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## **Bone marrow stromal cell : mediated neuroprotection for spinal cord repair**

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# Chapter 4

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## **Reducing Macrophages to Improve Bone Marrow Stromal Cell Survival in the Contused Spinal Cord**

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## **ABSTRACT**

We tested whether reducing macrophage infiltration would improve the survival of allogeneic bone marrow stromal cells (BMSC) transplanted in the contused adult rat thoracic spinal cord. Treatment with cyclosporine, minocycline, or methylprednisolone all resulted in a significant decrease in macrophage infiltration at 3 days postinjury. However, when BMSC were injected at that time point, survival 7 days later was similar between treatment groups and saline-injected controls. In fact, we found that the presence of BMSC resulted in a significant increase in macrophage infiltration into the contusion.

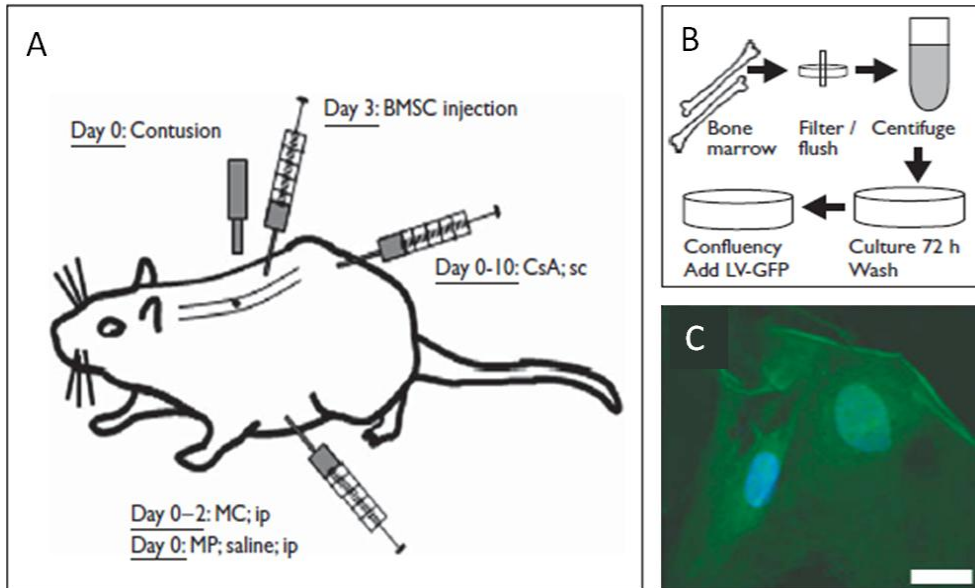
## INTRODUCTION

A contusive spinal cord injury causes immediate death of neural cells and disruption of axon circuits<sup>1</sup>. The number of macrophages in the injury site rapidly increases over the ensuing days and contribute to further destruction of local nervous tissue<sup>2</sup>. There is no therapy available that effectively improves function after spinal cord injury. Transplantation of bone marrow stromal cells (BMSC) has been explored for spinal cord repair<sup>3</sup>. In different model systems, BMSC transplantation resulted in tissue sparing and, in some cases, improved motor function<sup>4-7</sup>. These results warrant further investigation of BMSC for nervous system repair. Survival of allogeneic BMSC transplanted into the damaged spinal cord is low<sup>4,7,8</sup>. Several studies have pointed at a role of macrophages in the loss of BMSC after injection into the injured nervous system<sup>8,9</sup>. Thus, we reasoned that a reduction in macrophage number in an injury site would increase the survival of transplanted BMSC. Cyclosporine A (CsA), minocycline (MC), and methylprednisolone (MP) treatment decreases macrophage infiltration into a spinal cord lesion<sup>8,10-13</sup>. This study focuses on the efficacy of these three drugs to reduce macrophage infiltration and whether this would result in increased survival of subsequently transplanted BMSC.

