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Interferon-β delivery via human neural stem cell abates glial scar formation in spinal cord injury

Running title: Human neural stem cells in spinal cord injury

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Abstract

Glial scar formation is the major impedence to axonal regrowth after spinal cord injury (SCI), and scar-modulating treatments have become a leading therapeutic goal for SCI treatment. In this study human neural stem cells (NSCs) encoding interferon (IFN)-β gene were administered
intravenously to mice 1 week after spinal cord injury. Animals receiving NSCs encoding IFN-β exhibited significant neurobehavioral improvement, electrophysiological recovery, suppressed glial scar formation and preservation of nerve fibers in lesioned spinal cord. Systemic evaluation of SCI gliosis lesion site with lesion-specific microdissection, genome-wide microarray and MetaCore pathway analysis identified upregulation of toll-like receptor (TLR)-4 in SCI gliosis lesion site, and this led us to focus on TLR-4 signaling in reactive astrocytes. Examination of primary astrocytes from TLR-4 knockout mice, and in vivo inhibition of TLR-4, revealed that the effect of IFN-β on the suppression of glial scar formation in SCI requires TLR-4 stimulation. These results suggest that IFN-β delivery via intravenous injection of NSCs following SCI inhibits glial scar formation in spinal cord through stimulation of TLR-4 signaling.

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Introduction

Spinal cord injury (SCI) is a devastating clinical condition that results in permanent disability due to very limited regenerative capability of the adult human spinal cord. One major impediment to axonal regeneration in SCI is glial scar formation, a process mainly directed by reactive astrocytes (42). Normally, quiescent astrocytes in adults respond vigorously to injury; during the acute phase of injury, some of these responses have beneficial effects, such as isolating the injury site and minimizing the area of inflammation and cellular degeneration. Some astrocyte populations may even support axonal regrowth (6); however, astrocytes eventually become hypertrophied and proliferative, upregulate the expression of glial fibrillary acidic protein (GFAP), and form a dense network of glial processes around the

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injury site (12). Therefore, scar-modulating treatments have become a leading therapeutic goal for the treatment of SCI (9,37).

We have previously examined the ability of liposome-mediated IFN-β gene delivery to inhibit the formation of glial scar tissue in a SCI mouse model (12) and found that the IFN-β administration induced functional and structural recovery in injured spinal cord including regrowth of corticospinal tract (CST) axons.

Recently, there has been a great deal of interest in the potential use of stem cells in SCI treatment because of their abilities to self-renew, migrate, and differentiate into all types of neural cells (18). Neural stem cells (NSCs), in particular, are characterized by the capability to home in and deliver therapeutic genes (40), and constitute a promising source for cell replacement therapy (22,25,45).

In the present study, we attempted to attain neuronal regrowth via intravenous transplantation of human NSCs encoding genes for cytosine deaminase (CD) and IFN-β (F3.CD.IFN). We investigated whether human NSCs transduced with IFN-β gene can inhibit glial scar formation and improve spinal function after SCI, while the CD gene, a therapeutic suicide gene that converts non-toxic prodrug 5-fluorocytosine (5-FC) into toxic 5-fluorouracil (5-FU), provides a safe guard to allow removal of cells in cases of undesirable proliferation..

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Further, in order to clarify the function of IFN-β, we attempted to identify profile changes in SCI gliosis lesion site by using gliosis site-specific microdissection, genome-wide microarray, and MetaCore pathway analysis. This systematic data processing revealed the upregulation of toll-like receptor (TLR)-4, and we subsequently focused on the functional role of TLR-4 signaling cascades in reactive astrocytes. We here verify that the SCI-induced proliferation of reactive astrocytes in lesion is suppressed by the ligation of TLR-4 in the presence of IFN-β.

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Materials and methods

Neural stem cells

The HB1.F3 (F3) human NSC line was generated from human fetal telencephalon and immortalized by transfection with a retroviral vector encoding the v-myc oncogene, as described previously(21). It has been confirmed that this human NSC line is capable of self-renewal and has multipotent capacity to differentiate into neuronal or glial cell lineages both in vivo and in vitro(21). The F3 cell line was infected with a replication-incompetent retroviral vector encoding β-galactosidase (lacZ) and puromycin-resistance genes. The cell line was subsequently designated as F3.LacZ. In this study, the clonal F3.CD.IFN-β line (F3.CD.IFN) was derived from parental F3.CD cells, as previously described(1,11). F3.LacZ and F3.CD.IFN cells were cultured in Dulbecco's modified Eagle's medium (DMEM) with high glucose (Invitrogen, Carlsbad,
CA) supplemented with 10% fetal bovine serum (FBS), 100 U/mL penicillin, and 100 μg/mL streptomycin.

