

Research Article

# Adult Mouse Post Mortem Neural Precursors Survive, Differentiate, Counteract Cytokine Production and Promote Functional Recovery after Transplantation in Experimental Traumatic Spinal Cord Injury

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## Abstract

Spinal cord injury (SCI) is a debilitating clinical condition, characterized by a complex of neurological dysfunctions. Adult neural stem cells (NSCs) from the subventricular zone of the forebrain have been considered a potential tool for cell replacement therapies. We have recently isolated a subclass of neural progenitors from the cadaver of mouse donors. These cells, named Post Mortem Neural Precursor Cells (PM-NPCs), express both erythropoietin and its receptor and their EPO-dependent differentiation abilities produce a significantly higher percentage of neurons than regular NSCs. The aim of the present study was to compare the reparative properties of PM-NPCs and those expressed by NSCs in a mouse model of traumatic spinal cord injury. PM-NPCs and NSCs were administered intravenously, and then functional recovery and fate of transplanted cells were studied. Animals transplanted with PM-NPCs showed a more remarkably improved recovery of hind limb function than NSCs treated animals. The PM-NPCs effect was accompanied by reduced myelin loss, counteraction of the invasion of lesion site by macrophages, and attenuation of cytokine production. PM-NPCs migrate mostly at the injury site, where they survive at a significantly higher extent than classical NSCs.

**Keywords:** Spinal cord injury; Neural stem cells; Transplantation; Regenerative medicine; Animal behaviour; Inflammation;

## Introduction

Acute spinal cord injury (SCI), with an annual incidence of 22 to 59 cases per million population internationally, is a devastating disease that has a significant impact in society. Moreover, the majority of affected patients are young individuals (10-40 years old) resulting in a substantial burden of impairment and high costs to society [1,2]. There is currently no curative therapy, and the care in the acute phase is often limited to high-dose corticosteroid treatment, surgical stabilization and decompression aiming at the attenuation of further damage [1,3]. The pathophysiology of SCI is biphasic. Primary injury results from the immediate response to the trauma that cause axonal and blood vessels transection, lipid peroxidation and disruption of cell membranes. Secondary injury is due to the activation of degeneration that causes ischemia, inflammation, cytokine production, demyelination and death of multiple cell types [2,4]. A large number of studies have evaluated the effects of transplanting stem cells or stem cell-derived cells in spinal cord injury models, and remarkably, many studies using different strategies have indicated beneficial effects to a certain degree [5]. Transplanted cells can improve the recovery of function either by replacing partially the lost cells, or supplying a favorable environment that attenuates the effects of secondary degeneration thereby enhancing the amount of spared tissue at the injury site, i.e. transplanted stem cells must survive in such an unfavorable environment. We had recently reported the isolation of adult neural stem cells from SVZ several hours after

death of the mouse donor which are capable of surviving in such an unfavourable environment [6]. This procedure provides a population of NSCs, named post mortem neural precursors (PM-NPCs), that *in vitro* differentiate preferentially in neurons. Such a process is dependent on the autocrine EPO release and is prevented by exposure to EPO and EPOR antibodies [6]. In this work we make a comparison between adult mouse PM-NPCs and adult NSCs by evaluating their effects after intravenous transplantation in a mouse model of spinal cord injury. Here we report that after intravenous administration an high number of PM-NPCs migrate to spinal cord lesion site and survive for a long time, differentiate mostly into neuron-like cells and reduced tissue degeneration enhancing preservation of myelin fibres. These events are preceded by the counteraction of cytokine production and associated with the promotion of a stable recovery of hind limb function. Their action is apparently superior to that of regular adult NSCs.

## Materials and Methods

### Animal care

For this study we used adult CD1 male mice 25–30 g in weight (Charles River, Calco, Italy). All the procedures were taken with the approval of the Review Committee of the University of Milan and met the Italian Guidelines for Laboratory Animals which conform to the European Communities Directive of November 1986 (86/609/EEC). The animals were kept for at least 3 days before the experiments

in standard conditions ( $22 \pm 2^\circ\text{C}$ , 65% humidity, and artificial light between 08:00 a.m. to 08:00 p.m.).

