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Bone-Marrow-Derived Mononuclear Cells Relieve Neuropathic Pain after Spinal Nerve Injury in Mice

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Treating neuropathic pain is a critical clinical issue. Although numerous therapies have been proposed, effective treatments have not been established. Therefore, safe and feasible treatment methods are urgently needed. In this study, we investigated the therapeutic effects of autologous intrathecal administration of bone-marrow-derived mononuclear cells (MNCs) on neuropathic pain. We generated a mouse model of neuropathic pain by transecting the spinal nerve and evaluated neuropathic pain by measuring the mechanical threshold in the following 14 days. Mice in the MNC injection group had a higher mechanical threshold than those in the buffer group. We assessed the effect of MNC treatment on the dorsal root ganglia and spinal cord by immunohistochemistry, mRNA expression, and cytokine assay. The migration and accumulation of microglia were significantly suppressed in the MNC group, and the mRNA expression of inflammatory cytokines such as interleukin (IL)-6, IL-1β, and tumor necrosis factor alpha (TNF-α) was markedly downregulated. Furthermore, MNC administration tended to suppress various cytokines in the cerebrospinal fluid of the model mice. In conclusion, our results suggest that intrathecal injection of MNCs relieves neuropathic pain and might be a promising cell therapy for the treatment of this condition.

INTRODUCTION

Neuropathic pain is one of the most frequent uncontrollable diseases in daily medical care,[1,2] with a prevalence ratio of approximately 1%–7% in the general population.[3] This condition is primarily caused by mechanical compressive stimulation or inflammation of nerve roots due to spinal nerve damage, such as intervertebral disc herniation or nerve root extraction injury.[4–6] Nerve injuries inducehyperalgesia and allodynia, which are representative symptoms of neuropathic pain.[2,7,8]

There are various therapeutic approaches to treat patients with neuropathic pain: physiological therapy, including exercise, electronic stimulation, and rehabilitation; drug therapy, such as anti-inflammatory analgesics, cell excitation inhibitors, and nerve blocker injection with anesthetics; and surgical therapy.[2,9,10] Different therapies have been selected in the clinical scene. However, satisfactory guidelines for the treatment of neuropathic pain are still lacking. Therefore, new and effective therapies are required.

Recently, it was demonstrated that the immune system is deeply involved in the pathogenesis of neuropathic pain.[11] Immune cells such as the monocyte/macrophage lineage and microglia, as well as the cytokines produced by these cells, induce the activation of ion channels and the secretion of neurotransmitters related with neuropathic pain in the dorsal root ganglia (DRGs) and spinal cord.[11–14] Inflammatory cytokines such as interleukin (IL)-6, IL-1β, and tumor necrosis factor alpha (TNF-α) were reported to be upregulated and mediate neuropathic pain in a model mouse.[15–17]

We previously tested different gene therapies and molecular targeting drugs for neuropathic pain, focusing on specific molecules such as TNF-α in animal models.[16,17] However, inhibiting only one molecule resulted in limited effects because many cytokines are involved in the pathogenesis of neuropathic pain. In addition, there is a significant hurdle to ensure safety when viral vectors or molecular targeting drugs are employed in humans.[18]

We previously reported that intrathecal injection of autologous bone-marrow-derived mononuclear cells (MNCs) is a safe and effective treatment for spinal cord injury, and that it suppresses inflammatory cytokines.[19] The technique of MNC isolation and administration had been previously established as a treatment for spinal cord injury.[19–22] However, there were no reports about the effect of intrathecal injection of bone-marrow-derived MNCs on neuropathic pain. Therefore, in this study, we focused on this association. We treated neuropathic pain in a model mouse with bone-marrow-derived MNCs to observe the therapeutic effects and explore the possibility of a clinical application.

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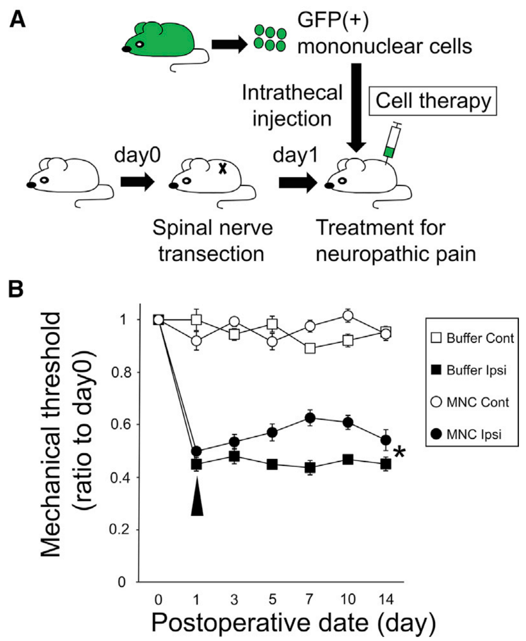

Figure 1. Effects of Bone-Marrow-Derived Mononuclear Cells for Neuropathic Pain

(A) Schematic representation of cell therapy for neuropathic pain. Spinal nerve transection (SNT) was performed on day 0 at the left side of the spinal nerve, and GFP-positive mononuclear cells (MNCs) were intrathecally administrated on day 1. (B) The mechanical threshold for hyperalgesia was measured on the ipsilateral (Ipsi) and contralateral (Cont) sides in mice administered buffer or MNCs for 14 days after SNT. White circles show the Cont side of the MNC group, black circles show the Ipsi side of MNC group, white squares show the Cont side of the buffer group, and black squares show the Ipsi side of the buffer group (n = 20 for each group). An arrowhead indicates the time of MNC or buffer injection. Data are represented as the mean ± SE. *p < 0.01 as compared with the buffer Ipsi and MNC Ipsi group. GFP, green fluorescent protein.

