Combination therapy with RvD1 and peripheral nerve-derived stem cell spheroids after spinal cord injury in rat

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Directed by Professor Keung Nyun Kim

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ABSTRACT

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Spinal cord injury (SCI) is primarily caused by trauma, resulting irreversible and permanent loss of motor, sensory and autonomic functions. Recently, various attempts have been made to overcome secondary injury, which is the main pathophysiology of SCI. Among these studies, research related to stem cells is being actively conducted, and the therapeutic effect has been proven. In previous studies, we demonstrated that peripheral nerve-derived stem cell (PNSC) spheroids, which have similar characteristics to neural crest stem cells (NCSC), have functional recovery and neuronal regeneration effects in the SCI model. However, since PNSC spheroids alone have a limited anti-inflammatory effect in SCI treatment, I investigated the effect of combination therapy with Resolvin D (RvD) 1, which has anti-inflammatory and neuroprotective effects. This study was conducted both *in vitro* and *in vivo* in rats. Through *in vitro* studies, I confirmed the pro-inflammatory cytokine inhibitory effect and anti-inflammatory effect of RvD1. After the SCI model was created, it was confirmed through spinal cord function, biological efficacy, qRT-PCR, and immunohistochemistry that the combination of RvD1 and PNSC spheroids had better neuronal regeneration and anti-inflammatory effects than the other monotherapies. Thus, Combination therapy with RvD1 and PNSC spheroids is expected to be a new treatment approach for SCI.

Key words : spinal cord injury, combination therapy, peripheral nerve-derived stem cell spheroid, Resolvin D1

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I. INTRODUCTION

Spinal cord injury (SCI) is primarily caused by trauma and results in irreversible and permanent post-injury complications in motor, sensory, and autonomic functions. Spinal cord injury occurs at a young age, causing many social, economic, and medical problems. National-level incidence studies have estimated the incidence of SCI to be between 40 and 80 per million individuals.[1-3]

SCIs are generally divided into primary and secondary injuries. Primary injury refers to damage to the nerve tissue and blood vessels caused by direct physical pressure from trauma. Secondary damage occurs as a result of mechanisms, such as inflammatory reactions, free oxygen, reactive oxygen species, ischemia, peroxidation, edema, and cell death that occur after primary injury.[4-7] High-dose corticosteroid administration within one day of injury, surgical decompression, and spinal immobilization are currently the only known treatments.[8-11] However, there is currently no treatment that can prevent and alleviate the secondary damage that occurs after primary injury and can regenerate the damaged spinal cord. The inefficiency and adverse effects of standard therapies highlight the need for new treatment strategies.[12,13] Owing their nerve regenerative and neuroprotective properties, stem cells are promising tools for the treatment of SCI.

Neutrophils, macrophages, T cells, and microglia infiltrate the spinal parenchyma and release pro-inflammatory cytokines such as, tumor necrosis factor alpha (TNF-α),

interleukin-1 beta (IL-1β), interleukin-1 alpha (IL-1α), and interleukin-6 (IL-6) in the injured cord.[14-17] Neuroinflammation triggers the development of necrotic cavities surrounded by glial scars, which block the progression of SCI.[17-19] Simultaneously, activated macrophages and microglia are involved in the phagocytosis of necrotic and damaged tissues. The rapid removal of this cellular debris is critical for creating an environment conducive to nerve regeneration. Thus, stem cell therapy for SCI aims to minimize the spread of secondary damage, enhance the function of the remaining cells, and promote the regeneration of neurons and glial cells. [20,21]

Currently, research is underway to achieve SCI recovery using various stem cell sources. Stem cell therapy is potentially protective and has been extensively studied in various SCI models. In particular, the regenerative potential of bone marrow-derived mesenchymal stem cells (BM-MSCs), cord blood-derived (CB) MSCs, adipose tissue-derived (AT) MSCs, neural stem cells (NSCs), and embryonic cell-derived oligodendrocyte progenitor cells (OPCs) in the putative treatment of SCI are discussed.[22,23] We successfully isolated, cultivated, and identified peripheral nerve derived stem cells (PNSCs) from adult peripheral nerves. PNSCs have characteristics similar to those of neural crest stem cells (NCSCs) and can differentiate into nervous and mesodermal lineage cells, and express and secret neurotrophic and growth factors from adult peripheral nerves. Additionally they have been applied as stem cell therapies for the regeneration of SCI. In particular, PNSCs have shown therapeutic potential by releasing neurotrophic factors including glial cell line-derived neurotrophic factor, insulin-like growth factor, nerve growth factor and neurotrophin-3. PNSC abilities were also enhanced due to their development into spheroids, which secrete neurotrophic factors several times more than non-spherical PNSCs and express multiple types of extracellular matrices. In a previous study, we confirmed that PNSC spheroid transplantation works effectively in functional recovery and neuronal regeneration a rat model of SCI.[24] However, since it is difficult to suppress the inflammation that occurs during secondary injury with PNSC spheroid transplantation alone, further treatments have been studied.

Various studies have attempted to suppress the inflammation that can occur after SCI. Recent studies have demonstrated that intrathecal injection of Resolvin D (RvD) 3 is effective for promoting inflammatory resolution, neuroprotection, functional neurological recovery, and for reducing thermal hyperalgesia after SCI.[25] These include the downregulation of pro-inflammatory cytokines, conversion of the macrophage phenotype into M2, preservation of tight junctions in the blood-spinal cord barrier, promotion of neuroprotection, and prevention of glial/fibrotic scar formation in the acute phase.[26-28]

In a mouse model of brain injury, administration of RvD1 was neuroprotective, attenuated inflammatory responses, and promoted functional recovery. RvD1 exerts its neuroprotective effects via the ALX/FPR2 receptor and its regulated microRNAs which mainly affect neuroinflammation.[29,30] However, the effect of RvD1 on SCI has not yet been studied. RvD1 is known to have anti-inflammatory and neuroprotective effects[29,31,32], and is thought to enhance the viability of PNSC spheroids and enhance regeneration function. In a rat model of spinal cord injury, the combination of RvD1 and PNSC spheroids may have a synergistic effect on the restoration of neurological function in injured rats.

II. MATERIALS AND METHODS

1. 2D cell culture protocol

The peripheral nerves (PNs) used for cell isolation in this study were obtained from common iliac nerve segments harvested from rat donors. The study protocol was reviewed and approved by the Institutional Review Board of Inje University Busan Paik Hospital. After removing the epineurium and surrounding connective tissue using a stereomicroscope, the PN segments were minced into 2- to 3-mm-long pieces using a razor blade. The pieces were then washed with phosphate-buffered saline (PBS, Cytiva, USA) and then suspended in a pre-chilled 0.25% neutral collagen solution. The resulting mixture was transferred into a 100 mm tissue culture dish and incubated in a humidified chamber at 37ºC for 2 hours to establish a neutral collagen hydrogel that would enable 3D encapsulation of the PN pieces. Organ cultures were performed under dynamic conditions in an orbital shaker at 25 rpm for two weeks, with the organ culture medium replenished three times per week. After 14 days of organ culture, the collagen hydrogels containing PN fragments and outgrown PNSCs were incubated with 0.01% collagenase type I. Once the hydrogels had degrade, the migrated/outgrown PNSCs released form the hydrogels were collected, centrifuged, and then suspended in cell culture medium. The obtained PNSCs were seeded in cell culture dishes and proliferated using conventional monolayer – culture conditions. The PNSCs obtained were seeded in cell culture dishes and proliferated using growth culture media composed of DMEM low glucose, supplemented with F12, 10% fetal bovine serum, 1% penicillin/streptomycin, 10 ng/mL IGF (Peprotech, Korea, Seoul, Korea), 10 ng/mL EGF (Peprotech), and 2 ng/mL bFGF (Peprotech). When the 150π culture dish reached 80% confluence, PNSCs were sub-cultured to the next passage and, detached using 0.5% trypsin-EDTA treatment.

2. 3D sphere-like cell culture

When the PNSCs reached 80% confluence, they were collected using 0.5% trypsin-EDTA treatment, and re-suspended in suspension culture media composed of DMEM

supplemented with bovine serum, DMSO, and penicillin/streptomycin. PNSCs grown in 150π cell culture plates were then seeded in a 100π ultra-low attachment cell culture plate (SPL3D^{TM} Cell Floater). Depending on their growth, the PNSCs 3D spheroids were cultured for 24- 72 h. PNSC passages 23- 24 were used throughout the experiments.

3. *In-vitro* and *in-vivo* inflammation

To test the anti-inflammatory effects of RVD1, PNSC spheroids were used. PNSC spheroids cultured in ultra-low culture dishes were seeded in 6-well plates. Twenty spheroids with size of 50 -100 μm were seeded and, incubated for a day at 37^{o}C for stabilization. LPS (0.5 μg/mL; Sigma Aldrich, St. Louis, MO, USA) was administered for 24 h to induce inflammation. Subsequently, RvD1 (Cayman Chemical Company, MI, USA), dissolved in 1X PBS, was used at four different concentrations (0 nM, 1 nM, 10 nM, and 100 nM). PNSC spheroids were incubated at 37^{o}C for 3 d, and the cells were collected for further analysis. RIPA buffer (Thermo Fisher Scientific, MA, USA) supplemented with protease inhibitors (Thermo Fisher Scientific) and phosphatase inhibitors (GenDEPOT, TX, USA) was used to extract protein from cells and tissue. The protein samples were electrophoresed in 10% and 15% SDS-PAGE gels and transferred to PVDF membranes. Transferred membrane were blocked with 5% skim milk for 1.5hr. Membrane was incubated in each primary antibody including NF-κB, p65 (1:1000, Santa Cruz Biotechnology, Inc., TX, USA), Anti-TNF-a (1:1000, abcam), Anti-IL-1 β (1:1000, abcam), and Anti-β-Actin (1:4000, abcam) for overnight in 4^{o}C. Total RNA was extracted from cells and tissues using TRIZOL (Thermo Fisher Scientific). cDNA synthesis was performed using the provided protocol of cDNA synthesis Platinum Master Mix (GenDEPOT). 1μg of RNA was used for cDNA synthesis and 50ng of cDNA and 200nM of primer were used per reaction for qPCR.