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In vitro assay of astrocyte suppression by F3.CD.IFN cells

The suppressive effects of F3.LacZ and F3.CD.IFN cells on the growth of primary-cultured astrocytes were quantified in the presence or absence of 5-FC (Sigma-Aldrich, St. Louis, MO). Primary astrocyte cultures were prepared from the cerebral hemispheres of fetal BALB-c nude mice (SLC, Shizuoka, Japan). The hemispheres were cleared of the meninges and choroid plexus and digested with 0.1% trypsin (Invitrogen) in phosphate-buffered saline (PBS) for 30 min at 37°C, followed by dissociation into single cells by repeated pipetting. A suspension containing 2×10^5 cells was seeded into 35-mm poly-L-lysine-coated Petri dishes, and the majority of cells
(90–95%) were confirmed to be astrocytes based on immunocytochemical analysis for GFAP expression. Astrocytes were cultured on Petri dishes for 1 week, after which F3.NSCs were

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added to the astrocyte cultures. Astrocytes were co-cultured with either F3.LacZ or F3.CD.IFN at various ratios of astrocytes to F3 cells (1:0, 20:1, and 40:1). F3 cells were prelabeled by incubating for 20 min in culture medium containing the cell tracker CM-Dil (Invitrogen), which emits 570-nm fluorescence. For experiments using F3.CD.IFN cells treated with 5-FC, 5-FC was added to the conditioned medium at a final concentration of 500 mg/mL following 24 h of culture, and the culture was maintained for24 hr. Cells were subsequently immunostained with anti-GFAP antibody (DAKO, Glostrup, Denmark) and Alexa 488-labeled IgG (Molecular Probes, Eugene, OR). The cultures were analyzed using an Olympus FV5-PSU confocal laser microscope (Olympus, Tokyo, Japan), and the total numbers of GFAP-positive cells were counted. Each experiment was performed in triplicate. The suppressive effects of F3.LacZ and F3.CD.IFN on the growth of primary-cultured TLR-4 deficient astrocytes were also quantified. Primary TLR-4 deficient astrocyte cultures were prepared from the cerebral hemispheres of fetal TLR-4 knock- out mice (Oriental Bio, Kyoto Japan). A suspension containing 2 × 105 cells was seeded onto 35-mm poly-L-lysine-coated Petri dishes. The majority of cells (90–95%) were immunocytochemically confirmed to be astrocytes and cultured on Petri dishes for 1 week, after which F3.NSCs were added to the astrocyte cultures. Astrocytes were co-cultured with either F3.LacZ or F3.CD.IFN cells at various ratios of astrocytes to F3 cells (1:0, 20:1, and 40:1). F3 cells were prelabeled by the cell tracker CM-Dil, and the culture was maintained for 2 days. Cells were subsequently immunostained with anti-GFAP antibody followed by Alexa 488-labeled IgG, and the total numbers of GFAP-positive cells were counted. Each experiment was performed in triplicate.

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Spinal transection procedure

Adult female Balb-c nude mice (8–12 weeks old; SLC) were used in this study. All experiments were performed in accordance with the ethical guidelines of the Nagoya University Institutional Animal Care and Use Committee. The mice were anesthetized with

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1.5% halothane and maintained on 1.25% halothane in an oxygen-nitrous oxide gas mixture. Laminectomy was performed at vertebral level T9–T10. The dura was opened, and the dorsal half of the spinal cord was transected to a depth of 1 mm with a pair of extra-fine microscissors. In the sham group, laminectomy was conducted without the accompanying SCI. Following spinal transection, the overlying muscle and skin were sutured. The mice were placed on soft bedding on a warming blanket held at 37°C for 1 hr after surgery. One week later, the motor function of each animal was evaluated according to the Basso Mouse Scale (BMS), as described below. Only animals with BMS scores below 4 out of 9 points were used. These were divided randomly into 4 treatment groups: (1) PBS group, SCI with intravenous administration of 100 µL PBS;