### Neural stem cell isolation and culture

Neural stem cells (NSCs) were isolated from sub-ventricular zone of adult mice brain [7,8]. Their maintenance in culture, their differentiation and their immunostaining were performed as described by Gritti and co-workers [7,8]. Briefly, 8 weeks old CD-1 albino were anesthetized by intraperitoneal (i.p.) injection of 4% chloral hydrate (0.1 mL/10 g body weight) and killed by decapitation. The brains were removed from adult mice and tissues containing the sub-ventricular zone (SVZ), were dissected out and transferred to a phosphate buffer solution containing penicillin and streptomycin 100 U/mL (Life Technologies), glucose (0.6%) at  $4^\circ\text{C}$  until the end of the dissection. Enzymatic digestion was performed by transferring the tissue to an Earl's balanced salt solution (EBSS) (Sigma-Aldrich, Milan, Italy) containing 1 mg/mL papain (27 U/mg; Sigma-Aldrich), 0.2 mg/mL cysteine (Sigma-Aldrich), and 0.2 mg/mL EDTA (Sigma-Aldrich), and incubated for 45 min at  $37^\circ\text{C}$  on a rocking platform. Tissue was then centrifuged at 123g and the supernatant was discarded. The pellet was re-suspended in 1 mL of EBSS and mechanically dissociated with a pipette, than cells were centrifuged at 123g and supernatant was discarded. This step was repeated three times as described [7]. Cells were counted and plated at 3500 cells/cm<sup>2</sup> in DMEM-F-12 (Euroclone, Pero, Milan, Italy) containing 2 mM L-glutamine (Euroclone), 0.6% glucose (Sigma-Aldrich), 9.6 gm/ml putrescine (Sigma-Aldrich), 6.3 ng/ml progesterone (Sigma-Aldrich), 5.2 ng/ml sodium selenite (Sigma-Aldrich), 0.025 mg/ml insulin (Sigma-Aldrich), 0.1 mg/ml transferrin (Sigma-Aldrich), and 2 µg/ml heparin (sodium salt, grade II; Sigma-Aldrich), bFGF (human recombinant, 10 ng/mL; Life Technologies) and EGF (human recombinant, 20 ng/mL; Life Technologies). Spheres formed after 5–7d were harvested, collected by centrifugation (10 min at 123g), mechanically dissociated to a single-cell suspension, and re-plated in medium indicated above [8]. The total number of viable cells was assessed at each passage by Trypan blue exclusion. Stem cells used in these experiments were between the fifth to the fifteenth passage in culture.

### Post Mortem Neural Precursor Cells derivation, differentiation and labelling

PM-NPCs were obtained from 6 weeks old CD-1 albino mice; their isolation, growth and characterization were performed following methods described for NSCs and set up by Gritti et al. [8]. Briefly, cells were isolated from the sub ventricular zone (SVZ) of adult male mice (CD1) six hours after their killing by cervical dislocation. Brains were removed and tissues containing the SVZ region were dissected, transferred to Earl's Balanced Salt Solution (Life Technologies, Monza, Italy) containing 1 mg/ml papain (27 U/mg; Sigma-Aldrich, Milan, Italy), 0.2 mg/ml cysteine (Sigma-Aldrich), and 0.2 mg/ml EDTA (Sigma-Aldrich) and processed as described above. At the end of isolation procedure cells were collected by centrifugation and resuspended in the same growth medium indicated above [7,8]. Differentiation of both NSCs and PM-NPCs was performed by plating the dissociated stem cells at the density of 40,000 cells/cm<sup>2</sup> in presence of adhesion molecules (Matrigel™, BD Biosciences, Buccinasco, MI, Italy) and bFGF (10ng/ml) for 48 hours, then cells were exposed to the same medium without bFGF and the addition of foetal bovine serum (1% vol/vol; Euroclone) for

the following 5 days as previously described [6,8]. Then, the extent of differentiation was determined by immunocytochemical staining [6]. For transplantation NSCs and PM-NPCs were labelled with PKH26 (Sigma-Aldrich) just before the injection, following manufacturer's instructions and previously described [9]. PKH26 is a not toxic cell dye characterized by long aliphatic tails (PKH26) that allow the dye incorporation in lipid regions of the cell membrane [10,11].

**Dead cells administration:** PM-NPCs ( $1 \times 10^6$  cells) were treated with 1 ml of 4% paraformaldehyde (Sigma-Aldrich) for 10 min at room temperature, then 10 mL of PBS were added and cells were spun down at 123g, and suspended in PBS. This washing step was performed twice, and, finally, the ready-to-use dead PM-NPCs were suspended at the final concentration of  $3.3 \times 10^5$  cells/50µl in saline solution.

