Administration of C5a Receptor Antagonist Improves the Efficacy of Human iPSCS-derived NS/PC Transplantation in the Acute Phase Of spinal Cord Injury

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Research

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Abstract

Background: We previously reported the efficacy of human-induced pluripotent stem cells derived neural stem/progenitor cells (hiPSC-NS/PCs) transplantation for spinal cord injury (SCI) in the subacute phase. However, this procedure is not effective in the acute phase due to the inflammatory response occurring immediately after SCI, which negatively impacts transplanted cell survival. C5a, which is one of the complement components, is a powerful chemoattractant which recruits inflammatory cells through binding of the C5a receptor. We hypothesized that suppression of the inflammatory response immediately after SCI using a C5a receptor antagonist (C5aRA) as an immunosuppressant would improve the efficacy of hiPSC-NS/PCs transplantation for SCI in the acute phase.

Methods: Immunodeficient SCID-Beige mice underwent contusion SCI at T10 and received C5aRA immediately after SCI. Inflammatory cytokines and inflammatory cells in the injured spinal cord tissue during the acute phase were quantified using quantitative PCR and flow-cytometry. Next, we randomly and blindly divided the SCI mice into 4 groups (PBS only, C5aRA only, PBS + transplantation (PBS+TP), C5aRA + transplantation (C5aRA+TP)). Immediately after SCI, C5aRA or PBS was injected intraperitoneally once a day for 4 consecutive days, and then, 5.0×10^5 hiPSC-NS/PCs were transplanted into the lesion epicenter on day 4 in the PBS+TP and C5aRA+TP groups. We evaluated cell survival rate, hindlimb motor function, and the differentiation profile of the graft hiPSC-NS/PCs.

Results: C5aRA administration significantly reduced several inflammatory cytokines such as IL-1b, IL-6 and TNFα, as well as inflammatory cells after SCI. Within the transplantation groups, the C5aRA+TP group had better functional improvement as compared to the PBS only group. The C5aRA+TP group also had a significantly higher cell survival rate compared to the PBS+TP group. There were no significant differences in the differentiation profiles of grafted hiPSC-NS/PCs between the C5aRA+TP and PBS+TP groups.

Conclusion: This study demonstrates that administration of C5aRA can suppress the inflammatory response during the acute phase of SCI, and also improve the survival rate of transplanted hiPSC-NS/PCs as well as enhance motor functional restoration. hiPSC-NS/PC transplantation with C5aRA is a promising treatment during the acute injury phase for SCI patients.

Background

A proportion of individuals who sustain a spinal cord injury (SCI) will suffer from lifelong and severe motor and sensory dysfunction, due to the body's limited ability for neurologic recovery post-injury[1]. To enhance recovery, much attention has been focused on cell transplantation therapies for SCI, especially the use of human-induced pluripotent stem cell (hiPSC). Indeed, we and other groups have demonstrated the efficacy of hiPSC-derived neural stem/progenitor cell (hiPSC-NS/PC) transplantation for conferring functional recovery in SCI [2–4], and these advancements are expected to reach clinical application in the near future [5].

However, critical issues remain surrounding the timing of cell transplantation and optimization of the injury site milieu. Currently, the optimal timing for transplantation is the subacute phase of SCI, which is about 7–14 days after SCI in rodents, after neuroinflammation subsides. Since the inflammatory reaction in the acute phase of injury does not allow transplanted cells to survive, researchers have been forced to wait until the subacute phase to transplant cells [6, 7]. During the acute phase of injury, several neurotoxic cytokines are upregulated and inflammatory cells intrude into the lesion area [8, 9]. As a consequence of this heightened inflammatory response, there is significant host cell death and a diminished ability for the spinal cord to recover, and thereby, functional improvement is limited even after cell transplantation. However, if this harmful inflammatory response were to be suppressed immediately after the injury, a favorable environment for the grafted cells could be created, and moreover, it may be possible to perform cell transplantation in the acute phase.

The complement system has a very important role in initiating secondary damage during the acute phase in SCI. The complement proteins activate various neurotoxic cytokines and inflammatory cells, and this complex inflammatory cascade lead to the exacerbation of neural damage [10–13]. Thus, the inflammation process is principally triggered by the complements, and among these proteins, C5a has recently received attention as a target of anti-inflammatory treatment. C5a is a small glycoprotein (74 amino acids, about 11 kDa) and generated by the cleaving of complement C5 [12]. C5a is an anaphylatoxin, which causes inflammatory cytokine activation and leukocyte infiltration through the C5a receptor (C5aR) [14]. Consequently, C5aR antagonist (C5aRA) could be a target as a treatment for inflammatory reaction after SCI. Among C5aRA, PMX205 (hydrocinnamate- (OPdChaWR)) is often used in the field of CNS disease experiment because PMX205 is able to penetrate blood-brain barrier and blood-spinal cord barrier [15, 16], and it has been shown that PMX205 administration after SCI suppresses inflammatory cytokines and macrophage infiltration into the lesion, decreases secondary damage, and improves the recovery of locomotor function [17]. These studies indicate that administration of C5aRA can inhibit the inflammatory reaction during the acute phase of SCI and prepare the injured microenvironment for receiving transplanted NS/PCs.

The purpose of the current study is to investigate whether C5aRA improves the inflammatory environment and enables increased survival of grafted cells when transplanted during the acute phase after SCI. We also evaluated the impact of a combined therapy of hiPSC-NS/PCs transplantation and C5aRA on locomotor functional recovery.

This study is particularly important as it is the first study to our knowledge which demonstrates immunosuppressant enables efficient hiPSC-NS/PCs transplantation during acute phase after SCI by increasing survival of grafted cells.

Materials And Methods

Animals

Adult female SCID-Beige mice (8–10 weeks, 17–22 g), which are deficient in lymphocytes and NK cells, were provided by Charles River Laboratory. All animals were housed in a temperature- and humidity-controlled environment. All experimental procedures were approved by the ethics committee of Keio University (Assurance No. 13020) and were in accordance with the Guide for the Care and Use of Laboratory Animals (National Institutes of Health, Bethesda, MD, USA). The number of mice used in this study is shown in Supplemental File 1.

Spinal cord injury

The mice were anesthetized with ketamine (60 mg/kg) and xylazine (10 mg/kg) intraperitoneally. Laminectomy was performed at the 10th thoracic spinal vertebra (T10), and the dorsal surface of the dura matter was exposed. Moderate (70 kdyn) contusion injury was induced at the level of T10 using an Infinite Horizon impactor (Precision Systems and Instrumentation, Fairfax Station, VA), as previously described [18]. The muscles were sutured and the skin was closed with wound clips. After spinal cord injury, ampicillin (12.5 mg/kg) was administrated subcutaneously.

C5a ELISA

A total of 25 mice were used in this experiment; the injured mice were sacrificed at 4 times (n = 5 /each survival time) with 5 naive controls. The times included 1, 4, 7, and 14 days post-injury. The mice were anesthetized with ketamine (60 mg/kg) and xylazine (10 mg/kg) intraperitoneally and transcardially perfused with PBS. Six millimeters of spinal cord, centered at the lesion epicenter, were dissected and immediately frozen in liquid nitrogen. As a control, samples of naïve spinal cords were harvested using the same protocol (N = 5). Dissected spinal cords were homogenized in lysis buffer with phosphatase and protease inhibitors and centrifuged at 10000 × g for 5 min. The supernatants were collected and protein concentration was evaluated using a Bradford assay. C5a concentration was determined using the ELISA kit (R&D system) according to the manufacturer's instructions.