4. 3D – Spinal cord injury and *in vivo* transplantation

The grown PNSCs were collected and suspended in PBS for transplantation. Each rat was

injected with 5 x10^5 cells in the form of spheroids with a volume of 10 μL, and 2 μL of 0.1 μg RvD1 dissolved in PBS. For the SCI/RvD1+PNSC group, cells were suspended in the required amount of RvD1 in cell culture media.

In this study, SD rats (200 ± 20g; OrientBio, Kyungki-do, Korea) were kept in an animal facility with the permission of the Association for Assessment and Accreditation of Laboratory Animal Care (AAALAC). All animals were anesthetized with ketamine (100 mg/kg; Yuhan, Seoul, Korea), rompun (10 mg/kg; Bayer Korea, Seoul, Korea) and isopropanol (Hanapharm, Kyungki-do, Korea). The spinal cords were exposed to laminectomy in all animals. The exposed spinal cords the rats were compressed at the T9 level for 30 s using self-closing forceps. After surgery, SCI rats were randomly divided into five groups: sham (n = 10), SCI/PBS (n = 20), SCI/PNSC spheroid (n = 30), SCI/RvD1 (n = 30), and SCI/RvD1+PNSC (n = 30). The transplantation of PBS, PNSC spheroids, RvD1, and PNSC+RvD1 were accomplished one week after injury using a 27-gauge Hamilton syringe. The injection rate was maintained with 1μL/minute and the needle was kept in place for an additional 2 min after each injection to prevent leakage. After all surgical procedures, cefazolin (25 mg/kg; Chong Kun Dang, Seoul, Korea) was administered for 5 d.

5. Open field testing

Post-injury motor behavior to evaluate functional recovery was assessed using the Basso, Beattie and Bresnahan (BBB) scale for 8 weeks. The BBB score was used to determine the rats' movements and quantify their motor recovery. BBB scores are composed of three-joint movement, weighted stepping, gait coordination, and tail movement, with a scoring range of 0 to 21. A score of 0 indicated complete paralysis whereas a score of 21 indicated normal movement.[24] BBB scores were evaluated by two different evaluators and averaged.

6. Ladder rung test

To evaluate the walking task, especially limb placement and coordination, rats were

placed in a metal rung with clear side wall ladder to walk back and forths twice a week. Evaluation was observed by two different evaluators, measuring correct placement steps out of the total steps, and converting scores into percentages.

7. Mechanical allodynia measurement

Mechanical allodynia was performed 3–4 weeks after spinal cord injury when rats were able to position their hind paws facing down. The rats were placed in an acrylic sectioned place on a wire-net-like platform. Before stimulation, all rats were allowed to stay for at least 10 min to familiarize themselves the environment. Stimuli were applied to hind paws using a Dynamic Plantar Aesthesiometer (Ugo Basile, Comerio, Varese, Italy). A 0.5 mm steel rod was used to gradually push the paws with increasing force, starting from 0 to 50 g. The force was gradually increased until the hind paw of the rat was removed from the rod. Once they removed their paws, the machine automatically recorded the duration along with the force.

8. Tissue sample preparation

Seven weeks after cell/drug injection and 3 weeks after injection for the cell viability group, the rats were perfused with saline and, fixed with 4% paraformaldehyde (PFA; Merck, Darmstadt, Germany). The spinal cords, especially regions around the T9 level, were isolated and, fixed in 4% PFA for an additional 24 h at 4^{o}C. Then, all samples were cryoprotected for one week in 30% sucrose at 4^{o}C. The cryoprotected tissues were placed in optimal cutting temperature compound (Sakura Finetek, Torrance, CA, USA) and placed in a deep freezer at -80^{o}C. I cut tissue samples of ±1mm proximal or distal from the injury site at a thickness of 20μm for further histological analysis. After staining the tissue with EC staining, I measured the total cord size using Image J with the section center tissue as the reference. I also measured cavity area, myelinated area, and gray matter area for each tissue.

9. Immunohistochemistry

Sectioned tissues were dried at room temperature (RT) for 30 min, washed three times with ice-cold 0.3% Tween 20 (Sigma-Aldrich) in PBS, and blocked with 10% normal donkey serum in PBS and 0.3% Triton X-100 (Sigma-Aldrich) for 1 h at RT. The tissue samples were then incubated overnight at 4°C with primary antibodies diluted in blocking buffer. The primary antibodies used were goat anti-GFAP (1:800; Ab53554; Abcam), mouse anti-CNPase (1:200; Ab6319; Abcam), and chicken anti-MAP2 (1:800; Ab5392; Abcam). After incubation, tissue samples were washed three times with ice-cold 0.3% Tween 20 (Sigma-Aldrich) and then treated with host specific secondary antibodies including DyLight™ 405 Donkey Anti-Mouse IgG (1:150, Jackson ImmunoResearch), Cy™3 Donkey Anti-Chicken IgY (IgG) (H+L) (1:400, Jackson ImmunoResearch), and Alex Fluor® 647 F(ab')$_2$ Fragment Donkey Anti-Goat IgG (H+L) (1:150, Jackson ImmunoResearch) for 1 h at RT. Sample tissues were then washed three times with 0.3% Tween 20 (Sigma-Aldrich) and, cover-slipped using mounting solution without DAPI (Vector Laboratories, Inc. Burlingame, CA, USA). PNSCs were initially tagged with GFP, and DAPI mounting solution was used for mounting for the cell viability group. Fluorescence images were obtained using a confocal laser microscope (LSM700; Carl Zeiss, Oberkochen, Germany).

10. Eriochrome cyanine - cresyl violet staining

Sectioned tissues were placed at RT for several hours to air dry. Tissue slides were then placed on slides in fresh acetone (Daejung Chemicals & Metals CO., Ltd., Gyenggi-do, Korea) for 5 min and air dried for 15 min. Tissues were then placed an eriochrome cyanine solution (MERCK, Kenilworth, NJ, USA) for 30 min at RT and rinsed with distilled water. Tissues were placed in 5% iron aluminum (MERCK) for 10 min to differentiate and visualize the gray matter. The tissues were then washed with distilled water and borax-ferricyanide solution (MERCK) was used for 3.5 minutes for complete differentiation. The tissues were washed with distilled water and placed in cresyl violet (Sigma-Aldrich) for 1

min, then differentiated using 95% ethanol with a few drops of 10% acetic acid (Sigma-Aldrich) solution for 5 min and dehydrated using graded ethanol solutions of 95% and 100%. The tissues were cleaned with xylene solution (Daejung Chemicals & Metals Co., Ltd) and a cover slip was placed using a permanent mounting medium (Fisher Scientific, NH, USA). A light microscope (IX71; Olympus, Tokyo, Japan) was used to obtain the stained images.

11. Statistical analysis

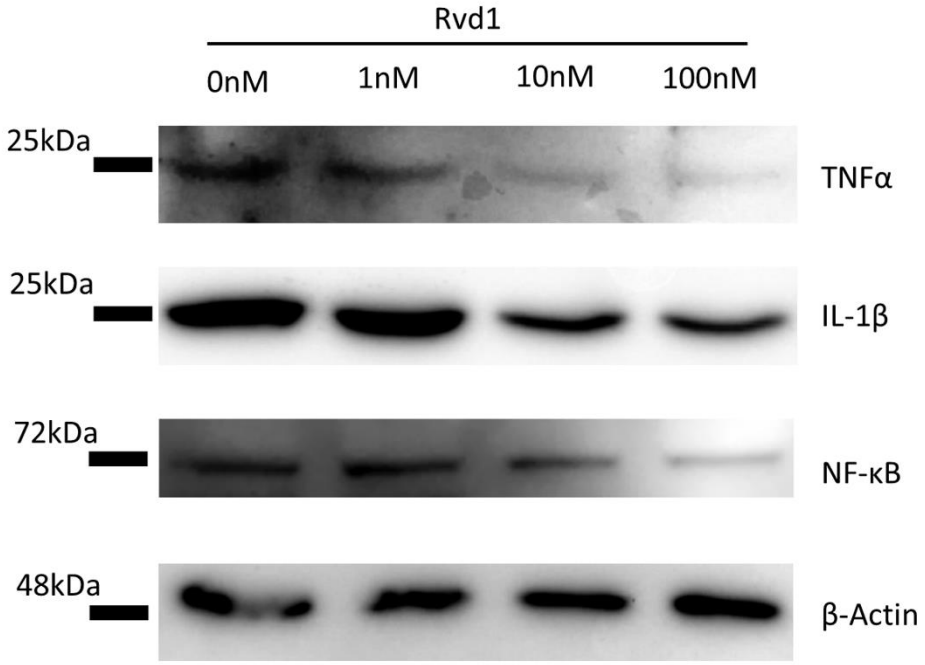
All data are expressed as mean ± standard error of the mean (SEM). Statistical differences between groups were measured using one-way or two-way analysis of variance (ANOVA) for multiple comparisons. One-way analysis of variance was performed using Tukey's post hoc test, and two-way analysis of variance was performed using a mixed-effects model. The p-values are indicated as follows: * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$ and **** $p < 0.0001$. All statistical analyses were performed using GraphPad PRISM 8.3 (GraphPad Software Inc., San Diego, CA, USA).

III. RESULTS

1. Anti-inflammatory effect of RvD1 in an *in vitro* study

Following the initiation of spheroid seeding under *in vitro* conditions, inflammation was artificially induced by lipopolysaccharide (LPS) stimulation, which led to the identification of pro-inflammatory cytokines. To normalize the analysis, β-actin was used as the housekeeping gene. In the western blot analysis, RvD1 was associated with a relative decrease in pro-inflammatory cytokines such as TNF-α, IL-1β, and nuclear factor kappa-light-chain-enhancer of activated B cells (NF-κB) at concentrations of 0 nM, 1 nM, 10 nM, and 100 nM compared to β-actin **(Fig. 1A)**. To measure the expression levels of TNF-α, IL-1β, and NF-κB, quantitative reverse transcription polymerase chain reaction (qRT-PCR) was performed. An inverse correlation was observed between the expression levels of all pro-inflammatory cytokines and the increasing concentration of RvD1 **(Fig. 1B)**. Furthermore, the expression levels of anti-inflammatory neurotrophic factors, specifically interleukin-10 (IL-10) and transforming growth factor beta (TGF-β), which are secreted by RvD1, were also assessed using qRT-PCR. The presence of these anti-inflammatory cytokines was confirmed in the RvD1-treated cells, with a significant upregulation in expression levels observed at 10 nM **(Fig. 1C)**. Overall, our data support the idea that RvD1 exhibits an anti-inflammatory effect by reducing the secretion of pro-inflammatory cytokines in an *in vitro* setting.

