

<sup>a</sup>Department of Orthopaedics and Rehabilitation Medicine, Faculty of Medical Sciences, University of Fukui, Matsuoka Shimoaizuki 23, Eiheiji, Fukui 910-1193, Japan; <sup>b</sup>Life & Health Sciences, Aston University, Aston Triangle, Birmingham B4 7ET, United Kingdom

\*Name and address of corresponding author: Kenzo Uchida, MD, PhD, Department of Orthopaedics and Rehabilitation Medicine, Faculty of Medical Sciences, University of Fukui, Matsuoka Shimoaizuki 23-3, Eiheiji, Fukui 910-1193, Japan. Voice: 81-776-61-8383; Facsimile: 81-776-61-8125, Email: [ku-uchida@u-fukui.ac.jp](mailto:ku-uchida@u-fukui.ac.jp); Conflict of interest: The authors declare no financial interest or any relationship with any of the commercial companies or institutions mentioned in this article

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## Early Transplantation of Mesenchymal Stem Cells after Spinal Cord Injury Relieves Pain Hypersensitivity Through Suppression of Pain-Related Signaling Cascades and Reduced Inflammatory Cell Recruitment

Shuji Watanabe, MD,<sup>a</sup> Kenzo Uchida, MD, PhD,<sup>a,\*</sup> Hideaki Nakajima, MD, PhD,<sup>a</sup> Hideaki Matsuo, RPT,<sup>a</sup> Daisuke Sugita, MD,<sup>a</sup> Ai Yoshida, MD,<sup>a</sup> Kazuya Honjoh, MD,<sup>a</sup> William E.B. Johnson, PhD,<sup>b</sup> and Hisatoshi Baba, MD, PhD<sup>a</sup>

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### ABSTRACT

Bone marrow-derived mesenchymal stem cells (BMSC) modulate inflammatory/immune responses and promote motor functional recovery after spinal cord injury (SCI). However, the effects of BMSC transplantation on central neuropathic pain and neuronal hyperexcitability after SCI remain elusive. This is of importance because BMSC-based therapies have been proposed for clinical treatment. We investigated the effects of BMSC transplantation on pain hypersensitivity in GFP-positive bone marrow-chimeric mice subjected to a contusion SCI, and the mechanisms of such effects. BMSC transplantation at day 3 post-SCI improved motor function and relieved SCI-induced hypersensitivities to mechanical and thermal stimulation. The pain improvements were mediated by suppression of PKC- $\gamma$  and p-CREB expression in dorsal horn neurons. BMSC transplants significantly reduced levels of p-p38 MAPK and p-ERK1/2 in both hematogenous macrophages and resident microglia, and significantly reduced the infiltration of CD11b and GFP double-positive hematogenous macrophages without decreasing the CD11b-positive and GFP-negative activated spinal-microglia population. BMSC transplants prevented hematogenous macrophages recruitment by restoration of the blood-spinal cord barrier, which was associated with decreased levels of: (i) inflammatory cytokines (TNF- $\alpha$ , IL-6); (ii) mediators of early secondary vascular pathogenesis (MMP-9); (iii) macrophage recruiting factors (CCL2, CCL5, CXCL10), but increased levels of a microglial stimulating factor (GM-CSF). These findings support the use of BMSC transplants for SCI treatment. Furthermore, they suggest that BMSC reduce neuropathic pain through a variety of related mechanisms that include neuronal sparing and restoration of the disturbed blood-spinal cord barrier, mediated through modulation of the activity of spinal-resident microglia and the activity and recruitment of hematogenous macrophages.

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## INTRODUCTION

Spinal cord injury (SCI) not only results in loss of normal sensation, but often debilitating neuropathic pain. Up to 80% of patients with SCI develop chronic neuropathic pain, which can be localized below, girdling, or above the level of the SCI lesion [1-4]. Beneficial effects of cell transplantation therapy in sensory recovery and reduced pain after SCI have been reported [5, 6], however other studies described harmful effects of cell transplants [7, 8]. Research has shown that autologous BMSC transplants increase sensory function after contusion SCI, but the effects of BMSC on SCI-induced chronic neuropathic pain remain contentious [9-12]. This is problematic because BMSC transplants for SCI treatments are being developed for clinical applications [13-15].

Neuropathic pain following SCI is associated with a number of molecular and cellular events. SCI results in a marked increase in extracellular concentrations of glutamate followed by activation of excitatory amino acid receptors and ion channels [16] with subsequent activation of intracellular downstream pathways that result in altered gene expression. These events are sufficient to produce persistent neuronal hyperexcitability of spinal dorsal horn neurons or central neuropathic pain [17]. The mechanism of neuropathic pain after SCI involves the activation of members of the mitogen activated protein kinase (MAPK) family, in particular, p38 MAPK, extracellular signal-regulated kinase (ERK) including ERK1/2, and c-Jun N-terminal kinase (JNK) [18]. Experimental models of peripheral neuropathic pain have also highlighted the roles of MAPK in neuronal hyperexcitability and demonstrated the selective expression of MAPK in activated microglia present in the spinal cord dorsal horn [19, 20]. Following thoracic SCI, both p38 MAPK and ERK are activated in spinal microglia, which contributes to neuronal hyperexcitability and central neuropathic pain [17, 21-23]. Furthermore, inhibition of microglial activation by minocycline (an anti-inflammatory drug) was reported to reduce SCI-induced pain below the injury site by attenuating p38 MAPK and ERK activation in microglia [23, 24].

Although activated microglia are thought to contribute to neuropathic pain symptoms through their release of modulators of neuronal excitability [25], these data derive largely from analyses of resident cells at sites remote and caudal to thoracic lesions [22, 26, 27]. However, following thoracic SCI the blood-spinal cord barrier (BSCB) is often disrupted and an influx of blood-borne inflammatory and other immune cells contribute to an intensive inflammatory response localized around the injured site [28]. Infiltrating macrophages have both detrimental [29, 30] and beneficial roles [31, 32] in spinal repair, although the net effect of their activity is unclear. In addition, it is not well-known how activated resident microglia and macrophages of different origins contribute to post-SCI dorsal horn hyperexcitability and

central neuropathic pain, particularly at the level of the lesion. One reason for the difficulty in differentiating microglia from infiltrating monocytes/macrophages relates to their morphological and phenotypic similarities [33]. However, in response to peripheral nerve injury there is increasing evidence that infiltration of peripheral immune cells, including macrophages, into the spinal cord contributes to the pathogenesis of neuropathic pain [34, 35]. Understanding the contribution of each cell type, i.e., of inflammatory cells of hematogenous monocytic lineage versus resident microglia to SCI-induced neuropathic pain is important to direct the search for novel therapies, especially involving BMSC transplants as these are known to influence macrophage activity.

In the present study, we investigated the effects of BMSC transplantation on post-SCI chronic neuropathic pain. Specifically, we focused on the effects of BMSC transplants on microglia and macrophages and MAPK signalling at the level of the lesion. Furthermore, we used chimeric mice wherein the bone marrow contained hematogenous cells that expressed green fluorescent protein (GFP). This was to determine the differential effects of transplanted BMSC on spinal-resident microglia and bone marrow-derived macrophages.

## MATERIALS AND METHODS

### Experimental Animals

All experiments followed the guidelines for the study of pain in awake animals established by the International Association for the Study of Pain [36]. Experiments were conducted in 453 adult male C57BL/6N mice (Nihon SLC, Shizuoka, Japan), aged 10-12 weeks, with a mean body weight of  $27.5 \pm 0.82$  g ( $\pm$  standard deviation, SD), and 20 adult male mice in which GFP expression was driven by the cytomegalovirus early enhancer  $\beta$ -actin (CAG) transgene (CAG-EGFP mice; Nihon SLC, Shizuoka, Japan), aged 6-8 weeks, with a mean body weight of  $19.4 \pm 0.47$  g ( $\pm$  SD). The experimental protocol was approved by the Ethics Committee for Animal Experimentation of Fukui University.