C5a receptor western blotting

A total of 16 mice were used in this experiment; the injured mice were sacrificed at 3 times (n = 4 /each survival time) with 4 naive controls. The times included 1, 4, and 7 days post-injury. Spinal cords centered at the lesion were dissected, as mentioned previously. Dissected spinal cords were homogenized in lysis buffer with phosphatase and protease inhibitors and centrifuged. The supernatant was dissolved in 4 × Laemmli sample buffer and heat-denatured at 95 °C for 5 min. As a control, samples of naïve spinal cords were harvested using the same protocol (N = 4). Samples were electrophoretically dissociated on 10% SDS-PAGE and transferred to membrane as described [19]. The membranes were blocked for 1 h at room temperature with block solution (Blocking one, nacalai tesque, Japan) and incubated with diluted C5aR antibody (1:1000, Rat, Bio-Rad, USA) at 4 °C overnight. After, the membranes were subjected to a reaction with HRP-conjugated anti-rat IgG at room temperature for 1 h. After the membranes were washed, the HRP activity was detected using an ECL kit. The image was scanned with the ImageQuant LAS4000 mini (GE Healthcare Life Sciences, USA), and the data were analyzed using ImageJ. β-actin (1:2000, Rat) was used as an internal control.

C5a receptor antagonist administration

We used PMX205 (Tocris Bioscience, England) as a C5a receptor antagonist, as previously reported [17]. Immediately after SCI, the mice randomly received PMX205(1 mg/kg body weight) [17] diluted in 1% ethanol or PBS containing 1% ethanol intraperitoneally once a day for 4 consecutive days, or until sacrificed.

quantitative RT-PCR

A total of 50 mice were used in this experiment; the injured mice were sacrificed at 4 times (C5aRA group; N = 4 each time point, PBS group; N = 5 each time point) with 5 naive controls. The times included 6 h, 12 h, 1 day, and 4 days after SCI. Six mm spinal cord sections centered at the lesion were harvested and immediately frozen in liquid nitrogen (C5aRA group; N = 4 each time point, PBS group; N = 5 each time point). Total RNA was isolated from the frozen spinal cords using the RNeasy Mini Kit (Qiagen Inc., Hilgen, Germany), in accordance with the manufacturer's instructions. As a control, total RNA was isolated from naïve spinal cords by the same protocol (N = 5). A reverse transcription reaction was performed using Reverse transcriptase kit (ReverTra Ace; Toyobo, Japan). RT-PCR was performed using primers specific to the genes of interest and TaqMan Fast Advanced Master Mix (Thermo Fischer Scientific, USA). The data were normalized to the expression level of β−actin (ACTB).

The following manufactured primers (Applied Biosystems; http://www.appliedbiosystems.com) against mouse DNA sequences were used: interleukin-1β (Il1β)-Mm00434228_m1, interleukin-6 (Il6)-Mm00446190_m1, TNF-Mm00443258_m1, ACTB-Mm02619580_g1.

mRNA-Seq

A total of 22 mice were used in this experiment; the injured mice were sacrificed at 4 times (C5aRA group; N = 2 each time point, PBS group; N = 2 each time point) with 2 naive controls. The times included 3 h, 6 h, 12 h, 1 day, and 4 days after SCI. Six mm spinal cord sections centered at the lesion were harvested and samples were processed for RNA preparation and library preparation for mRNA-SEq. Samples for mRNA-Seq were prepared using the TruSeq RNA Sample Prep Kit (Illumina) in accordance with the manufacture's instructions. As a control, samples were prepared from naïve spinal cords using the same procedure. The sequencing library was sequenced with the HiSeq 2500 (Illumina). Base-calling and chastity filtering were performed using Real-Time Analysis Software version 1.18.61 and raw reads were mapped to reference genome mm9 using Sailfish (v0.7.6) with default settings. All gene expression profiles were evaluated using Exatlas (https://lgsun.irp.nia.nih.gov/exatlas/). The extracted data were visualized with Morpheus (https://software.broadinstitute.org/morpheus).

Flow cytometry

After the mice were transcardially perfused with PBS, 6 mm spinal cord sections centered at the lesion were harvested (Neutrophils; N = 6 each group, Macrophage; N = 6 each group), digested with collagenase (Accumax; Innovative cell technologies, USA), and passed through a wire mesh screen (Sigma-Aldrich

Canada Ltd., Canada) to acquire a single-cell suspension. The cells were incubated on ice for 30 min with Fc blocker followed by an additional 30 min on ice with fluorescent antibodies. To exclude the dead cells, 7-AAD was added. Flow cytometric analysis was carried out using a FACS Verse (Becton Dickinson, USA), and the data were analyzed using Cell Quest software. The samples were immunolabeled with rat anti-CD11b-BV421 (1:200; BD Horizon, USA), rat anti-LY6G-PE (1:200; BD Horizon, USA) and rat anti-CD45-APC (1:200; BD Horizon, USA). Macrophages were defined as $CD45^{high}$ $CD11b^{+}$ LY6G- population, and neutrophils were defined as $CD45^{high}$ CD11b- $LY6G^{+}$ population.

Cell culture, lentivirus transduction

Cell culture of hiPSC (414C2) was performed as described previously, with subtle modifications [20]. Briefly, hiPSC grown on gelatin-coated (0.1%) tissue culture dishes were used for EB formation. EBs were then enzymatically dissociated into single cells and cultured in serum-free media hormone mix (MHM) for 10–12 days to allow the formation of neurospheres. Neurospheres were dissociated into single cells and cultured in the same method for passage. A lentivirus that expressed ffLuc, which is a fusion protein consisting of yellow variant of Aequorea GFP and a firefly luciferase [21] under the control of an EF promoter to enable the detection of the grafted cells in living mice and in fixed sections, was prepared [22], and transduced into hiPSC-NS/PCs as previously described [2].

Neural differentiation and immunohistochemistryDissociated third-passage ffLuc induced NS/PCs (1.0×10^5/well) were plated in poly-L-ornithine /fibronectin coated 8 well chamber slides (Thermo Fischer Scientific, USA), and PMX205 (20μ g/well) or PBS was added to each chamber. These cells were cultured in medium without growth factors at 37 °C under 5% CO2 for 14 days in total. Differentiated cells were fixed at 0.1M PBS containing 4% PFA and stained with the following primary antibodies overnight at 4 °C; anti-βIII Tubulin (mouse IgG2b, 1:300; SIGMA, T8660), anti-O4 (mouse IgM, 1:200; Thermo Fischer, MAB1326) and anti-GFAP (Rabbit, 1:2000; Proteintech, AG10423). These sections were incubated with Alexa Fluor 488-, 555-, or 647-conjugated secondary antibodies (Thermo Fisher Scientific, USA), and Hoechst 33258 (10 μg/ml, Sigma-Aldrich) as a nuclear counterstaining. All images were obtained using a fluorescence microscope (BZ 9000; Keyence Co., Japan) or a confocal laser scanning microscope (LSM 700; Carl Zeiss, Germany). For quantification of βIII Tubulin-, O4-, and GFAP-positive cells, five regions within the chamber were randomly selected and counted under × 40 magnification.

Cell transplantation

Four days after SCI, the mice were randomly and blindly divided into four groups based on their BMS score to ensure equivalent deficits across the groups: (PBS only group, C5aRA only group, PBS + transplantation (PBS + TP) group, C5aRA + transplantation (C5aRA + TP) group), and re-anesthetized with isoflurane. hiPSC- NS/PCs (5×10^5cells/2μ l) or PBS were transplanted into the lesion epicenter using a metal needle at a rate of 1 μl/minute (PBS only;N = 20, C5aRA only;N = 19, PBS + TP;N = 15, C5aRA + TP;N = 16). After transplantation, the skin was closed with wound clips and ampicillin (12.5 mg/kg) was injected subcutaneously. These mice were sacrificed 42 days after the SCI.

Bioluminescence imaging

A Xenogen-IVIS spectrum CCD optical macroscopic imaging system (PerkinElmer, USA) was used in vitro and in vivo for bioluminescence imaging (BLI) to confirm the survival of the transplanted hiPSC-NS/PCs, as described previously [23] .

In vitro, the hiPSC-NS/PCs were plated to 8-chamber wells and D-luciferin (1 mg/well) was then added to each well. The luminescent signal was detected immediately using a Xenogen-IVIS spectrum cooled charged-coupled device (CCD) optical macroscopic imaging system (Caliper Life Sciences, USA) (N = 3 each group).