(2) F3.LacZ group, SCI with intravenous administration of F3.LacZ (2 × 106 cells in 100 µL of PBS); (3) F3.CD.IFN group, SCI with intravenous administration of F3.CD.IFN (2 ×10^6 cells in 100 µL of PBS); and (4) F3.CD.IFN+5-FC group, SCI with intravenous administration of F3.CD. IFN (2 × 10^6 cells in 100 µL of PBS) and, beginning 2 days later, 10 intraperitoneal injections of 5-FC daily (900 mg/kg). In order to elucidate the mechanism by which IFN-β elicits effects following SCI, we used the same spinal transection procedure and assigned the mice to 2 treatment groups 1 week later: (1) F3.LacZ group, SCI with intravenous administration of F3.LacZ

(2 × 10^6 cells in 100 µL of PBS); (2) F3.CD.IFN group, SCI with intravenous administration of F3.CD.IFN (2 × 10^6 cells in 100 µL of PBS). In addition, the F3.CD.IFN group was randomly divided into 2 further groups 1 week after the initial group assignment: (2-a) F3.CD.IFN group, (2-b) F3.CD.IFN+OxPAPC group, SCI with intravenous administration of F3.CD.IFN (2 × 10^6 cells in 100 µL of PBS), and 7 days later, an intraperitoneal injection of 50 µg of OxPAPC (TLR4 Inhibitor; InvivoGen, San Diego, CA). Finally, we compared the functional recovery in F3.LacZ, F3.CD.IFN and F3.CD.IFN+OxPAPC groups.

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Laser-captured microdissection and microarray

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On day 10 after surgery, 5 animals from the laminectomy only and plain SCI groups were deeply anesthetized with barbiturate overdose and intracardially perfused with PBS. Their spinal cords were removed and immediately frozen in Tissue-Tek OCT medium (Sakura Finetek, Tokyo, Japan). The spinal cords were sectioned in the sagittal plane onto uncoated slides. A PixCell II LCM instrument (Arcturus, Mountain View, CA) was used to dissect the injury site, and RNA was extracted from the microdissected samples using the PicoPure RNA Isolation Kit (Arcturus), according to the manufacturer's instructions. Total RNA was pooled from the 5 animals from each group and amplified and labeled using the Amino Allyl MessageAmp aRNA Kit (Ambion,
Austin, TX). Briefly, after reverse transcription (2 mg total RNA/sample), double-stranded cDNA was transcribed in vitro using the amino allyl cRNA. The RNA was amplified twice, and the purified and concentrated cRNA (5 mg) was coupled with either Cy3 or Cy5 dyes (GE Healthcare). The dye-labeled aRNA was purified from uncoupled dye using Micro Bio-Spin P-30 Tris chromatography columns (Bio-Rad, Hercules, CA) and Microcon YM-30 centrifugal filter devices (Millipore, Billerica, MA). The cRNA was fragmented in a fragmentation buffer (40 mmol/L Tris acetate (pH 8.1), 100 mmol/L potassium acetate, and 30 ʃmol/L magnesium acetate) at 94°C for 15 min and purified with Microcon YM-10 (Millipore). An oligonucleotide-based mouse DNA microarray, AceGene (mouse Oligo Chip 30K; DNA Chip Research, Yokohama, Japan) was preblocked with 1% bovine serum albumin (BSA) solution. The fragmented cRNA was added to the microarray in hybridization solution and subsequently hybridized at 42°C for 16 h. The arrays were then washed, scanned at a pixel size of 10 mm, gridded, and analyzed (GenePix 4000B; Axon Instruments, Union City, CA). The background was subtracted, and the medium sum intensity (CH1 and CH2) of <100 absorbance units was excluded. Data were normalized by the trimmed mean at 10% to account for the differences in the amounts of labeled RNA or labeling efficiencies.

