**Transplantation of Mesenchymal Stem Cells Promotes the Alternative Pathway of Macrophage Activation and Functional Recovery after Spinal Cord Injury**

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**ABSTRACT**

Mesenchymal stem cells (MSC) derived from bone marrow have the potential to reduce the acute inflammatory response in spinal cord injury (SCI) and this is beneficial to the recovery of neurological function. However, the precise mechanisms through which transplanted MSC attenuate inflammation after SCI are still unclear. The present study was designed to investigate the effects of MSC transplantation especially focused on their potential influence on the alternative pathway of macrophage activation after SCI. We produced a T9-T10 SCI by contusion in rats, 3 days after which 1.0 x 106 cells PKH26-labeled MSC were transplanted into the contusion epicentre. The transplanted MSC migrated within the injured spinal cord, but did not differentiate into glial or neuronal elements. MSC transplantation was associated with major alterations in the SCI environment, significantly increasing levels of IL-4 and IL-13 whilst reducing levels of TNF-α and IL-6. This simultaneously was associated with an increase in the prevalence of alternatively activated macrophage (M2 phenotype: arginase-I or CD206-positive macrophages) and a decrease in the prevalence of classically activated macrophages (M1 phenotype: iNOS or CD16/32-positive macrophages). Furthermore, a higher degree of locomotion recovery was evident in the MSC transplanted group which correlated with increased axonal regeneration, less scar tissue formation and increased myelin sparring. Our results suggested that the acute transplantation of MSC after SCI modifies the inflammatory environment by shifting the macrophage phenotype from M1 to M2, and that this may reduce the effects of the inhibitory scar tissue in the subacute/chronic phase after injury to provide a permissive environment for axonal extension and functional recovery.

[Word Count: 250]

**Key Words:** mesenchymal stem cell; bone marrow; spinal cord injury; transplantation; macrophage

**INTRODUCTION**

A number of experimental studies have attempted to establish a feasible method to improve pinal cord function after neural injury. For example, supplementation of neurotrophic factors (Nakajima H, 2007, 2010), prevention of the effects of anti-inflammatory cytokines (Chen KB, 2011), or suppression of nerve-inhibitory factors (Chen MS, 2000) could enhance spinal cord regeneration. One of the most promising therapeutic approaches for spinal cord injury (SCI) is cellular transplantation (Murray M. 2004). A number of different cell types have been evaluated, among them adult mesenchymal stem cells derived from bone marrow (MSC). MSC have been shown to promote anatomical and functional recovery in animal models of SCI by promoting tissue sparing (Himes BT, 2006; Sheth RN, 2008)and axonal regeneration (Wu S, 2003), suggesting that the therapeutic effects of MSC are primarily due to their secretion of soluble factors and the provision of an extracellular matrix that provides neural protection and support, although MSC-mediated remyelination (Akiyama Y, 2002) and neural differentiation have also been reported [refs]..

MSC are attractive candidates for transplantation into human patients because they can be easily harvested, expanded and banked, or derived directly from the patient allowing for autologous transplantation, avoiding the immunological and ethical problems that associated with transplantation of other types of stem cells such as embryonic stem cells (McDonald JW, 1999) or neural stem cells (Ogawa Y, 2002). Evidence suggests that MSC areimmunosuppressive (Di Nicola M, 2002; Jiang X-X, 2005), i.e. they possess anti-inflammatory activity and have been used to abrogate graft-versus-host disease [refs]. This property, in particular, may contribute to their evident capacity to reduce the acute inflammatory response to SCI and hence reduce cavity formation as well as decrease astrocyte and microglia/macrophage reactivity (Neuhuber B, 2005; Himes BT, 2006). Importantly, MSC transplantation has been shown not only to enhance tissue preservation after SCI, but also to associate with a reduction in injury-induced sensitivity to mechanical stimuli in an experimental SCI model, which is functionally indicative of anti-inflammatory activity (Abrams MB, 2009).