In vivo, the mice were injected D-Luciferin (300 mg/kg body weight) intraperitoneally, and placed in a light-tight chamber 15 minutes after injection. The peak of the signal intensity was between 15 and 30 minutes after injection. The integration time was 5 seconds to 2 minutes, depends on the intensity of signals emitted from luciferase-expressing grafted cells. BLI signals were quantified in maximum radiance units (photons/second/centimeter squared/steradian photons/sec/cm^3/sr) and presented as log10 values (PBS + TP;N = 15, C5aRA + TP;N = 16).

Tissue Immunohistochemistry

The mice were anesthetized and transcardially perfused with 0.1 M PBS containing 4% PFA. Their spinal cords were harvested and immersed in 0.1 M PBS containing 4% PFA at 4 °C for 24 h. The tissues were transferred into 10% sucrose for 24 h followed by 30% sucrose for 24 h. The sections were embedded in Optimal Cutting Temperature compound (Sakura Finetek Japan, Japan) and immediately frozen by liquid nitrogen and stored at -30 °C. The tissues were sectioned at 20 μm in axial or 16 μm sagittal planes using a cryostat (Leica CM3050 S; Leica Microsystems, Germany). The sections were stained with the following primary antibodies overnight at 4 °C; anti-CD88(Rat, 1:400; Biorad, MCA2456), anti-C5R1 (Rabbit, 1:1000; Abcam, ab59390), anti-Iba1 (Rabbit, 1:400; WAKO, CDP0133), anti-LY6G (Rat, 1:1000; Novusbio, NBP200441), anti-GFP (Rabbit, 1:2000; MBL), anti-human nuclear antigen (mouse IgG, 1:100; Millipore, MAB4383), anti-human Nestin (Rabbit, 1:200; IBL: 18741), anti-Ki67 (rabbit IgG, 1:200; Leica, NCL-Ki67p), anti-pan-ELAVL (human IgG, 1:1000, a gift from Dr. Robert Darnell; The Rockefeller University, New York, USA)[24], anti-APC (mouse IgG2b, 1:200; Millipore, OP80), and anti-GFAP (Rabbit, 1:2000; Proteintech, AG10423). anti-CD88 and anti-C5R1 antibodies were used as a C5a receptor detection. These sections were incubated with Alexa Fluor 488-, 555-, or 647-conjugated secondary antibodies (Thermo Fisher Scientific, USA), and Hoechst 33258 (10 μg/ml, Sigma-Aldrich, USA) as a nuclear counterstaining. All images were obtained using a fluorescence microscope (BZ 9000; Keyence Co., Japan) or a confocal laser scanning microscope (LSM 700; Carl Zeiss, Germany). For Quantification of survival rate of grafted cells, anti-GFP (Rabbit, 1:2000; MBL) and Hoechst 33258 (10 μg/ml, Sigma-Aldrich, USA) as a primary antibody and the appropriate secondary antibodies were used. The images were captured at the lesion epicenter and 0.2, 0.4, 0.6, 0.8, and 1.0 mm rostral and caudal to the epicenter in axial sections using a fluorescence microscope (BZ 9000; Keyence Co., Japan), and the area of survived grafted cells was assessed by measuring the GFP positive areas on each cross section using ImageJ (https://imagej.nih-.gov/ij/). For quantification of APC-, GFAP-, pan-ELAVL-, Ki67-, Nestin- and OCT4-

positive cells, five regions within the area 2 mm rostral and caudal to the lesion epicenter were randomly selected and counted under × 63 magnification.

Locomotor function analysis

The hindlimb locomotor function analyses were evaluated using the Basso Mouse Scale (BMS) score at 0, 1, and 4 days as well as weekly after SCI, up to 35 days [25] (PBS only;N = 20, C5aRA only;N = 19, PBS + TP;N = 15, C5aRA + TP;N = 16). Motor coordination was evaluated using a rotating rod apparatus (Rotarod, Muromachikikai Co., Japan), which is composed of a 3 cm diameter plastic rod rotated at 20 revolutions per min. The mice were tested by monitoring the time spent on the rod (PBS only;N = 20, C5aRA only;N = 19, PBS + TP;N = 15, C5aRA + TP;N = 16). The gait performance of the mice was assessed through treadmill gait analysis (DigiGait system; Mouse Specifics, USA) (PBS only;N = 11, C5aRA only;N = 11, PBS + TP;N = 8, C5aRA + TP;N = 8).

Statistical analysis

All data are presented as means ± SEM. A Mann-Whitney U test was used to identify any significant differences between groups with respect to the results of flowcytometry and immunohistochemistry. One-way analyses of variance (ANOVA) followed by Tukey–Kramer tests for multiple comparisons were used to detect significant differences in stride length, stance angle, and rotarod score between the four groups. Two-way repeated-measures ANOVA followed by Tukey–Kramer tests were used for the others. For all statistical analyses, the significance level was set at $p < 0.05$. Microsoft Excel 2016 and IBM SPSS Statistics (ver. 25) were used for all calculations.

Results

C5a and C5aR expression in the spinal cord after injury

To investigate expression of C5a in the spinal cord, we evaluated the protein levels of C5a before and after injury at different time points. The results revealed that C5a protein levels were significantly increased 1 day after injury (8.16 ± 0.83 pg/μg protein) and maintained the high amount of protein compared with the one prior to injury(2.43 ± 0.45 pg/μg protein) at 4 (6.69 ± 0.99 pg/μg protein), 7 (9.72 ± 0.85 pg/μg protein) and 14 days (8.24 ± 0.98 pg/μg protein) (Fig. 1A).

Next, we investigated the level and distribution of C5a receptor expression in the injured spinal cord using western blotting and immunohistochemistry. Western blotting showed that the level of C5a receptor expression were upregulated at 1 day after injury, then decreased at days 4 and 7 (1 day: 14.02 ± 01.54, 4 days: 4.18 ± 0.85, 7 days: 2.08 ± 0.16) (Fig. 1B-C). To examine what types of cells expressed the C5a receptor, immunohistochemical analysis was performed for the injured spinal cord. At 1 and 4 days after SCI, $Iba1^+$ macrophages, activated microglia and some $LY6G^+$ neutrophils expressed C5a receptor (Fig. 1D-E). These results suggest that C5a protein was produced immediately after SCI, and that C5a receptors were expressed in inflammatory cells such as macrophages, activated microglia and neutrophils during the acute phase after SCI.

Administration of a C5aR antagonist reduced the inflammatory response after SCI

To investigate the influence of C5aRA on the production of inflammatory cytokines in spinal cord tissue after SCI, C5aRA (PMX205, 1 mg/kg body weight) were injected into the mice immediately after SCI and the expression of cytokines in spinal cord tissue was evaluated using quantitative RT-PCR. IL-1β and IL-6 were significantly reduced by the antagonist administration compared to PBS administration at 6 hours (IL-1β: 60.9 ± 11.5 vs 30.5 ± 5.2, IL-6: 1228.8 ± 380.1 vs 112.8 ± 25.0, $p < 0.05$) and 12 hours (IL-1β: 108.5 ± 23.1 vs 36.1 ± 2.4, IL-6: 433.3 ± 102.7 vs 177.2 ± 3.6 $p < 0.05$) (Fig. 2A-B). TNFα significantly reduced at 3 hours (32.6 ± 6.9 vs 10.6 ± 2.2, $p < 0.05$) and 6 hours (21.5 ± 4.1 vs 5.5 ± 1.2, $p < 0.05$) after SCI (Fig. 2C). Next, mRNA-Seq analysis was performed to analyze the influence of C5aRA on the gene expression profile in the injured spinal cord. mRNA sequencing revealed that the expression of genes associated with inflammatory cytokines was suppressed by administering C5aRA at 12 hours after SCI (Fig. 2D). With regard to the apoptotic and necroptotic markers, administration of C5aRA also downregulated several apoptotic (Caspase8 and Pidd1) and necroptotic (RIPK3 and MLKL) markers 4 days after injury (Fig. 2E).