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Pathway analysis

We next attempted to identify the biological pathways involving novel gene networks that are activated in reactive astrocytes after SCI. For this purpose, we analyzed the genes whose expressions were upregulated in the lesion by at least a factor of 2.0, relative to their respective expressions in the normal spinal cord. The functional mapping tool MetaCore (GeneGO, St Joseph, MI) was used in this analysis. MetaCore is a web-based computational platform designed primarily for the analysis of high-throughput experimental data in the context of mouse regulatory networks and pathways. It includes a curated database of protein interactions, metabolism, and bioactive compounds. For a network of a particular size, MetaCore can be used to calculate statistical significance based on the probability of the network's assembly from a random set of nodes (genes) that is of the same size as the input list (p value).

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In vivo migration of DiI-labeled NSCs

F3.LacZ cells were prelabeled by incubating for 20 min in culture medium containing Cell Tracker CM-DiI (Invitrogen). We evaluated whether DiI-labeled NSCs could migrate to the SCI site in adult mice after intravenous transplantation. Seven days after the induction of transection injury, the injured animals were injected via the tail vein with 2×10^6 DiI-labeled F3.LacZ NSCs in 100 μL of PBS. The control injured animals were injected with PBS alone. Seven days after NSC transplantation, the animals were euthanized and transcardially perfused with 4% paraformaldehyde (PFA).

RT-PCR for IFN-β and TLR-2 and -4 expression at the SCI site

The total RNA extracted from the harvested tissues was subjected to DNase (Invitrogen) treatment prior to reverse transcription using a Transcriptor First-Strand cDNA Synthesis Kit (Roche, Mannheim, Germany), according to the manufacturer's protocol. PCR amplification

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was performed using GoTaq DNA polymerase (Promega, Madison, WI) with the following primers: human IFN-β, 5′-GCCGCATTGACCATCTATGAGA-3′ (sense) and 5′-GAGATCTTCAGTTTCGGAGGTAAC-3′ (antisense); mouse TLR-2, 5'-ACAGCTACCTGTGTGACTCTCCGCC-3' (sense) and 5'-GGTCTTGGTGTTCATTATCTTGCGC-3' (antisense); mouse TLR-4, 5'-ACCTGGCTGGTTTACACGTC-3' (sense) and 5'- CAGGCTGTTTGTTCCCAAAT-3' (antisense); mouse β-actin, 5'-GACATGGAGAAGATCTGGCACCACA-3' (sense) and 5'-ATCTCCTGGTCGAAGTGTAGAGCAA-3' (antisense); human and mouse GAPDH (product length; 454 bp), GACCACAGTCCATCGCATCA (sense) and GTCCGCCACCCTGTTGCTGT (antisense).

Immunohistochemistry

The mice were deeply anesthetized with an overdose of barbiturate and intracardially perfused with PBS followed by 4% PFA in PBS. The lesioned spinal cord region was removed and postfixed overnight in the same fixative. The fixed tissues were embedded in paraffin, and 3-μm sections were prepared for immunohistochemistry. Immunohistochemical staining was performed as per the manufacturer's instructions. Briefly, sections were preblocked with the blocking reagent (DAKO REAL Peroxidase-Blocking Solution, DAKO), and antigen retrieval was performed for GFAP. After sections were stained with rabbit anti-GFAP (DAKO), mouse anti-neurofilament (NF) (Nichirei, Tokyo, Japan), and a polymer reagent (Chemomate Envision kit/HRP, DAKO), color was developed with diaminobenzidine (DAB). Finally, sections were counterstained with hematoxylin. The corresponding tissue sections were routinely stained by hematoxylin and eosin for morphological evaluation. Five areas of immunostained sections were randomly selected, and the color intensity was measured using NIH image software as reported(44). Background intensity was subtracted from the mean intensity of the images, and the means were used for statistical analysis.

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Basso Mouse Scale for locomotion

The recovery of hindlimb motor function in 7 mice in each treatment group was measured using the BMS for locomotion, as previously described(2). The behavior of each animal was videotaped for 5 min, and 2 investigators provided a score (on a scale of 0–9) for each hindlimb at 1, 2, 3, 4, 6, and 8 weeks after injury. The scores from the 2 hindlimbs were averaged to obtain a single value per animal for each time point.