Considering the inflammatory response after SCI, different macrophages populations have been documented based on their phenotypes and activity, but two subtypes have become of great interest; classically activated macrophages (termed the M1 phenotype) or alternatively activated macrophages (M2 phenotype) (Popovich PG, 1999; Schwartz M, 1999; Gordon S, 2003; Mantovani A, 2004; Laskin DL, 2009; Busch SA, 2011). Classically activated macrophages are the product of exposure to T helper 1 (Th1) cytokines, e.g. interferon gamma (IFN-γ) and tumor necrosis factor alpha (TNF-α). Alternatively activated macrophages are the product of T helper 2 (Th2) cytokines, e.g. interleukin (IL)-4 and IL-13,; (Gordon S, 2003; Ma J, 2003).

Alternatively activated macrophages not only have enhanced phagocytic capacity but also possess anti-inflammatory activity, and hence are capable of generating beneficial effects in the recovery of SCI (Bomstein Y, 2003; Kigerl KA, 2009; Schwartz M, 2010; Busch SA, 2011). MSC transplantation into SCI lesions similarly attenuates acute inflammation and this is beneficial to the recovery of function following SCI. However, the precise mechanisms through which the transplanted MSC attenuates the inflammation after SCI are still unclear.Therefore, the present study was designed to investigate the effects of MSC transplantation and was especially focused on their potential influence on the alternative pathway of macrophage activation after SCI.

**MATERIALS AND METHODS**

***Preparation of MSC***

Commercially obtained human MSC (Lot. #PT-2501; Lonza, Walkersville, MD), which had a CD immunoprofile consistent with the MSC phenotypes (CD105+, CD166+, CD29+, CD44+, CD14-, CD34-, CD45-), were cultured at 37°C in a humidified atmosphere of 5% CO2 in mesenchymal stem cell basal medium (MSCBM) with 10% fetal calf mesenchymal cell growth supplement, 200mM L-glutamine, 25units penicillin and 25μg streptomysin (Lot. #PT-3001; Lonza). When the proliferating colonies had reached near confluence, the adherent cells were lifted by incubation in 0.25% trypsin solution for 5-10 minutes. After 3-4 passages, we used the cells for transplantation. For *in vivo* tracing, the MSC were pre-labeled with the membrane dye PKH26 according to the manufacturer’s instructions (Sigma-Aldrich).

***Animal Model of Spinal Cord Injury***

Experiments were conducted in 69 adult male Sprague-Dawley rats (Clea, Tokyo), aged 8-10 weeks with a mean body weight of 271±29.1 g (±SD). Following anaesthesia using isoflurane (Forane®, Abbot, Tokyo, Japan), laminectomy was performed at the T10 levels under a surgical microscope (VANOX-S, Olympus, Tokyo), taking utmost care in avoiding dura matter laceration. At the T9-10 vertebral level, the dorsal surface of the spinal cord was compressed extradurally using the Infinite Horizons Impactor (Precision Systems and Instrumentation LLC, Fairfax, VA) with an impact force of 200 kilodynes (kdyn). All the subjects were housed under a 12-hour light-dark cycle in a bacteria-free biologically clean room with access to food and water *ad libitum*, where they received manual bladder expression twice daily until the control of sphincters was recovered. The experimental protocol was approved by the Ethics Committee for Animal Experimentation of Fukui University.

***MSC Transplantation***

Three days after the injury, MSC were pre-labeled with the membrane dye PKH26, then 5 μl of MSCBM containing MSC at a concentration of 1.0 x 106 cells/ml was injected into the contusion epicenter using a microsyringe; hence a total of 5 x 103 MSC were administered in the treatment group. 5 μl of MSCBM alone (no cells) was also injected into rats at 3 days after SCI and used as non-treatment (control) groups.

