the Animal Care and use Committee guideline at University of Michigan. A mouse hemisection model of SCI was performed as described previously[8, 18]. C57/BL6 female mice (4-6 weeks old; The Jackson Laboratory, Bar Harbor, ME, USA) were anesthetized using isoflurane (2%). A dorsal laminectomy was performed at T9-T10 level then a 2mm long lateral of the midline spinal cord segment was removed to create a hemisection SCI mice model (Supplementary Fig 1b). ~~Then, b~~Bridges were implanted in the injury site and covered using Gelform (Pfizer, New York, NY, USA). Muscle was sutured together, then the skin was ~~stabled~~stapled. For ~~the~~ postoperative

animal care, Baytril (enrofloxacin 2.5 mg/kg SC, once a day for 2 weeks), buprenorphine (0.1 mg/kg SC, twice a day for 3 days), and lactate ringer solution (5 mL/100 g, once a day for 5 days) were administrated. Bladders ~~was~~ were manually expressed twice a day until bladder reflexive function was observed.

Enzyme-linked Immunosorbent Assay (ELISA)

To measure the recombinant human IL10 and IL4 protein level from transfected host cells and tissues, a 6mm long section of spinal cord, both rostral and caudal to the bridge, was removed, homogenized in RIPA lysis and extraction buffer (Thermo Fisher Scientific, Waltham, MA, USA) with 10 μL of Halt Inhibitor Cocktail (Thermo Fisher Scientific) per milliliter of sample directly. Then, ~~incubate~~ the homogenized tissue sample was incubated for 10 minutes at room temperature, and centrifuged at 14,000 rpm for 30min at 4 $^{\circ}C$ to remove tissue debris. ~~Protein~~ The protein concentration of each sample was estimated using Pierce BCA Protein Assay (Thermo Fisher Scientific), and then samples were diluted accordingly. Human IL10 and IL4 concentration was subsequently assayed by Human IL10 Quantikine ELISA Kit (R&D system, Minneapolis, MN, USA) and Human IL4 ELISA Kit (LSbio, Seattle, WA, USA) based on the manufacturer's instructions. Herein, we measured the human IL10 and IL4 protein levels to eliminate any confounding results with the expression of murine IL10 and IL4 protein by delivered lentiviral vectors. ELISA kits used for IL10 and IL4 quantification allow for distinguishing human IL10 and IL4 from murine IL10 and IL4, since the antibodies were not cross reactive as reported by the manufacturer.

RNA isolation and cDNA microarray

To isolate RNA, the spinal cord tissue~~s~~ were removed from sham, SCI only, SCI+Bridge, SCI+Bridge+Fluc (vCtrl), SCI+Bridge+vIL10, and SCI+Bridge+vIL4 groups (n=5-6/group/time point; POD 3,7,14, and 28) and cut into 6mm segments centered on 2mm of bridge region~~. All~~ ; samples were not pooled. We performed RNA isolation based on ~~the~~ a previous study[6]. ~~In b~~Briefly, the spinal cord samples were homogenized using 1 ml of Trizol reagent (Invitrogen, Carlsbad, CA) with a tissue grinder. RNA isolation was followed by chloroform extraction and isopropanol precipitation. The extracted RNA was dissolved in 30 μl of RNase-free distilled water then, we measured RNA concentration using a NanoDrop 2000C (Thermo Scientific, Newark, DE) with A260/A280 ratios between 1.9 and 2.1 for all samples. Total isolated RNA was stored at -80 $^{\circ}C$ ~~deep freezer~~ until ~~in~~ use. For the cDNA microarray, gene expression analysis from each condition was conducted using the Mouse Gene ST 2.1 microarray platform (Affymetrix). Data was processed using the oligo R package. Raw output from the multi-array was converted to expression values through robust microarray averaging[52]. Data quality was assessed by principle component~~s~~ analysis. Sample in each group was normalized to expression values from a sham group or bridge+vCtrl group and log transformed. Probe set annotation was downloaded from BioConductor. Gene ontology analysis was performed using PANTHER[53].

Quantitative Real-Time PCR

For the gene expression analysis, we performed ~~the~~ quantitative real-time PCR (qRT-PCR). cDNA was synthesized using an iScript™ cDNA Synthesis kit (Bio-Rad, Hercules, CA) according to the manufacturer's ~~manual~~ guidelines. One microgram of RNA was used from each sample to synthesize cDNA. Primers were designed for qRT-PCR based on the previous study (~~supplementary~~ Supplementary ~~table~~ Table 1)[5, 15, 54, 55]. 18s-rRNA was used as an internal control. The qRT-PCR products were measured using the accumulation level of iQ™ SYBR Green Supermix (Bio-Rad) fluorescence following a manufacturer's protocol on CFX Connect™ Real-Time PCR Detection System (Bio-Rad). The gene expression level was normalized by the expression of 18s-rRNA and differences in gene expression were presented as fold ratios from sham spinal cord samples. Relative quantification was calculated as $X = 2^{-\Delta\Delta Ct}$, where $\Delta\Delta C_t = \Delta E - \Delta C_t$ and $\Delta E = C_{t,exp} - C_{t,18s\text{-}rRNA}$, $\Delta C_t = C_{t,\,sham} - C_{t,18s\text{-}rRNA}$[56].