**Cell quantification at the lesion site:** Cells were considered PKH26 positive, when fluorescence was visible by the confocal microscope (Leica TSC2; Leica Microsystems, Heidelberg, Germany) and if the spots had an emission wave length of 567 nm which correspond to the emission of PKH26. As negative reference the confocal analysis we used sections obtained from animals transplanted with unlabeled PM-NPCs. The counting of the cells was performed assessing the PKH26-positive cells in the transversal sections in a region of 4 mm centered at the lesion site. The lesion epicenter was defined in cross sections as the region with the minimum tissue sparing. The PKH26-positive cells present in a group of three consecutive sections (10 µm thick) were averaged, and such count was repeated every 400 µm. The total number of labeled cells was obtained integrating the curve calculated by the average of each sections across a 4 mm span across the epicenter of the lesion [12].

### Spinal Cord Injury, experimental groups and cell administration

The traumatic SCI was performed using a commercially available Infinite Horizon (IH; Precision Systems and Instrumentation, LLC, Lexington, KY, USA) spinal cord injury device [13] at the T8 level. Surgery on the animals was performed as described elsewhere [13]. Briefly, animals were anesthetized with 2.5% isoflurane in oxygen (1 L/min; Farmagricola, San Donato, Milan, Italy) for 5 minutes before surgery. A dorsal vertical incision was made through the skin from T7 to T12, superficial fat pad was removed and T7 and T10 bilateral paravertebral muscles were cut. Laminectomy was performed and spinal cord exposed. The impactor tip was then positioned just above the cord following the constructor instructions, the force of 70 Kdynes was applied for 1 s to the cord. After the contusion the animal muscles were sutured and the skin closed by means of clips (2Biological Instruments, Besozzo, Varese, Italy). Experimental animals were divided into four groups: 1) Laminectomies mice (n=18); 2) Lesioned mice treated by i.v. route with phosphate buffer (PBS, n=24); 3) Lesioned mice transplanted by i.v. route with PM-NPCs (n=24); 4) Lesioned mice transplanted by i.v. route with dead PM-NPCs (n=12); 5) Lesioned mice transplanted by i.v. route with NSCs (n=24). Intravenous administration was performed by injections in the tail vein. PM-NPCs, PBS, NSCs or dead PM-NPCs were administered after spinal cord lesion. The first treatment was a slow i.v. injection of 50 µl in the tail vein performed within 30 min after injury, followed by a second injection 6 h later and a third one 18 h after the lesion. Each cellular administration consisted of  $3.3 \times 10^5$  cells in PBS for a

total of  $1 \times 10^6$  cells. The choice of a time limit of 18 h after SCI for administering PM-NPCs was determined by the optimal permeability of the blood brain barrier at this time [14]. PM-NPCs between the 5th and the 9th passage in culture were used for these experiments, the cultures were tested for proliferation and differentiation ability before being transplanted [6]. Up to now we observed that transplanted animals can survive until 90 days after grafting, then were sacrificed to perform further investigations.

### Behavioural tests and hind limb function

All outcome measures were assessed in a blinded fashion. Neurological function was evaluated first 24 h after injury and then twice a week for the first 4 weeks. The methods utilized are well known in the field of behavioural evaluation of recovery of function after SCI. Locomotor function and hind limb recovery after contusion were evaluated with the open field test according to the Basso mouse rating scale [15]. For behavioural experiments we used 5 animals in laminectomies group, and at least 12 animals for other groups. Allodynia-like responses in the unaffected forepaw were assessed by means of standard hotplate test and cold stimulation. For hotplate testing, mice were placed on hotplate and the latency to licking was measured. Non-responders were removed after 60s. The response to cold was tested by the application of ethyl chloride spray (Gebauer Company, Cleveland, OH, USA) to the palm surface. The response was rated 1 (no response), 2 (brief withdrawal with licking), and 3 (vocalization, withdrawal with licking, and aversion) [16].