### The Contusion SCI Model

Each mouse was anesthetized prior to surgery with isoflurane (Forane<sup>®</sup>; Abbot, Tokyo, Japan), and the spinal cord was exposed by laminectomy at the T9-T10 vertebral level using a surgical microscope (VANOX-S; Olympus Optical, Tokyo). A contusion SCI was produced using the Infinite Horizon Impactor (Precision Systems and Instrumentation LLC, Fairfax, VA; <http://www.presysin.com/>) with an impact force of 60 kilodynes (kdyn) [37]. In a sham SCI operation group, each mouse underwent a laminectomy only at the T9-T10 vertebral level, with no SCI performed.

### Preparation of Bone Marrow-Derived Mesenchymal Stem Cells

BMSC were isolated and cultured from the bone marrow of the femur and tibia bones collected from young adult male C57BL/6N or CAG-EGFP transgenic mice. The prepared bone marrow cells were placed into dishes with Dulbecco's Modified Eagle's Medium (DMEM; GIBCO, Grand Island, NY) supplemented with 10% fetal bovine serum (FBS; GIBCO). Dissociated BMSC were isolated according to preferential adherence to tissue culture plastic as described in Supporting Information Methods. Before all experiments using BMSC transplants, flow cytometric analysis confirmed that these cells were phenotypically BMSC based on positive immunostaining for CD44, CD90, and CD29 and negative immunostaining for CD45 and CD11b (Supplemental Fig. 1).

### BMSC Transplantation

We divided animals into four groups according to the time of transplantation of BMSCs that were isolated from C57BL/6N mice. The BMSC transplantation was performed on days 1, 3, 7 and 14 post-SCI. The treatment group received  $2.0 \times 10^5$  BMSC in  $3 \mu\text{l}$  of DMEM, which were injected into the middle of the contusion site, identified as the middle point of the laminectomy area (identified by a hematoma at day 3) [38]. To maximize engraftment of all injected BMSC into the spinal cord, the needle was not disconnected from the spinal cord for 5 minutes after the injection. Control mice were injected with  $3 \mu\text{l}$  of DMEM alone (i.e., no cells) at each time point post-SCI. Sham-operated mice were not injected with cells or with DMEM.

### Assessment of transplanted BMSC survival

The relationship between the survival rates of transplanted BMSCs with the time after SCI that the BMSCs were transplanted was initially assessed. The transplantation for these survival studies were at 1, 3, 7, and 14 days post-injury. GFP-positive BMSC survival was examined at 15 minutes, 1, 3, 7, 14, and 28 days after transplantation with  $2.0 \times 10^5$  BMSCs in  $3 \mu\text{l}$  of culture medium. To assess BMSC survival, we examined midsagittal sections prepared from the injured portion of the spinal cord. The numbers of GFP-positive BMSCs in these sections were counted at each time point as described in the section on Semi-Quantitative Analysis of Tissue Staining. These data have been expressed as the survival rate (%) of BMSCs at each time point indicated compared with the number of GFP-positive BMSCs present at 15 minutes after transplantation.

### Bone Marrow-Chimeric Mice

We generated bone marrow-chimeric mice using highly purified, genetically marked bone marrow cells as reported previously [34, 39], enabling us to limit our studies to hematopoietic lineages. Isolated unfractionated marrow cells ( $5.0 \times 10^6$  cells) from donor CAG-EGFP

transgenic mice were injected intravenously into the tail vein of a recipient mouse that had been irradiated at 9.0 Gy for 30 min (4.5 Gy/15 min twice) after covering the head with a lead plate. Their successful engraftment and induction of chimerism was confirmed after 4 weeks by Dual-laser FACS Calibur (BD Biosciences) analysis of recipient peripheral blood to identify donor GFP<sup>+</sup>-bone marrow-derived cells. Bone marrow chimeric mice were used for SCI surgery at the age of 10-12 weeks.

### Behavioral and Sensory Testing

The Basso Mouse Locomotor Scale (BMS) is a 0 to 9 point scale, which was developed to describe recovery of locomotor function in mice after thoracic spinal cord contusion or transection injury [40]. BMS scores were recorded at times indicated post-SCI for the left and right hind limbs, and averaged to obtain a single calculated value per mouse per test.

Mechanical allodynia and thermal sensitivity were recorded at times indicated post-SCI by two independent examiners blinded to the experimental conditions. Mechanical allodynia was assessed using the Dynamic Plantar Aesthesiometer (Ugo Basile, Comerio, Italy; <http://www.ugobasile.com/>) [41]. The withdrawal threshold (expressed in grams) was measured five times and the reported value represents the mean of these five evaluations. Thermal sensitivity of the plantar hind-paws was tested according to the method described previously [42] (Plantar Test Apparatus, Ugo Basile) (See Supporting Information Methods). Latency (in seconds) to withdrawal from the heat source was recorded, together with any other behavior indicating attendance to the stimulus, including sniffing, licking, looking at the affected paw or attacking the stimulus. High and low latencies were dropped for each paw, and the remaining six latencies were averaged for each mouse [43].

We used the same technique to transplant  $2.0 \times 10^5$  BMSCs in  $3 \mu\text{l}$  DMEM versus  $3 \mu\text{l}$  DMEM alone (as controls) at day 3 post-SCI for detailed analysis of biochemistry related to pain hypersensitivity. Previous reports indicate that allodynia is present in approximately 81% of experimental mouse models after SCI [44] and approximately 75% of experimental rat models after contusion SCI [45]. For the present study, 142 mice underwent contusion SCI and were examined using sensory tests for the presence of allodynia and 111 (78.2%) of these were selected based on the presence of significant sensory differences when compared with the sham-operated group.

### Immunohistochemistry

Perfused segments of the spinal cord obtained from separate animals were embedded in optimal cutting temperature compound (Sakura Finetek, Torrance, CA) and cut on a cryostat for the preparation and analysis of axial sections (for assessment of dorsal horn and gray matter after SCI) or sagittal sections (for assessment of the distribution of transplanted BMSCs and blood-

derived cells). All frozen sections were 20µm thick and serially mounted on glass slides (See Supporting Information Methods). All digitized images of immunopositivity were obtained using a fluorescence microscope (Olympus AX80; Olympus Optical) or a confocal laser scanning microscope (model TCS SP2; Leica Instruments, Nussloch, Germany).

### Semi-Quantitative Analysis of Tissue Staining

For semi-quantitative analysis of dorsal horn neuronal hyperexcitability, 10 axial sections randomly selected at a distance up to 2 mm rostral and caudal to the site of the lesion were immunostained with antibodies specific for putative markers, i.e. for protein kinase C gamma (PKC-γ) or phospho-cyclic AMP response element binding protein (p-CREB), both of which are upregulated in association with at-level allodynia after contusion SCI [21, 27]. For PKC-γ staining in the superficial laminae I-III of spinal dorsal horn, the area and density of pixels within the threshold value representing immunoreactivity were calculated. The extent of immunopositivity for each antibody was calculated from the product of pixel density and image area, which was determined using the color image analyzer (MacSCOPE; Mitani, Fukui, Japan). For p-CREB staining, the numbers of p-CREB- and neuronal nuclei (NeuN) -double-positive cells in the superficial laminae I-III of the spinal dorsal horn were counted, as previously reported [46].

The number of GFP-positive BMSCs, CD11b and GFP positive cells, CD11b-positive and GFP-negative cells, and p-38 MAPK and p-ERK1/2 colocalized with GFP positive cells were counted. For this analysis, 3 mid-sagittal sections through the injured portion of the spinal cord were randomly selected and 20 high magnification (400x) non-overlapping digitized images were captured using a confocal microscope (TCS SP2) within a region that was 2 mm rostral and caudal to the site of lesion. Quantification of cells in each image was performed by MacSCOPE as described previously [47].