## METHODS

### Spinal cord contusion and postsurgery care

Adult female Sprague–Dawley rats (n=76, 200–230 g; Harlan, Indianapolis, Indiana, USA) were anaesthetized with an intraperitoneal injection of 60 mg/kg of ketamine HCl (Phoenix Pharmaceuticals, St Joseph, Maryland, USA) and 0.4 mg/kg medetomidine (Domitor, an  $\alpha$ -2- adrenergic agonist; Orion Corp., Espoo, Finland). The 10th thoracic spinal cord segment was exposed and contused using the Infinite Horizon Impactor (Precision System & Instrumentation, Lexington, Kentucky, USA) at a force of 200 kDyn (Fig. 1a). Consistency between animals was guaranteed by registering the impact force and spinal cord displacement. The wound was closed and the rats were given antisedan (atipamezole hydrochloride; 1.25 mg/kg, intramuscular), an  $\alpha$ -2-adrenergic antagonist that reverses



**Fig. 1.** Schematic representation of the experiments and BMSC harvest and transduction. A. Rats were contused at the 10th thoracic spinal cord segment and then divided into 4 groups that received CsA, MC, MP or saline. B. BMSC were harvested, transduced to express GFP using lentiviral vectors, and grown in DMEM. C. GFP-expressing BMSC in a passage 3 culture which were used for transplantation into the epicenter of the 3-day old contusion. Abbreviations: BMSC, bone marrow stromal cells; CsA, cyclosporine A; ip, intraperitoneal; MC, minocycline; MP, methylprednisolone; sc, subcutaneous. Bar in C represents 10  $\mu$ m.

sedative and analgesic effects of medetomidine. All surgical procedures were performed by the same investigator. Postsurgery maintenance was as described earlier<sup>7</sup>.

### Drug administration

Contused rats were divided into four groups that received CsA, MC, MP, or saline (n=18 each; Fig. 1a) starting 5 min after the contusion. All injections were performed by the same investigator. CsA (Bedford Labs, Bedford, Ohio, USA) was administered subcutaneously once per day at a dose of 30 mg/kg for the first three days and 15 mg/kg for the next 7 days<sup>8</sup>. MC (Sigma-Aldrich, St Louis, Missouri, USA) was administered intraperitoneally at a dose of 50 mg/kg twice a day for the first two days<sup>12</sup>. MP (Sigma-Aldrich) was administered intraperitoneally once at a dose of 30 mg/kg<sup>10</sup>. Saline was given to controls following the same regime as for MP.

### **Bone marrow stromal cell culture and lentiviral transduction**

BMSC were obtained from femurs of adult female Sprague-Dawley rats (n=4) as described earlier<sup>7,14</sup> (Fig. 1b). BMSC at passage 0 were transduced overnight using lentiviral vectors encoding for green fluorescent protein (LV-GFP) at an MOI of 150<sup>7,15</sup>. Transduced BMSC were cultured in Dulbecco's modified Eagle's medium with 10% fetal bovine serum at 37°C/5% CO<sub>2</sub>. BMSC from the third passage (Fig. 1c) were used for the transplantation experiments. The transduction rate of the BMSC was determined using a FACScan/FACSorter [Becton Dickinson Immunocytometry Systems (BDIS) Biosciences, San Jose, California, USA]. The number of viable GFP-positive cells relative to the total number of cells was 63%.

### **Transplantation of bone marrow stromal cells**

At 3 days after injury, 24 rats (six rats from each group) were anaesthetized with intraperitoneal injections of 60mg/kg of ketamine HCl (Phoenix Pharmaceuticals) and 0.4mg/kg of medetomidine (Domitor; Orion Corporation). The 10th thoracic spinal cord segment was exposed and 5 ml Dulbecco's modified Eagle's medium with  $1 \times 10^6$  BMSC (Fig. 1a) was injected into the contusion epicenter<sup>14</sup>. Four additionally-contused rats were similarly injected and perfused with fixative (see below) 15min later. These rats were used to determine the number of BMSC in the contusion at 15min postinjection. All BMSC injections were given by the same investigator. After the injections, the rats were maintained as described previously<sup>7</sup>.