Inclined plane test

We constructed an inclined plane as previously described(36), and tested the ability of each animal to maintain its position on the incline at 1, 2, 3, 4, 6, and 8 weeks after injury. The maximum inclination at which a mouse could maintain itself for 5 s was recorded; this value was considered to represent the functional ability of the mouse. In practice, the angle was either increased or decreased by 5° intervals until the mouse could maintain its position on the inclined plane for 5 s without falling.

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Electrophysiology

To measure signal conduction in mouse motor pathways following SCI, transcranial electrical MEPs were measured at 4 and 8 weeks after injury. To record MEPs, mice were anesthetized with 1.5% halothane and maintained in 1.0% halothane in oxygen. All recordings were performed using standard clinical electromyographical analysis with a 3000-Hz hi-cut filter and a 30-Hz low-cut filter (Nihon Kohden, Tokyo, Japan). Electrical stimulation (1 Hz, 15 mA) was delivered over the cranium at an area 1 mm lateral to bregma. Evoked responses were recorded with needle electrodes from the contralateral femoral muscle. Response amplitudes were measured from base to peak.

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In vivo MRI

To investigate the status of injured neural tissue at the SCI site in a noninvasive manner, MRI was performed using 7.0-T MRI (superconducting magnet: Kobelco and Jastec, Japan; console: Avance-I system, Bruker BioSpin, Germany), with a volume coil for transmission (Bruker BioSpin), and 2-ch phased array coil for reception (Rapid Biomedical, Germany) dedicated to examining spinal cord lesions in live mice. DTI, and T2-weighted (T2WI) MRI were conducted 4 weeks after injury in mice assigned to the PBS, F3.LacZ, and F3.CD.IFN groups. DTI data sets were acquired with a respiratory-gated gradient-echo echo-planar diffusion-weighted pulse sequence, based on the Stejskal-Tanner diffusion preparation(41). Scanning parameters of the DTI were as follows: repetition time (TR), 3000 ms; echo time (TE), 34 ms; flip angle, 90°; field of view (FOV), 25.6 × 12.8 mm; data matrix, 128 × 128; reconstructed image resolution,
200 × 100 μm^2; slice thickness, 1.0 mm; b-value, between 0.68 and 1000 s/mm^2; motion-probing gradient (MPG) orientations, 30 axes and 5 B0 images; number of averaging (NA), 1; number of EPI segment, 4; fat saturation pulse, on. T2WIs were acquired through rapid acquisition with relaxation enhancement (RARE) sequence as follows: TR, 4200 ms; TE, 12 ms; effective TE, 36 ms; FOV, 25.6 × 12.8 mm; data matrix, 256 × 128; reconstructed image resolution, 100 × 100 μm2; slice thickness, 1.0 mm; fare factor, 8; NA, 8; fat saturation pulse, on. Mice were placed under anesthesia with a mixture of 1.5% isoflurane, 40% oxygen, and 58% air delivered via tracheal intubation prior to T2WI and DTI analysis. Vital signs (respiration, heart rate, and body temperature) of the anesthetized animals were monitored using a MRI-compatible monitoring and gating system (Model 1025; SA Instruments Inc., Stony Brook, NY). Respiratory-gated acquisition was used to increase the image quality by minimizing breathing-related image artifacts whenever necessary. To reduce motion artifacts, animals were immobilized on an acrylic bed, and a specially designed head positioner (Rapid Biomedical) for gated imaging was attached to each animal's front thorax. Diffusion tensor analysis was performed using ParaVision with Jive software (Bruker BioSpin). Three

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eigenvectors (*e*1, *e*2, *e*3) associated with the largest eigenvalue (λ1) were visualized as a color map (left-right, red; posterior-anterior, green; superior-inferior,blue) (34).

Statistical analysis

The statistical significance of observed differences was determined by ANOVA (StatView; SAS Institute, Cary, NC), and Bonferroni's correction was employed for multiple comparisons. All reported P-values are two-tailed; a value of $P < 0.05$ was considered statistically significant.