We assessed blood-spinal cord barrier (BSCB) function at days 4, 7 and 14 post-SCI, because we previously observed that the infiltration of blood-derived macrophages peaked between 1-2 weeks after SCI. BSCB was assessed as follows; 10 axial sections were randomly selected at a distance up to 2 mm rostral and caudal to the site of lesion and stained for albumin or immunolabelled for platelet-derived growth factor receptor alpha (PDGFR-α). Digitised images of the stained sections were collected and MacSCOPE was used to calculate the relative intensity of albumin- and PDGFR-α-positive signal intensity in the gray matter. The mean pixel density of positivity was expressed as a percentage of that for the total fields analyzed (pixel area/total field) [48].

### Flow Cytometric Analysis

At day 14 post-SCI, the damaged area of the spinal cord (2.5 mm either side of the lesion) was surgically dissect-

ed for flow cytometry, as described previously [49] (See Supporting Information Methods). GFP<sup>positive</sup>/CD45<sup>high</sup>/CD11b<sup>high</sup>/GR-1<sup>negative</sup> cells were designated hematogenous macrophages and GFP<sup>negative</sup>/CD45<sup>high</sup>/CD11b<sup>high</sup>/GR-1<sup>negative</sup> cells were designated endogenous activated microglia [49]. For intracellular staining [50], these cells were resuspended in Fixation buffer (Santa Cruz Biotechnology, Santa Cruz, CA) and treated with Permeabilization Buffer (Santa Cruz Biotechnology), followed by resuspension in ice-cold PBS and incubation for 1 hour with rabbit phycoerythrin (PE) (1:200, Santa Cruz Biotechnology) conjugated rabbit anti-p-p38 MAPK antibody (1:200, Cell Signaling Technology, Danvers, MA) or rabbit anti-p-ERK1/2 antibody (1:200, Cell Signaling Technology). Flow cytometry was performed immediately using a FACS Canto™ II (BD Biosciences) using forward scatter to further eliminate any cellular debris from analysis.

### Immunoblot Analysis

Immediately after deep anesthesia, the damaged spinal cord (2.5 mm either side of the lesion) was removed at days 4 or 14 post-SCI and stored immediately at -80°C in liquid nitrogen. Western blotting was performed as described in Supporting Information Methods. Images for immunoblot analysis were captured on an Image Quant LAS 4000 mini chemiluminescence imaging analyzer (GE Healthcare Life Science, Piscataway, NJ) and the band intensity was quantified using Image Quant TL software (GE Healthcare Life Science). The intensity of each band was expressed relative to that of β-actin. Kaleidoscope Prestained Standards (Bio-Rad Laboratories, Hercules, CA) were used as molecular weight controls.

### Statistical Analysis

All values are expressed as mean ± standard deviation (SD). Differences between groups were examined for statistical significance using one-way factorial analysis of variance (ANOVA). A  $p < .05$  denoted the presence of significant difference with Tukey's *post-hoc* analysis. The above tests were conducted using SPSS software version 11.0 (SPSS, Chicago, IL).

## RESULTS

### Relationship between Survival Rate of Transplanted BMSCs and Time of Transplantation

There was a decrease in the presence of GFP-positive transplanted BMSCs with time after transplantation such that by 28 days post BMSC transplant very few viable BMSCs were observed. However, the number of surviving BMSCs that had been transplanted at 1 or 3 days after SCI were markedly greater than the number of surviving BMSCs that were transplanted at 7 or 14 days after SCI, with the greatest survived number seen when BMSCs were transplanted at 3 days (Supplemental Fig. 2).

### Early transplantation of BMSCs after SCI Increases Locomotor Function and Decreases Pain Hypersensitivity to Mechanical and Thermal Stimulation

The effects of BMSC transplants were examined at the acute, sub-acute and chronic stages after SCI (days 1, 3, 7 and 14). This was to examine whether effects of the transplants on pain hypersensitivity may be time dependent, and potentially related to inflammatory reactions.

After BMSC transplantation at 14 days post-SCI, there were no significant effects in behavioral or sensory test outcomes. In contrast, the mice that were treated with BMSCs at 3 days post-SCI showed the greatest improvement in their BMS locomotor scores compared with the control group, which was significantly increased by day 14 post-SCI and persisted up to week 6 after injury (Fig. 1A). Furthermore, the most marked improvements in mechanical and thermal sensitivity scores were also seen in the 3 day BMSC-treated group (Fig. 1B, 1C) (see also Supplemental Fig. 3). Subsequent analyses were performed on 3 day BMSC-treated mice versus control or sham-operated mice.

### BMSC Transplantation Reduced PKC- $\gamma$ - and p-CREB Protein Levels Spinal Cord Dorsal Horn Neurons

To examine whether BMSC transplantation reduces localized putative markers of hyperexcitability in neurons, we assessed immunopositivity for PKC- $\gamma$  and p-CREB colocalized with the neuronal marker, NeuN in the dorsal horn post-SCI. In the control group, SCI caused an increase in PKC- $\gamma$ -positivity in NeuN labelled neurons in the dorsal horn of the spinal cord around the injury site. In contrast, BMSC transplantation significantly suppressed the observed expression of PKC- $\gamma$  in NeuN labelled cells in these locations (Fig. 2A, 2B). The number of cells positive for both p-CREB and NeuN in the dorsal horn post-SCI was also increased in the control group, especially in the deeper lamina, whereas BMSC transplantation significantly attenuated this increase in p-CREB in NeuN labelled cells (Fig. 2E, 2F). Immunoblot analysis of extracted proteins from the lesion site following SCI confirmed that the protein levels of PKC- $\gamma$  and p-CREB were decreased in the BMSC transplantation group compared with the control group (Fig. 2C, 2D, 2G, 2H).

### BMSC Transplantation Decreased the Upregulation of p-p38 MAPK- or p-ERK1/2- in CD11b Positive Cells in the Spinal Cord Dorsal Horn

We mapped the presence of the MAPK family, i.e., p-p38 MAPK, p-ERK1/2 and p-JNK in the spinal cord after SCI. Although the observed levels of p-p38 MAPK, p-ERK1/2 and p-JNK were weak in the intact spinal cord (data not shown), marked increases in p-p38 MAPK, p-ERK1/2 and p-JNK were noted in all laminae within the

spinal cord dorsal horn at day 14 post-SCI in the control group. The observed increase in expression of p-p38 was robust and tightly colocalized with cells that were positive for CD11b, but almost negligible with NeuN and glial fibrillary acidic protein (GFAP) positive neurons and astrocytes, respectively. Similarly, p-ERK1/2 colocalized with CD11b positivity, and there was also some colocalization of p-ERK1/2 with GFAP-labelled cells, but very little colocalization with NeuN-positive cells. P-JNK positivity colocalized with some GFAP-positive cells, but was also seen, albeit weakly, with some NeuN and CD11b-positive cells (Supplemental Fig. 4).

We evaluated the effects of BMSC transplantation on p-p38 MAPK- and p-ERK1/2 levels in CD11b positive cells by examining tissue sections post-SCI following dual immunohistochemical labelling for these markers and by immunoblotting of extracted proteins. Axial sections were used for the evaluation of MAPK activities in the injured spinal dorsal horn, where a reduction in the number of dual-labelled cells, especially in the spinal dorsal horn, was seen in the mice that received BMSC transplants compared with the control group. In contrast, the levels of expression of p-p38 MAPK and p-ERK1/2 did not change in sham-operated group (Fig. 3A, 3B, 3E, 3F). In addition, protein levels of p-p38 MAPK and p-ERK1/2 were decreased in the BMSC transplantation group compared with the control group (Fig. 3C, 3D, 3G, 3H).

### BMSC Transplantation Inhibited the Recruitment of CD11b and GFP- Positive Hematogenous Cells to the Lesion after SCI

To determine whether BMSC transplantation affects the recruitment of bone marrow-derived cells into the injured spinal cord, the contusive SCI was reproduced in chimeric mice wherein the hematogenous cells were GFP-labelled. Immunostaining of mid-sagittal sections of the spinal cord post-SCI revealed a transient increase in the numbers of CD11b positive cells that colocalised with GFP positive cells in the control group and BMSC treated group, both of which peaked at 14 days after injury. However, the distribution of cells that were dual labelled for CD11b and GFP was localized more to the lesion site in the BMSC transplantation group (Fig. 4). In contrast, CD11b positivity was weak and there was little evidence of migration of GFP-positive cells into the spinal cord in the sham-operated group. In addition, there were significantly fewer CD11b and GFP dual labelled cells in the spinal cord at days 14 and 28 post-SCI in the BMSC transplanted group compared to the control group (Fig. 4D). In contrast, the numbers of CD11b-positive and GFP-negative cells in the spinal cord tended to be higher in the BMSC transplanted group than in the control group, albeit insignificantly (Fig. 4E).