Immunofluorescence – should indicate somewhere below that staining and quantification were done blind to group.

Spinal cords containing multichannel bridge were collected at 14, 28 and 84 days after SCI and were snap frozen in isopentane and embedded in Tissue Tek O.C.T compound (Sakura Finetek, Torrance, CA, USA) with 30% sucrose. Spinal cords were cryosectioned ~~in~~ at 18 µm thickness in the ~~transversely~~transverse plane. The following antibodies were used for immunofluorescence: neurofilament 200 (NF200, 1:200, Sigma Aldrich), myelin basic protein (MBP, 1:500, Santa Cruz Biotech, Dallas, TX, USA), P-zero myelin protein (P0) (1:250, Aves Labs, Tigard, OR), F4/80 (1:200, AbD Serotec, Raleigh, NC, USA), and arginase I (1:100, clone N20, Santa Cruz Biotech). The number of immune-positive cells and axons within the implant bridge area were manually counted. Multiple markers by co-staining ~~was~~ were examined by evaluating pixel overlap of different channels in NIH ImageJ (Bethesda, MD, USA). The total number of macrophages and M2 phenotype macrophages were evaluated by determining $F4/80^+$ cells and $F4/80^+$/$arginase^+$ cells. Macrophages were identified by localizing $Hoechst^+$ staining to $F4/80^+$ immunoreactive cells and labeling them in ImageJ. Then the cell~~s~~ number~~s~~ were normalized to the counted area in each tissue section. To investigate the numbers of regenerated and myelinated neurofilaments, we used $NF200^+$, $NF200^+/MBP^+$, and $NF200^+/MBP^+/P0^+$ to identify the numbers of axons, myelinated axos, and myelinated axons by infiltrating Schwann cells, respectively. We picked tissues from rostral, middle, and caudal bridge regions (Supplementary Fig. 1c) then averaged the counts over the bridge. The percentage of myelinated axons was determined from the ratio of the number of myelinated axons ($NF200^+/MBP^+$) divided by the number of axons ($NF200^+$) within the bridge area. To determine the source of the myelination, we performed ~~at~~ triple staining of NF200, MBP and P0. $NF200^+/MBP^+/P0^-$ neurofilaments were considered as oligodendrocyte-derived myelination and $NF200^+/MBP^+/P0^+$ neurofilaments were myelinated neurofilaments by infiltrated Schwann cells. The fraction of oligodendrocyte-derived myelination was calculated as $NF200^+/MBP^+/P0^-$ neurofilaments divided by $NF200^+/MBP^+$ neurofilaments.

Behavioral test for locomotor function

Motor recovery in the ipsilateral hindlimb was accessed using open field Basso Mouse Scale (BMS) locomotor rating scale after SCI[26]. The score is based on the hindlimb locomotor ability of SCI mice. SCI mice (n=15 per group) were observed in an open field for 4 minutes after they

had gently adapted to the field. Only the ipsilateral hindlimb side was assessed at days 3 and then weekly after SCI for 12 weeks. The score was obtained by taking an average value of results from each experimental group. A baseline was determined prior to SCI. Animals were randomly assigned to treatment group and assessed on the BMS by observers blind to group.

Statistical analysis

Unless noted otherwise, a one or two-way ANOVA was used for multiple comparisons. For the statistical significance between groups, we performed Tukey post hoc testing or Šidák correction for the multiple comparisons. Pearson's correlation coefficient was used for determining the statistical significance of regression data. To achieve reasonable statistical power analyses, type II errors were controlled at 0.2 level for all the statistical tests. Equal variance (ANOVA Model) was validated and assumed for each study. Given the above parameters, appropriate sample size for each study and all statistical analysis analyses were performed using OriginPro (OriginLab Corporation, Northampton, MA, USA) or Prism (GraphPad Software, La Jolla, CA, USA). $P<0.05$ was considered as statistically significant and the all values were expressed in mean $\pm$ SEM.

Data availability

~~The~~ All data ~~that support the findings of~~from~~f~~ this study are available from the corresponding author upon reasonable request.

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Author contributions
Conceptualization and design the study, J.P. and L.D.S.; Methodology, J.P., J.T.D., D.J.M., and L.D.S.; Investigation, J.P., J.T.D., D.J.M., and D.R.S.; Draft writing and review, J.P., J.T.D., D.R.S., B.J.C. and L.D.S.; Funding, B.J.C., A.J.A., and L.D.S.; Supervision, L.D.S.

Competing interests
The authors declare no competing ~~competing~~ interests.