Results

Molecular signature of reactive astrocytes following SCI

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A laser-captured microdissection technique was used to procure specific areas from each heterogeneously damaged tissue structure. In order to analyze the molecular signaling of reactive astrocytes following SCI, the sites of lesions that were expected to form a glial scar were captured, and a microarray was used to profile the expression of 30,000 genes on day 10 after dorsal hemisection of the spinal cord at the T9–T10 level. We evaluated gene expression as either significantly upregulated or significantly downregulated by using the fold-level criteria (>2-fold change: the significance of upregulation was set at >2 and that of downregulation at <0.5). Data analysis identified 488 significantly upregulated genes and 226 downregulated genes in several different categories. Complete lists of genes demonstrating significant expression changes are listed in Supplementary Table 1. Only 1.6% of the probe set corresponding to individual genes was expressed at a level >2.0-fold after SCI. After the microarray data were imported into MetaCore, functional gene networks were generated to integrate reactions and interactions around the identified genes of interest. Significantly activated pathways are displayed in Fig. 1A ($p < 0.05$). In this study, we focused on TLRs

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that may be related to IFN-β as reverse transcriptase-polymerase chain reaction (RT-PCR) and quantitative RT-PCR revealed that TLR-4 was markedly upregulated in the lesioned astrocytes (Fig. 1B).

F3 cells migrate to SCI lesion sites in mice

We evaluated the efficient migration of intravenously injected F3 NSCs tagged with DiI or lacZ to SCI sites in mice (Fig. 2A). It was revealed that a large number of DiI-labeled F3 cells migrated to the lesion site (Fig. 2B), and immunohistochemical staining with anti-β-galactosidase (β-gal) antibody demonstrated that β-gal-positive cells were also extensively distributed in the lesioned area (Figs. 2C, D). All NSCs were found to be located in or around the injured parenchyma, indicating that there was specificity of migration. Conversely, in the control injured animals injected with PBS alone, no β-gal-positive cells were observed in the spinal cord (Figs. 2E, F). IFN-β expression was also detected at the SCI site of the animals injected with 2×10^6 F3.CD.IFN by RT-PCR (Fig. 2G).

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F3.CD.IFN cells inhibit growth of primary astrocytes, but not TLR-4-deficient astrocytes

To quantify the inhibitory effect of F3.CD.IFN on astrocyte growth, DiI-labeled F3 cells were co-cultured with primary normal astrocytes in different ratios. As shown in Fig. 3A and B, the number of astrocytes significantly decreased with a reduction in the astrocyte:F3 ratio in the F3.CD.IFN group. When they were co-cultured with F3.CD.IFN with added 5-FC (F3.CD.IFN + 5-FC), there was no growth inhibitory effect on cultured astrocytes. To evaluate whether TLR-4 is necessary for IFN-β to exert this inhibitory effect on astrocyte growth, DiI-labeled NSCs were also co-cultured with astrocytes derived from TLR-4-deficient mouse in 40:1 ratio. As shown in Fig. 3C and D, the number of TLR-4-deficient astrocytes did not change. Thus, the inhibitory effect by the F3.CD.IFN cells was not exerted in the absence of TLR-4.

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Intravenous administration of F3.CD.IFN-β NSCs spares host neural tissue and enhances axonal regeneration and TLR-4 blockade cancels regeneration

To determine the effects of the intravenous administration of F3.CD.IFN cells on neuronal regeneration and the inhibition of glial scar formation, the expression of glial fibrillary acidic protein (GFAP) and neurofilament (NF) at the injury site was immunohistochemically evaluated. SCI was shown to result in a severe loss of neurons at the injury site, as well as the extensive growth of reactive astrocytes forming a glial scar. Hematoxylin and eosin (HE) staining revealed severe destruction of the dorsal half of the spinal cord above the central canal (Fig. 4A), and the formation of a granular scar was clearly detected at the injury site. Immunohistochemical staining for GFAP showed hypertrophy of GFAP-positive reactive astrocytes even in the area ventral to the central canal (Fig. 4B). The intensity of GFAP staining was significantly decreased in the F3.CD.IFN-treated group as compared to that observed in the other groups. GFAP density was calculated quantitatively using NIH imaging (mean ± SEM): sham group, 17 ± 0.5; PBS, 38 ± 2.9; F3.LacZ, 35 ± 2.3; F3.CD.IFN, 28 ± 1.2; F3.CD.IFN + 5FC, 35 ± 1.1 (Fig. 4C). In the PBS and F3.LacZ groups, NF-stained transverse sections demonstrated significant loss of neural fibers extending to the ventral side (Fig. 4D), while only partial preservation of neural axons was detected in the F3.CD.IFN + 5FC group. Compared to these findings, sections from the F3.CD.IFN group revealed the dramatic preservation of fiber number and alignment, which was confirmed by the quantification of NF density: sham, 31 ± 0.4; PBS, 21 ± 1.5; F3.LacZ, 21 ± 1.3; F3.CD.IFN, 30 ± 1.8; F3.CD.IFN + 5FC, 24 ± 3.5) (Fig. 4E). Next, the expression of GFAP and NF at the injury site was compared in mice treated with F3.CD.IFN in the presence or absence of the TLR-4 inhibitor OxPAPC. While the dorsal half of the spinal cord was destroyed equally in every group, the anterior half of the spinal cord was found to be markedly preserved in the F3.CD.IFN group when compared to the F3.CD.IFN+OxPAPC group (Fig. 5A). The intensity