### Tissue collection and processing, histology and immunohistochemistry

At the end of the experimental period, animals were anesthetized by i.p. injection of clorinium hydrate (Sigma-Aldrich) 4% in distilled water, and perfused with 4% paraformaldehyde in phosphate buffer (PB) 0.1 M pH 7.4 by transcardial perfusion. Spinal cords were post-fixed overnight in the same fixative, cryoprotected with 30% sucrose (Sigma-Aldrich), and quickly frozen, stored at  $-80^{\circ}\text{C}$  and sectioned by means of a cryostat (Leica). Every twentieth section was stained with thionin (Sigma-Aldrich). Cross-sections containing the lesion epicenter and the complete T8 segment cavitations were analyzed by computer-assisted image analysis. The percent of lesion was calculated as the area of the injured tissue divided by the area of the total cross-section at the level of the injury. Cryostat coronal sections ( $15 \mu\text{m}$ ) were also collected onto glass slides and processed for immunocytochemistry. Sections were rinsed with PBS (Euroclone), treated with blocking solution (Life-Technologies) and incubated with primary antibodies overnight at  $4^{\circ}\text{C}$ . After treatment with primary antibodies, the sections were washed with PBS and incubated with appropriate secondary antibodies (Alexa Fluor<sup>®</sup> 488, Molecular Probes<sup>®</sup>, Life Technologies) for 2 hours at room temperature. Sections were washed in PBS, nuclei were stained with DAPI ( $1 \mu\text{g}/\text{ml}$  final concentration, 10 minutes at room temperature; Sigma-Aldrich) and then mounted using the FluorSave Reagent (Calbiochem, Merck Chemical, Darmstadt, Germany) and analyzed by confocal microscopy. In control determinations, primary antibodies were omitted and replaced with equivalent concentrations of unrelated IgG of the same subclass. The following primary antibodies were used:  $\beta$ -Tubulin III (1:150; Covance). For immunofluorescence, the following secondary antibodies were used: 488 goat-anti-mouse IgG (1:200; Alexa), 488 donkey-anti-rabbit IgG (1:200; Alexa).

### Assessment of myelin preservation

In order to perform a homogeneous analysis, the staining was carried out on sections of non lesioned, Lesion+PBS and Lesion+cells animals placed on the same coverslip. Myelin preservation was evaluated comparing the levels of myelin in the ventral white matter at 0.4 mm (rostral and caudal) laterally from the lesion epicenter in healthy, saline and cells treated animals. The choice of the ventral white matter was based on the knowledge that the reticular spinal pathway descends mostly in the ipsilateral dorso- and ventro-lateral funiculi and is directly involved in the regulation of the movement of the mouse foot [17]. We previously reported that the quantification of the spared ventral myelin evaluated in a semi-thin section gave comparable results when fluoromyelin was used (FluoroMyelin Green, Molecular Probes, Life Technologies). Confocal microscope images for laminectomy animals, saline- and cell-treated mice were obtained using the same intensity, pinhole, wavelength and thickness of the acquisition. As reference we used sections close to the ones analyzed and not treated with fluoromyelin. Briefly, the procedure of the staining was carried out by incubating the cryosections with fluoromyelin diluted 1:300 in PBS for 20 minutes; slides were then washed three times for 10 min each with PBS and mounted with FluorSave (Merck, Darmstadt, Germany), and qualitatively and quantitatively analyzed by confocal microscopy (Leica TSC2; Leica Microsystems, Heidelberg, Germany).

### Estimate of the macrophages number at the site of lesion

ED1- positive cells were counted in transversal sections made at the lesion epicenter (1 mm extension) and 0.4 mm rostral. As negative reference for the confocal analysis we used a consecutive section that was stained by omitting primary antibody anti rat macrophages/monocytes and replacing it with equivalent concentrations of unrelated IgG of the same subclass. The zero level was adjusted on this reference and used for all the further analysis (we used a new zero reference for each new staining). The ED-1 positive cells present in a group of three consecutive sections ( $10 \mu\text{m}$  thick) were averaged, and we repeated this count each  $100 \mu\text{m}$ . The total number of ED1-positive cells was obtained integrating the average within volume analyzed, i.e. the 1 mm around the epicenter of the lesion [12].

### RNA Isolation and Real-Time PCR Analysis

Mice ( $n=6$  48h post injury group,  $n=6$  1 week post injury group) were anesthetized [4,7,18] and killed by decapitation. Laminectomy was performed at the T5–T12. The spinal cord region corresponding at the lesion site was removed (we took 4 mm of tissue rostral and 4 mm of tissue caudal to the lesion epicenter). The tissue was put in 1 ml of Trizol<sup>®</sup> Reagent (Life Technologies), shock frozen, and kept at  $-80^{\circ}\text{C}$  until performing the RNA isolation. Total RNA was isolated by using Trizol<sup>®</sup> Reagent (Life Technologies) in accordance with the manufacturer's instructions. The genomic DNA was removed by DNase I treatments ( $2 \text{ U}/\mu\text{g}$  of RNA) (Ambion, Austin, Texas, USA). The synthesis of single-strand cDNA was carried out on  $1 \mu\text{g}$  of RNA, using U M-MLV Reverse Transcriptase III (Life Technologies) following the manufacturer's instructions.