### BMSC Transplantation Suppressed the Activation of p-p38 MAPK and p-ERK1/2 in Bone Marrow-derived Macrophages and Resident Microglia after SCI, and Prevented Recruitment of Hematogenous Macrophages into the Injured Spinal Cord

In control mice, SCI resulted in an observed increase in the presence of cells that were dually labelled with p-p38 MAPK and GFP- or p-ERK1/2 and GFP within the spinal cord dorsal horn. In contrast, BMSC transplantation not only appeared to prevent the recruitment of GFP-positive bone marrow-derived cells into the spinal cord dorsal column, it also appeared to decrease their co-expression of p-p38 MAPK and p-ERK1/2 (Supplemental Fig. 5).

Flow cytometry confirmed the observed effects of BMSC transplantation in attenuating the infiltration of hematogenous macrophages and inhibiting the activation of those fewer cells that were recruited as well as the resident microglia. The number of CD11b and GFP dual-labelled cells was significantly lower in the BMSC transplantation group compared with the control group (Fig. 5A, 5B, 5C). In the sub-population of cells that was GFP<sup>positive</sup>/CD11b<sup>positive</sup>/CD45<sup>positive</sup>/Gr-1<sup>negative</sup> (i.e., hematogenous macrophages), the number (percentages) of activated p-p38 MAPK and p-ERK1/2-positive cells were 2778 ± 55 (97.6%) and 2792 ± 45 (98.1%) in the control group, and 976 ± 286 (65.6%) and 686 ± 153 (46.1%), respectively, in the BMSC transplantation group; these represented significant differences (Fig. 5D, 5E, 5F, 5G). In the sub-population of cells that were GFP<sup>negative</sup>/CD11b<sup>positive</sup>/CD45<sup>positive</sup>/Gr-1<sup>negative</sup> (i.e., resident microglia), the number (percentages) of activated p-p38 MAPK and p-ERK1/2-positive cells were 9101 ± 267 (96.5%) and 7950 ± 329 (84.3%) in the control group, and 1920 ± 391 (58.2%) and 1023 ± 478 (31.0%), respectively, in the BMSC transplantation group; these also represented significant differences (Fig. 5H, 5I, 5J, 5K).

### The Effects of BMSC Transplantation on Blood-spinal Cord Barrier (BSCB) Function

The effects of BMSC transplantation on the BSCB was evaluated by examining the presence of albumin, which leaks into the parenchyma after central nerve system injury and PDGFR- $\alpha$ , which localizes on perivascular astrocytic endfeet [49]. After the initial injury, BMSC transplantation significantly reduced leakage of albumin into the injury site at day 7 post-SCI compared to the control group, whilst at day 14 post-SCI only minor leakage of albumin was identified in both groups (Figs. 6A, 6C). Similarly, a significant reduction in PDGFR- $\alpha$  immunoreactivity at the lesion site was seen at day 7 post-SCI in the BMSC transplantation group compared with the control group, with a further reduction observed in both groups at day 14 (Fig. 6B, 6D).

### BMSC Transplantation Moderated the Cytokine Profile at the Lesion Site after SCI

Finally, we measured protein levels of tumor necrosis factor alpha (TNF- $\alpha$ ) and Interleukin (IL) -6 (as proinflammatory cytokines), matrix metalloproteinase 9 (MMP-9) (as a mediator of early secondary vascular pathogenesis), chemotactic cytokine ligand (CCL) 2, CCL5, CXC chemokine (C-X-C motif) ligand 10 (CXCL10) (as macrophage recruiting factors), and granulocyte-macrophage colony-stimulating factor (GM-CSF) (as a local microglial stimulating factor) at day 4 post-SCI (i.e. day 1 after transplantation) by immunoblot analysis. BMSC transplantation reduced the intensities of the bands for TNF- $\alpha$ , IL-6, MMP-9, CCL2, CCL5, and CXCL10, but increased that of GM-CSF, compared with the SCI control group (Fig. 7).

## DISCUSSION

Transplantation of BMSCs after SCI has been shown consistently to improve motor function following SCI [51, 52], however the effect of BMSC transplants on neuropathic pain or pain hypersensitivity is less clear [9-12]. Understanding the possible outcome and mechanisms of action of BMSC transplantations in terms of pain is an important issue, especially as BMSCs are being considered for clinical treatments of SCI. The present study demonstrated significant improvements in motor function as well as mechanical allodynia and thermal hyperalgesia in a contusion model of thoracic SCI as a result of BMSC transplantation. The findings were most marked in SCI animals that were transplanted with BMSCs at 3 days after injury, with no significant improvement seen when animals were transplanted at 14 days post-SCI. Previously, we demonstrated that the greatest survival of transplanted BMSCs was also achieved when these cells were transplanted at day 3 post-SCI, compared with transplantation at days 1, 7, and 14 post-SCI; furthermore, we and others found that day 3 post-SCI is the time point when the numbers of major inflammatory cells are still increasing in the injury site [39, 53]. Based on these previous findings, as well as the present results, we suggest that transplantation of BMSCs in the acute as opposed to sub-acute phase after SCI will have beneficial effects on post-SCI chronic neuropathic pain, at least in the short-term, which supports their early use in clinical treatments. Further research into methods designed to enhance the survival of transplanted BMSCs is warranted. We have previously demonstrated that blocking IL-6 signaling enhances BMSC survival and immunomodulatory and neuroprotective outcomes following BMSC transplantation [38]. Such a strategy may also prove beneficial to improve the effects of BMSC transplantation on neuropathic pain. In addition, a better understanding of the mechanisms of action of BMSCs in suppressing pain after SCI is essential to their successful therapeutic application.

Activation of the MAPK signaling pathway in dorsal horn neurons and glial cells contributes to the development and maintenance of neuropathic pain, which promotes long-term potentiation (LTP) and central sensitization. MAPK signaling cascades are indicated through phosphorylation and hence activation of ERK1/2, JNK, and p38 MAPK; this in turn can lead to phosphorylation and activation of the transcription factor, CREB [19]. Activated CREB regulates the NR1 subunit on the NMDA channel [19] and is upregulated in spinothalamic tract cells in association with the development of neuropathic pain [54]. Phosphorylated PKC- $\gamma$  is another important intracellular signaling protein found in a specific subset of excitatory inter-neurons in the inner region of lamina II, which receive input from myelinated, non-noxious afferents [55]. PKC- $\gamma$  has similarly been associated with neuropathic pain behavior [56]. The present results indicated that early BMSC transplantation suppressed the activation of p38 MAPK and ERK1/2 in microglia and macrophages, as well as that of CREB and PKC- $\gamma$  in dorsal horn neurons in and around the lesion. This demonstrates that early BMSC transplants after SCI suppressed several pain signaling pathways, and raises the question as to how BMSCs might bring about these effects.

Several studies using animal models of neural damage showed that MSC, including BMSCs, inhibit microglial activation and inflammatory responses [9, 57, 58]. *In vitro* experiments have demonstrated that MSC reduce the pro-inflammatory potential of dendritic cells and natural killer cells by inhibiting the production of inflammatory factors, including TNF- $\alpha$  and interferon  $\gamma$  (IFN- $\gamma$ ) [59] and they also inhibit the activation of microglial cells [23, 60]. We have previously reported that BMSCs enhance neuroregeneration through overexpression of neurotrophic factors, as well as through modulation of immune responses following SCI from an inflammatory (M1) to an alternatively activated (M2) macrophage phenotype. This was associated with downregulation of T helper 1 (Th1)-type cytokines TNF- $\alpha$  and IFN- $\gamma$  and increased levels of Th2-type cytokines IL-4 and IL-13 at the site of injury [39, 52]. Hence, this and previous work has provided compelling evidence that early BMSC transplants after SCI suppress pain signaling pathways and pain hypersensitivity through their anti-inflammatory and immunomodulatory activity.