***Immunofluorescence Staining***

Following injection of MSC or MSCBM, the rat spinal cord was perfused and fixed with 4% paraformaldehyde in 0.1M phosphate-buffered saline (PBS) and post-fixed in the same fixative (24 hours), 10% sucrose in 0.1M PBS (24 hours), and 20% sucrose in 0.1M PBS (24 hours). Segments of the spinal cord (between T8 and T12 cord segments) were embedded in optimal cutting temperature compound (OCT) and cut on a cryostat into serial 25 μm-thick sagittal and transverse frozen sections. For immunofluorescence staining, the sections were incubated at 4°C with anti-neuronal nuclei (NeuN) monoclonal antibody (1:400, mouse IgG; Chemicon International, Temecula, CA) for neurons, anti-reactive immunology protein (RIP) monoclonal antibody (1: 100,000, mouse IgG; Chemicon International) as a mature oligodendrocyte-specific marker, anti-glial fibrillary acidic protein (GFAP) monoclonal antibody (1:400, mouse IgG; Chemicon International) for astrocytes, anti-microglia monoclonal antibody (OX42, CD11b, 1:400, mouse IgG; Abcam plc) for microglia, anti-iNOS polyclonal antibody (1:200, mouse IgG; Abcam plc), anti-CD16/32 polyclonal antibody (1:200, mouse IgG; Abcam plc), anti-arginase-I polyclonal antibody (1:200; Santa Cruz Biotechnology, Santa Cruz, CA), anti-CD206 polyclonal antibody (1:200; Santa Cruz Biotechnology), anti-growth-associated protein (GAP)-43 polyclonal antibody (GAP-43; 1:500, rabbit IgG; Abcam plc), or monoclonal antineurofilament 200 kD (RT97, 1:1000, rabbit IgG; Abcam plc) diluted in Antibody Diluent with Background Reducing Components (Dako Cytomation). The secondary antibodies were donkey anti-goat Alexa Flour® 488-conjugated antibody, goat anti-rabbit Alexa Flour® 488-conjugated antibody, goat anti-mouse Alexa Flour® 488/fluorescein-conjugated antibody, or goat anti-mouse Alexa Flour® 350/fluorescein-conjugated antibody (1:250; Molecular Probes, Eugene, OR), applied for 1 hour at room temperature.

All images were obtained using a fluorescence microscope (Olympus AX80, Olympus Optical, Tokyo) or a confocal laser scanning microscope (model TCS SP2, Leica Instruments, Nusslosh, Germany). Some sections were counterstained with nuclear marker DAPI (Abbott Molecular, Des Plaines, IL).

***Flow Cytometry Analysis***

Immediately after being terminally anesthetized, each rat was intracardially perfused with 200ml of ice-cold 0.1M PBS and the spinal cords were harvested. The injured portion of each spinal cord (1cm around the epicentre) was surgically dissected and dissociated with collagenase (175U/ml; Sigma-Aldrich; St. Louis, MO) for 1 hour at 37°C. Cells were washed in Dulbecco’s modified Eagle’s Medium (Invitrogen Life Technologies, Carlsbad, CA) containing 10% fetal bovine serum and filtered through a 40µM nylon cell strainer (BD Biosciences, San Jose, CA) under centrifugation to remove tissue debris, obtaining a single-cell suspension, following previous protocols (Saiwai, H., et al., 2010).

Cells were then incubated for 1 hour on ice with the following fluorescently tagged antibodies: APC anti-mouse CD45 (0.25 µg: 1ml; BioLegend, San Diego, CA), Pacific BlueTM anti-mouse Ly-6G/Ly-6C (equivalent to Gr-1) (1.0 µg: 1ml; BioLegend) and PerCP-CyTM 5.5 Rat anti-mouse CD11b (0.25 µg: 1ml ; BD PharmigenTM). For intracellular staining (Stirling, D.P., et al., 2008), the cells were resuspended in 1% buffered formalin (Fixation Buffer, Santa Cruz Biotechnology) and permeabilized with methanol (Permeabilization Buffer, Santa Cruz Biotechnology) followed by resuspension in ice-cold PBS to the proper concentration and incubation for 1 hour with arginase-I (1:200; Santa Cruz Biotechnology) secondarily conjugated to FITC (1:200; Santa Cruz Biotechnology) and PE iNOS antibody (3 µg/ml; Abcam plc, Cambridge, UK). Samples with cells alone and cells incubated with isotype-matched irrelevant antibodies were prepared to be used as controls to eliminate non-specific binding and autofluorescence.