To investigate whether C5aR inhibition blocked infiltration of neutrophils and macrophages after SCI, flow cytometric analysis was performed. The results showed that administration of C5aRA reduced the number of $CD45^+/LY6G^+$ neutrophils and $CD45^+/CD11b^{high}$ macrophages in the spinal cord compared to administration of PBS (Neutrophils: 151 ± 12 cells/2×10^5events vs 270 ± 25 cells/2×10^5events Macrophage: 1198 ± 111 cells/2×10^5events vs 2128 ± 173 cells/2×10^5events, $p < 0.05$) (Fig. 2F-G). These findings indicate that the acute inflammatory response after SCI occurs through C5a receptor activation, and inhibition of the C5a-C5aR axis suppressed inflammatory cytokine production and infiltration of inflammatory cells.

C5aR antagonist injection does not affect cell differentiation in vitro.

To investigate the influence of C5aRA on hiPSC-NS/PCs, we evaluated the differentiation profiles and expression of the C5a receptor in these cells. The hiPSC- NS/PCs with or without C5aRA presented similar differentiation patterns; β III-tubulin$^+$ neurons (49.38 ± 3.87% vs. 44.87 ± 5.14%, $p = 0.275$), $GFAP^+$ astrocytes (1.01 ± 1.01% vs. 2.53 ± 1.29%, $p = 0.246$), and $O4^+$ oligodendrocytes (0 ± 0% vs. 0 ± 0%, $p = 1.00$) (Fig. 3A-C), and there were no significant differences in all cell types between the groups (Fig. 3C). In addition, the hiPSC-NS/PCs rarely expressed C5a receptor (Fig. 3D). These findings suggest that C5aR antagonist did not change the differentiation properties of hiPSC-NS/PCs.

Inhibition of C5aR increases the survival rate of grafted NS/PCs

To examine the effect of C5aRA on the survival rate of grafted cells, we assessed the luminescence of the grafted cells using BLI (Fig. 4A). The BLI analyses demonstrated that the luminescence of the grafted

cells in the C5aRA + TP group was significantly higher than that of grafted cells in the PBS + TP group at 14 days post-SCI and thereafter (C5aRA + TP vs PBS + TP; day14: 4.86 ± 0.71 E + 07 vs 3.24 ± 0.51 E + 07, day21: 4.93 ± 0.94 E + 07 vs 2.64 ± 0.51 E + 07, day28: 6.85 ± 0.94 E + 07 vs 2.39 ± 0.46 E + 07, $p < 0.05$) (Fig. 4B).

We also evaluated grafted cell survival using immunohistochemistry. In the C5aRA + TP group, GFP^+ area had a tendency to increase compared to PBS + TP group in each axial section (Fig. 4C), and total volume of GFP + areas was significantly larger compared to the PBS + TP group (C5aRA; 0.29 ± 0.05mm^3, PBS; 0.13 ± 0.03mm^3, $p < 0.05$) (Fig. 4D). These results indicate that inhibition of C5aR improves the survival and proliferation of hiPSC-derived NS/PCs after the transplantation.

Administration of C5aR antagonist does not affect cell differentiation in vivo

In order to evaluate the neural differentiation and proliferation of the grafted cells in vivo, we performed immunohistochemical analyses using antibodies specific for human nuclear antigen (HNA) and cell type specific markers. Immunohistochemistry revealed that the HNA positive grafted cells in the C5aRA + TP and PBS + TP groups differentiated into pan-$ELAVL^+$ neurons (37.0 ± 3.6% and 36.8 ± 3.9%, $p = 0.827$), $GFAP^+$ astrocytes (46.2 ± 6.0% and 33.1 ± 3.2% $p = 0.127$), and APC^+ oligodendrocytes (8.0 ± 1.2% and 11.6 ± 1.5%, $p = 0.127$) (Fig. 5A-B). The differentiation rates of neurons, astrocytes, and oligodendrocytes did not significantly differ between the C5aRA + TP and PBS + TP groups (Fig. 5C). The proportions of $Ki67^+$ cells (2.5 ± 0.7% vs. 2.7 ± 0.8%, $p = 0.43$) and $OCT3/4^+$ cells (0 ± 0% vs. 0 ± 0%, $p = 1.0$) were not significantly different between the two groups (Fug5C).

C5aR inhibition at the acute phase improved locomotor function

We assessed hindlimb locomotor function using the BMS score, Rotor-rod test, and treadmill gait analysis with the Digigait system. The average BMS scores of the C5aRA + TP group were significantly higher than those of the PBS group at day 28 (3.5 ± 0.3 vs. 2.4 ± 0.2, $p < 0.05$) and day 35 (3.8 ± 0.3 vs. 2.4 ± 0.2, $p < 0.05$) after SCI (Fig. 6A). In the Rotor-rod test, which was evaluated at day 35, the running time of mice in the C5aRA + TP group was significantly longer than the time in the other groups (C5aRA + TP:17.5 ± 4.0sec; PBS + TP: 9.7 ± 1.3sec; C5aRA: 9.5 ± 1.2sec; PBS: 7.5 ± 0.8sec, $p < 0.05$) (Fig. 6B). Using the treadmill Digigait system to analyze gait performance, only the mice in the C5aRA + TP groups showed significant improvement in stride length (C5aRA + TP: 4.17 ± 0.16 cm; PBS + TP: 3.74 ± 0.16 cm; C5aRA: 3.65 ± 0.08 cm; PBS: 3.43 ± 0.13 cm, $p < 0.05$)) or paw angle (C5aRA + TP: 23.5 ± 3.3°; PBS + TP: 32.8 ± 3.8° C5aRA: 29.1 ± 3.7°; PBS: 41.9 ± 4.5°) compared with the PBS group ($p < 0.05$) (Fig. 6C-D).

Discussion

As shown previously, anti-inflammatory treatment is needed to enable efficient cell transplantation therapy during acute phase because transplanted cell rarely survives in an inflammatory environment in spinal cord during acute phase after SCI, but effective immunosuppressant which increase grafted cell survival has not been found. Therefore, we hypothesized that C5aRA could be a novel immunosuppressant which strongly suppressed inflammatory reaction after SCI and enables efficient hiPSC-NS/PCs transplantation during acute phase. The present study demonstrates that C5aRA administration after SCI significantly reduces upregulation of IL-1β, IL-6 and TNFα, as well as the infiltration of neutrophils and macrophages. C5aRA also downregulated the expression of inflammatory cytokines and apoptosis markers, which was demonstrated by RNA sequence analysis. The combined therapy of hiPSC-NS/PCs transplantation and C5aRA decreased the grafted cell death compared to cell transplantation monotherapy. Consequently, this combined therapy significantly improved locomotor function after SCI. Our findings show that C5aRA could be a promising medication to enhance the efficacy of hiPSC-NS/PCs when transplanted during the acute phase of SCI.

It is well known that inflammatory reactions play a critical role in the exacerbation of secondary damage in the SCI microenvironment [6, 8, 10, 26]. In particular, IL-1β and TNFα contribute to upregulation of several inflammatory mediators, recruiting neutrophils and macrophages, and resulting in the apoptosis of neurons and oligodendrocytes after SCI [27–31]. IL-6 also promotes cytotoxic macrophage infiltration into the lesion and increases secondary damage [32, 33]. Neutrophil and macrophage infiltration into the lesion area is a detrimental factor for resident cell survival and functional recovery after SCI [34–37]. A previous study showed that these inflammatory cytokines and cells were regulated by the complement C5a-C5aR axis, and that inhibition of these factors decreased resident cell death, thus improving functional restoration after SCI [17]. In the present study, we demonstrated that administration of C5aRA reduced secretion of various cytokines, infiltration of inflammatory cells, and apoptosis of resident cells in the spinal cord, suggesting that the C5aRA comprehensively suppressed main factors which contribute to secondary damage after SCI. Therefore, the intervention using C5aRA could prevent the expansion of secondary damage and create a hospitable environment for cell survival after transplantation in SCI.