RESULTS

Effects of Bone-Marrow-Derived MNCs on Neuropathic Pain Induced by Spinal Nerve Transection

The mouse model of neuropathic pain was generated by spinal nerve transection (SNT) on the left side of the spinal nerve at the level of the fifth lumbar (L5) vertebra (ipsilateral side) on day 0 (Figure 1A). The right side of the L5 spinal nerve was exposed and closed without transection as a contralateral side. After confirmation of the SNT-induced severe mechanical hyperalgesia in the ipsilateral hind paw on day 1, bone-marrow-derived, green fluorescent protein (GFP)-positive MNCs obtained from transgenic (Tg) mice were intrathecally injected into SNT mice on the same day (Figures 1A and 1B).

The hind paw withdrawal thresholds against mechanical stimulation were measured on both sides of the MNC-injected mice and control buffer-injected mice for 14 days (Figure 1B). On day 1, the mechanical threshold in the ipsilateral side of the buffer and MNC groups was significantly lower than that of the respective contralateral sides before injection of the buffer or MNCs. After day 1, the threshold of the MNC group showed partial remission, whereas the threshold of the buffer group remained constant for 14 days (Figure 1B). Besides, any impairment of motor function and worsening of hyperalgesia did not occur by the injection procedure.

Distribution of Transplanted MNCs in the Neuronal Tissues of the Neuropathic Mouse Model

We performed a histological analysis of MNC-injected mice to investigate how the transplanted MNCs were distributed in the neuronal tissues. The GFP signal, corresponding to GFP-positive injected MNCs, was examined in the spinal cord and DRGs at the level of the fourth lumbar (L4) and L5 vertebrae of the MNC-injected mice on day 14 (Figure 2). GFP-positive cells were not recognized in the spinal cord (Figure 2A), but in the L4 and L5 DRGs on the ipsilateral side (Figures 2B and 2C). MNCs mainly accumulated at the L5 DRGs, where the spinal nerve had been transected, and to a lesser extent, at the L4 DRGs in the ipsilateral side of MNC-injected mice (Figure 2D). No GFP-positive cells were observed in the contralateral side, either in the spinal cord or DRGs (Figure 2). These results indicated that bone-marrow-derived MNCs accumulated at the injury area and that a small number migrated to the L4 DRGs.

MNCs Suppressed Microglial Migration in Ipsilateral L5 DRGs after SNT

To better understand the mechanisms underlying the effect of MNC injection, we investigated the migration of microglia, which is intimately related to inflammation and injury, in the regions where GFP-positive cells were observed in MNC-injected mice (L4 and L5 DRGs). We performed immunohistochemistry of the microglia marker Iba1 in the ipsilateral side of L4 and L5 DRGs on MNC-injected and buffer-injected mice on day 7 (Figure 3). Many Iba1-positive cells were observed around the neurons of ipsilateral L4 and L5 DRG tissues of SNT mice (Figure 3A). Iba1-positive cells were markedly increased in the ipsilateral side when compared with the contralateral side (Figure S1).

For the quantitative evaluation of Iba1 staining, Iba1-positive cells were counted, and the intensity of the Iba-1-positive area was quantitated in the images binarized by ImageJ (Figures 3A, right panels, 3B, and 3C). The number of Iba1-positive cells was significantly lower in the ipsilateral L5 DRGs of MNC-injected SNT mice than in the buffer-injected SNT mice (Figure 3B). The average intensity of the Iba1-positive area in the L5 DRGs was significantly lower in the MNC group than in the buffer group (Figure 3C). In contrast, there was no significant difference in the number of Iba1-positive cells and the intensity of the positive area in the L4 DRGs between the MNC and buffer groups (Figures 3B and 3C). These results showed that MNCs suppressed the migration of microglia induced by spinal nerve injury.

MNCs Suppressed the Expression of Inflammatory Cytokines in the Ipsilateral L5 DRGs after SNT

We evaluated the expression of several cytokines in the ipsilateral L5 DRGs of MNC-injected or buffer-injected SNT mice on day 7 to analyze the inflammatory process further. We performed quantitative