### **General histology**

Three days (n=24) and 10 days (n=48) after injury, rats were anaesthetized with an intraperitoneal injection of 90 mg/kg of ketamine HCl (Phoenix Pharmaceuticals) and 0.6 mg/kg of medetomidine (Orion Corporation). After deep sedation was confirmed, 0.1 ml heparine (500 IU; Henry Schein, Melville, New York, USA) was injected into the left ventricle of the heart. Then, 500 ml saline followed by 500 ml ice-cold 4% paraformaldehyde in phosphate buffer (PB; 0.1 M, pH 7.4) was pumped through the vascular system. Spinal cords were removed without damaging the anatomical integrity,

postfixed for 24 h in the same fixative, and transferred to 30% sucrose in phosphate-buffered saline (PBS; 0.1 M, pH 7.4) for 48 h. A 12-mm-long spinal cord segment centered at the contusion was cut into 20-mm-thick horizontal cryostat sections, which were mounted on glass slides.

### **Immunocytochemical procedures**

For characterization of the BMSC, eight-well chamber glass slides (BD Falcon; BD Biosciences, Bedford, Massachusetts, USA) were coated with 100 µl/ml poly-D-lysine for 1 h at room temperature. After washing 2 x 5 min with double-distilled water, 3000 BMSC in 250 µl D-10 medium were plated per well. After 2 days at 37 °C/ 5% CO<sub>2</sub> the cultures were washed 3 x 5 min with PBS and fixed with 4% paraformaldehyde in PB (10 min, room temperature). Next, the cultures were washed 5 x 5 min with PBS, incubated with 5% normal goat serum (NGS) in PBS for 30 min, and then incubated overnight at 4 °C with antibodies against CD90 (1 : 100; Immunotech, Brussels, Belgium), CD105 (1 : 100; N1G1, Becton Dickinson), CD34 (1 : 100; 8G12 clone IgG1, Becton Dickinson), CD45 (1 : 100; H130 clone IgG1, Becton Dickinson), and HLA-DR (1 : 100; Dako, Clostrup, Denmark) diluted in PB with 5% NGS. Some cultures were incubated with PB with 5% NGS only and served as negative (no primary antibody) controls to exclude a false-positive outcome. Next, cultures were washed 3 x 5 min with PB and then incubated with goat-anti-mouse IgG-Alexa 594 (1:500 in PB; Molecular Probes, Carlsbad, CA) for 2 h at room temperature. Afterwards, cultures were washed 3 x 5 min with PB and covered with a glass slip with Vectashield and DAPI (Vector Laboratories, Inc., Burlingame, CA). The slides were examined and images were taken with an Olympus Fluoview FV1000 confocal microscope.

For immunostaining of activated macrophages, every 10th cryostat section was pre-incubated at room temperature for 30 min in 5 % NGS and 0.3 % Triton X-100 in 0.01 M PBS (pH 7.4) and then incubated with antibodies against ED1 (1:200; Serotec, Raleigh, NC) in 5 % NGS for 2 h at room temperature followed by overnight incubation at 4 °C. After washing 3 x 5 min with PBS, sections were incubated with goat anti-mouse Alexa 594 antibodies (1:200; Molecular Probes) in PBS (0.01 M; pH 7.4) at room temperature for 2 h.



The sections were then washed and covered with a glass slip in Vectashield with DAPI (Vector Laboratories, Inc.). The coverslips were sealed with nail polish. All sections were stored at -20 °C until analysis.