Previous studies have revealed that the acute inflammatory reaction after CNS injury contributes to rejection of grafted cells in spinal cord tissue [38–40]. To address this challenge, several researchers have tried to improve cell engraftment by inhibiting acute inflammatory factors, but few studies have reported successful improvement in cell survival after SCI. For example, depletion of neutrophils was found to decrease the astrogliosis of the transplanted cells, but did not change the survival rate [41]. A reduction in macrophages also failed to favorably affect cell engraftment [37]. It is inferred from those results that the blockade of a single inflammatory factor cannot decrease grafted cell death. Therefore, a strong immunosuppressant capable of blocking several inflammatory reactions after SCI is indispensable for the improvement of cell survival. C5aRA was an efficacious drug against rejection of hiPSC-NS/PCs transplantation because this medication was able to suppress multiple inflammatory cells, and our results demonstrated amelioration of the cell survival rate. We can deduce from this result that C5aRA is more effective for improving the efficacy of cell transplantation into the injured spinal cord than other

immunosuppressants, because the C5a-C5aR axis regulates several inflammatory reactions in the acute phase after SCI [12].

Our results demonstrated that, compared to NS/PC transplantation only, cell engraftment with C5aRA administration decreased cell death and maintained the number of cells, which contributed to functional restoration after SCI. In other words, the intensive inflammatory reaction during the acute phase of SCI did not allow transplanted cells to survive sufficiently and exert their efficacy when C5aRA was not used. Although the in vivo differentiation profiles were comparable between groups with or without the C5aRA (Fig. 5), our results indicate that it is necessary to secure a certain number of survived transplanted cell for the differentiated cells to play functional roles. By using C5aRA, it is inferred that more NS/PCs differentiated into axons to construct functional circuits, or differentiated into oligodendrocytes for remyelination, and these mechanisms led to functional recovery [2, 42, 43]. If cell transplantation is possible in the acute phase of injury, earlier interventions could become feasible and bring the benefits of regenerative medicine to the SCI field.

For the transplantation studies using human-derived cells, immunodeficient animals were employed to avoid rejection of the grafted cells. For example, NOD-SCID mice which generally used in transplantation experiment lacked lymphocytes and impaired innate immune system such as neutrophils, macrophage and complement[23, 43–46]. However, NOD-SCID mice are not suitable for our current study because normal activation of the complement system is necessary to evaluate the effect of C5aRA. Immunocompetent mice are also not appropriate for the present study owing to lymphocytes, which strongly reject xenograft [40, 47]. In this study, we selected SCID-Beige mice, which lack lymphocytes but maintain a functional complement system. We demonstrated that the complement system was activated normally after SCI in SCID-Beige mice, and we could perform evaluating the influence of C5aRA on the grafted cells and locomotor function recovery. Thus, the SCID-Beige mouse was a reasonable tool to evaluate the impact of complements on the transplanted cells in SCI.

Importantly, as it is known that T cells and NK cells can express C5a receptor[48, 49], this study using SCID-Beige mice might be insufficient to accurately evaluate the effect of C5aRA against cell transplantation after SCI. Therefore, further study using immunocompetent mice with lymphocyte depressants are necessary to enhance our understanding.

In this study, we showed the efficacy of the combined therapy C5aRA and hiPSC-NS/PCs transplantation for SCI in SCID-Beige mice. However, in order to apply this treatment in a clinical setting, it is necessary to perform further studies using C5aRA, which has already been clinically applied, instead of PMX205 that is not approved for marketing [16]. For example, CCX168 has completed phase trials [50] and is one of the most advanced C5aRA in clinical development. However, because CCX168 is effective only in human C5aR, further studies using human C5a knock-in mice are necessary.

Conclusion

The present study demonstrates that the administration of C5aRA suppresses the inflammatory response during the acute phase of SCI. This beneficial effect led to improved transplanted hiPSC-NS/PCs survival rates as well as the enhancement of motor functional restoration. These findings suggest that administration of C5aRA makes it possible to transplant hiPSC-NS/PCs during the acute phase of SCI. This work opens a potentially novel strategy wherein the transplantation of neural stem cells could be combined with early decompressive surgery as a one stage treatment for severe SCI.

Abbreviations

BLI
bioluminescence imaging; BMS:Basso-Beattie-Bresnahan; CCD:Charge Coupled Device; C5aRA:C5a receptor antagonist; CD:cluster of differentiation; EB:embryoid body; ECL:Enhanced chemiluminescence; ELISA:Enzyme-linked immunosorbent assay; GFAP:Glial fibrillary acidic protein; HRP:Horseradish peroxidase; IL:Interleukin; IVIS:In vivo imaging system; hiPSC-derived NS/PCs:human induced pluripotent stem cell derived neural stem/progenitor cells; PBS:Phosphate-buffered saline; qPCR:Quantitative polymerase chain reaction; RHI:Rump-height Index; RIP3:Receptor-interacting serine-threonine kinase 3; RNA:Ribonucleic acid; SCI:Spinal cord injury; SDS-PAGE:Sodium dodecyl sulfate Polyacrylamide gel electrophoresis; SEM:standard error of the mean; TNF:Tumor necrosis factor; TP:Transplantation

Declarations

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Availability of data and materials

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

Authors' contributions

R.S., N.N., K.M. M.F., M.N. and H.O. designed the project. R.S. performed most of the experiments and acquired and analyzed the data. R.S. and N.N. wrote the manuscript. Technical assistance and experimental support were provided by N.N., S.I., K.K., S.N., J.K., and M.M. Supervision of the overall project and preparation of the final manuscript were performed by N.N., S.N., K.M., J.K., M.F., M.M., M.N. and H.O.

Ethics approval

All animal experiments were performed in accordance with guidelines fromthe Keio University Ethics Committee(Assurance No. 13020).

Consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interestsrelated to the present study.