(A)



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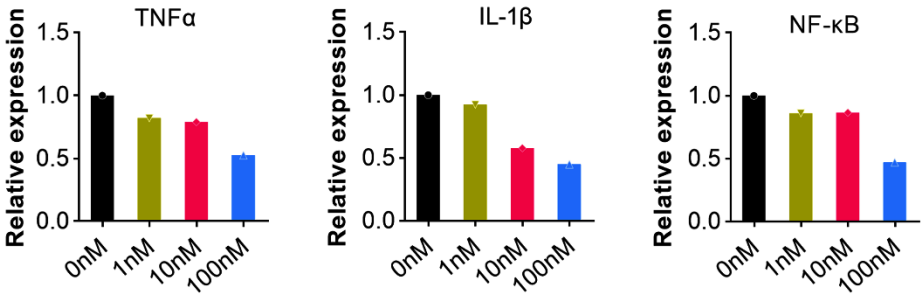
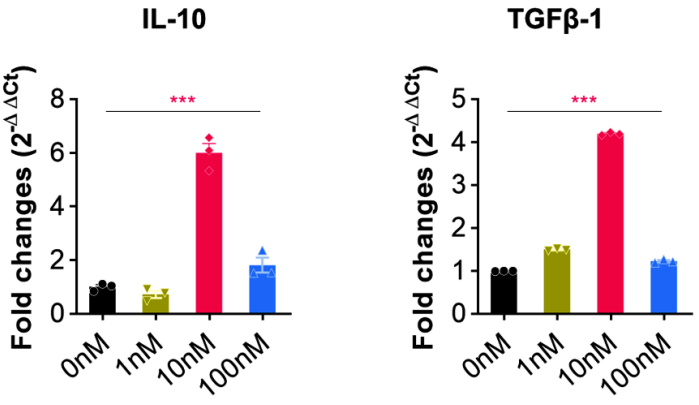


(C)



(D)

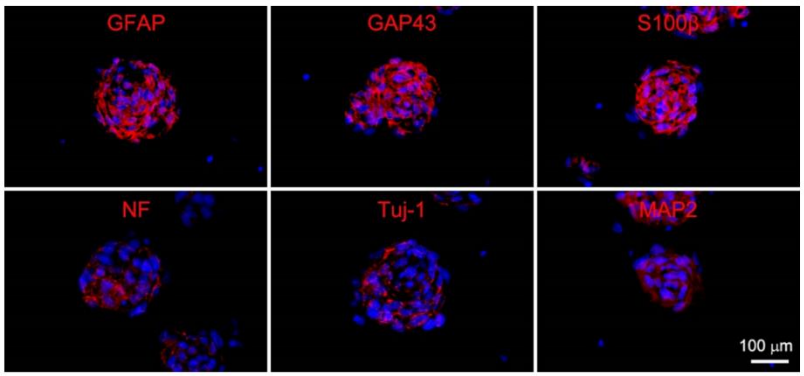


Figure 1. RvD1 demonstrates substantial *in vitro* anti-inflammatory effects

(A) Western blot analysis confirmed the expression of pro-inflammatory cytokines (TNF-α, IL-1β, and NF-κB) in response to RvD1 treatment at concentrations of 0 nM, 1 nM, 10 nM, and 100 nM (n=2). **(B)** The expression of pro-inflammatory cytokines TNF-α, IL-1β, and NF-κB, as determined by qRT-PCR, in response to RvD1 treatment at concentrations of 0 nM, 1 nM, 10 nM, and 100 nM. **(C)** The expression of anti-inflammatory cytokines IL-10 and TGF-β, as assessed by qRT-PCR, following RvD1 treatment at concentrations of 0 nM, 1 nM, 10 nM, and 100 nM. **(D)** Spheroid PNSCs expressed markers for committed glial cells (GFAP, S100 β and GAP43) and neuronal cells (neurofilament; NF, Tuj-1, and MAP2). Blue means DAPI. * $p < 0.05$, ** $p < 0.01$, and *** $p < 0.001$.

2. Functional recovery and tissue regeneration in combination therapy

To investigate the therapeutic potential of PNSC spheroids and RvD1, a compressive SCI rat model was employed. One week after injury, PNSC spheroids were directly administered as single-cell spheroids. These PNSC spheroids, which exhibit stability, immune expression properties similar to NCSCs, and the ability to differentiate into neuronal and mesenchymal cells, continuously secrete various neurotrophic and anti-inflammatory factors. We tested their potency for differentiation into neuroglial cells by employing a 3D spheroid culture system that enables the activation of the endogenous canonical Wnt pathway signaling and the intrinsic differentiation cascades. PNSCs, when seeded on culture dishes that prevent cell attachment, aggregated with each other and assembled spontaneously into multicellular spheroids within 1 d of culture. Under the spheroid culture conditions, PNSC spheroids expressed glial cell-related markers, including GFAP, GAP43, and S100b, without any requirement for differentiation inducers. At 7 d of culture, more than 80% of the PNSCs expressed GFAP (83.7 ± 6.5%), GAP43 (89.5% ± 8.2%), and S100b (84.9 ± 7.3%), indicative of the PNSCs showing an innate propensity to differentiate in vitro into glial cell lineage without a requirement for differentiation inducers. By inducing cells with neurogenic differentiation inducers, such as NGF, BDNF, and cAMP, PNSCs expressed neural cell markers, including Tuj1+ (15.4 ± 6.8%), MAP2 (1.1 ± 2.7%), and NF200 (26.3 ± 3.4%), at 7 d of culture (**Fig. 1D**), indicating that PNSCs have the ability to differentiate to neural cells but preferentially differentiate into glial cells, thus demonstrating therapeutic potential in SCI models.

Open field tests were performed weekly, and motor function was assessed at the 8-week mark using the Basso, Beattie, and Bresnahan (BBB) scale. Following SCI, the SCI/phosphate-buffered saline (PBS) group showed a significant decline in motor function. Although the SCI/RvD1 group exhibited minor behavioral improvement, the change was not significant. In contrast, the SCI/PNSC group demonstrated a gradual and significant increase in the BBB score, with the most notable enhancement observed in the SCI/RvD1+PNSC combination group. At 8 weeks post-injury, the average BBB score for

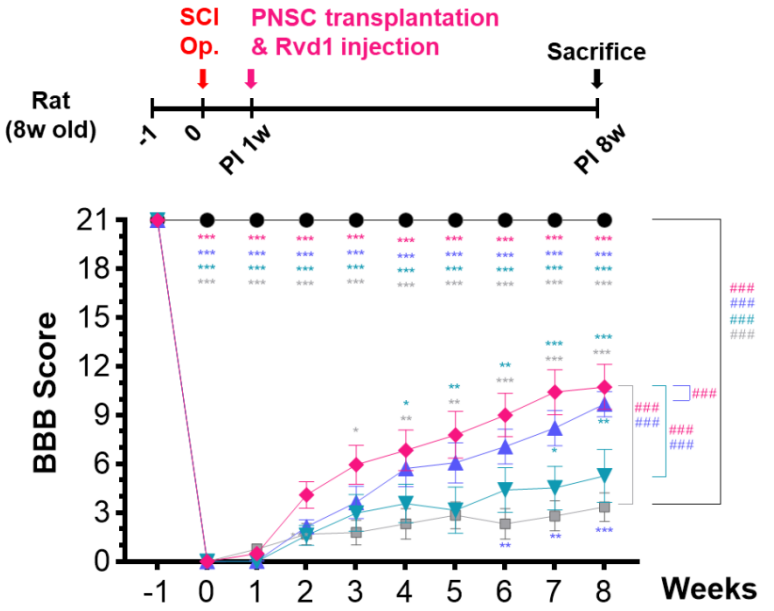
the SCI/RvD1+PNSC group was 10.75 ± 1.38, indicative of hind limb weight-supported walking **(Fig. 2A).** These results suggest that RvD1 may enhance the effects of PNSCs.

A ladder rung test was utilized to qualitatively assess locomotion and limb coordination. Video recordings were carefully examined on a frame-by-frame basis. Measurements were taken weekly over an 8-week period following SCI for all groups. Two independent observers determined the ratio of accurately placed steps to total steps, converting the score to a percentage. In the SCI/PBS group, no functional recovery was observed for the 8 weeks after SCI. However, ladder rung test scores gradually improved in the SCI/RvD1 and SCI/PNSC groups. The greatest increase was observed in the SCI/RvD1+PNSC group, with an average score of 52.5 ± 2.5 at 8 weeks post-injury **(Fig. 2B)**. The best outcomes in locomotion and limb coordination assessment were confirmed in the SCI/RvD1+PNSC combination group.

To investigate the relationship between functional recovery and tissue regeneration, spinal cord samples from each group were stained with cresyl violet and eriochrome cyanine (EC) at 4 and 8 weeks post-injury. The sham group showed no evidence of spinal cord degeneration. In contrast, substantial cavitation and tissue contraction were observed in the SCI/PBS group. Tissue shrinkage was also apparent in the spinal cords of the SCI/PNSC and SCI/RvD1 groups, albeit with a reduced, yet still considerable, cavity presence **(Fig. 2C)**. Conversely, the SCI/RvD1+PNSC group exhibited an increase in the total cord, myelinated, and gray matter areas, along with a significant reduction in the cavity area compared to other groups **(Fig. 2D)**. This indicates the occurrence of remyelination and the inhibition of demyelination near the injury site. As a result, spinal cord tissue regeneration in the combination group was similar to that observed in the sham group. Additionally, a well-connected tissue structure was confirmed around the injury site.

These observations correspond closely with the BBB scores, indicating that PNSC spheroids, when combined with RvD1, can promote spinal cord regeneration and contribute to functional recovery.