### **Quantitative assessments**

For analysis of GFP-positive BMSC, every 10th cryostat section was covered with a glass slip with Vectashield mounting medium with DAPI (Vector Laboratories, Inc). Stereoinvestigator (MicroBrightField Inc., Colchester, VA, USA) was used to determine the numbers of surviving BMSC in the contusion<sup>16</sup>. The sections were 200  $\mu$ m apart spanning the width of the spinal cord. In every section containing GFP positive cells, the transplanted area was outlined manually at 4 X magnification and covered by a 250 x 250  $\mu$ m grid. At 60 X magnification with oil immersion, GFP-positive cells with a discernable DAPI-positive nucleus were marked using the optical fractionator with a 60 x 60  $\mu$ m counting frame. Numbers were corrected for the 63 % transduction rate of the BMSC with LV-GFP. For each of the groups, BMSC survival was calculated as the number of BMSC relative to the number of BMSC at 15 min after transplantation (which was 168159  $\pm$  31129; SEM, n=4). The effect of treatment on BMSC survival was assessed by expressing BMSC survival for each group as a percentage of that in controls. For analysis of macrophages we employed a method described by Hayashi and colleagues<sup>17</sup>. This method uses three sections per rat for examination: one section through the center of the contusion/transplant (with the densest cellular staining), and sections 200  $\mu$ m dorsal and ventral to the center. The area fraction of staining in these sections was determined using SlideBook 4.1.0.12 (Intelligent Imaging Innovations, Inc, Santa Monica, CA, USA) and expressed as a percentage of that in control animals.

### **Statistical analysis**

Sigmastat® (Systat Software, Inc., San Jose, CA, USA) was used for statistical analyses using one-way ANOVA and the Bonferroni post-hoc test. Differences were accepted at p<0.05.

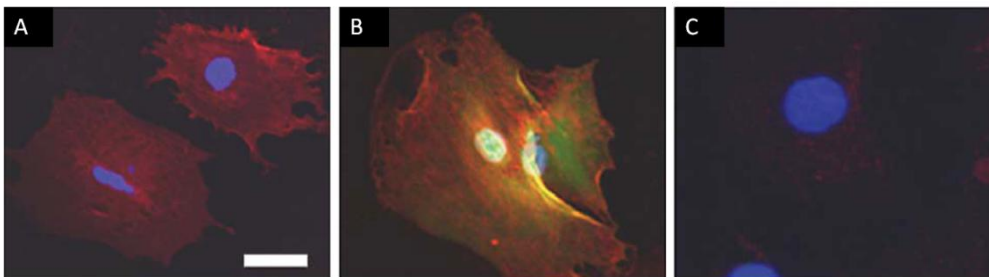
## Ethics and surgical approval

All rats used in this study were housed according to the guidelines of the National Institute of Health and United States Department of Agriculture. The described animal procedures were approved by the Institutional Animal Care and Use Committee at the Johns Hopkins University.

## RESULTS

### Characterization of BMSC in vitro

Cultured GFP-positive cells expressed CD90 (Fig. 2a) and CD105 (Fig. 2b). These two extracellular molecules are both well-known BMSC markers<sup>18,19</sup>. None of the cells expressed the blood cell markers, CD34 (Fig. 2c) and CD45 or the immune cell marker, HLA-DR. No staining was visible if the primary antibody was omitted. The data characterize the cells used for transplantation as BMSC.



**Fig. 3.** Characterization of bone marrow stromal cells (BMSC) in vitro. Cultured cells expressed the BMSC markers, CD90 (A) and CD105(B), but not the blood cell marker, CD34 (C). Scale bar =10 mm.

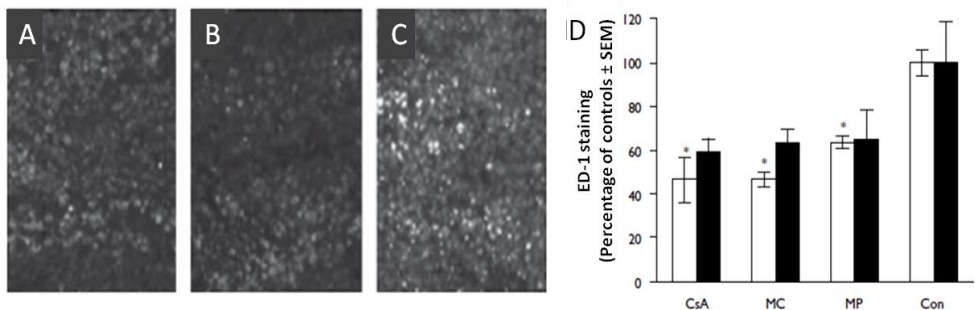
### CsA, MC, and MP reduce macrophage infiltration into the spinal cord contusion