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Figures

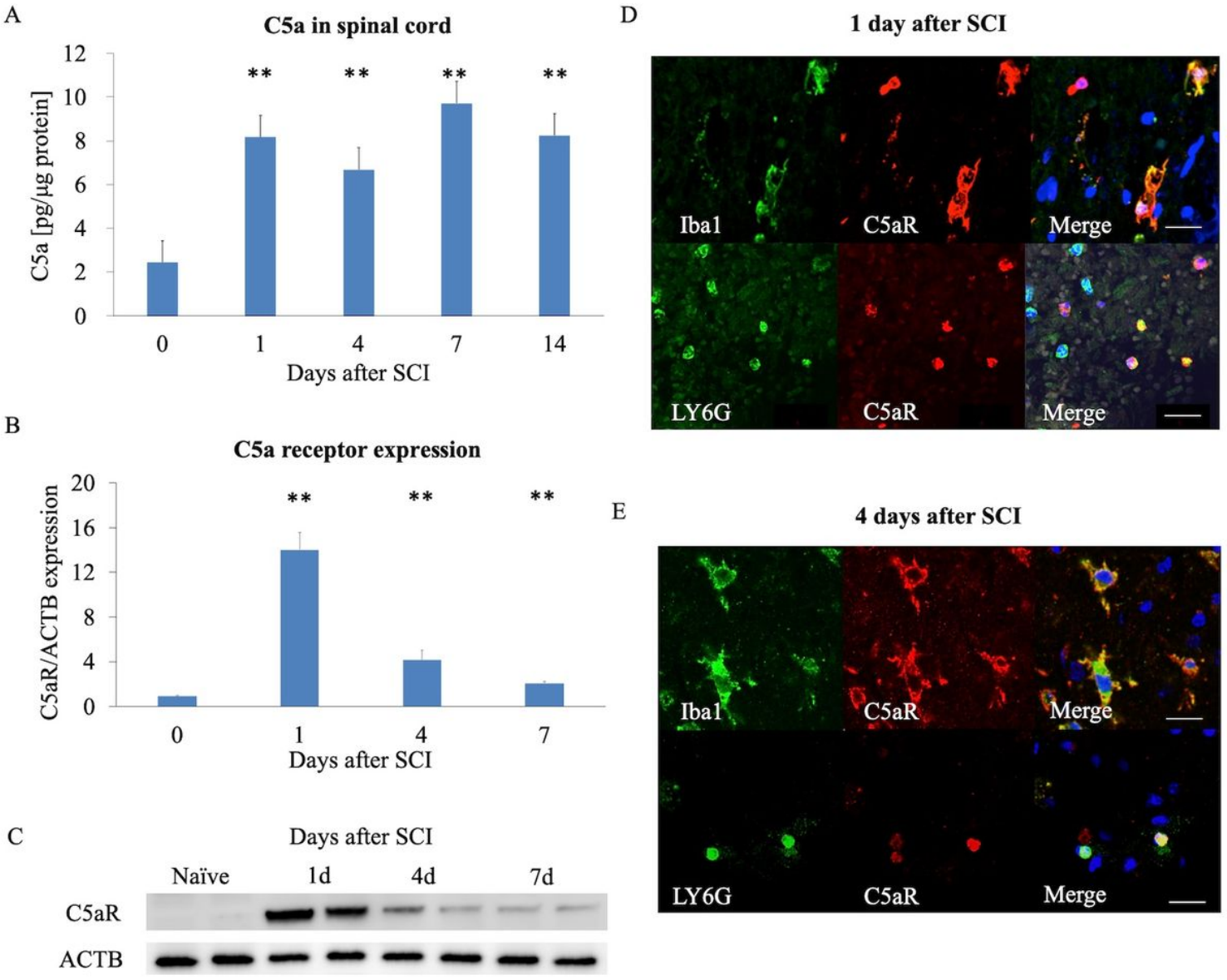

Figure 1

Determination of C5a production and C5a receptor expression after spinal cord injury A: ELISA analysis of C5a production at 0, 1, 4, 7, and 14 days after spinal cord injury. **; $p<0.05$ B: Western blot analysis of C5a receptor expression following spinal cord injury at 0, 1, 4, and 7 d. **; $p<0.05$ C: Representative Western blots demonstrating C5aR expression at 0, 1, 4, 7 d after SCI. D-E: Representative images of sagittal sections stained for Iba1, LY6G and C5aR at 1 and 4 d after SCI. Scale bars;20µm.

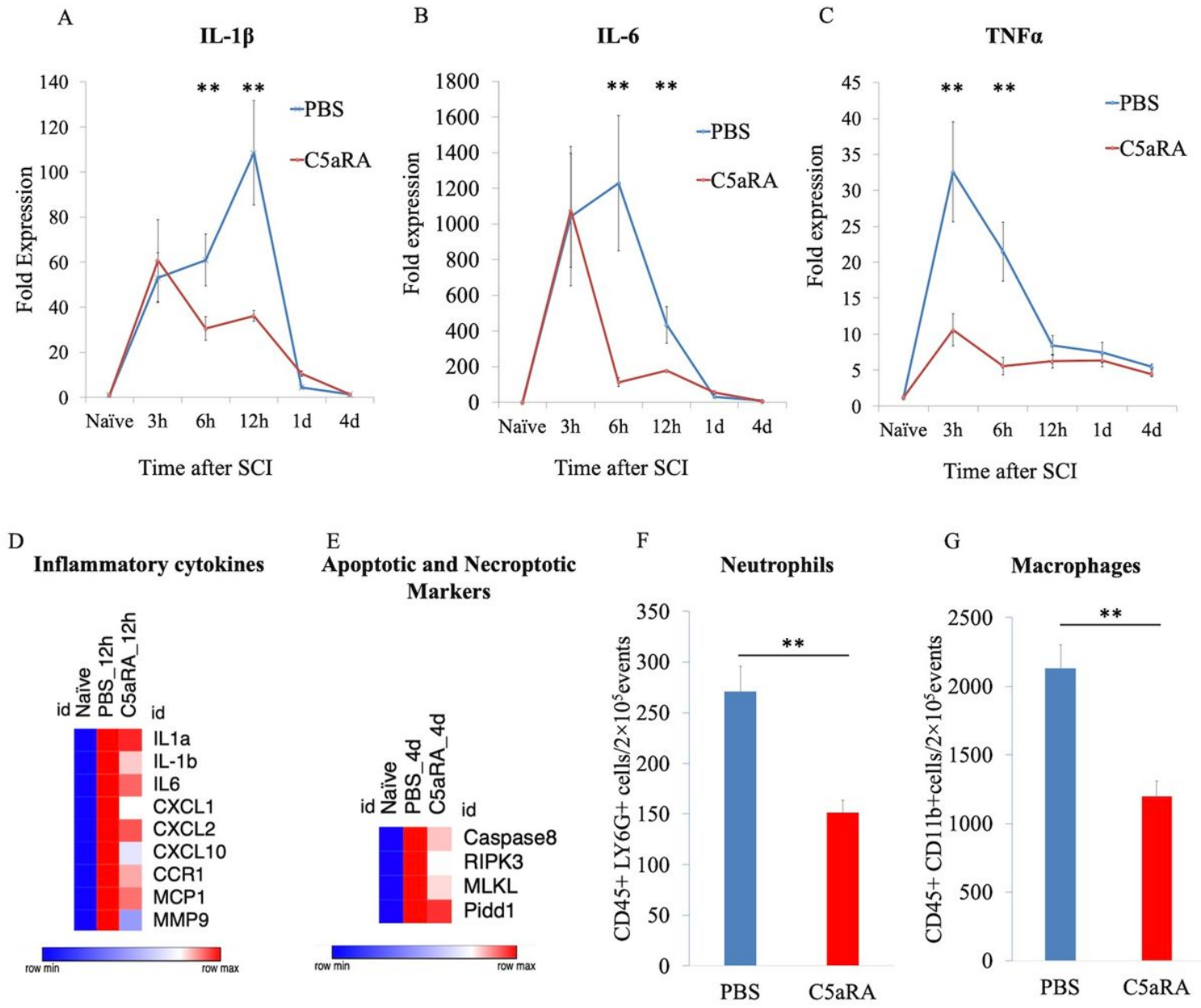

Figure 2

Inflammatory reaction after spinal cord injury with C5aR antagonist. A-C: Inflammatory cytokine (IL-1β, IL-6, TNFα) activation at 3h, 6h, 12h, 1d, and 4d after SCI by RT-PCR. **; p<0.05 D: Analysis of inflammatory cytokines at 4d after SCI by RNA sequencing. Heat map of expression profiles for the 9 significant genes involved in inflammation as assessed by RNA-sequencing. E: Analysis of apoptotic necroptotic markers at 4d after SCI by RNA sequences. Heat map of expression profiles for the 4 significant genesinvolved in apoptotic and necroptotic markers as assessed by RNA-sequencing. F-G: Analysis of neutrophils and macrophages at 4d after SCI using flow cytometry. **; p<0.05