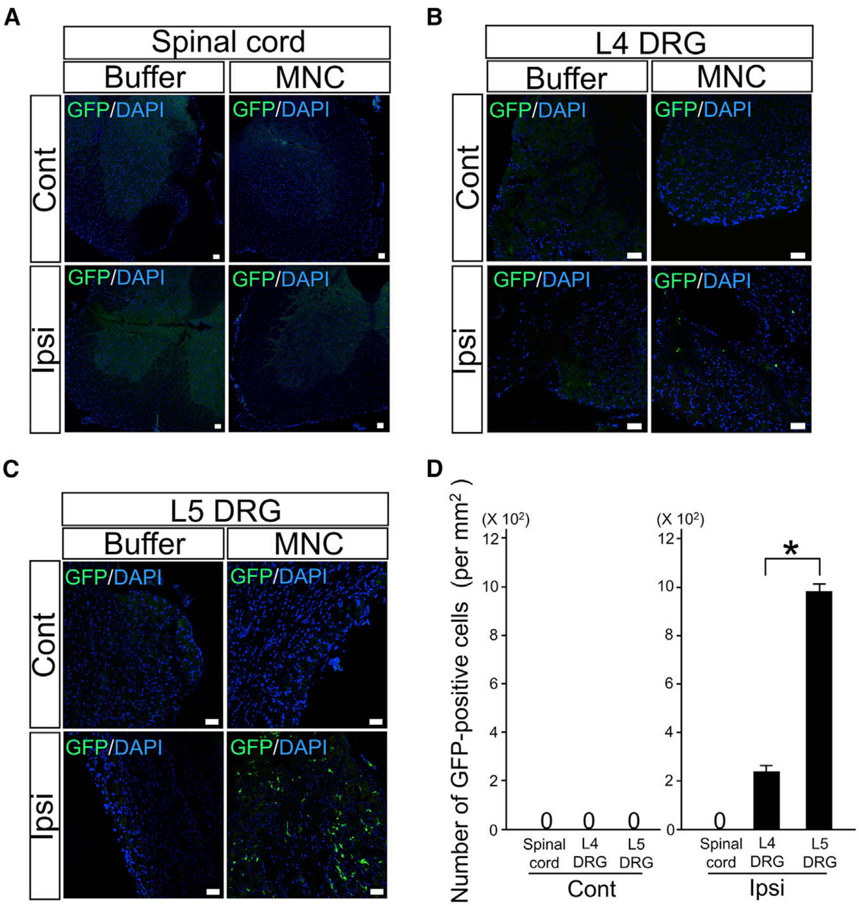

Figure 2. Distribution and Abundance of GFP-Positive MNCs in Mice on Day 14 after SNT and MNC Injection

(A–C) GFP-positive cells (green) and DAPI (blue) were observed in (A) the spinal cord, (B) L4 DRGs, and (C) L5 DRG sections under a confocal microscope. (D) Cell number of GFP-positive cells per square millimeter in the Ipsi and the Cont sides of the spinal cord, L4 DRGs, and L5 DRGs (n = 3 for each group). Data are represented as the mean + SE; *p < 0.01. Scale bars, 50 μm. DAPI, 4′, 6-diamidino-2-phenylindole; DRG, dorsal root ganglion.

PCR (qPCR) of representative inflammatory cytokines, IL-6, IL-1β, and TNF-α (Figure 4). The expression levels of the three cytokines were significantly lower in the MNC group than in the buffer group (Figure 4). These results indicated that MNC injection inhibited the inflammation associated with microglial migration and nerve transection.

MNCs Suppressed Microglial Migration and the Expression of Inflammatory Cytokines in the Spinal Cord after SNT

Next, we evaluated microglial migration and the expression of inflammatory cytokines in the dorsal horn of the spinal cord, which is the nearest afferent tract from the spinal nerve and DRGs. Immunohistochemistry of Iba1 was performed in the spinal cord of MNC-injected and buffer-injected mice (Figure 5). Iba1-positive cells accumulated at the dorsal horn of the ipsilateral side in the spinal cord of both groups but were hardly observed at the contralateral side (Figure 5A).

The accumulation of Iba1-positive cells was significantly suppressed in the MNC group when compared with the buffer group (Figures 5A and 5B). We performed qPCR of IL-6, IL-1β, and TNF-α in the ipsilateral spinal cord of MNC-injected or buffer-injected SNT mice on day 7 (Figure 6). The expression levels of the three cytokines were significantly suppressed in the MNC group when compared with the buffer group (Figure 6). These results were consistent with those of the DRG tissue and suggested that MNCs affected microglial migration and suppressed inflammation in the spinal cord.

Cytokine Expression in the Cerebrospinal Fluid after MNC Therapy for Neuropathic Pain

To investigate the influence of MNC injection on the humoral factors associated with spinal nerve injury, we examined the protein expression profile of cytokines in the cerebrospinal fluid (CSF) of MNC-injected and buffer-injected SNT mice by ELISA. We measured 32 cytokines, 25 of which were detectable in the spinal cord of both groups (Figure 7). The comparison analysis found that 8 cytokines were increased (Figure 7, red bars), 15 were decreased (Figure 7, blue bars), and 2 were unchanged (Figure 7) in the MNC group when compared with the buffer group. Interestingly, the eight cytokines that increased after MNC injection showed a less than 2-fold elevation. In contrast, 4 cytokines among the 15 that decreased showed a reduction of over 2-fold (Figure 7). Overall, MNC injection tended to suppress cytokine expression.