(A)



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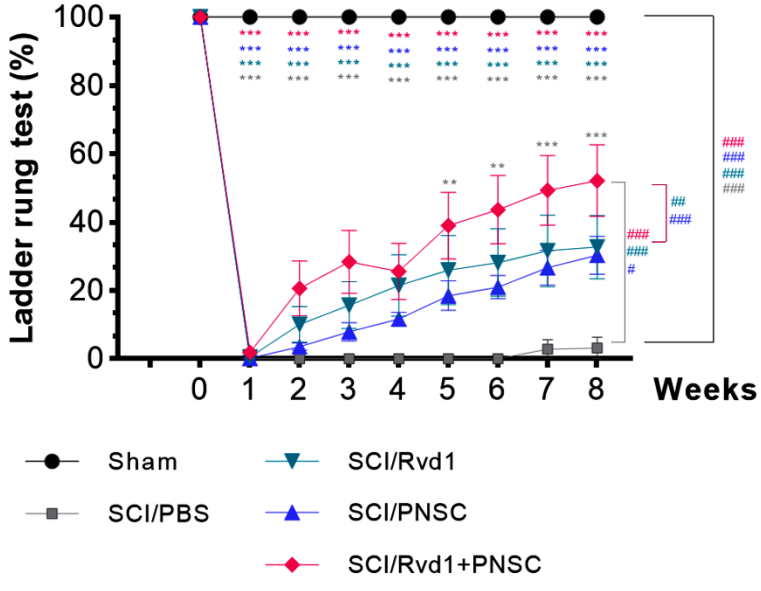


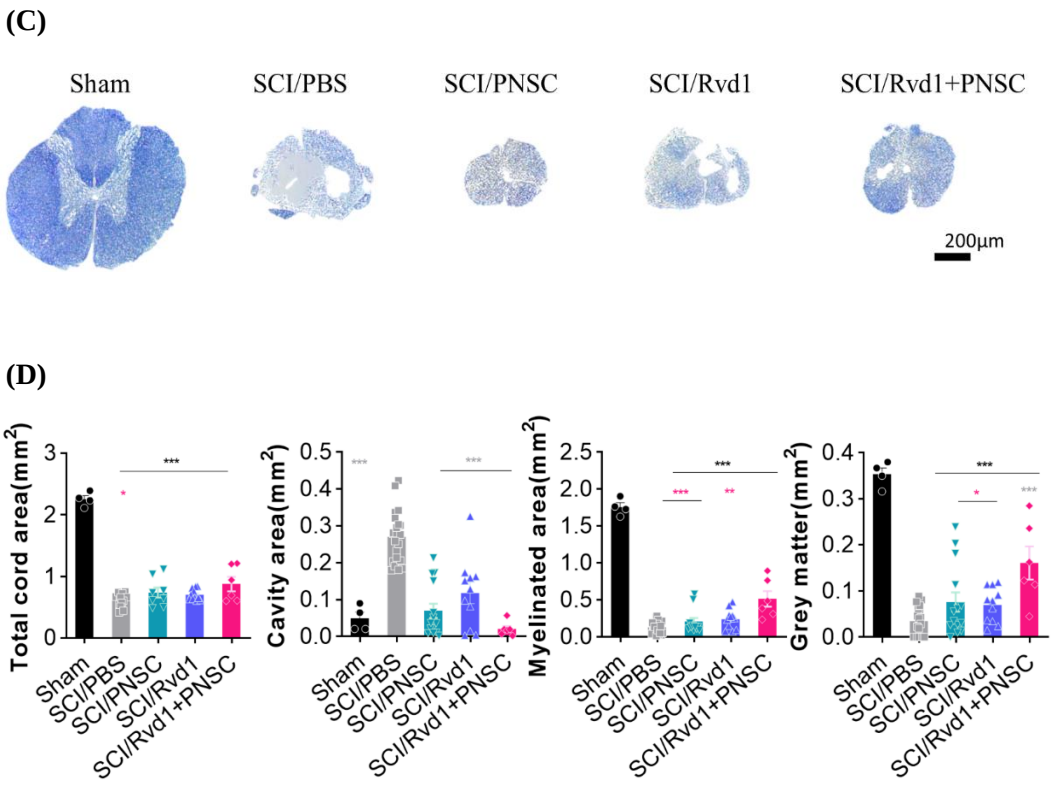


Figure 2. Functional recovery and tissue regeneration in combination therapy

(A) BBB scores post-SCI for all groups (sham, SCI/PBS, SCI/RvD1, SCI/PNSC, SCI/RvD1+PNSC), from 1 week pre-injury to 8 weeks post-injury. **(B)** Ladder rung test results post-SCI for all groups from the day of injury to 8 weeks later. **(C)** Eriochrome cyanine-cresyl violet staining of spinal cord cross-sections for myelin in all groups (sham, n = 3; SCI/PBS, n = 4; SCI/ RvD1, n = 6; SCI/PNSC, n = 6; and SCI/RvD1+PNSC, n = 6) for the assessment of tissue regeneration at 4 and 8 weeks post-SCI. **(D)** Evaluation of total cord area, cavity area, myelinated area, and gray matter area from eriochrome cyanine staining across all groups. All data were processed using two-way analysis of variance with the Tukey multiple comparisons test. The *p*-values are indicated as follows: * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$, and **** $p < 0.0001$.

3. Alleviation of mechanical allodynia through combined therapy

Mechanical allodynia was assessed using the dynamic plantar test. Mechanical allodynia was measured beginning 3–4 weeks after SCI when the hind paws were facing down. The hind paw of the sham manipulation group was retracted using an average force of 33.34 g. Three weeks after injury, the animals in all groups were hypersensitive to mechanical stimuli. Eight weeks after injury, the hind paws were removed at 12.4 g, 11.6 g, and 15.1 g, as they were still hypersensitive in the SCI/PBS, SCI/RvD1, and SCI/PNSC groups, respectively. In contrast, the SCI/RvD1+PNSC group withdrew its paw at 17.46 g and 8 weeks after injury, indicating lower sensitivity to stimuli than the other groups. Overall, the SCI/RvD1+PNSC group showed near-sham mechanical allodynia improvement eight weeks after SCI **(Fig. 3)**.

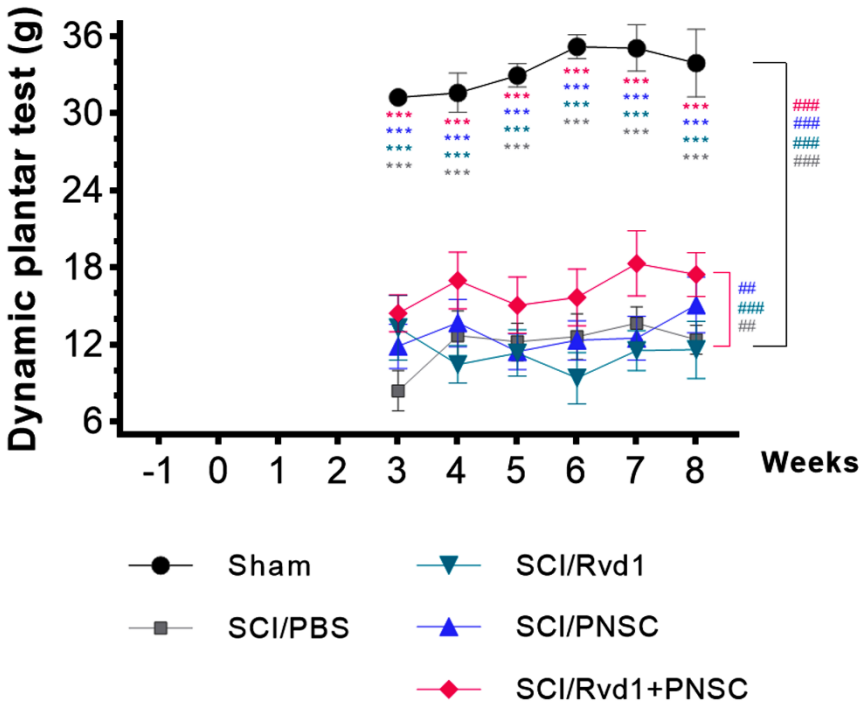


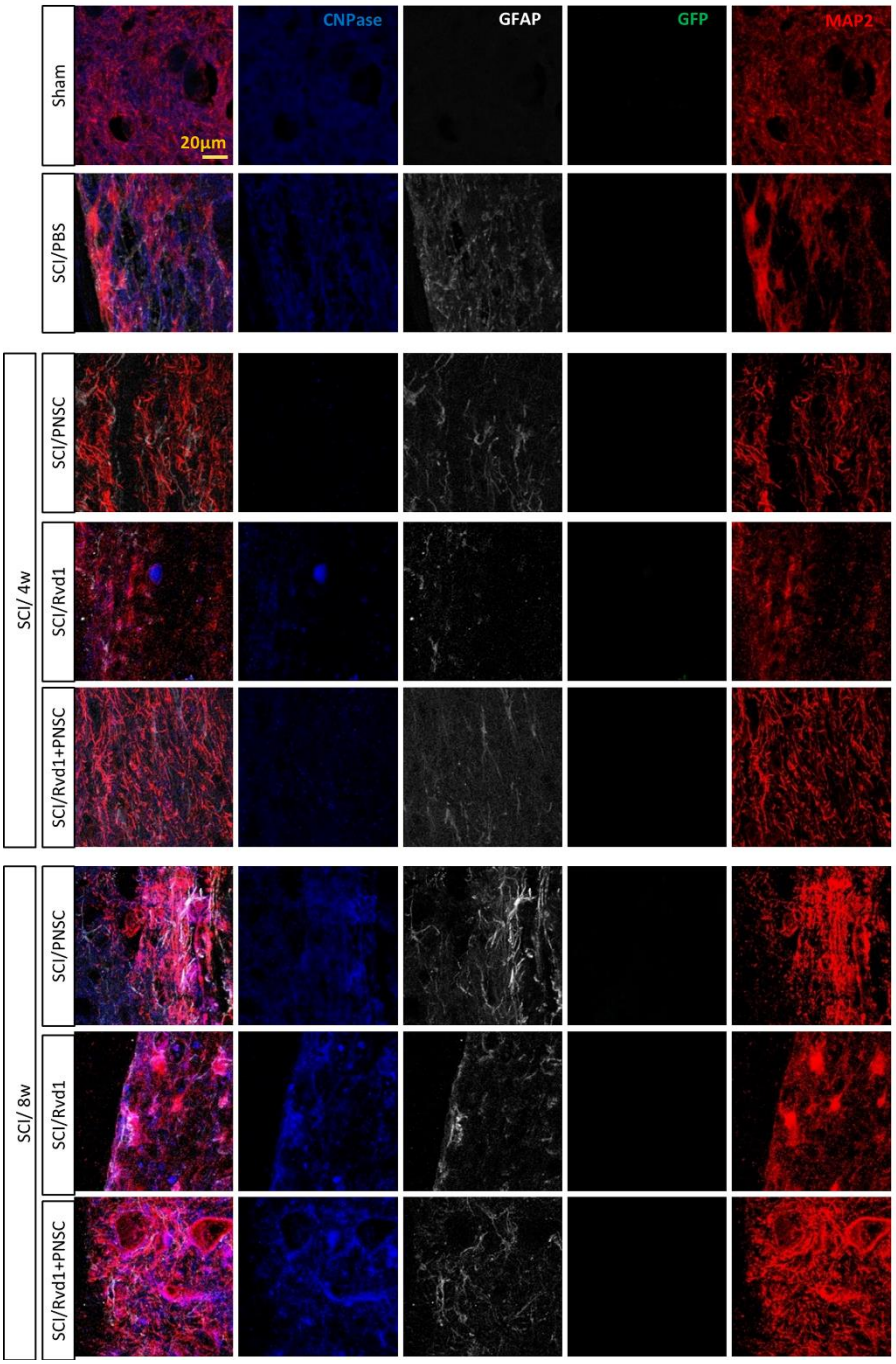
Figure 3. Pain reduction in combination therapy