Flow cytometry analysis was performed using a FACS CantoTM II (Becton Dickinson Biosciences, San Jose, CA) using forward scatter to remove cellular debris. In each test a minimum of 250, 000 cells were analyzed and the data processed using BD FACSDiva software (Becton Dickinson Biosciences). The different cells in the suspension were classified accordingly to the combination of the expressed antigens, stated in previous reports as follows: CD45 high/CD11b high/GR-1 high identified neutrophils (Fleming, T.J., et al.,1993; Lagasse, E., et al.,1996); CD45 positive/CD11b high/GR-1 negative identified macrophages (Ho, M.K., et al., 1983); and CD45 positive/CD11b low/GR-1 negative identified microglia (Sedgwick, J.D., et al., 1991). The phenotype of such macrophages was corroborated through quantification of iNOS or arginase-1 expression.

***Immunoblot Analysis***

MSC or MSCBM injected thoracic spinal cord segments (10-mm long) centred on the site of injury (epicenter) were obtained at 1 week after spinal cord injury and stored in liquid nitrogen. The samples were solubilized in RIPA buffer (50 mM TRIS-HCl, pH 7.5, 150 mM NaCl, 1% Triton X-100, 0.5% sodium deoxycholate, 20 μg/ml leupeptine and 1 mM phenylmethylsulfonylfluoride [PMSF]), homogenized and then stored at –80°C. The protein concentration was analyzed using the Bio-Rad DC protein assay kit (No. 500-0116, Bio-Rad Laboratories, Hercules, CA). Laemmli sodium dodecylsulphate buffer samples containing proteins were boiled and subjected to immunoblot analysis. Total protein (80 μg/lane) was subjected to sodium dodecylsulphate polyacrylamide gel (15%) electrophoresis (SDS-PAGE) and transferred onto polyvinylidene difluoride membrane (PE Applied Biosystems, Foster, CA) for 70 minutes in a semi-dry blot apparatus. The membranes were then washed twice in PBS containing 0.05% Tween 20, blocked with 5% skimmed milk for 1 hour, and subsequently probed with antibodies against the following cytokines; anti-TNF-α (0.2µg/ml; Abcam plc), anti-IL-6 (1:200; Santa Cruz Biotechnology), anti-IL-4 (1:200; Santa Cruz Biotechnology, Santa Cruz, CA), or anti-IL-13 (1:200; Santa Cruz Biotechnology). Each primary antibody was diluted as indicated and incubated with the membranes overnight at 4°C; membranes were then washed prior to incubation with respective secondary HRP-conjugated antibodies, i.e. anti-goat (1:1000), anti-rabbit (1:5000), or anti-rat (1:1000) (Santa Cruz Biotechnology) for 3 hours. After subsequent triple washing in PBS, the membranes were sunk in a commercially available kit for enhanced chemiluminescence(ECL; supplier needed, Amersham?) for 1 minute to take an X-ray film for visualization of peroxidase activity.

***Measurement of Injury Magnitude and Histological Analysis***

For a semi-quantitative analysis of the extent of cavitation and demyelination 5 weeks after SCI, images of axial sections stained with Haematoxylin and eosin (HE) and Luxol fast blue (LFB) (for myelination) were obtained. The cavitation area on HE stained sections and the extent of LFB-positive areas in ventro-lateral funiculus at the epicenter, 4mm rostral and caudal to the epicenter, were analyzed by a color image analyzer (MacSCOPE; Mitani, Fukui, Japan). The LFB-positive area in which the density of stain significantly exceeded the threshold of each background was calculated as a percentage of the cross-sectional area of residual tissue.