DISCUSSION

This study showed that intrathecal injection of bone-marrow-derived MNCs improved the mechanical threshold and suppressed neuropathic pain in mice with spinal nerve injury. MNCs mainly accumulated in the L5 DRGs and not in the spinal cord of treated mice. However, microglia accumulation and inflammatory cytokine expression were significantly suppressed in the DRGs and spinal cord after MNC injection. Therefore, MNCs not only affected the DRGs directly and the spinal cord indirectly.

In previous reports, MNCs showed therapeutic effects by suppressing several cytokines in rodent models of spinal cord injury and stroke.[17,20,23,24] Humoral factors that originated from MNCs probably provided these therapeutic effects. Our study also indicated the involvement of non-cell direct effects, which is consistent with previous studies.[17,20,23,24] MNC injection therapy has the potential to affect

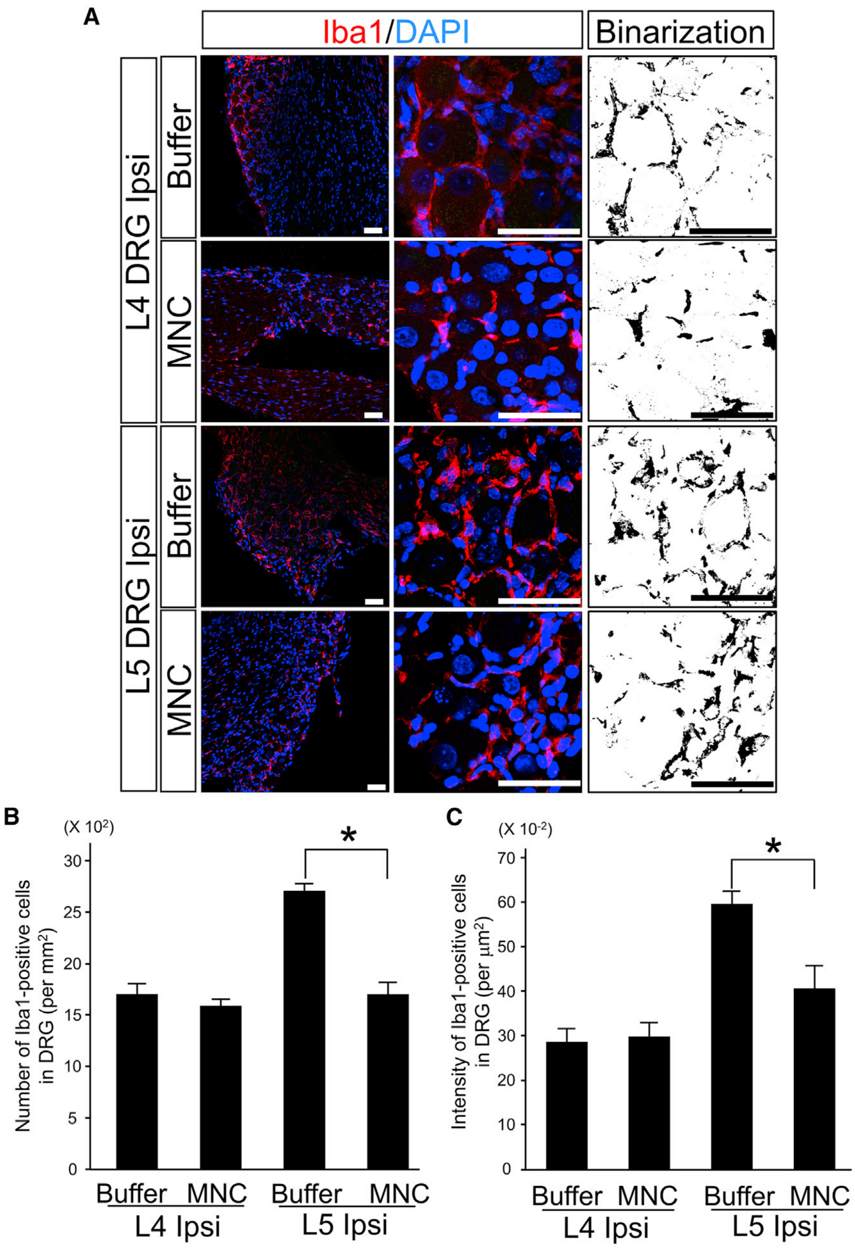

Figure 3. Immunohistochemical Analysis of Microglia in DRG Tissue on Day 7 after SNT and MNC Injection

(A) The left (low magnification) and middle panels (high magnification) show the immunostaining of anti-Iba1 (red) and DAPI (blue). The right panels show the binarization images of the Iba1-positive area. Scale bars, 50 μm. (B) Bar graphs show the number of Iba1-positive cells in L4 and L5 DRGs from the buffer and MNC groups (n = 3 for each group). (C) Bar graphs show the intensity of Iba1-positive cells in L4 and L5 DRGs from the buffer and MNC groups (n = 3 for each group). $^{*}p < 0.01$. Bars represent the mean + SE.

different tissues, cells, and the environment around injuries by suppressing several cytokines in neuronal tissues. In this study, MNC therapy caused partial pain remission for 14 days, and the therapeutic effect tended to diminish slightly by day 14. Thus, MNCs in ipsilateral L5 DRGs are expected to decrease after 14 days or more. However, changes in administrative strategies, such as increasing cell number or injection frequency, could result in a higher effect. Therefore, MNC injection therapy might provide a breakthrough in the treatment of intractable neuropathic pain.