Real-time (RT)-PCR was performed in an MJ Opticon 2 (Biorad, Segrate, Milan, Italy) using Brilliant SYBR Green qPCR Master Mix (Stratagene, La Jolla, CA, USA) following manufacturer's instructions. The housekeeping gene GAPDH (glyceraldehyde 3-phosphate



**Table 1: Locomotor activity evaluation after i.v. treatment with PM-NPCs; PBS, NSCs and killed PM-NPCs.** The open field locomotion was the test employed for the determination of motor function recovery, the score is determined according to the Basso Mouse Scale [2]. Experimental animals, tested the day prior to the injury, scored the maximum (9 points) in the BMS scale, then BMS score fell to 0 following SCI and gradually recovered thereafter. The groups were randomized, the analysis was performed in double blind fashion. Values represent average  $\pm$  SD.

Days	LAM	PBS	PM-NPCs	NSCs	Killed PM-NPCs
-1	9.0 $\pm$ 0	9.0 $\pm$ 0	9.0 $\pm$ 0	9.0 $\pm$ 0	9.0 $\pm$ 0
1	8.0 $\pm$ 0.05	0.0 $\pm$ 0.87	0.0 $\pm$ 0.10	0.0 $\pm$ 0.10	0.0 $\pm$ 0.10
6	8.5 $\pm$ 0.05	2.0 $\pm$ 0.230	3.0 $\pm$ 0.20 ( $p < 0.01$ vs PBS; $p < 0.01$ vs killed)	3.0 $\pm$ 0.10	2.0 $\pm$ 0.10
13	8.5 $\pm$ 0.13	2.5 $\pm$ 0.140	3.5 $\pm$ 0.30 ( $p < 0.001$ vs PBS; $p < 0.001$ vs killed)	3.5 $\pm$ 0.40	2.0 $\pm$ 0.40
18	9.0 $\pm$ 0.06	2.5 $\pm$ 0.130	4.0 $\pm$ 0.30 ( $p < 0.001$ vs PBS; $p < 0.001$ vs NSCs; $p < 0.001$ vs killed)	3.5 $\pm$ 0.30	3.0 $\pm$ 0.20
24	9.0 $\pm$ 0.06	3.0 $\pm$ 0.220	4.5 $\pm$ 0.45 ( $p < 0.001$ vs PBS; $p < 0.001$ vs NSCs; $p < 0.001$ vs killed)	3.5 $\pm$ 0.10	3.0 $\pm$ 0.30
30	9.0 $\pm$ 0.06	3.0 $\pm$ 0.170	5.0 $\pm$ 0.35 ( $p < 0.001$ vs PBS; $p < 0.001$ vs NSCs; $p < 0.001$ vs killed)	3.5 $\pm$ 0.30	3.0 $\pm$ 0.25

dehydrogenase) was used for normalization of cytokine expression. The relative expression of cytokine genes, with GAPDH as reference gene, was determined using the  $2^{-\Delta\Delta Ct}$  method. We used this method because both target and reference genes were amplified with similar efficiencies near 100%. The primer design was performed using the DNASTAR Lasergene program. Primers used were the following:

GAPDH (F: cgactcaacagcaactcccactcttc; R: tgggtgtccagggtttctactctt), BDNF (F: cattacctctcatctgttgg; R: cgtggacgtttacttcttcatgg), IL-6 (F: gacaaccacggccttcctac; R: cgtgttcataatcagaattgcc), NGF (F: tgggcccaataaagttttgcc; R: tgggcttcaggacagagctcc), TNF $\alpha$  (F: tctatggcccagaccctcacac; R: cagcactccagctctctc), MIP2 (F: acgccccaggaccctactg; R: ggacagcagcccaggctctcc), LIF (F: aacgtggaaaagctatgtg; R: gcgaccatccgatacagctc).