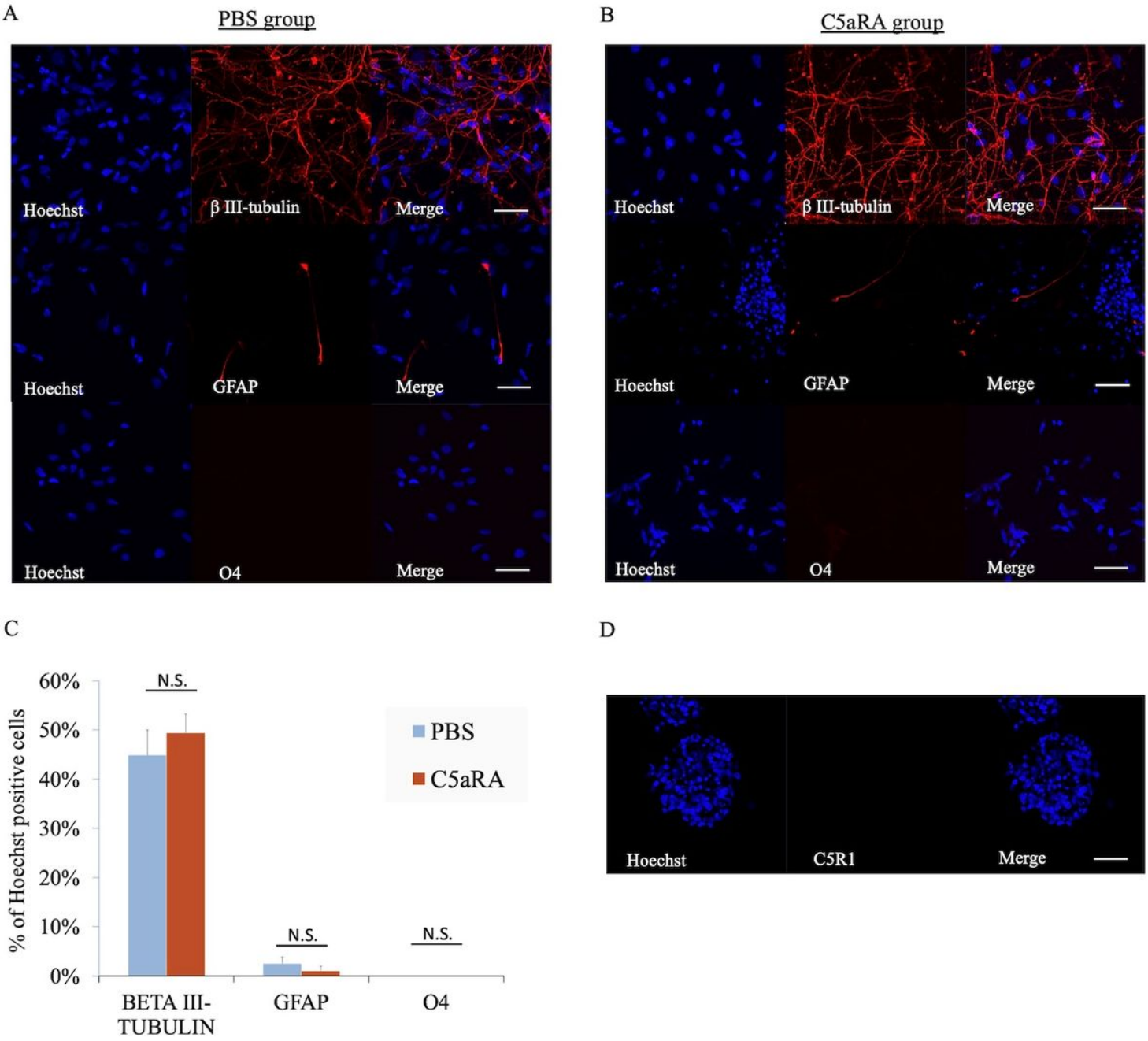

Figure 3

hiPSC-NS/PCs differentiation and proliferation in vitro. A-B: Representative images of hiPSC-NS/PCs stained with O4 (oligodendrocytes), GFAP (astrocytes), and □□III-tubulin(neurons). Scale bars; 40μm. C: Percentage of cell-type-specific marker positive cells among Hoechst+ cells. NS: Not Significant D: Representative images of C5a receptor expression of hiPSC-NS/PCs in vitro. Scale bars; 40μm.

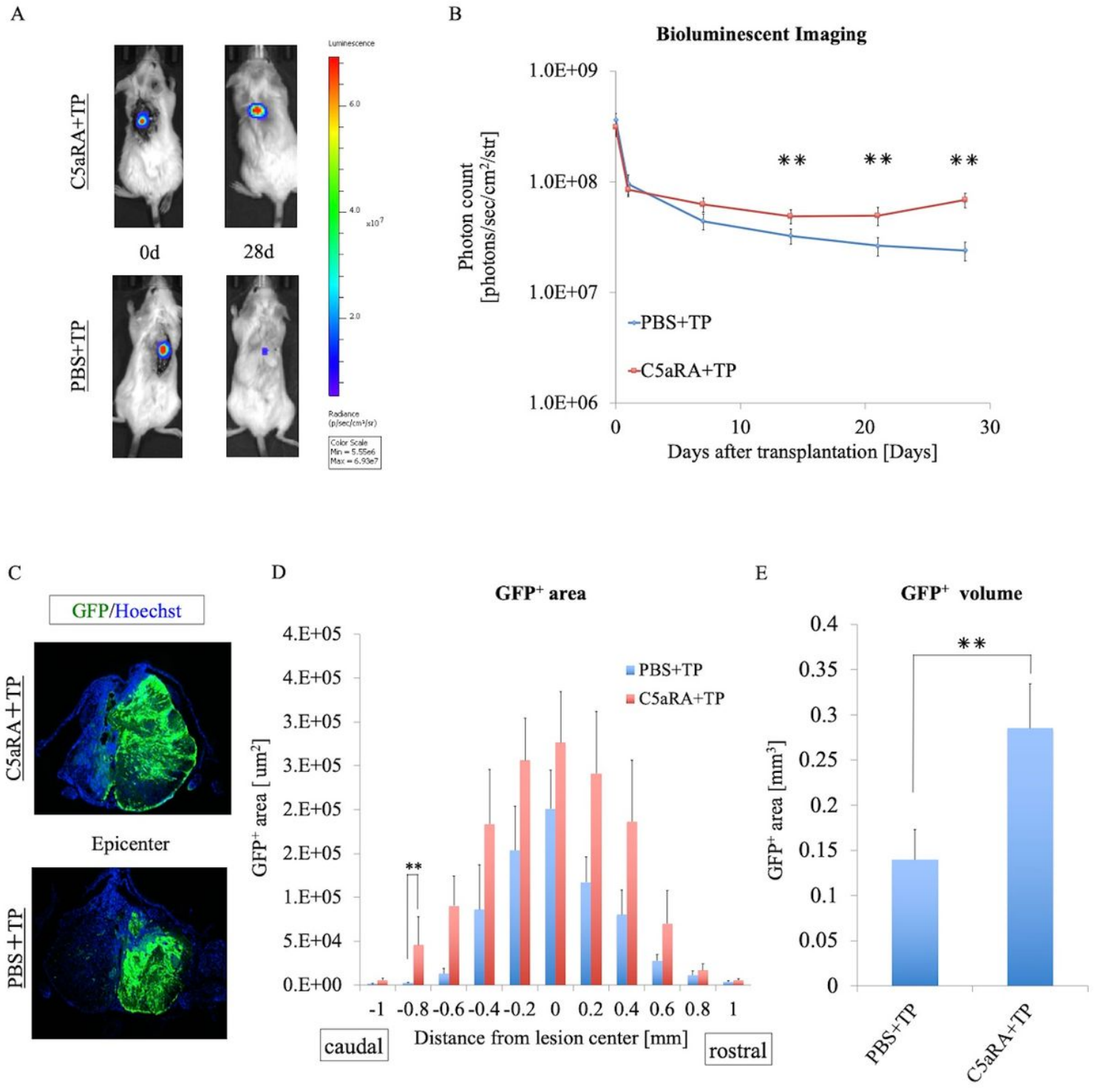

Figure 4

Survival rate of the transplanted cells. A-B: Representative in vivo BLI of PBS+TP and C5aRA+TP group at 0d and 28d after transplantation. **; p<0.05 B: Quantification of photon count in both PBS+TP and C5aRA+TP groups. C: Representative images of GFP+ area at epicenter of the lesion. D-E: Quantification of GFP+ area and volume. **; p<0.05