The pathogenesis of neuropathic pain and central sensitization after peripheral nerve injury seems to involve spinal cord microglia as well as macrophages derived from hematogenous monocytes [34, 35]. We used GFP-labelled bone marrow chimeric mice in the present study for a better understanding of the relative roles of these different cell types in SCI. The prevalence of activated resident microglia and infiltrating macrophages increased after SCI especially at day 14 when the most severe pain hypersensitivity was noted in the control group (Figs 4 and 5). BMSC transplantation decreased p-p38 MAPK and p-ERK1/2 positivity both in the resident microglia (GFP-negative cells) and hematogenous mac-

rophages (GFP-positive cells), indicating that BMSCs suppressed the activation of both cell types (Fig. 5). Furthermore, BMSC transplantation significantly reduced the recruitment of CD11b- and GFP-dual-labelled hematogenous macrophages at days 14 and 28 to the injury site (Fig. 4D). The decrease in hematogenous macrophage infiltration into the injured spinal cord after BMSC transplantation was associated with modifications in the secreted profile of inflammatory cytokines and chemokines known to increase endothelial permeability and attract inflammatory cells to wound sites, i.e. TNF- $\alpha$ , IL-6, MMP-9, CCL2, CCL5, and CXCL10 [35, 61-63], as well as direct evidence of a restored BSCB, as shown through decreased cavitation in the lesion site, reduced staining for albumin, and evidence of tight junctions in the astrocytic endfeet and endothelial cells following PDGFR- $\alpha$  immunolocalization and electron microscopy at day 7 after SCI (Fig 6) [49, 64]. Of these changes, it is noteworthy that CCL2, a member of the chemokines family that has been recently identified as a possible key integrator of neuropathic pain in peripheral nerve injury and SCI [35] was downregulated following BMSC transplantation in our study. In contrast to the recruitment of hematogenous macrophages after SCI, proliferation of resident microglia is mainly regulated by colony-stimulating factors [66]. GM-CSF also facilitates axonal regeneration and functional recovery after SCI [31]. The local increase in GM-CSF at the injury level observed in our study could have stimulated the proliferation of microglia, which tended to increase the proportion of CD11b-positive and GFP-negative activated spinal cord microglia at the lesion site, albeit insignificantly (Fig. 4E).

## CONCLUSION

In this study, we have demonstrated that early BMSC transplants not only decreased the activation of MAPK signaling in injured spinal cord, but also altered the localized presence of inflammatory mediators, which will have contributed to a decrease BSCB disruption and reduction in the recruitment of harmful blood-borne macrophages. The net effect of these changes is likely to play a major role in reducing pain hypersensitivity in the BMSC treated SCI animals.

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## AUTHOR CONTRIBUTIONS

S.W.: conception and design, collection and/or assembly of data, data analysis and interpretation, manuscript writing; K.U.: conception and design, administrative support, data analysis and interpretation, manuscript writing; S.W. and K.U.: contributed equally to this work; H.N. data analysis and interpretation, manuscript writing; H.M., D.S., A.Y., and K.H.: collection and/or assem-

bly of data; W.E.B.J.: conception and design, manuscript writing; H.B.: conception and design, administrative support, final approval of manuscript.

## DISCLOSURE OF POTENTIAL CONFLICTS OF INTEREST

The authors indicate on potential conflicts of interest.

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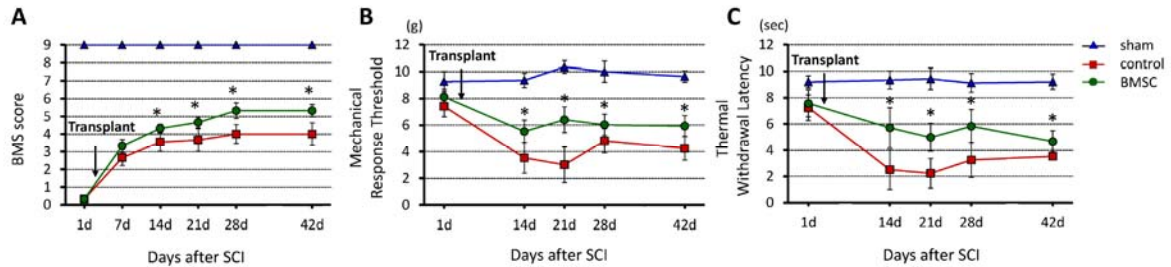


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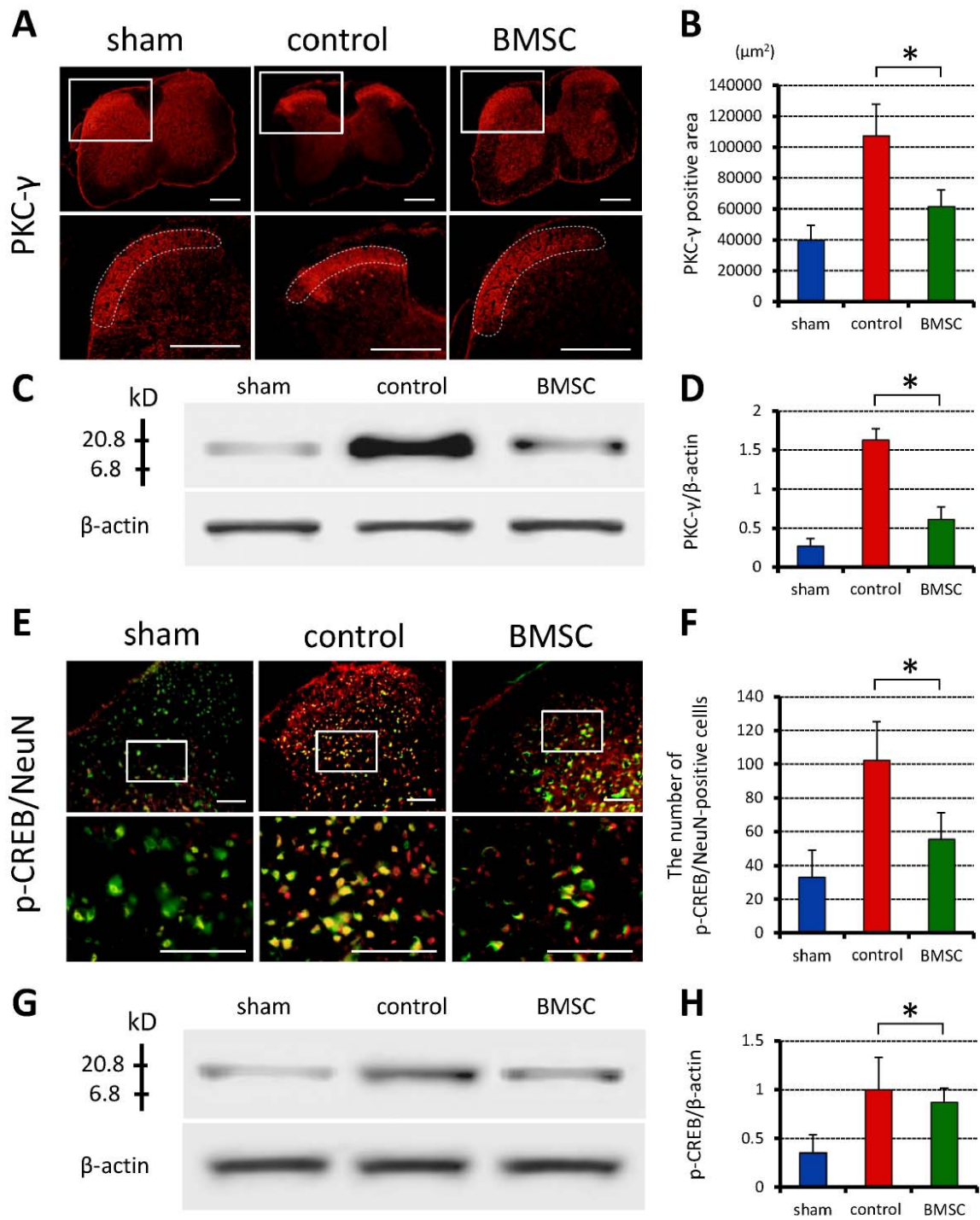