Microscopic analysis of macrophage presence in the contusion revealed high numbers in controls (Fig. 3a) compared to treated rats (Fig. 3b) at 3 days after injury. The numbers appeared increased in treated rats at 10 days (Fig. 3c) compared to 3 days (Fig. 3b) after contusion. Quantitative analysis demonstrated that at three days post-injury, relative to controls, macrophage infiltration was  $46 \pm 10$  % with CsA-,  $47 \pm 3$  % with MC-, and  $63 \pm 3$  % with MP-treatment (Fig. 3d). ANOVA revealed that the number for each of the

treatment groups was significantly smaller ( $p < 0.001$ ) than that for controls. Thus treatment-induced reduction was 54 % with CsA, 53 % with MC, and 37 % with MP compared to controls. Treatment-induced reduction was not significantly different from controls at ten days post-injury (Fig. 3d). These results showed that all three drugs when administered following the treatment regime described above reduced macrophage infiltration into the adult rat spinal cord contusion at three days post-injury.

### **BMSC survival in contusion is not affected by CsA, MC, or MP treatment**

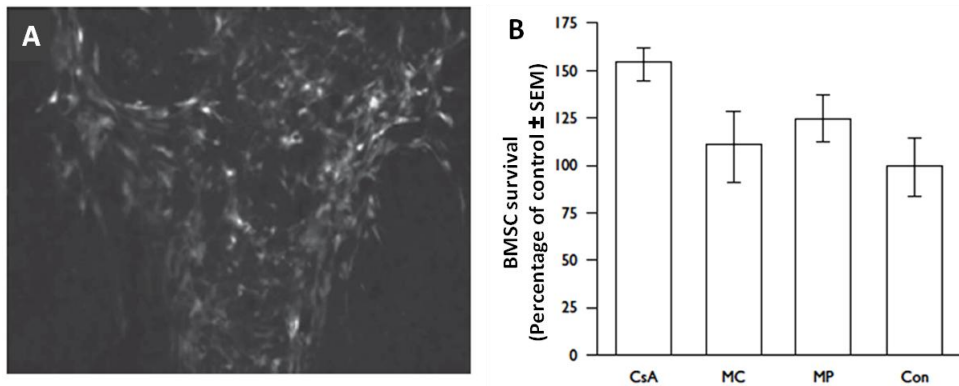
BMSC were present in the contusion at seven days post-injection (Fig. 4a). Quantitative analysis revealed that BMSC survival in the contusion at seven days post-injection was  $27 \pm 4$  % with CsA,  $24 \pm 4$  % with MC, and  $33 \pm 2$  % with MP treated rats. In saline-injected control rats, BMSC survival was  $21 \pm 7$  %. To assess the effects of treatment, we expressed BMSC survival in each treatment group relative to that in the control group (Fig. 4b). We found that BMSC survival was  $126 \pm 17$  % with CsA,  $111 \pm 19$  % with MC, and  $155 \pm 12$  % with MP treated rats relative to control rats (Fig. 4b). ANOVA revealed no difference in BMSC survival between treatment and control groups ( $p = 0.16$ ).



**Fig. 4.** Cyclosporine A (CsA), minocycline (MC), and methylprednisolone (MP) treatment reduced macrophage infiltration into the contusion. Photomicrographs of ED-1-positive cells in control rats (A) and CsA-treated rats (b) at 3 days postcontusion and in CsA-treated rats at 10 days postcontusion (B). In panel (C) the more intense staining was found associated with cellular debris. (D) Bar graph shows that at 3 days postinjury (open bars) relative to controls (Con), macrophages infiltration in the contusion was decreased significantly with CsA, MC, and MP treatment. At 10 days postinjury (solid bars), the decrease in macrophage presence in the contusion of treated rats was not statistically different from that in Con. \*Significant difference between treated and Con groups at 3 days postinjury with  $P < 0.001$ .