Mechanical allodynia was evaluated using the dynamic plantar test. Testing began 3 weeks after SCI and continued until the 8-week mark. All data were statistically processed using two-way analysis of variance coupled with the Tukey multiple comparisons test. Multiple comparisons results expressed by *** $p < 0.001$. And analysis by linear mixed effects model is expressed to $^{\#\#}$ $p < 0.01$, and $^{\#\#\#}$ $p < 0.001$.

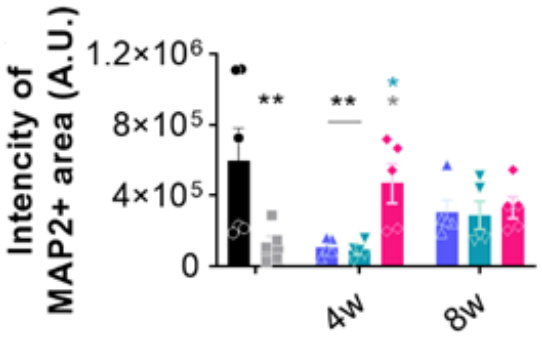
4. Outcomes of immunohistochemical analysis

In our previous report, we confirmed that PNSC spheroids express NS lineage markers including nestin, p75NTR and CD105. In addition, NC-specific transcription factors (such as Sox2, Sox9 and Sox10) were also retained in PNSCs, and markers indicating commitment to the Schwann cell lineage (such as GFAP, GAP43, and S100b) were also expressed in PNSC spheroids. In this study, I investigated neurons, oligodendrocytes, and astrocytes using antibodies against MAP2, CNPase, and GFAP at 4 and 8 weeks after injury **(Fig. 4A)**. No differentiated PNSC group was observed, as the cells were not viable for 7 weeks after transplantation. Although differentiated cells were not observed, the expression of neuronal and glial cell markers was significantly altered. MAP2 is a mature neuronal marker that is characteristically identified in neuronal cells at a relatively early stage. In this study, MAP2 was expressed at the highest level in the combination group, indicating that neuronal regeneration was the most effective **(Fig. 4B)**. Although the expression level of MAP2 at 8 weeks was slightly lower than that at 4 weeks, it is possible that other differences may be observed when cells at later stages are analyzed. CNPase, an oligodendrocyte marker, did not show any difference from the SCI/PBS group at 4 weeks, but at 8 weeks, it showed a significant increase in expression level in the SCI/RvD1 group, SCI/PNSC group, and SCI/RvD1+PNSC group **(Fig. 4C)**. This suggests that remyelination increased more in 3 groups than in the control group. GFAP, an astrocyte marker, is mainly expressed in reactive astrocyte, but not in naive astrocytes. Glial scars were most prominent in the PBS group, and the expression of GFAP was the highest, which was confirmed to interfere with neuronal regeneration. This was supported by the observation of the lowest expression level of MAP2 in the PBS group. In the RvD1 group, the expression of GFAP was the lowest, but the expression of MAP2 and CNPase were also low, indicating that although glial scar formation was inhibited, it was insufficient for neuronal regeneration. In the combination group, the expression level of GFAP was relatively low, while the expression of MAP2 and CNPase were highest, indicating that the formation of glial scars was suppressed and the neuronal regeneration effect was the most prominent **(Fig. 4D)**.

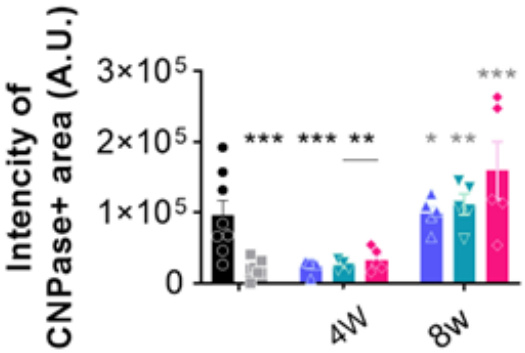
(A)



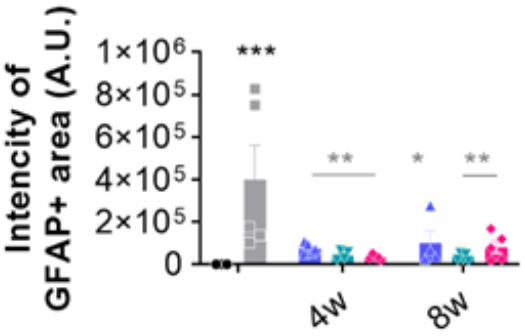
(B)



(C)



(D)



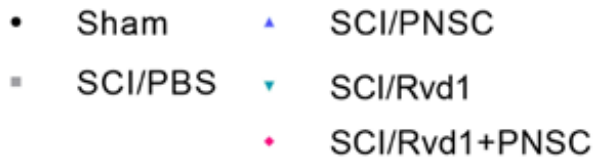


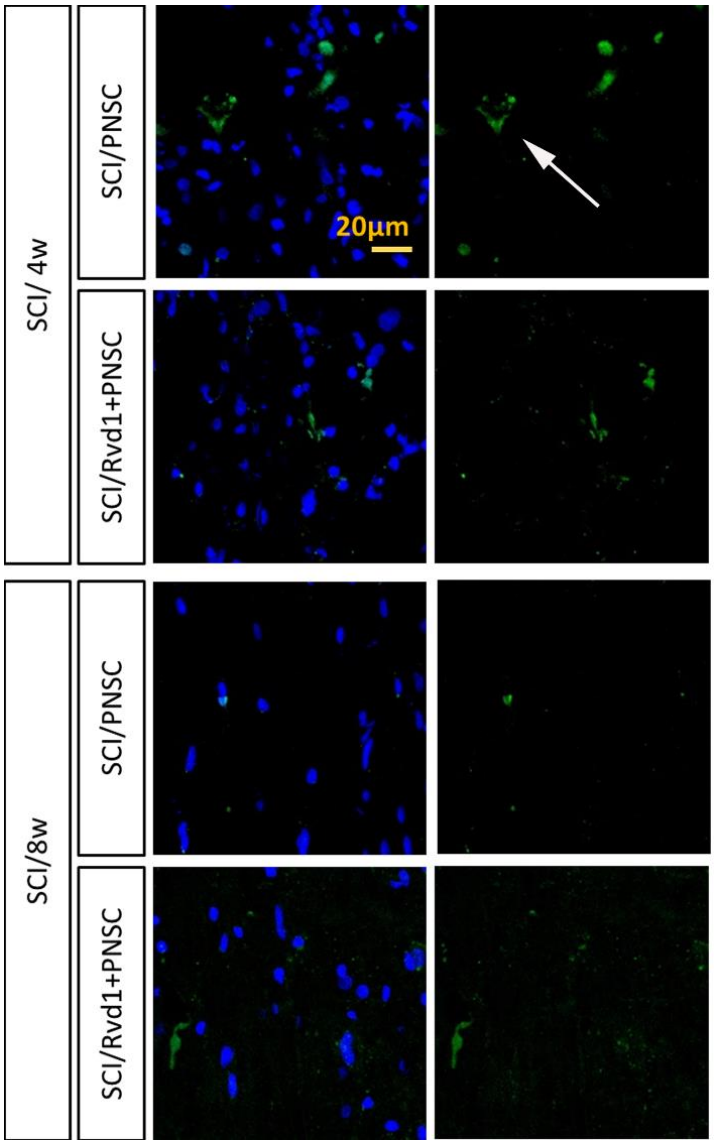
Figure 4. Outcomes of *in vivo* immunohistochemical analysis

(A) Representative images depict staining for CNPase (blue), GFAP (gray), GFP (green), and MAP2 (red) at 4 and 8 weeks following SCI. **(B)** Aggregate MAP2 intensity across all study groups (sham, n = 5; SCI/PBS, n = 10; SCI/RvD1, n = 20; SCI/PNSC, n = 20; and SCI/RvD1+PNSC, n = 20). Analysis was conducted using ordinary one-way ANOVA with the Tukey multiple comparisons test. **(C)** Overall intensity of CNPase staining across all groups (sham, n = 5; SCI/PBS, n = 10; SCI/RvD1, n = 20; SCI/PNSC, n = 20; and SCI/RvD1+PNSC, n = 20). **(D)** Total intensity of GFAP staining across all groups (sham, n = 5; SCI/PBS, n = 10; SCI/RvD1, n = 20; SCI/PNSC, n = 20; and SCI/RvD1+PNSC, n = 20). Analysis was conducted using ordinary one-way ANOVA with the Tukey multiple comparisons test. All data are presented as mean ± standard error of the mean; * $p < 0.05$, ** $p < 0.01$, and *** $p < 0.001$.