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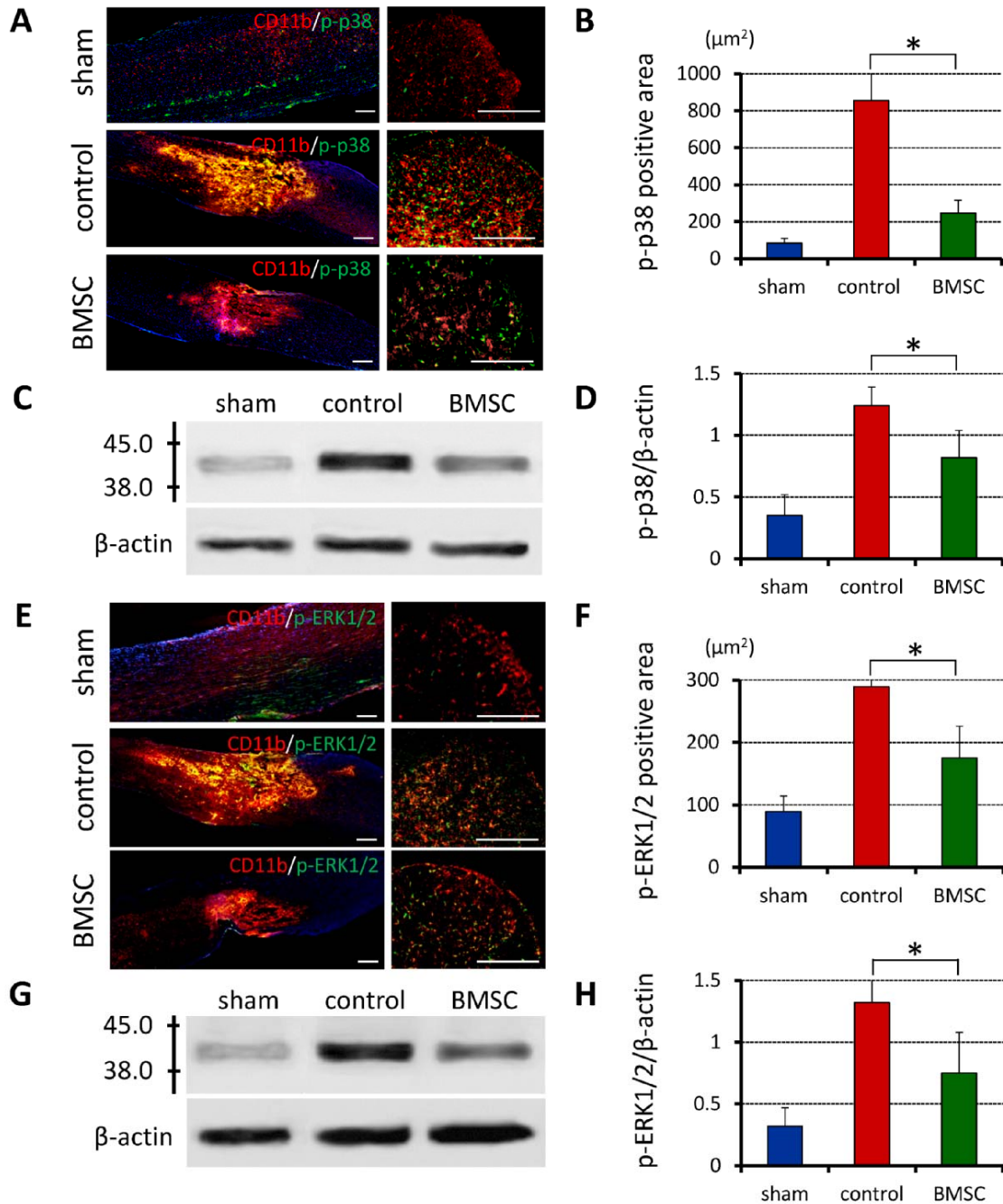
**Figure. 1. BMSC transplantation improved the locomotor BMS score and hypersensitivities to mechanical and thermal stimulation after SCI. (A):** Significant improvement in hind-limb motor function was noted in the BMSC transplantation group compared with the control group from day 14 post-SCI. The control group and BMSC transplantation group demonstrated similar increases in responsiveness to both stimuli from day 14 post-SCI (sham, n=18; control, n=36; BMSC, n=36; \*, p< .05). Data shown are means±SD. **(B, C):** SCI-induced hypersensitivities to mechanical and thermal stimulation improved in the BMSC transplantation group from day 14 post-SCI (sham, n=15; control, n=30; BMSC, n=30; \*, p< .05). Data shown are means±SD. (A, B, C) one-way factorial ANOVA. Abbreviations: BMS, Basso Mouse Scale; SCI, spinal cord injury; BMSC, bone marrow-derived mesenchymal stem cells.



**Figure. 2. BMSC transplantation reduced PKC- $\gamma$  and p-CREB immunoreactivity in the dorsal horn after SCI. (A-D):** PKC- $\gamma$  was found primarily in a band of cells restricted to the inner portion of lamina II in the sham group. High magnification pictures taken from respective boxed area. **(A):** PKC- $\gamma$  immunoreactivity after SCI was increased within the spinal dorsal horn around the site of the lesion at day 14 post-SCI. **(B):** In the BMSC transplantation group, the PKC- $\gamma$ -positive area was significantly smaller than the control group (n=3 each; \*, p< .05). **(C, D):** Western blotting samples from the BMSC transplantation group showed significantly lower protein levels of PKC- $\gamma$  (n=3 each; \*, p< .05). **(E, F):** Representative immunofluorescent staining of axial sections showing colocalization of p-CREB staining (red) with NeuN staining (green) around the injured spinal cord at day 14 post-SCI. Colocalization of p-CREB and NeuN neurons in the dorsal horn was significantly reduced in the BMSC transplantation group compared with the control group (n=3 each; \*, p< .05). High magnification pictures taken from respective boxed areas. **(G, H):** Western blotting samples from the BMSC transplantation group showed significantly lower protein levels of p-CREB (n=3 each; \*, p< .05). Data shown are means $\pm$ SD. Scale bars=100  $\mu$ m in (A) and 50  $\mu$ m in (E). (B, D, F, H) one-way factorial ANOVA. Abbreviations: BMSC, bone marrow-derived mesenchymal stem cells; PKC- $\gamma$ ; protein kinase C gamma; p-CREB, phospho- cyclic AMP response element binding protein; SCI, spinal cord injury.