Immune cells such as the monocyte/macrophage lineage and microglia have been reported to play a crucial role in inflammation-induced neuropathic pain and its pathogenesis.[11] SNT induces the secretion of inflammatory cytokines, such as TNF-α, IL-1β, IL-6, and interferon regulatory factor (IRF) 5,[25] which increase neuronal excitability and activate ATPases and voltage-gated sodium and calcium channels in the sensory nervous system.[26] The studies above considered inflammatory cytokines as potential treatment targets for neuropathic pain.[25,26]

We have tested and reported the results of anti-TNF-α drugs and gene therapies as treatments for neuropathic pain.[16,17,25,27] In this study, bone-marrow-derived MNC administration was superior to treatments that target a specific molecule because it suppressed many cytokines. Our strategy represents an advantage over the use of artificial vectors or chemicals for gene delivery because it preserves the physiological conditions and can be administrated autologously, which could minimize the side effects and ensure the safety of this molecular therapy.

Other cell therapies, such as mesenchymal stem cells (MSCs), have been frequently used for the treatment of neuropathic pain.[28] Intrathecal injection of MSCs showed therapeutic effects against neuropathic pain in previous studies.[28,29] MSCs have the potential to differentiate into multiple cell types, including endothelial, bone, muscle, and lipid cells, which gives them an advantage in terms of repair and regenerative ability. Previous studies showed that MSCs improved synaptic transmission and neuronal networks, and had anti-proliferation, anti-inflammation, and anti-apoptosis effects, which helped to treat neuropathic pain.[28] However, dealing with MSCs requires a cell sorting and cell processing center. In contrast, bone-marrow-derived MNCs can be collected through a simple and short procedure. MNCs could be administered to neuropathic pain patients in the clinical scene without the need for expensive facilities.[30]

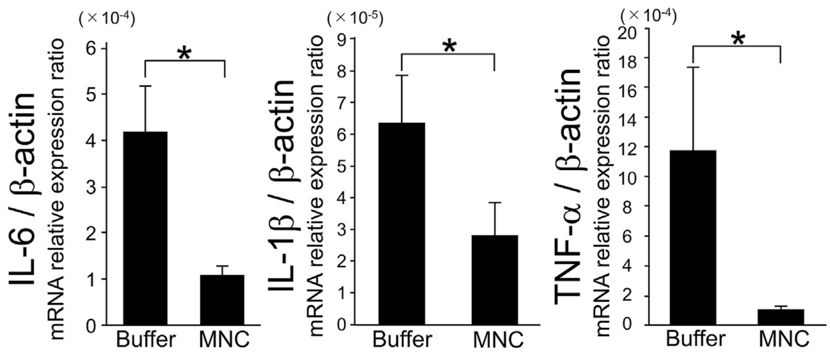

Figure 4. Relative mRNA Expression of Inflammatory Cytokines in DRGs from the MNC Injection Group

We calculated the mRNA expression of IL-6, IL-1β, and TNF-α in the L5 DRGs on day 7 after SNT and injection of bone-marrow-derived MNCs (n = 10 for each group). Data were standardized by β-actin mRNA expression. *$p < 0.05$. Bars represent the mean + SE.

The application route of cell therapies is one of the most important points to consider. Various animal and clinical studies on the application of MNCs have been conducted. MNCs have been administrated into mouse disease models through several routes, such as intravenous, intramuscular, and intrathecal injection.[31–37] The appropriate route depends on the therapeutic target tissues. Therapies for neuropathic pain should target nervous tissues, including the spinal cord, DRGs, peripheral nerves, and nociceptors. In the study, we targeted the DRGs and spinal cord because the spinal nerve was transected. Therefore, we selected intrathecal injection as our application route. Our results showed that the injected MNCs could directly access the DRGs through the CSF.

The safety of the administration method must be taken into account in clinical applications. Intravenous injection is a minimally invasive procedure for cell administration.[38] However, it has systemic effects and may affect non-desired organs. Intrathecal injection is a local administration method, limited to the intrathecal space, which has minimal side effects. Some studies even reported no side effects.[20,22] Lumbar puncture is often performed in the clinical practice,[30,39] and is a safe and effective administration method that has been used for drug injection to treat neuropathic pain.[40–42] In our study, intrathecal administration was performed for MNC injection and showed its effect without major side effects of motor sensory function. No mice died until day 14 after SNT and cell therapy.

MNCs have been used to treat different diseases in clinical and animal studies. MNC therapy has been reported to be effective for arteriosclerosis obliterans, Buerger disease, cerebral infarction, and spinal cord injury because of trophic effects, angiogenesis, and neurogenesis.[22,32–34,36,37,43–45] We are currently performing a clinical trial of intrathecal MNC administration for spinal cord injury, and preliminary results showed therapeutic effects.[30] Therefore, the safety of intrathecal administration of MNCs has been recognized and seems to be a feasible treatment for neuropathic pain, although we should confirm the effectiveness and its effective duration in clinical study. Here, we showed for the first time that intrathecal administration of MNCs relieved neuropathic pain caused by SNT by suppressing inflammatory cytokines in the DRGs and spinal cord. Autologous intrathecal administration of MNCs might be a promising cell therapy for the treatment of neuropathic pain.