5. Assessment of *in vivo* cell survival and expression of neurotrophic factor following transplantation

I assessed cell survival at 4 and 8 weeks after transplantation. The transplanted PNSC spheroids were identified using GFP because they were originally isolated from murine stem cells. Only a small number of cells survived in the SCI/PNSC and SCI/RvD1+PNSC groups 4 weeks after transplantation. However, at 8 weeks after transplantation, few GFP-positive cells were observed in all the groups **(Fig. 5A and 5B)**. This finding suggests that although the cells within the SCI/PNSC and SCI/RvD1+PNSC groups may not demonstrate long-term survival, they continue to impact the spinal environment through paracrine effects, ultimately contributing to improved functional behavior.

(A)



(B)

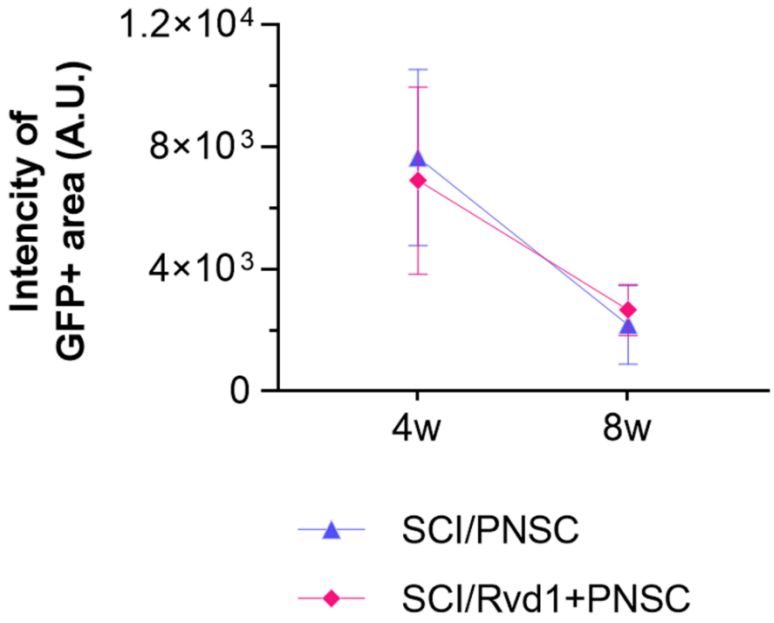
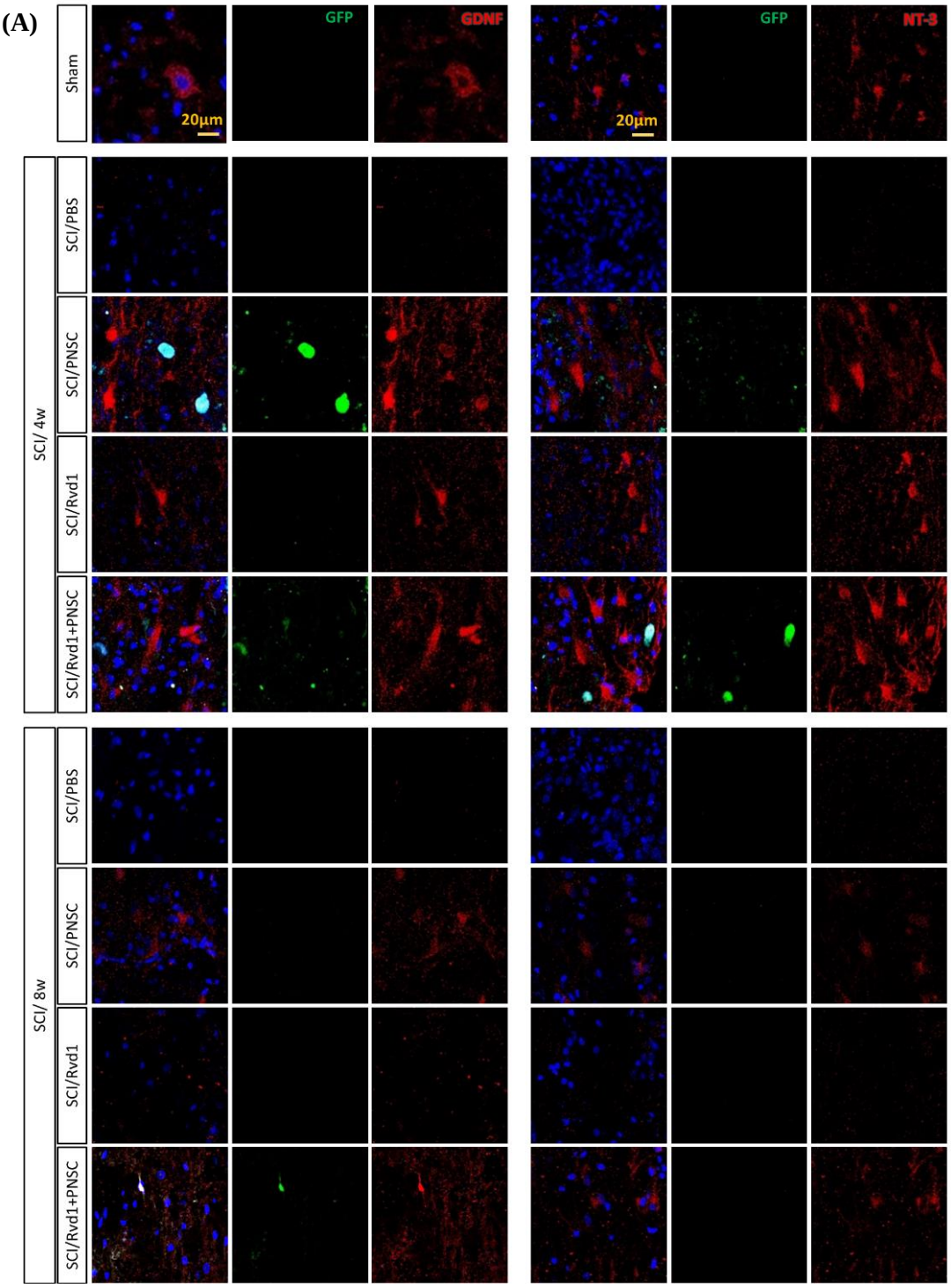


Figure 5. Viability of transplanted cells in an *in vivo* study

Viability of transplanted peripheral nerve-derived stem cells (PNSCs) and RvD1+PNSC. **(A)** Representative confocal images showing GFP (white arrow) and DAPI (blue) (sham, n = 5; SCI/PBS, n = 10; SCI/RvD1, n = 20; SCI/PNSC, n = 20; and SCI/RvD1+PNSC, n = 20) staining four weeks, and eight weeks after injury. **(B)** Total intensity of GFP+ cells per mm^2 is shown for all groups. Data were analyzed using one-way ANOVA followed by the Tukey post-hoc test for multiple comparisons.

6. The RvD1 extend the expression of GDNF and NT-3 after SCI

Furthermore, our previous report provided evidence of the expression of various neurotrophic factors, including brain-derived neurotrophic factor (BDNF), glial-derived neurotrophic factor (GDNF), insulin-like growth factor (IGF), IL-6, nerve growth factors (NGF), and neurotrophin-3 (NT-3), in PNSCs. Notably, we observed an increased expression of GDNF and NT-3 in spheroid forms of PNSCs, suggesting that PNSC spheroids have a prolonged and heightened influence on their expression, particularly in the context of SCI. This current study aims to verify the impact of Rvd1 on the expression of GDNF and NT-3 by PNSC spheroids. Our results indicate a significant increase in the expression of GDNF and NT-3 in all groups, including PNSC spheroids, Rvd1, and Rvd1 combined with PNSC spheroid transplants, at 3 weeks post-transplantation (4 weeks after injury) **(Fig. 6A-6D)**. Notably, the group receiving Rvd1 and PNSC co-transplantation demonstrated the most substantial increase in GDNF expression compared to other groups. Furthermore, our previous study has already demonstrated the ability of PNSC spheroids to maintain the secretion of neurotrophic factors for a more prolonged period compared to single cells. Confirming these findings, our current study demonstrates that Rvd1 contributes to an extended secretion of GDNF and NT-3 compared to the group transplanted with PNSC spheroids alone, with notable emphasis on the sustained effect on NT-3 at 8 weeks after injury.



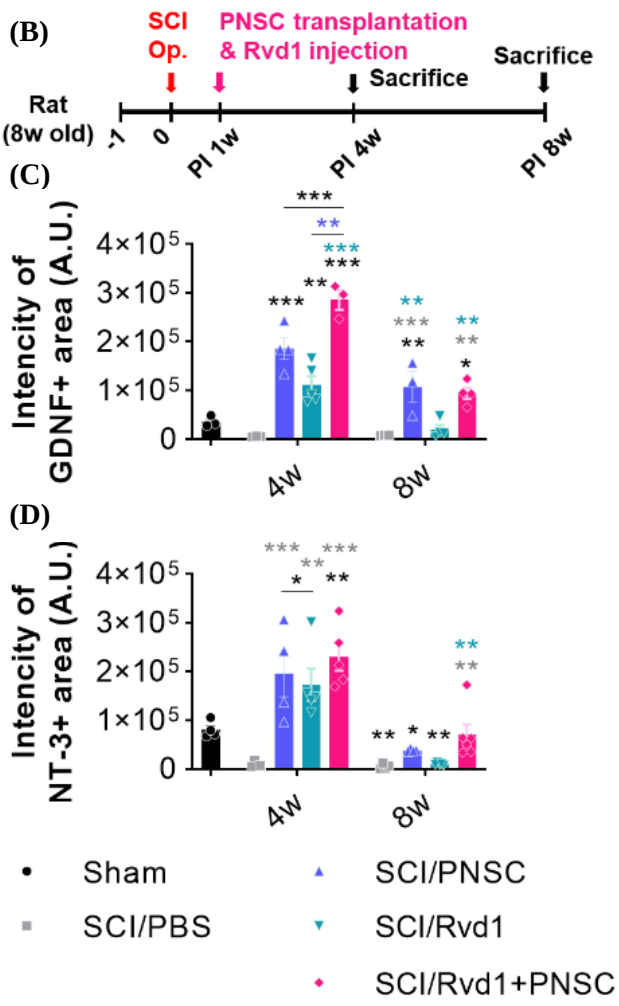


Figure 6. The RvD1 extend the expression of GDNF and NT-3 after SCI

(A) Representative confocal imaging, with blue indicating DAPI, green indicating GFP, and red indicating either GDNF or NT-3. **(B)** Schematic diagram of experiments. **(C)** Total intensity of GDNF. **(D)** Total intensity of NT-3. Data were analyzed using one-way ANOVA. Multiple comparisons results expressed by * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$, and **** $p < 0.0001$.