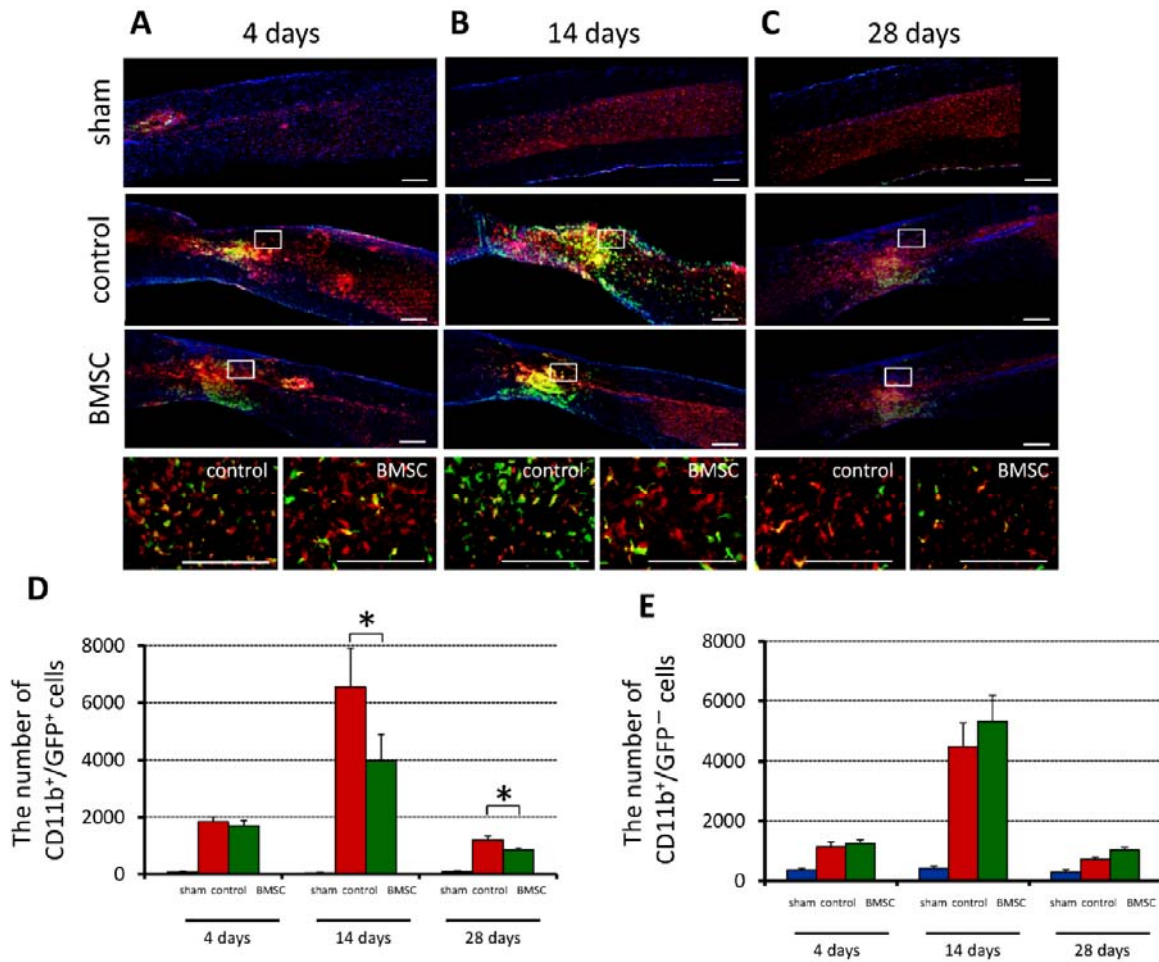


**Figure 3. BMSC transplantation reduced the prevalence of p-p38 MAPK- and p-ERK1/2-positive CD11b cells. (A, B, E, F):** Representative sagittal and axial immunofluorescent staining showing colocalization of p-p38 MAPK and p-ERK1/2 with CD11b at day 14 post-SCI. The presence of p-p38 MAPK and p-ERK1/2 colocalized with CD11b immunolabelled cells was reduced in the BMSC transplantation group, especially in the spinal dorsal horn. Representative sagittal and axial immunofluorescent staining in sham group showed little colocalization of p-p38 MAPK and p-ERK1/2 with CD11b. Scale bars=200  $\mu\text{m}$ . **(C, D, G, H):** Western blotting samples from the BMSC transplantation group showed significantly lower protein levels of p-p38 MAPK and p-ERK1/2 compared to the control group. Each graph shows the relative band intensity normalized to that of  $\beta$ -actin ( $n=3$  each; \*,  $p < .05$ ). Data shown are means  $\pm$  SD. (B, D, F, H) one-way factorial ANOVA. Abbreviations: BMSC, bone marrow-derived mesenchymal stem cells; MAPK; mitogen activated protein kinase; ERK, extracellular signal-regulated kinase; SCI, spinal cord injury.