MATERIALS AND METHODS

Ethics Statement

All experimental animal protocols were approved by the Institutional Animal Care and Usage Committee at Shiga University of Medical Science. All procedures were performed in accordance with the guidelines of the Research Center for Animal Life Science of Shiga University of Medical Science.

Animals

Male 8- to 9-week-old C57BL6 mice (SLC, Shizuoka, Japan) weighing 19.0–22.0 g were used in this study. C57BL/6-Tg (UBC-GFP)$^{30scha/J}$ mice were purchased from The Jackson Laboratory (Bar Harbor, ME, USA). The mice were housed in separated cages and maintained under a 12-h light and dark cycle. Food and tap water were available *ad libitum*.

Surgical Procedures

A neuropathic pain model by L5 SNT was generated following a previously described procedure.[46] Mice were anesthetized by intraperitoneal administration of sodium pentobarbital (5 mg/kg). After a midline incision of the mouse back skin, the bilateral L5 transverse processes of the lumbar spine was removed. We exposed the bilateral L5 spinal nerves. Only the left side of the spinal nerve was transected at the distal to the L5 DRGs; the right spinal nerve was exposed without transection to serve as a negative control. After confirmation of hemostasis, the spine was restored, and the subcutaneous tissue and skin were sutured. The next day, mechanical hyperalgesia was confirmed.

Behavioral Test

Mechanical hyperalgesia was evaluated before the surgery and 1, 3, 5, 7, 10, and 14 days after SNT with a dynamic planter aesthesiometer (Ugo Basile, Gemonio, Italy) as described previously.[25,27] We evaluated paw withdrawal in response to mechanical stimulation. Each mouse was put in a box with a metallic mesh floor and allowed to acclimatize to the testing environment for at least 1 h. A filament probe for stimulation was positioned under the hind paw and applied gradually until the mice withdrew their paw. The pressure was increased at about 10 g/mm^2/s. The test was performed on the right and left hind paws. The withdrawal threshold was calculated as the average of three tests. The threshold at the ipsilateral side was

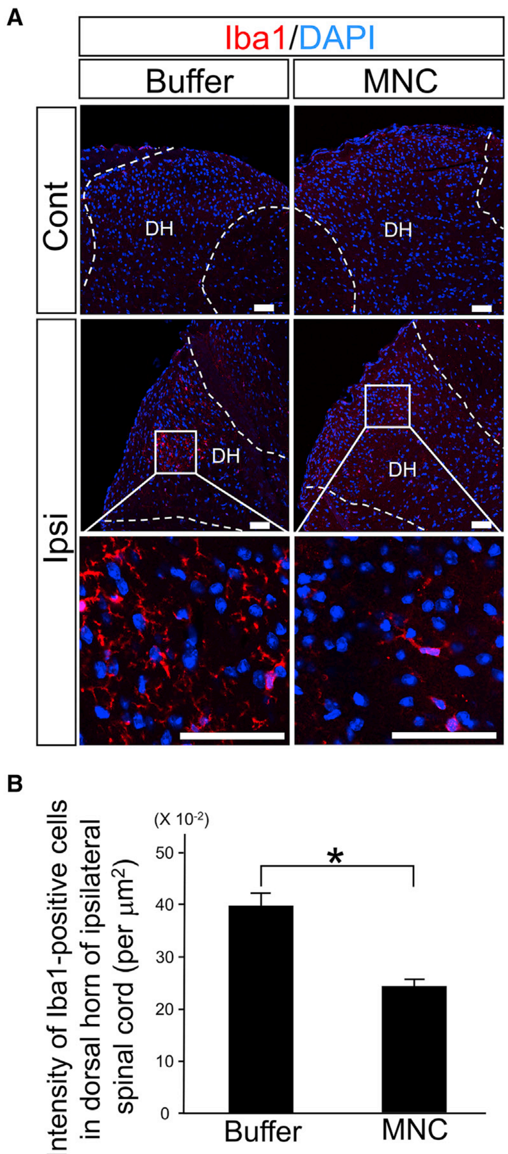

Figure 5. Immunohistochemistry of Microglia in Spinal Cord Tissue on Day 7 after SNT and MNC Injection

(A) The upper panels show the sections of the spinal cord at the Cont side in the buffer and MNC groups. Middle panels show the Ipsi side of the spinal cord in the buffer and MNC groups. The broken white lines show the spinal dorsal horn (DH) area. Lower panels show the Iba1-positive area (red) with DAPI (blue) at the enlarged image of the white square in the Ipsi side of the spinal cord. (B) Bar graphs show the intensity of Iba1-positive cells in the spinal cord after SNT and the injection of MNCs (n = 3 for each group). $^{*}p < 0.01$. Bars represent the mean + SE. Scale bars, 50 μm.

evaluated to compare the ratio against the pressure in the contralateral side on day 0.