***Assessment of Locomotor Behavior***

To assess the behavior of each rat after SCI and recovery of locomotor function, 10 rats from each of the groups were investigated at 3 days, 1, 2, 3, 4, and 5 weeks post injury. Locomotor function of the hind limbs was graded using the Basso-Beattie-Bresnahan (BBB) hind limb locomotor rating scale.The BBB rating scale (Basso DM, 1995) is a 21-point system based on operationally-deﬁned behavioral features which follow the recovery progression from complete paralysis to normal locomotion. The rating scale ranges from 0 to 21, with a score of 0 indicating complete hind limb paralysis, while a score of 21 denotes completely normal locomotor function. Scores of 0–20 indicate an animal’s altered ability to move the hind limb joints, to bear weight, and to coordinate forelimb and hind limb movement.

***Statistical Analysis***

All values are expressed as mean± standard deviation (SD). Differences between groups were examined for statistical significance using the paired-t test. A *P*-value<0.05 denoted the presence of a significant difference. The above tests were conducted using SPSS software version 11.0 (SPSS, Chicago, IL).

**RESULTS**

***The distribution of transplanted MSC in the injured spinal cord***

To the distribution of PKH26-labelled MSC in the injured spinal cord was assessed at 1 and 5 weeks after transplantation in harvested sagittal tissue sections.. At 1 week post injury, the transplanted MSC were distributed only around the injured lesion (Fig. 1C). While a number of cells extended out of the injured lesion at 5 weeks (Fig. 1A, B).

Immunostaining of spinal cord sections 5 weeks after transplantation of MSC was performed for NeuN, RIP, GFAP, and OX-42. The transplanted cells were identified by the distribution of PKH26. There was no obvious colocalization of NeuN, RIP, GFAP, or OX-42 with PKH26-identified MSC (Fig. 2). These results indicated that although the MSC were distributed in areas rich in glial and neuronal processes, there was no evidence of the MSC themselves differentiatin into glial or neuronal cells.

***The effects of MSC transplantation on the phenotype of infiltrated macrophages after spinal cord injury***

Higher numbers of classically activated macrophages located mainly in the gray matter around the injured site of the spinal cord, expressing iNOS and CD16/32 colocalized with OX-42, were found in the control group than in the MSC transplanted group. These positive cells presented small compact cytoplasm with occasional unipolar extensions (Fig. 3A, B, E, and F). On the other hand, consistently higher numbers of alternatively activated macrophages, expressing arginase-I or CD206 colocalized with OX-42, were found in the MSC transplanted group, with a more localized distribution at the injury site in the spinal cord. These positive cells showed a typically enlarged cytoplasm encasing vacuolar structures. In the control group, arginase-I or CD206/OX42 positive cells were barely found (Fig. 3C, D, G, and H).

The profiles of the macrophages present (CD45positive/CD11b high/GR-1negative cells) were also analyzed quantitatively using flow cytometry. The proportion of CD45positive/CD11b high/GR-1negative cells (macrophage) within the injured spinal cord cells had increased in both MSC (24.5 ± 1.9%) and control group (30.4 ± 2.1%) compared with sham (laminectomy only) group (4.5 ± 1.3%) at 1 week after SCI. In the control group, at 1 week after spinal cord injury, 93.4 ± 5.6% of these cells were iNOS positive classically activated macrophages (M1 phenotype) and no arginase-I positive alternatively activated macrophages (M2 phenotype) were present. While in the MSC transplanted group, only 11.7 ± 1.3% of these cells were iNOS positive and 32.2 ± 1.9% were arginase-I positive (Fig. 4).

***Immunoblot Analysis of Cytokine Expression after MSC Transplantation***

Western blotting was performed to evaluate the effects of MSC transplantation on protein levels of TNF-α, IL-6, IL-4, and IL-13 in the region of the SCI at 1 week after injury. In the MSC transplanted group, the intensity of the band in TNF-α and IL-6 were attenuated, whereas that of IL-4 and IL-13 were increased compared with the control group (Fig. 5A). The protein levels of TNF-α and IL-6 were lower (IL-6 dominant) and that of IL-4 and IL-13 were higher (IL-13 dominant) of MSC transplanted group compared with the control group (Fig. 5B).