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of GFAP staining was also significantly decreased in the F3.CD.IFN group as compared to that observed in the F3.CD.IFN+OxPAPC and F3.LacZ groups (Fig. 5B). NF-stained transverse sections also demonstrated a significant loss of neural fibers in the F3.CD.IFN+OxPAPC and F3.LacZ groups, while neural fiber was dramatically preserved in sections from the F3.CD.IFN group (Fig. 5C).

Behavioral studies

To determine whether F3.CD.IFN administration improved motor function after SCI, we assessed functional recovery using two independent behavioral tests: the Basso mouse scale (BMS) and the inclined plane test. Animals were evaluated for locomotor recovery at 1, 2, 3, 4, 6, and 8 weeks after injury.

One week after SCI, on the day of PBS or NSC administration, all injured animals exhibited a mean BMS score of <3, indicating extensive ankle movement without stepping ability. Animals with SCI receiving only PBS (PBS group) or F3.LacZ.NSCs (F3.LacZ group) spontaneously recovered to a BMS score of 3–4 (plantar placing of the paw or occasional plantar stepping) by 4 weeks after injury, and further recovery was limited. Animals treated with F3.CD.IFN followed by 5-FC administration (F3.CD.IFN + 5-FC group) had slightly better outcomes. In contrast, mice injected with F3.CD.IFN in the absence of 5-FC (F3.CD.IFN group) showed significant improvement in BMS scores compared to the other 3 groups, whereby the F3.CD.IFN group exhibited a mean BMS of 4.9 ± 0.5 at 4 weeks, and the PBS, F3.LacZ, and F3.CD.IFN + 5-FC groups attained mean scores of 3.7 ± 0.4, 3.6 ± 0.5, and 4.0 ± 0.5, respectively. Differences between the mean BMS scores of the F3.CD.IFN group and the other groups were statistically significant 4 weeks after injury and thereafter ($p < 0.05$) (Fig. 6A).

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The inclined plane test was performed to examine additional post-SCI locomotor function. There was significant recovery of performance 2 weeks following the injury in the F3.CD.IFN

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group, whereas the performance of the other 3 groups remained severely impaired ($p < 0.05$). None of the groups recovered to pre-injury levels of performance (Fig. 6B).
Finally, the improvements in the BMS scores and inclined plane test results that were observed in the F3.CD.IFN group were shown to be inhibited by the OxPAPC TLR-4 inhibitor (Fig. 6C, D).

Electrophysiology

The mean motor nerve evoked-action potentials (MEP) amplitude observed in the sham operation group was approximately 600 μV at both 4 and 8 weeks after injury. This amplitude was reduced to a range of approximately 150–200 μV in the PBS, F3.LacZ, and F3.CD.LacZ+5-FC groups at 4 and 8 weeks, while animals in the F3.CD.IFN group showed significantly better recovery, to approximately 300 μV at both 4 and 8 weeks after injury ($p < 0.05$) (Fig. 7A).
However, OxPAPC administration in the F3.CD.IFN group significantly worsened the mean MEP amplitude to 178 μV and 228 μV at 4 and 8 weeks, respectively ($p <0.05$)(Fig. 7B).