Isolation and Injection of MNCs

Total bone marrow cells were isolated from a GFP Tg mouse, and MNCs were isolated from bone marrow cells using a Ficoll-Paque (GE Healthcare, Chicago, IL, USA). Total bone marrow cells were mixed in 3 mL phosphate-buffered saline (PBS) and carefully layered upon 3 mL of Ficoll separation medium. Ficoll gradients were centrifuged for 30 min at 20°C and 400 × *g* (brake was turned off). The layer of whole MNCs was collected with a sterile pipette, washed with PBS, mixed with trypan blue, and counted by hemocytometer. The concentration was adjusted for injection. For the treatment of neuropathic pain, a midline incision of the skin was made at the lumbar region of the back side in day 1 SNT mice under deep anesthesia. After exposure of spine, 1×10^6 MNCs/10 μL were intrathecally injected using a Hamilton syringe with a 30G needle at intervertebral space of lumbar level. We confirmed by the evoked tail flick in mice whether the tip of the needle inserted into the subarachnoid space.[47] PBS (10 μL) was injected into the buffer control group. Buffer and whole MNCs were intrathecally injected over approximately 2 min.

mRNA Expression Analysis

The spinal cord and DRGs were extracted from mice under deep anesthesia and immediately frozen in liquid nitrogen. Total RNA was extracted from frozen tissues with the RNeasy mini kit (QIAGEN, Hilden, Germany) with DNase I (RNase-free DNase set; QIAGEN) treatment. Reverse transcription was performed from 100 ng of total RNA in each tube using the PrimeScript RT reagent Kit with gDNA Eraser (Perfect Real Time; Takara Bio, Kusatsu, Japan). The quantitative real-time PCR assay was performed using a LightCycler 480 with SYBR Green (Roche Diagnostics, Manheim, Germany) according to a manufacturer's protocol. The following primers were used: IL-6, forward, 5′-ACGGCCTTCCCTACTTCACA-3′ and IL-6 reverse, 5′-CATTTCCACGATTTCCCAGA-3′; IL-1β forward, 5′-CAACCAACAAGTTGATATTCTCCATG-3′ and IL-1β reverse, 5′-GATCCACACTCTCCAGCTGCA-3′; TNF-α forward, 5′-CACGTCGTAGCAAACCACCAAGTGG-3′ and TNF-α reverse, 5′-GATAGCAAATCGGCTGACGGTGTGG-3′; β-actin forward, 5′-CGTGCGTGACATCAAAGAGAA-3′ and β-actin reverse, 5′-TGGATGCCACAGGATTCCAT-3′. The normalization and the relative expression analysis of target genes were performed using the comparative cycle threshold method with β-actin as a control.

Histological Analysis

Animals were deeply anesthetized by intraperitoneal administration of 0.3 mg/kg medetomidine, 4.0 mg/kg midazolam, and 5.0 mg/kg butorphanol, and perfused with PBS followed by a fixative containing 4% paraformaldehyde in 0.1 M phosphate buffer. After perfusion fixation, animal tissues were kept in the same fixative at 4°C overnight. The fixative was replaced with 15% sucrose buffer the next day. The DRGs and spinal cord were isolated, embedded in Optimal Cutting Temperature compound (Tissue Tek, Sakura, Tokyo, Japan), frozen with liquid nitrogen, and cut to 10-μm sections on a cryostat. After mounting the sections in Vectashield medium with 4′, 6-diamidino-2-phenylindole (DAPI; Vector, Burlingame, CA, USA), GFP-positive cells were counted under a Leica TCS SP8 X confocal microscope with the Leica Application Suite X software (Leica, Tokyo, Japan). For immunohistochemical analysis, other sections were

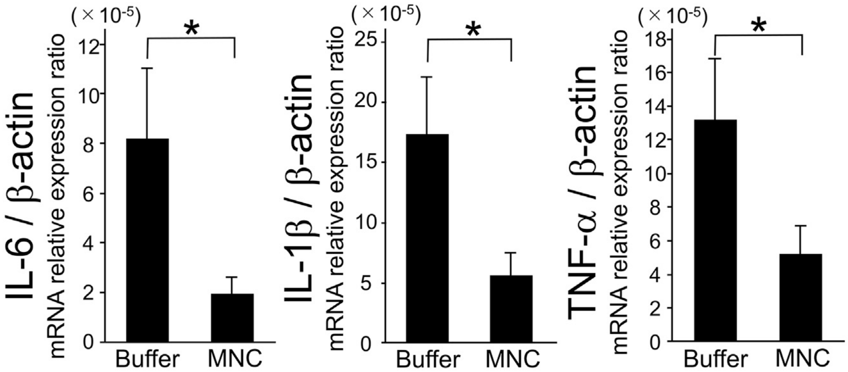

Figure 6. Relative mRNA Expression of Inflammatory Cytokines in the DRGs from the MNC Injection Group

We calculated the mRNA expression of IL-6, IL-1β, and TNF-α in the L5 DRGs on day 7 after SNT and bone-marrow-derived MNC injection (n = 10 for each group). Data were standardized by β-actin mRNA expression. *$p < 0.05$. Bars represent the mean + SE.

blocked with 3% normal goat serum in PBS at room temperature for 30 min. Anti-Iba1 antibody (1:1,000; Abcam, Cambridge, UK) and Alexa Fluor 555 antibody (1:1,000; Abcam) were used as primary and secondary antibodies, respectively. The sections were mounted in Vectashield medium with DAPI (Vector). Fluorescence images were observed under a Leica TCS SP8 X confocal microscope with the Leica Application Suite X software (Leica). To evaluate all the sections, we prepared at least three consecutive sections (each of 30-μm intervals) and evaluated at least three scenes in each section. GFP-positive cells were counted in a 100 μm × 100 μm visual field in the spinal cord and the L4 and L5 DRGs of each group, and Iba1-positive cells were counted in the L4 and L5 DRGs. For the quantification of the intensity of Iba1-positive staining, red color in the images was isolated, converted to black-white binary images, and measured the intensity by ImageJ 1.52a (National Institutes of Health, Bethesda, MD, USA).