***Histological Evaluation of the Injured Spinal Cord after MSC Transplantation***

The severity of trauma at the injury epicenter site was evaluated in both groups at 5 weeks after SCI. HE staining in the MSC transplanted group showed a significant decrease in the total cavity areas at epicenter and at 4mm rostral and caudal to the epicenter compared with the control group (Fig. 6A). On LFB-stained samples in the MSC transplanted group, images demonstrated significantly smaller areas of cystic cavity formation and enhanced staining in both gray and white matters compared with that of the control group. Quantitative analysis of the myelinated areas revealed significant differences between the two groups at the injury epicenter and at 4mm rostral and caudal to the epicenter (Fig. 6B).

To determine the effects of MSC transplantation on axonal growth and regeneration after SCI, we examined tsagittal sections of the spinal cords following immunostaining with anti-GAP-43 and anti-RT97 antibodies at 5 weeks after SCI. In the MSC transplanted group, GAP-43-positive or RT97-positive fibers were significantly increased and their neurites were also significantly elongated compared with the control group (Fig. 6C).

***Evaluation of Locomotor BBB Score***

The degree of motor disturbance in the hind limbs was assessed between day 3 and 5 weeks after SCI. Rats with SCI who received no MSC treatment (control group) had significant motor disturbances in their hind limbs, with some degree of recovery evident that reached a functional plateau (BBB score 9.2±2.1) at 5 weeks after SCI. In contrast, the MSC transplanted group showed a markedly better functional recovery such that their the BBB locomotor score was significantly higher than that of the control group from 1 week after SCI (Fig. 7).

**DISCUSSION**

The object of this research was to study the possible beneficial effects and mechanisms of MSC transplantation in the injured spinal cord, specifically with regards to the effects of MSC on macrophage phenotype and function. The main points of our research and the effects of MSC transplantation could be summarized as follows; 1) MSC transplanted into the injured site migrated into the adjacent nervous tissue and embedded in areas rich in glial and neuronal processes, but did not differentiate into glial or neuronal elements; 2) MSC transplantation favoured the development of a population of alternatively activated macrophages (M2 phenotype) whilst preventing the development of a population of classically activated macrophages (M1 phenotype); 3) MSC transplantation was associated with a decrease in the presence of TNF-α and IL-6 and an increase in the presence of IL-4 and IL-13; 4) MSC transplantation was associated with the formation of smaller injury sites, less scar tissue formation and increased myelin sparing; 5) these morphological findings correlated with increased axonal growth and improved locomotor function in the MSC transplanted group compared with the control group.

Several studies report that MSC differentiate into neurons and astrocytes (Ankeny DP, 2004; Zurita M, 2008), myocytes, and Schwann cells in vitro (Dezawa M, 2005). In our study, transplanted MSC migrated into the adjacent injured spinal cord, but did not differentiate into glial or neuronal elements. Current thinking is that the potential beneficial effects of MSC in SCI is not as a result of their neuronal or glial differentiation, but from their secretion of growth factors or cytokines (Sasaki N, 2009), which can provide for neuroprotection (Chen X, 2002; Parr AM, 2007), induction of axonal sprouting (Shen LH, 2006), neovascularization (Onda T, 2008), and immunomodulation (Ohtaki H, 2008; Bai L, 2009). MSC may also promote axonal regeneration or encourage functional plasticity by establishing an environment that supports axonal growth, for example, by abrogating the inhibitory influence of the chondroitin sulphated proteoglycans (CSPG) or myelin debris present in the injury site and glial scar (Wright KT et al, 2007). MSC synthesize a number of neurotrophic cytokines that stimulate nerve growth, including brain-derived neurotrophic factor (BDNF), nerve growth factor (NGF), and vascular endothelial growth factor (VEGF) (Neuhuber B, 2005; Crigler L, 2006). Because MSC can alter their gene expression profile in response to the surrounding environment (Mosser DM, 2011; Ide C, 2010; Yamaguchi S, 2006), we consider that transplanted MSC do not differentiate into neural cells at least in the injured spinal cord environment, but instead that they bring about CNS functional recovery by modifying the SCI environment to directly affect the endogenous cells present.