### Statistical analysis

Data were expressed as the mean  $\pm$  SD. Multiple group comparison was made by ANOVA with a post hoc Tukey test. The analyses were performed using Prism 3.0 software (GraphPad Software, Inc.). Statistical significance was accepted for a  $P < 0.05$ .

## Results

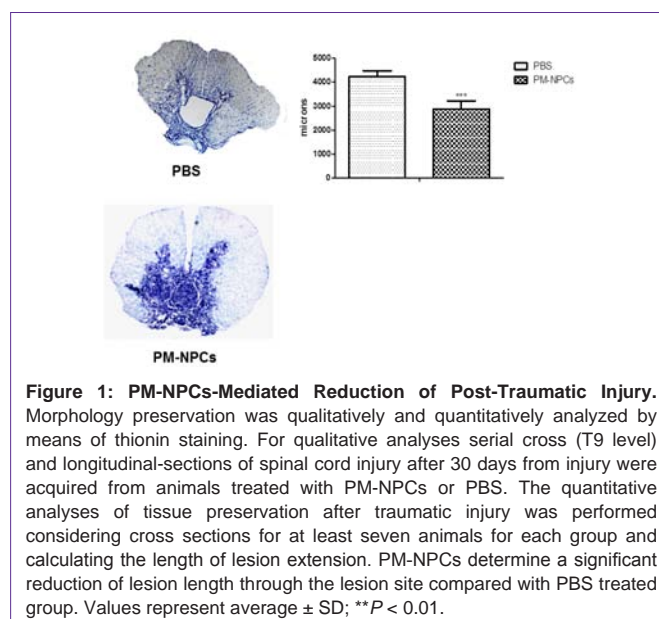
### PM-NPCs improve recovery of hind limb function

The 70 Kdynes traumatic impact to the mouse cord caused a transient loss of ability in hind limb function, that was followed by a progressive gradual recovery reaching the maximum extent within 2-3 weeks ( $3.0 \pm 0.220$  points of the BMS scale;  $n = 12$ ) (Table 1). The recovery was far better and reached the extent of  $5.0 \pm 0.35$  at day 30 ( $n = 12$ , corresponding to frequent or consistent plantar stepping without coordination, or frequent or consistent plantar stepping with some coordination) when injured mice were treated with adult PM-NPCs (see Materials and Methods). The behavioral improvement was particularly evident during the first 3 weeks after SCI, the recovery was then steadily improving. Monitoring was done up to 90 days ( $n = 6$ ). The injection of adult neural stem cells (NSCs) determined an

early locomotor recovery of hind limb function at day 7 that improved also during the following week. However the locomotor tests failed to show further improvements in the following weeks (Table 1). Differently, the application of killed PM-NPCs used as controls, failed to promote recovery of hind limb function and the rate of recovery was comparable to that of the saline group (Table 1). No signs of allodynia-like forelimb hypersensitivity [16] were recorded at any time in any experimental group throughout the observational period.

### PM-NPCs-mediated tissue sparing

Tissue sparing at lesion site was analyzed by means of quantitative analysis (see Materials and Methods) following thionin staining at 30 days after lesioning. The post-traumatic tissue loss is far more evident in the cord of PBS-treated lesioned mice (Figure 1), than in the cord of those treated with PM-NPCs, and this is confirmed



**Figure 1: PM-NPCs-Mediated Reduction of Post-Traumatic Injury.** Morphology preservation was qualitatively and quantitatively analyzed by means of thionin staining. For qualitative analyses serial cross (T9 level) and longitudinal-sections of spinal cord injury after 30 days from injury were acquired from animals treated with PM-NPCs or PBS. The quantitative analyses of tissue preservation after traumatic injury was performed considering cross sections for at least seven animals for each group and calculating the length of lesion extension. PM-NPCs determine a significant reduction of lesion length through the lesion site compared with PBS treated group. Values represent average  $\pm$  SD;  $**P < 0.01$ .

**Table 2: Cytokines mRNA expression levels 2 and 7 days after lesioning and i.v. treatment with PBS, PM-NPCs, NSCs.** Expression of cytokines mRNA levels was evaluated by real time RT-PCR. Each group was composed of six mice, and we performed three quantitative (Q)-RT-PCRs in each experiment, we run a duplicate per each sample and a parallel PCR for the gene of interest and the reference gene Glyceraldehyde-3-Phosphate Dehydrogenase. Values represent average  $\pm$  SD.