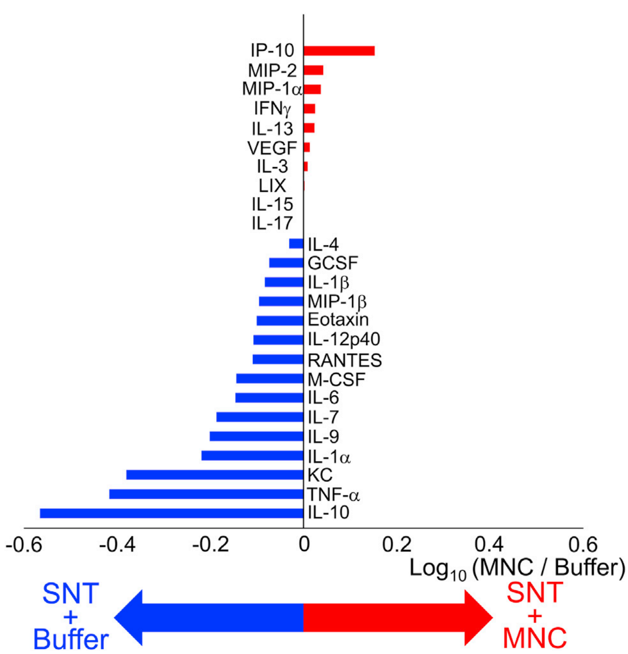

Figure 7. Quantitative Analysis of Cytokines in Cerebrospinal Fluid after MNC Treatment

We measured 25 cytokines in the cerebrospinal fluid of buffer- or MNC-injected mice. The concentration of cytokines in the cerebrospinal fluid of both groups was compared by calculating the logarithm ratio of the MNC group with the buffer group. Red bars show the elevated cytokines, and blue bars show the decreased cytokines in the MNC group when compared with the buffer group.

Cytokine Assay in CSF

CSF was collected from cistern magna at day 7 after SNT.[48] Under deep anesthesia, a midline incision of the skin was made from occipital to neck region. After exposure of the dura mater, CSF was collected by a 30G needle. CSF was combined from three mice in the buffer or MNC groups. At least 15 μL of CSF was harvested from each mouse. The 32-cytokine assay in CSF was outsourced to GeneticLab (Sapporo, Japan). CSF was used for a multiplex assay running, and the concentration of 32 cytokines was measured with a Milliplex MAP kit HCYTMAG-70K-PX32 (Millipore, Burlington, MA, USA) and a Luminex 200 System (Luminex, Austin, TX, USA) by ELISA technology. The procedure was done according to the assay protocols and guidelines provided by Millipore. The cytokines included in the kit were as follows: G-CSF (granulocyte-colony-stimulating factor), Eotaxin, GM-CSF (granulocyte macrophage-colony-stimulating factor), IFN-γ (interferon-γ), IL-1α, IL-1β, IL-2, IL-3, IL-4, IL-5, IL-6, IL-7, IL-9, IL-10, IL-12p40, IL-12p70, LIF (leukemia inhibitory factor), IL-13, LIX (lipopolysaccharide [LPS]-induced CXC chemokine), IL-15, IL-17, IP-10 (interferon-γ-induced protein-10), KC (keratinocyte-derived chemokines), MCP-1 (monocyte chemoattractant protein-1), MIP-1α (macrophage inflammatory protein-1α), MIP-1β, M-CSF (macrophage-colony-stimulating factor), MIP-2, MIG (monokine induced by INF-γ), RANTES, TNF-α, and VEGF (vascular endothelial growth factor). The results were calculated by MasterPlex software (Hitachi Solutions America, Irvine, CA, USA).

Statistical Analysis

All data are expressed as the means + or ± standard error (SE). One-way ANOVA, followed by Tukey's test was used to calculate the statistical significance for multiple datasets. For behavioral analyses, two-way ANOVA and Scheffe's tests were used. A p value below 0.05 was considered significant.

SUPPLEMENTAL INFORMATION

Supplemental Information can be found online at https://doi.org/10.1016/j.omtm.2020.03.020.

AUTHOR CONTRIBUTIONS

H.T. conducted the experiments, analyzed the data, and wrote the manuscript. T.T. advised on the experimental procedures, designed the study, and helped to write and revise the manuscript. H.K., S.I., Y.S., J.O., M.K., and K.M. advised on the experimental design and techniques, and provided expertise and feedback. All authors have read and approved the final manuscript.

CONFLICTS OF INTEREST

The authors declare no competing interests.

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