7. Anti-inflammatory effect of RvD1 in an *in vivo* study

Following spheroid implantation, five groups (sham, SCI, SCI/PNSC, SCI/RvD1, and SCI/RvD1+PNSC) were assessed for inflammatory markers using western blotting. Consistent with our *in vitro* findings, we observed a significant increase in pro-inflammatory cytokines (TNF-a, IL-1β, and NF-κB) after SCI. Furthermore, suppressing the secretion of pro-inflammatory cytokines proved difficult with PNSC transplantation alone. RvD1, which has anti-inflammatory properties, showed some effectiveness in reducing the expression of pro-inflammatory markers, as well as the recruitment of the CD68 macrophage marker and MCP1-macrophage marker. However, the most potent effect was seen with the combination of RvD1 and PNSC, which also led to a decrease in overall macrophage recruitment **(Fig. 7A and 7B)**.

The expression of the anti-inflammatory cytokine TGF-β and pro-inflammatory cytokines NF-κB, IL-1β, and TNF-a across all groups was analyzed using qRT-PCR. A significantly higher level of TGF-β was observed in the SCI/RvD1+PNSC group, while no differences were detected among the other groups. The expression of pro-inflammatory cytokines (NF-κB, IL-1β, STAT3 and TNF-a) was considerably higher in the SCI/PBS and sham groups compared to the SCI/PNSC, SCI/RvD1, and SCI/RvD1+PNSC groups. However, no statistically significant differences were found among these three groups **(Fig. 7C)**.

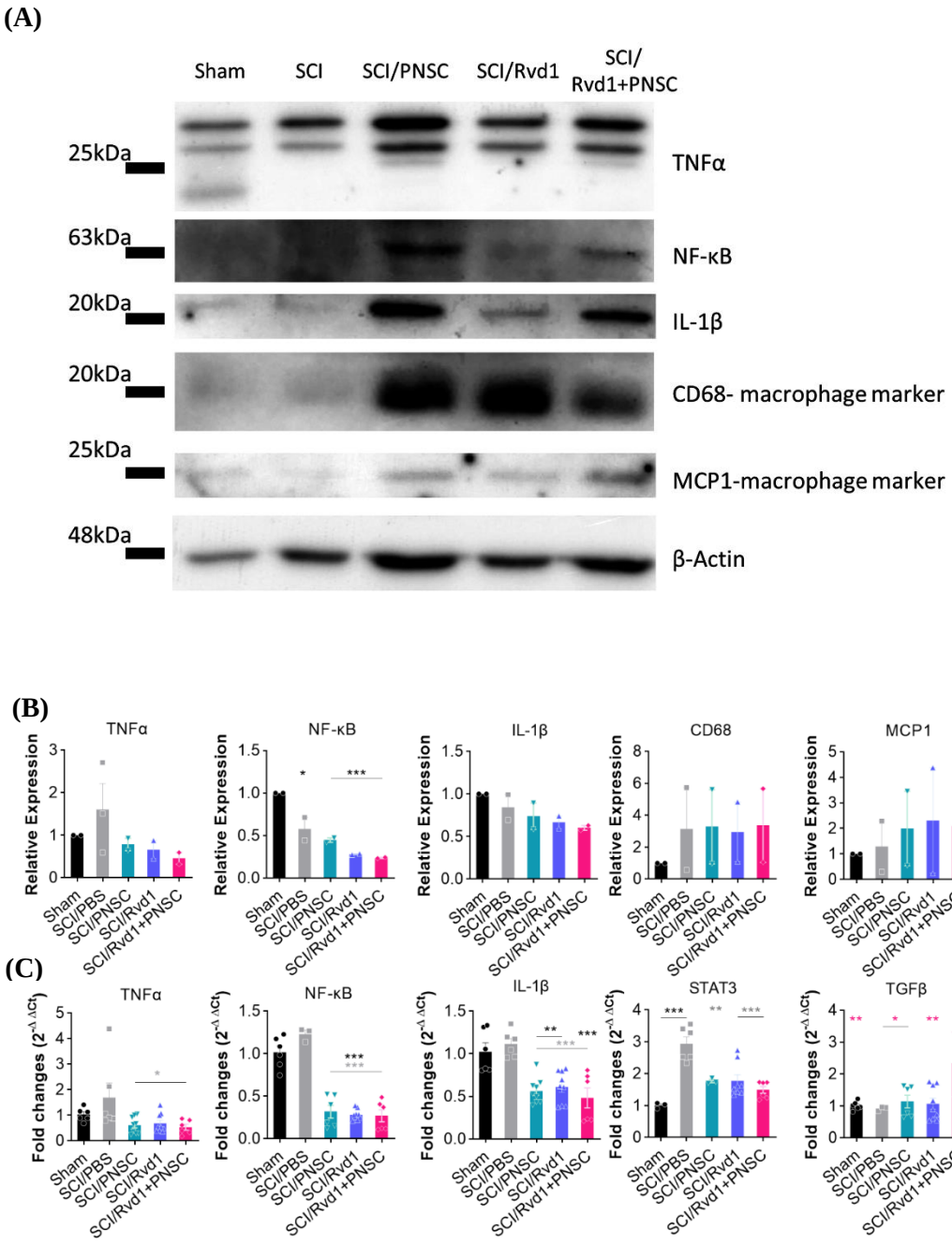


Figure 7. Demonstration of RvD1's potent anti-inflammatory action *in vivo*

(A) The production of pro-inflammatory cytokines (TNF-a, NF-κβ, IL-1β and macrophage markers CD68 and MCP1) was analyzed in all groups (sham, n = 2; SCI/PBS, n = 2; SCI/RvD1, n = 2; SCI/PNSC, n = 2; and SCI/RvD1+PNSC, n = 2) using western blotting. **(B)** Quantitative analysis of western blot. All data is normalized with sham group. **(C)** The SCI/RvD1+PNSC group displayed the highest expression of the anti-inflammatory cytokine TGF-β, as determined by qRT-PCR. In contrast, a significantly greater expression of pro-inflammatory cytokines (NF-κB, IL-1β, STAT3 and TNF-α) was observed in the SCI/PBS group compared to the other three groups. Data were analyzed using one-way ANOVA. Multiple comparisons results expressed by * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$, and **** $p < 0.0001$.

IV. DISCUSSION

Cells transplanted into a barren microenvironment (such as excessive inflammatory reactions, free radicals, and hypoxia induced after spinal cord injury) require the development of cell therapy or cell transplantation technology that can improve the survival rate and functionality.[19,33-37] To secure the original technology for PNSC separation and culture, and a culture system in which NCSC can voluntarily form 3D microspheres in a suspension culture environment, we developed a tissue homeostasis-mimicking 3D organ culture system from the peripheral nerves of brain -death and patients.[24] SCI regeneration was achieved by the secretion of factors including BDNF, GDNF, IGF, IL-6, NGF, and NT-3.

I hypothesized that the combination of RvD1 and PNSC spheroids promotes myelin regeneration and functional recovery in SCI by regulating the inflammatory response and promoting of angiogenesis, compared to a single treatment. In the present study, I tested the effect of the combination of RvD1 and PNSC spheroids on spinal cord regeneration *in vitro* and in a rat model of SCI.

Various stem cell components, including BM-MSCs, CB-MSCs, AT-MSCs, NSCs, and OPCs have been employed as primary stem cell treatments for SCI, with numerous clinical studies conducted. BM-MSCs are multipotent adult stem cells that originate from the bone marrow stroma, contributing to hematopoiesis and bone regeneration.[38-41] They can be directly transplanted into the damaged spinal cord or administered intravenously due to their homing properties.[39,42,43] CB-MSCs were obtained from the blood or umbilical cord. In an animal model of SCI, CB-MSCs showed a promising profile of neurotrophic, anti-apoptotic, and anti-inflammatory effects.[44-46] Yao *et al*. reported autonomic restoration and changes in somatosensory evoked potential after intravenous and intrathecal infusion of human umbilical cord blood stem cells in patients with traumatic SCI.[47] AT-MSCs are obtained from adherent cultures of the stromal-vascular fraction separated from fatty tissues and have characteristics similar to BM-MSCs.[48-50] It is capable of multilineage differentiation into fat, bone, cartilage, smooth muscle, hepatocytes, and nerve cells.[51-53]

NSCs are multipotent, self-renewing progenitor or stem cells isolated from the subventricular zone of the hippocampus and a region of the central canal of the spinal cord.[54] These cells can differentiate into specific neuronal or glial phenotypes, and several studies have confirmed their functional recovery after NCSC transplantation in SCI models. NSCs were able to reconstitute lost neuronal and glial tissue with trophic support.[55-57]

NCSCs are cells that migrate from the neural tube during development to all organs and tissues in the body to form tissues and cells of the peripheral nervous system (PNS). Although known to disappear after birth, recent studies have confirmed that NCSC-like cells in various tissues of the adult body are also found in the bone marrow, skin, teeth, olfactory nerves, cornea, gastrointestinal tract, palate, and carotid artery.[48,58-60] NCSC-like stem cells isolated, cultured, and identified in adults are the only adult cells capable of differentiating into neurons and Schwann cells, and have mechanisms that can be applied to spinal cord injury due to their ability to secrete neuronal activity. Skin-derived precursor (SKP) and olfactory nerve-derived cells (olfactory ensheathing cells, OECs) are representative NCSC-derived cell sources, and these cell sources are being studied as treatments for spinal cord injury.[61-64] However, it is difficult to produce sufficient therapeutic amounts in the skin or olfactory nerves. In particular, side effects of developing cysts after transplantation due to contamination of nasal mucosal cells have been reported in the case of olfactory nerve-derived cells; therefore, safety should be ensured. Cells responsible for tissue regeneration after peripheral nerve injury generate repair phenotype Schwann cells through reverse differentiation or reprogramming mechanisms. Theses repair Schwann cells secrete nerve-activating factors and anti-inflammatory factors essential for peripheral nerve regeneration. It was confirmed that the recovered Schwann cells have characteristics similar to those of Schwann progenitor cells or NCSCs.