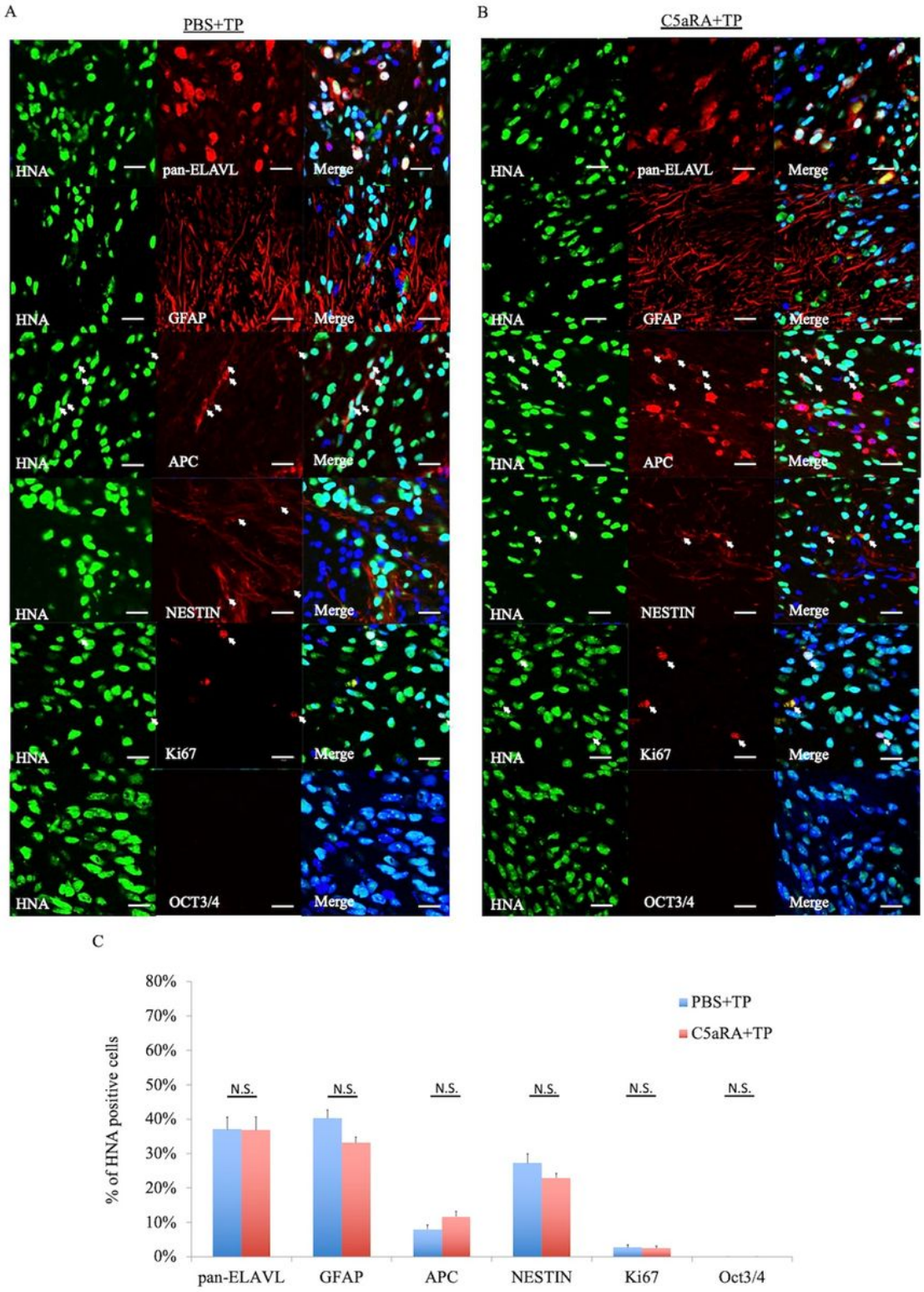

Figure 5

Transplanted hiPS-NS/PCs differentiation and proliferation. A-B: Representative images of HNA+ transplanted cells labeled with APC (oligodendrocytes), GFAP (astrocytes), pan-ELAVL(neurons), NESTIN (neural stem cells), Ki67, and OCT4. C: Percentage of cell-type-specific marker positive cells among HNA+ transplanted cells. NS; Not Significant

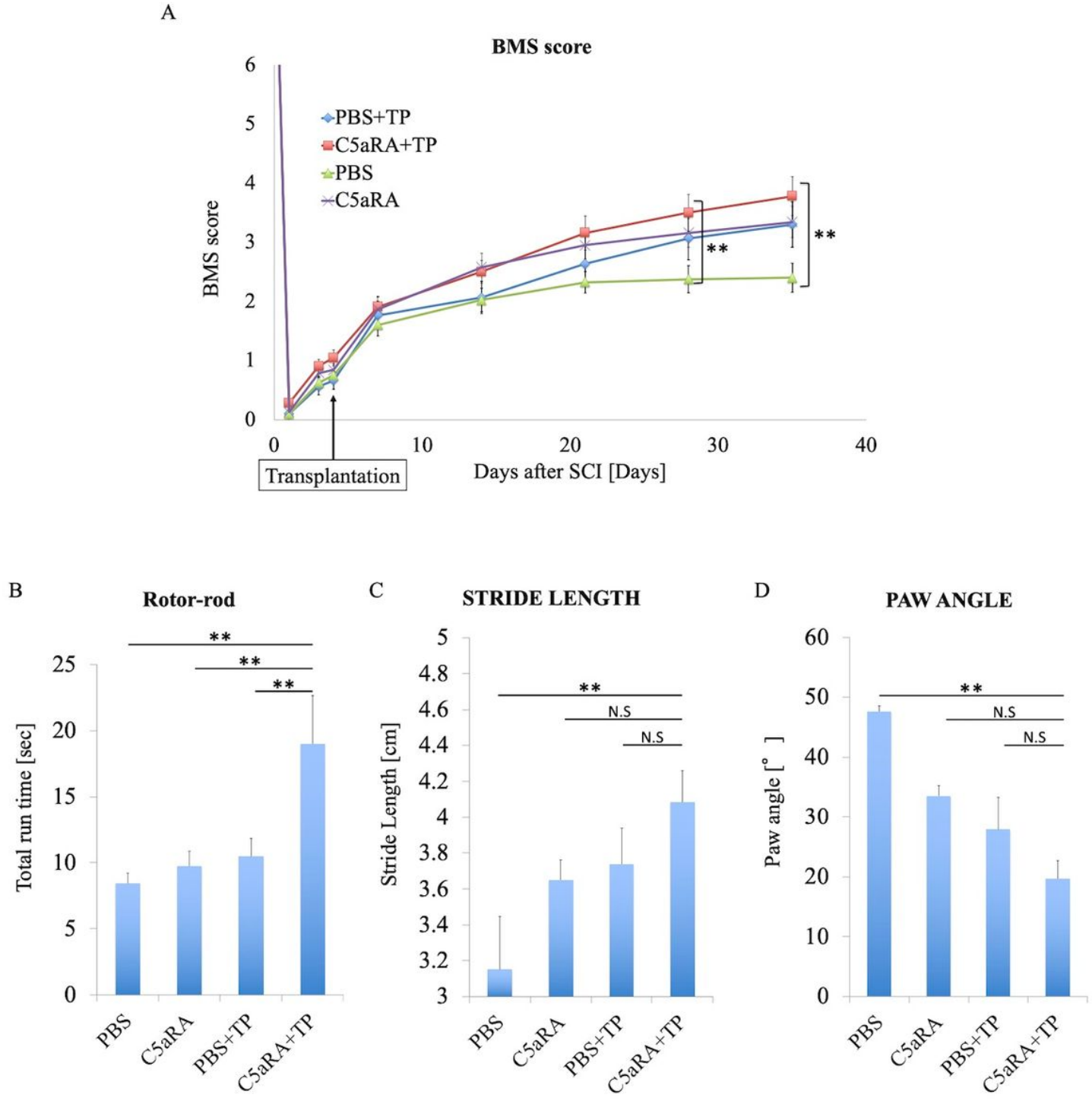

Figure 6

Hindlimb motor function after transplantation A: Hindlimb motor function was assessed weekly with the BMS score.**;p<0.05. B: Total run time on the Rotor-rod in each group at 35 days after SCI. **;p<0.05 C-D: Stride length and paw angle acquired from Treadmill gait analyses using the DigiGait System in each group at 35 days after SCI. **;p<0.05

Supplementary Files

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