Hematogenous macrophages and microglia are the major players in the inflammatory pathology of SCI. Some investigators indicated that hematogenous macrophages are regarded as more detrimental, whereas microglia are believed to be relatively beneficial for spinal cord repair owing to their higher phagocytotic activity and expression of various neurotrophic factors (Mukaino M, 2010). On the other hand, some investigators indicated that the distinct hematogenous macrophage subset, i.e. alternatively activated macrophages (M2 phenotype) have beneficial effects on injured spinal cord, but also have a distinct role from activated resident microglia. The resident microglia are embedded in the CNS prior to the injury and immediately activated by the insult, whereas alternatively activated macrophages (M2 phenotype) do not encounter the injured CNS tissue prior to their delayed arrival to the damage site (Shechter R, 2009). Subsequent studies have correlated such divergent effects to the presence of different macrophages populations with contrasting functions, where classically activated macrophages (M1 phenotype) are the predominant type after SCI, with deleterious effects for the injured tissues, and alternatively activated macrophages (M2 phenotype) have only a short term response, dissipating within 3 to 7 days after injury. That prevalence of the M2 phenotype over the M2 phenotype may be partially responsible for the lack of functional recovery after SCI (Gordon S,2003; Mantovani A,2004).

Previous studies identified TNF-α and IL-6 as the most significant factors to generate classically activated macrophage (Kigerl KA, 2009), and IL-4 and IL-13 to generate alternatively activated macrophages (Loke P, 2002), while identifying microglia as the main source of IL-4 (Ponomarev ED, 2007) and T cells of IL-13 (Offner H, 2005). In our study, we found that 3 days after MSC transplantation following a SCI, the profile of cytokines present changed into an alternative activating environment, with significant increase in the levels of IL-4 and IL-13 and simultaneous reduction of TNF-α and IL-6. The increased protein levels of Th2 cytokines (IL-4, IL-13) will activate resident macrophages and hematogenous macrophages through the IL-4Rα/Jak1/STAT signaling pathway into alternatively activated macrophages (M2 phenotype), where the reduced levels of Th1 cytokines (TNF-α, IL-6) will prevent the macrophages of shifting back into classically activated macrophages (M1 phenotype) (Gordon S,2003; Mosser DM, 2008).

It has been proposed that MSC act as guiding strands for regenerating axons across the lesion site in the injured cord and along spinal cord tracts *in vivo*. Transplanted MSC were seen to form bundles that bridged the lesion, which were also populated with immature astrocytes and nerve fiber outgrowths (Hofstetter CP, 2002). Alternatively activated macrophages (M2 phenotype) have increased phagocytic abilities due to the presence of endosomes/lysosomes and pinocytic structures with digestive enzymes to remove scar tissue and growth inhibitors present in the myelin debris allowing axonal regeneration (Kigerl KA, 2009; Schwartz M, 2010). In our study, regenerated axons were identified at 5 weeks after MSC transplantation by staining their specific proteins including GAP-43, which is present in the growth cones of axons, and neurofilament 200kD (through the RT97 antibody). It is possible that such regenerating axons might have at least partly contributed to locomotory improvement in the present study. The acute or subacute environment of the injured spinal cord may influence the mechanism by which the MSC graft might induce tissue protection/repair in a manner that differs to the chronic phase; i.e., in the acute phase MSC transplantation may have beneficial effect through their anti-inflammatory activity, whereas in the subacute/chronic phase after SCI the MSC may be used for neurostimulatory and cell bridging effects (Wright KT, 2011).

In conclusion, our results suggested that the transplantation of MSC after SCI shifts the phenotype of macrophage response after injury from the formation of classically activated macrophages (M1 phenotype) to that of alternatively activated macrophages (M2 phenotype) in the acute phase; this was associated with the presence of relevant cytokine profiles, a reduction in inhibitory scar tissue/cavity formation in the subacute/chronic phase, and the provision of a permissive environment for axonal extension and functional recovery.