We successfully isolated undifferentiated PNSCs similar to NCSCs using 3D culture. PNSCs are known to have the ability to differentiate into neuronal and mesenchymal cells. These cells release neurotrophic and anti-inflammatory factors. The effect of PNSC is based on the paracrine effects of neurotrophic factor expression. GDNF and NT-3 are

constantly expressed at high levels in PNSCs, and these factors are known to have neuroprotective effects and induce neuronal regeneration. PNSCs have been proven to have axon regeneration and remyelination abilities. However, it is known that the growth of axons behind the graft is restricted due to glial cell scarring. In general, a prominent inflammatory response occurs after SCI, involving resident microglia and macrophages as the key players. Indeed, their selective regulation is critical for disease outcome. The pro-inflammatory environment recruits many peripheral monocytes/macrophages with different phenotypes. Some potentially have deleterious effects, such as pro-inflammatory M1 macrophages that exacerbate neurodegeneration and tissue loss. There are also beneficial M2 macrophages that support neuroprotection and regeneration.[23,26-28,65] In this study, we confirmed that PNSC spheroids have functional recovery and repair effects in SCI models. However, it is difficult to inhibit inflammation, an important mechanism in secondary injury, with the use of PNSC spheroids alone. Thus, additional treatments for neuroprotection have been studied.

In SCI, neuroprotective agents are most commonly used in clinics and are mainly classified into calcium antagonists, glutamate release inhibitors, neurotrophic factors, free radical scavengers, cell membrane stabilizers, anti-inflammatory factors, glutamate antagonists, excitatory amino acid antagonists, GABA receptor agonists, and leukocyte adhesion inhibition agents based on their respective mechanisms of action.[4,66,67] Unfortunately, most of these drugs are ineffective or have adverse effects. Even the suppression of inflammation would be beneficial for neuroprotection, as most of the neurological injury results from the subsequent inflammation. As mentioned in the Introduction section, we studied the role of RvD1 in various neuroprotective substances.

Mounting evidence indicates that the resolution of acute inflammation is an active process involving the production of specialized pro-resolving mediators (SPMs), such as resolvins, protectins/neuroprotectin, and maresins. RvD-series (e.g., RvD1, RvD2) and E-series (e.g., RvE1) are derived from the omega-3 polyunsaturated fatty acids docosahexaenoic acid (DHA) and eicosapentaenoic acid (EPA), respectively. They exhibit potent anti-

inflammatory and pro-resolution effects in various animal models of inflammation and infection.[30,68-70] SPMs regulate inflammation and resolution via the activation of different cell surface G-protein-coupled receptors (GPCRs), which rapidly transport signals and activate intracellular pathways to control a number of biological functions.[71] Considering that the inflammatory response is one of the major components of the secondary injury process following SCI, SPMs may regulate the inflammation resulting from SCI.

One study showed that the RvD group had an anti-inflammatory effect and was effective for nerve regeneration. RvD3 and RvD4 had no effect on the inhibition of postoperative pain after bone fracture. However, RvD1, RvD2, and RvD5 decreased mechanical allodynia.[72-74] Previously, Juri Kim *et al*. reported that intrathecal injection of RvD3 effectively in promoted inflammatory resolution, neuroprotection, functional nerve recovery and reduced thermal hyperalgesia in a SCI model. In this study, the authors demonstrated that RvD3 has effects such as down-regulation of pro-inflammatory cytokines, migration of macrophage phenotype to M2, preservation of tight junctions of the blood-spinal cord barrier, promotion of neuroprotection, and prevention of glial/fibrotic scar formation in the acute phase.[25]

RvD1 has potent anti-inflammatory effects and has been shown to promote resolution of inflammation in several studies.[75,76] RvD1 dose-dependently (10-500 nM) reduced the secretion of pro-inflammatory cytokines IL-6, IL-6Ra, MCP-1, and leptin.[75] It is known to reduce pro-inflammatory molecule gene expression by inducing M2 macrophage polarization in chronic inflammatory autoimmune diseases[77] and uncontrolled inflammatory acute respiratory distress syndrome (ARDS).[78] E. Bisicchia *et al*. reported that RvD1 activates the receptor ALX/FPR2 to create a new resolution circuit that specifically regulates miR-219a-1-3p and miR-146b, their downstream targets (TLR4 and IL6R), and (to a lesser extent), NF-κβ and CD200. These reports are consistent with other studies in mice, where RvD1 downregulated microRNA-regulated expression of NF-κβ and its downstream pro-inflammatory cytokines in a brain damage model.[29]

In this study, I found that RvD1 induces a decrease in pro-inflammatory cytokines (such

as TNF-a, IL-1β, and NF-κB) and promotes the secretion of anti-inflammatory cytokines (such as TGF-β) in the SCI model. Other studies have reported that aspirin-induced (AT)-RvD1 treatment reduced leukocyte recruitment, neutrophil-platelet interactions, and downregulation of pro-inflammatory mediators (such as IL-1β, IL-6, IL-8, and TNF-a).[30,77] This may be related to the rapid recognition of RvD1-stimulated macrophage swiftly recognized necroptotic cells.

When spinal cord injury occurs, 80% of patients in the acute phase and 30–50% of patients in the chronic phase experience neuropathic pain. In SCI, demyelination occurs due to secondary damage, and hyperalgesia and allodynia are known to occur due to hyperexcitability of C-fibers. Lee *et al*. reported that by injecting PNSC spheroids into spinal cord injury model rats, high NT-3 expression was induced. Moreover, a remyelination effect was generated to reduce neuropathic pain.[24] In this study, I demonstrated that the combination of PNSC spheroids and RvD1 reduced hyperalgesia and allodynia and increased motor function. BBB scoring was performed to confirm the effect of RvD1 on PNSC functional recovery and neuroregeneration after SCI; it was confirmed that RvD1 enhances the effect on PNSCs. In addition, allodynia is a representative neurogenic pain often accompanied spinal cord injury. Eight weeks after spinal cord injury, the combination of RvD1 and PNSCs showed the greatest reduction in mechanical allodynia compared to the use of RvD1 or PNSCs alone. This is thought to be caused by the combination therapy, which increases remyelination and reduces demyelination through an anti-inflammatory effect.

In this study, I examined the effects of co-administering RvD1 and PNSC spheroids in a rat model of SCI. our findings revealed several significant outcomes, including functional recovery, neuronal regeneration, and reduced mechanical allodynia, which demonstrated greater therapeutic benefits compared to other monotherapy strategies in a rat model. It is hypothesized that these effects are due to the enhanced paracrine action of stem cells, as co-administration therapy both *in vivo* and *in vitro* has been shown to suppress inflammation following SCI and increase the secretion of various neurotrophic factors.

However, it is essential to recognize certain limitations and inconsistencies observed during this study. Although RvD1 was administered once at 1 week post-SCI to achieve an anti-inflammatory effect, further research is needed to evaluate the impact of multiple injections. Moreover, this study was conducted using rats, and insufficient long-term data currently exist regarding the applicability to humans and potential oncogenic effects associated with the use of stem cells, which represent the limitations of this study.

V. CONCLUSION

In our previous research, we found that, when PNSC spheroids were transplanted to the injured site in a spinal cord injury model, they differentiated into neurons and improved neurological function in spinal cord injured rats with SCI. The neurological function was improved from the initial stage of transplantation, and it was confirmed that the neurological function was remarkably improved after 8 weeks of transplantation. In *in vitro* and *in vivo* studies, pro-inflammatory cytokines are greatly increased after spinal cord injury. However, reducing these cytokine secretions proved difficult through the sole transplantation of PNSC spheroids. While RvD1, known for its anti-inflammatory properties, showed some effectiveness in decreasing pro-inflammatory marker expression and macrophage recruitment, the most substantial impact was observed when RvD1 was combined with PNSC spheroids. This combination approach resulted in a reduction in overall macrophage recruitment and an increase in the expression of anti-inflammatory markers. Future research should prioritize strategies that promote cell differentiation, specifically focusing on transplanted PNSC spheroids, to enhance their ability to differentiate into neural cells. In this study, we performed the transplantation of RvD1 1 week after SCI. As a result, it is crucial to further investigate the anti-inflammatory mechanism by which RvD1 inhibits demyelination in the early stages of SCI, aiming to identify potential interventions that could further minimize neuronal damage.

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ABSTRACT(IN KOREAN)

랫드에서 척수손상 후 RvD1과 말초신경 유래 줄기세포구의 복합 치료 요법

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정 승 영

척수 손상은 주로 외상에 의해 발생하며 비가역적이고 영구적인 운동, 감각 및 자율신경 기능의 상실을 일으키는 것으로 알려져 있다. 최근 척수 손상의 주된 병태생리인 이차성 손상을 극복하기 위한 다양한 시도가 이루어지고 있으며, 여러 줄기세포를 이용한 연구에서 치료 효과가 입증되고 있다. 이전 연구에서 우리는 신경 능선 줄기세포와 유사한 특성을 갖는 말초 신경 유래 줄기세포구가 척수 손상 모델에서 기능 회복 및 신경 재생 효과를 갖는다는 것을 입증하였다. 그러나 말초 신경 유래 줄기세포구의 단독으로는 항염증 효과가 제한적이기 때문에 항염증 및 신경보호 효과가 있는 RvD1과의 병용 요법의 효과를 연구하였다. 이 연구는 랫드를 사용하여 시험관내 및 생체 내에서 수행되었다. 시험관 내 연구를 통하여 RvD1의 전염증성 사이토카인 억제 효과와 항염증 효과를 확인하였다. 척수손상 모델 생성 후 척수 기능, 생물학적 효능, qRT-PCR, 면역조직화학요법을 통해 RvD1과 말초 신경 유래 줄기 세포구의 조합이 다른 단일 요법보다 우수한 신경 재생 및 항염증 효과를 가짐을 확인하였다. RvD1과 말초 신경 유래 줄기 세포구의 병용요법은 척수손상에 대한 새로운 치료 접근법이 될 것으로 예상된다.

핵심되는 말 : 척수 손상, 복합 치료 요법, 말초신경 유래 줄기세포, RvD1