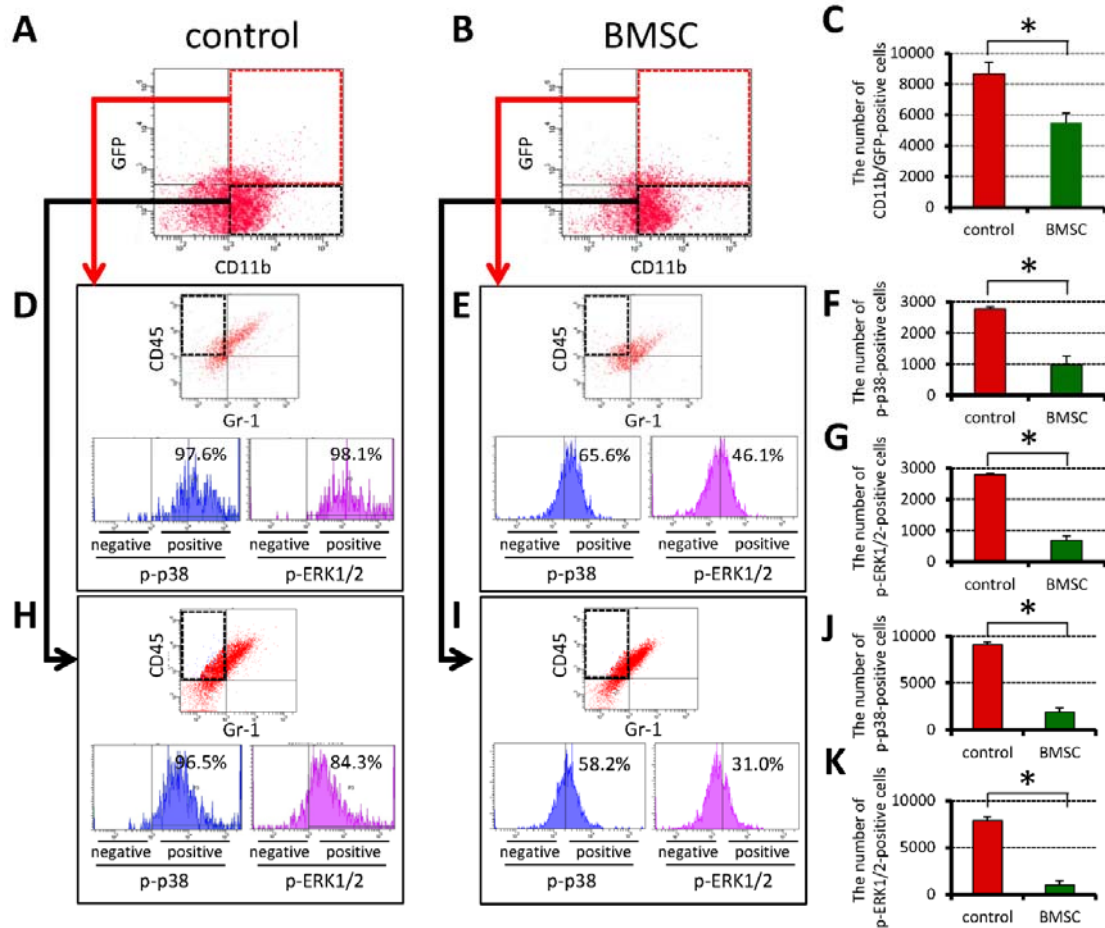




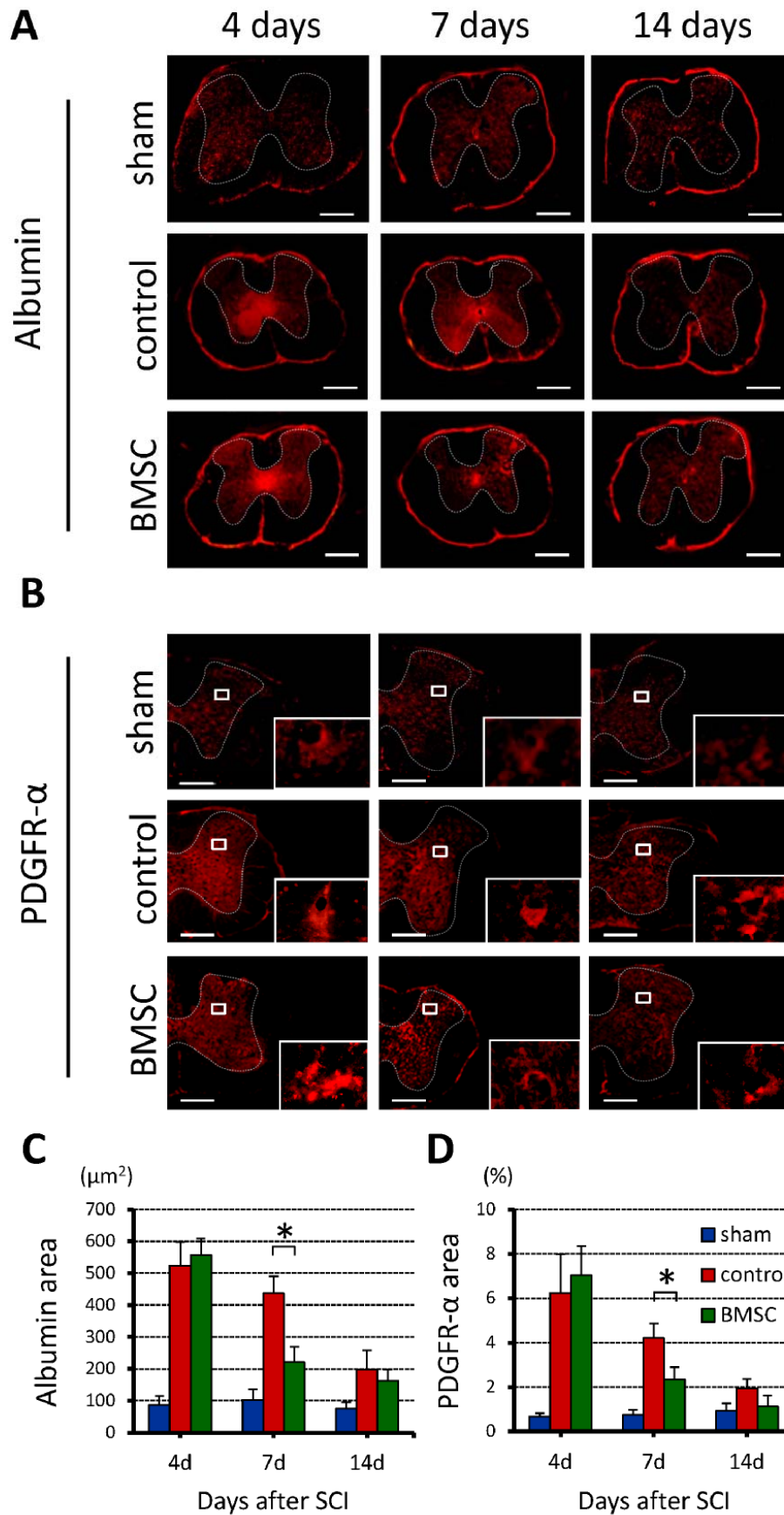
**Figure 4. BMSC transplantation reduced the prevalence of CD11b and GFP double-positive cells. (A, B, C):** The number of CD11b-positive cells were scored in equal areas of tissue in the BMSC transplanted group and control group. The prevalence of these cells reached a peak value at day 14 post-SCI. A more focalized lesion with the centralized presence of CD11b and GFP double-positive cells was a common finding in the BMSC transplantation group, compared to a larger lesion seen in the control group. High magnification pictures taken from respective boxed area. There are a few CD11b and GFP positive cells in sham operated model. Scale bars=200  $\mu$ m. **(D, E):** BMSC transplantation tended to reduce the prevalence of CD11b and GFP double-positive cells at the lesion site, and tended to increase the prevalence of CD11b-positive and GFP-negative cells, albeit insignificantly ( $n=3$ , each; \*,  $p < .05$ ). Data shown are means  $\pm$  SD. (D, E) one-way factorial ANOVA. Abbreviations: BMSC, bone marrow-derived mesenchymal stem cells; GFP, green fluorescent protein; SCI, spinal cord injury.



**Figure 5. BMSC transplantation suppressed the presence of pain-related proteins in the injured spinal cord: flow cytometry analysis for the p-p38 MAPK and p-ERK1/2 signaling pathways. Representative histograms of flow cytometry at day 14 post-SCI for the control and BMSC transplantation groups after SCI are shown. (A-C):** The number of CD11b and GFP dual labelled cells was lower in the BMSC transplantation group compared with the control group. Specifically, BMSC transplantation prevented the migration of hematogenous cells into the injured spinal cord (n=3 each; \*, p< .05). **(D-G):** The number of activated p-p38 MAPK and p-ERK1/2-positive cells in the population of GFP<sup>positive</sup>/CD11b<sup>positive</sup>/CD45<sup>positive</sup>/Gr-1<sup>negative</sup> cells (hematogenous macrophages) were lower in the BMSC transplantation group than in the control group (n=3 each; \*, p< .05). **(H-K):** The number of activated p-p38 MAPK and p-ERK1/2-positive cells in the population of GFP<sup>negative</sup>/CD11b<sup>positive</sup>/CD45<sup>positive</sup>/Gr-1<sup>negative</sup> cells (resident microglia) were also lower in the BMSC transplantation group than the control group (n=3 each; \*, p< .05). Data shown are means±SD. (C, F, G, J, K) one-way factorial ANOVA. Abbreviations: BMSC, bone marrow-derived mesenchymal stem cells; MAPK; mitogen activated protein kinase; ERK, extracellular signal-regulated kinase; SCI, spinal cord injury; GFP, green fluorescent protein.



**Figure 6. BMSC transplantation reduced disruption of the BSCB after SCI. (A, B):** Representative immunofluorescent staining around the site of SCI lesion showing the prevalence of albumin and PDGFR- $\alpha$  in the injured spinal cord at days 4, 7 and 14 post-SCI. There was comparatively little evidence of albumin leakage and PDGFR- $\alpha$  expression in the sham group (high magnification images for PDGFR- $\alpha$  positivity were taken from the respective boxed areas). Scale bars=200  $\mu$ m. **(C, D):** The albumin- and PDGFR- $\alpha$ -positive areas in the gray matter (dotted line) were significantly smaller in the BMSC transplanted group compared with the control group at day 7 post-SCI, but no significant differences were found between these two groups at days 4 and 14 post-SCI (n=3 each; \*, p< .05). Data shown are means $\pm$ SD. (C, D) one-way factorial ANOVA. Abbreviations: BMSC, bone marrow-derived mesenchymal stem cells; BSCB, blood-spinal cord barrier; SCI, spinal cord injury; PDGFR- $\alpha$ , platelet-derived growth factor receptor alpha.



**Figure 7. BMSC transplantation decreased protein levels of inflammatory cytokines and macrophage recruiting chemokines, while increasing that of GM-CSF.** Each graph shows the relative band intensity of each protein normalized to that of  $\beta$ -actin. Western blotting showed significantly lower protein levels of TNF- $\alpha$  and IL-6 (inflammatory cytokines), MMP-9 (a mediator of early secondary vascular pathogenesis), and CCL2, CCL5 and CXCL10 (all macrophage recruiting factors), and significantly higher protein levels of GM-CSF (a microglial stimulating factor) in the BMSC transplanted group compared with the control group at day 4 post-SCI (n=3 each; \*, p<.05). Data shown are means $\pm$ SD. (A, B, C) one-way factorial ANOVA. Abbreviations: BMSC, bone marrow-derived mesenchymal stem cells; GM-CSF, granulocyte-macrophage colony-stimulating factor; TNF- $\alpha$ , tumor necrosis factor alpha; IL-6, interleukin 6; MMP-9, matrix metalloproteinase 9; CCL, chemotactic cytokine ligand; CXCL10, chemokine (C-X-C motif) ligand 10; SCI, spinal cord injury.