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**FIGURE LEGENDS**

**Figure 1. The distribution of MSC transplanted after spinal cord injury.** Photomicrograph showing the distribution of PKH26 labeled transplanted MSC counterstained with DAPI staining for nuclei at 1 week and 5 weeks after spinal cord injury. Transplanted PKH26 induced fluorescent MSC survive and distributed only around the injured site at 1 week after spinal cord injury (C), whereas a number of PKH-positive cells extended out of the injured lesion at 5 weeks after spinal cord injury (A, B). (B) High-power photomicrograph of the box area of (A). Scale bar= 500 m (A, C), 50 m (B).

**Figure 2. Transplanted MSC did not differentiate to form neuronal or glial cells.** Images are shown of colocalization of cell specific markers (green, second column; NeuN,RIP,GFAP, and OX-42) and MSC labeled with PKH26 (red, first column) in the injured spinal cord at 5 week after spinal cord injury. MSC did not express markers for neurons, oligodendrocytes, astrocytes, or microglia/macrophage. Scale bar= 20 m.

**Figure 3. The prevalence and distribution of M1 and M2 macrophages in MSC transplanted versus control injured spinal cords.** Immunofluorescent staining showing the difference of the expression of iNOS and CD16/32 (green) for classically activated macrophages (M1 phenotype) and arginase-I and CD206 (green) for alternatively activated macrophages (M2 phenotype) colocalized OX-42 (blue) after MSC transplantation in the injured spinal cord at 1 week after spinal cord injury. In the MSC transplanted group, the number of iNOS and CD16/32-positive cells decreased and that of arginase-I and CD206 increased compared with those of the control group. Scale bar= 20 m.

**Figure 4. Quantitative analysis of the presence of M1 and M2 macrophages present in SCI versus control.** (A) There was an increase in the proportion of macrophages present (CD45positive/CD11b high/GR-1negative cells) after SCI, with evident differences in the presence of iNOS positive or arginase-I positive cells within the CD45positive/CD11b high/GR-1negative (macrophage) population between MSC transplanted and control group. (B) Quantitation of the flow cytometry data demonstrated that MSC transplantation decreased the proportion of iNOS positive cells (blue; M1 phenotype) present and the macrophage phenotype shifted to arginase-I positive cells (red; M2 phenotype) at 1week after spinal cord injury. Data are mean±SD. \*, p<0.05.

**Figure 5. Immunoblot analysis of TNF-α, IL-6, IL-4 and IL-13 protein levels at 1 week after spinal cord injury.** (A): representative Western blots (B): graphs indicating relative band intensities compared with that of β-actin . In the MSC transplanted group, TNF-α and IL-6 levels were significantly lower whereas IL-4 and IL-13 levels, were significantly higher, compared to the control group. Data are mean±SD. \*, p<0.05.

**Figure 6. Histological evaluation of the extent of spinal cord injury.** (A) HE staining of mid-sagittal and axial sections of the lesion epicenter showed a remarkably smaller area of damage and cavity formation in the MSC transplanted group. Significant differences in the total cavitation areas in the axial sections at the epicenter and at 4mm rostral and caudal to the epicenter were observed between the two groups. (B) The axial sections stained with Luxol fast blue (LFB) showed a remarkable reduction in the area of demyelination in the MSC transplanted group compared with the control group. Quantification of LFB-positive myelinated areas showed a significant difference between the two groups at all of the examined sites. (C) Representative images of mid-sagittal sections through an area 4mm caudal to the epicenter showed a greater abundance of GAP-43-positive fibers and RT97-positive fibers in the MSC group compared with that in the control group (5 weeks after injury).

**Figure 7. Analysis of locomotor BBB score after spinal cord injury.** A significant improvement in hindlimb motor function was observed in the MSC transplanted group compared with the control group from 1 week and thereafter after the injury (n=10 for each time point). \**P* <0.05.