### BMSC presence initiates macrophage infiltration into the spinal cord contusion.

We assessed the effect of a BMSC transplant on macrophage presence in the contusion at ten days post-injury relative to that at three days post-injury. With a BMSC transplant, macrophage infiltration relative to controls was 2.4 fold in CsA- and MP-treated rats, and 6.9 fold in MC-treated rats. These differences were statistically significant ( $p < 0.001$ ). In the absence of a BMSC transplant, macrophage presence in treated rats relative to controls was unchanged. Our data demonstrated that the presence of a BMSC transplant significantly increased macrophage infiltration into the contused adult rat spinal cord.



**Fig. 5.** Cyclosporine A (CsA), minocycline (MC), and methylprednisolone (MP) treatment did not improve bone marrow stromal cell (BMSC) survival. (a) Photomicrograph of green fluorescent protein-positive cells within the contusion at 7 days postinjection. (b) Bar graph showing BMSC survival relative to controls (Con). The differences were not statistically different, although there was a trend towards higher numbers in CsA-treated rats.

### DISCUSSION

Quantitative investigations have demonstrated that survival of BMSC transplanted into the contused adult rat spinal cord is low<sup>4,7</sup>. Previously, it was proposed that macrophages which are naturally present within an injury site are involved in the loss of BMSC transplanted into the central nervous system<sup>9</sup>. Our present results showed that a decreased presence of activated macrophages at the time of BMSC injection (three days post-injury) does not increase survival of grafted BMSC. It is possible that our treatment regimens failed to lower macrophage infiltration to a level where BMSC survival would have been improved. The maximum reduction which was achieved here was 54%. Because

macrophage invasion into a spinal cord injury site is typically large<sup>1,2,11</sup>, this reduction may still leave many macrophages that could potentially contribute to BMSC loss. An additional observation is that the treatment effect which was present at three days post-injury was not significant at ten days post-injury. Although we used treatment protocols known to effectively reduce the presence of macrophages<sup>8,10-13</sup> they might not have been effective enough for sufficient and prolonged reduction of macrophages.

An alternative explanation for the observed lack of improved BMSC survival would be that any treatment-induced reduction in macrophage presence was masked by a subsequent increase in macrophage infiltration due to the introduction of BMSC into the environment. This notion is supported by our data because in animals with a BMSC transplant we found that macrophage infiltration was drastically increased. It is likely that these extra macrophages have exacerbated the loss of BMSC<sup>20</sup>. It is important to keep in mind that other factors than invaded macrophages are most likely also involved in transplanted BMSC loss such as the lack of oxygen and/or nutrients within the damaged tissue<sup>21</sup>.

Previously, it was reported that BMSC are hypo-immunogenic; they suppress the proliferation and function of T-cells, B-cells, natural killer cells, and dendritic cells<sup>22</sup>. However, these publications did not investigate possible effects of BMSC on macrophage invasion. It is possible that the immunosuppressive properties of BMSC affect only the adaptive immunity due to the low expression level of human leukocyte antigen (HLA) major histocompatibility (MHC) class I and the absence of co-stimulatory molecules<sup>22</sup>. This would explain why the recruitment of macrophages (acquired immunity) would not be affected by BMSC.

Lowering the number of macrophages in the injured spinal cord needs to be addressed with caution. It is well known that macrophages can support spinal cord repair by promoting axon regeneration and myelination which may be accompanied by improved function<sup>23,24</sup>. These constructive effects occur while macrophages also exert destructive effects such as neural cell death<sup>25</sup>. Because of this dual role, decreasing the number of

macrophages within a spinal cord injury could lead simultaneously to beneficial and detrimental effects<sup>24</sup>. Thus it is important to aim for a reduction in macrophages that would not jeopardize their positive contributions to spinal cord repair.

## CONCLUSION

After spinal cord contusive injury, concurrent macrophage reducing treatments did not improve survival of transplanted BMSC. The presence of BMSC in the contusion site resulted in a four-fold increase in macrophage numbers.

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