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Assessment of regenerative sprouting of CST axons by 7-T MRI

Four weeks after dorsal hemisection, in vivo proton diffusion tensor imaging (DTI) was performed for noninvasive evaluation of regenerative axonal sprouting (Fig. 8). We visualized the diffusion tensor of the thoracic spinal cord. The eigenvectors associated with the tract were depicted as colors according to their orientation: blue illustrates a longitudinal (superior-inferior) orientation. The continuity of this longitudinal tract demonstrated functional regeneration of the axons. DTI was performed 4 weeks after the injury in mice that received PBS, F3.LacZ, or F3.CD.IFN. In the PBS and F3.LacZ groups, the injury site and its surrounding area displayed high signal intensity in T2-weighted sagittal sections, indicating

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spinal cord edema or necrotic tissue alteration, whereas this high intensity was not detected in the F3.CD.IFN group. The DTI of the injured spinal cord in the PBS and F3.LacZ groups revealed discontinuity of longitudinal fibers, which were mostly visible in the F3.CD.IFN group.

Discussion

In the present study, we transplanted intravenously human NSCs transduced with IFN-β gene in SCI mice and the animals exhibited extensive suppression of glial scar formation and preservation and/or regeneration of nerve fibers in the lesioned spinal cord. Significant neurobehavioral and electrophysiological recovery was attained, as measured by the Basso mouse scale for locomotion, inclined plane test and transcranial MEPs. Evaluation with 7.0-T MRI also confirmed axonal regeneration. Systematic evaluation of spinal cord 10 days after SCI with gliosis-specific microdissection, genome-wide microarray and MetaCore pathway analysis revealed the upregulation of TLR-4 in the spinal cord lesion site. We therefore focused on the functional role of TLR-4 signaling in reactive astrocytes, as there is a strong relationship between TLR-4 and IFN-β in glia (8). TLR-4 in the presence of IFN-β was shown to suppress the SCI-induced proliferation of reactive astrocytes within lesion site. Furthermore, as mice that were administered with IFN-β-expressing NSCs and TLR-4 inhibitor simultaneously gained less functional recovery than mice in which only IFN-β-expressing NSCs were injected, we have verified that TLR-4 plays an extremely important role in the effects of IFN-β during astrocytic gliosis. These results suggest that IFN-β delivery by intravenous injection of genetically engineered NSCs is capable of inhibiting glial scar formation after SCI, and promotes functional recovery in the presence of TLR-4.

Benefit of the utilization of NSCs in the treatment of SCI

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Three different delivery methods can be considered for SCI cell therapy: intralesional, intrathecal, and intravenous. A cardinal feature of NSCs is their exceptional migratory ability, which has led to their emergence as a therapeutic paradigm in various animal models of neurodegeneration, stroke, and brain tumors (23,24,28,47). Previously, we and others have demonstrated that the transplantation of genetically engineered F3 human NSCs into sites of SCI lesions induces functional improvements in rat SCI models (10,17,18,26). We have also demonstrated that there is

glial and neuronal differentiation of NSCs, indicating that intravenous NSC delivery activates the microenvironment of the injured spinal cord to stimulate self-regeneration (44). Thus, intravenous injection of human NSCs is a promising tool for replacing depleted cells or for delivering therapeutic genes in the treatment of SCI, with the potential to be used in clinical applications. We have also demonstrated that intravenously injected F3 human NSCs migrate preferentially to the SCI site (44). As a novel strategy for SCI treatment, we combined these two studies and examined whether functional recovery and axonal regeneration could be achieved through the intravenous administration of IFN-β-expressing NSCs. F3 NSC is a clonally isolated, multipotent human neural stem cell line that has the ability to self-renew, stably express genes, and

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differentiate into neurons, astrocytes, and oligodendrocytes (21). F3 NSCs have previously been used in a clinical trial for gene therapy in glioma patients (A pilot feasibility study of oral 5-Fluorocytosine and genetically-modified neural stem cells expressing E Coli cytosine deaminase for trearment of recurrent high grade glioma-City of Hope National Medical Center, Duarte, CA, USA, June 2010). Inhibition of glial scar formation is a promising approach for the treatment of SCI. Recent studies have focused on the multifaceted roles of astrocytes in response to SCI.

It has previously been demonstrated that selective depletion or ablation of a subpopulation of reactive astrocytes causes widespread tissue disruption and pronounced cellular degeneration (6). Another study has reported that the emergence and migration of reactive