2 DAYS	CTRL	LAM	PBS	PM-NPCs	NSCs
IL-6	1.00 $\pm$ 0.84	3.035 $\pm$ 1.54	86.75 $\pm$ 35.17 ( $p < 0.001$ vs CTRL; $p < 0.001$ vs LAM)	17.95 $\pm$ 7.37 ( $p < 0.001$ vs CTRL; $p < 0.001$ vs LAM; $p < 0.001$ vs PBS)	78.50 $\pm$ 34.84 ( $p < 0.001$ vs CTRL; $p < 0.001$ vs LAM; $p < 0.001$ vs PM-NPCs)
MIP-2	1.00 $\pm$ 0.38	0.53 $\pm$ 0.50	6.26 $\pm$ 1.85 ( $p < 0.001$ vs CTRL; $p < 0.001$ vs LAM)	1.80 $\pm$ 0.55 ( $p < 0.05$ vs LAM; $p < 0.01$ vs PBS)	5.8 $\pm$ 2.33 ( $p < 0.001$ vs CTRL; $p < 0.001$ vs LAM; $p < 0.01$ vs PM-NPCs)
TNF-alpha	1.00 $\pm$ 0.23	0.72 $\pm$ 0.15	24.10 $\pm$ 1.40 ( $p < 0.001$ vs CTRL; $p < 0.001$ vs LAM)	1.57 $\pm$ 0.20 ( $p < 0.001$ vs PBS)	23.035 $\pm$ 11.5 ( $p < 0.001$ vs CTRL; $p < 0.001$ vs LAM; $p < 0.001$ vs PM-NPCs)
7 DAYS	CTRL	LAM	PBS	PM-NPCs	NSCs
IL-6	1.00 $\pm$ 0.26	1.16 $\pm$ 0.31	16.7 $\pm$ 0.44	2.68 $\pm$ 1.34 ( $p < 0.01$ vs PBS)	3.5 $\pm$ 2.45 ( $p < 0.01$ vs PBS)
MIP-2	1.00 $\pm$ 0.15	1.64 $\pm$ 0.11 ( $p < 0.05$ vs CTRL)	8.79 $\pm$ 0.28 ( $p < 0.001$ vs CTRL, $p < 0.001$ vs LAM)	1.10 $\pm$ 0.09 ( $p < 0.05$ vs LAM; $p < 0.001$ vs PBS)	5.9 $\pm$ 1.75 ( $p < 0.05$ vs PBS; $p < 0.001$ vs PM-NPCs)
TNF-alpha	1.00 $\pm$ 0.26	1.28 $\pm$ 0.07	28.086 $\pm$ 4.21 ( $p < 0.001$ vs CTRL, $p < 0.001$ vs LAM)	1.93 $\pm$ 0.56 ( $p < 0.01$ vs CTRL, $p < 0.01$ vs LAM, $p < 0.01$ vs PBS and $p < 0.01$ vs NSCs)	21.035 $\pm$ 6.24 ( $p < 0.001$ vs CTRL, $p < 0.001$ vs LAM; $p < 0.001$ vs PM-NPCs)

by the quantitative analysis (Figure 1). The lesser tissue loss is likely due to tissue sparing. The reticulospinal tract is an important pathway in eliciting locomotion, it descends mainly through the ipsilateral dorso- and ventro-lateral funiculi. Its function in the coordination of rhythmic stepping movements was demonstrated by electrophysiological studies [19,20]. The condition of myelin in these descending structures was evaluated by quantitative estimates of FluoroMyelin™ staining by means of confocal quantitative analysis as detailed in Materials and Methods [17,21]. Myelin preservation was quantified 30 days after lesioning, sections were taken at the center of the lesion and 2 mm caudally to the lesion site. Only intact myelin was assessed, and the degenerated myelin was not considered. The traumatic lesion caused a marked loss of myelin in close proximity of the lesion that was even higher in the more caudal region. The administration of PM-NPCs reduced such a loss in remarkable and highly significant manner, while NSCs appeared less effective (Figure 2).

### PM-NPCs homing to site of injury, survival, and differentiation

Four weeks after injury serially sectioned cords of cell treated mice were analyzed at the edge of the lesion by means of confocal microscopy to detect PKH26 and determine the amount of engrafted cells. We find out that the total number of PM-NPCs was 245.000  $\pm$  60.000 per cord, while as previously reported the amount of NSCs was 10 fold lower and on the order of 23.000  $\pm$  7.500 [7].

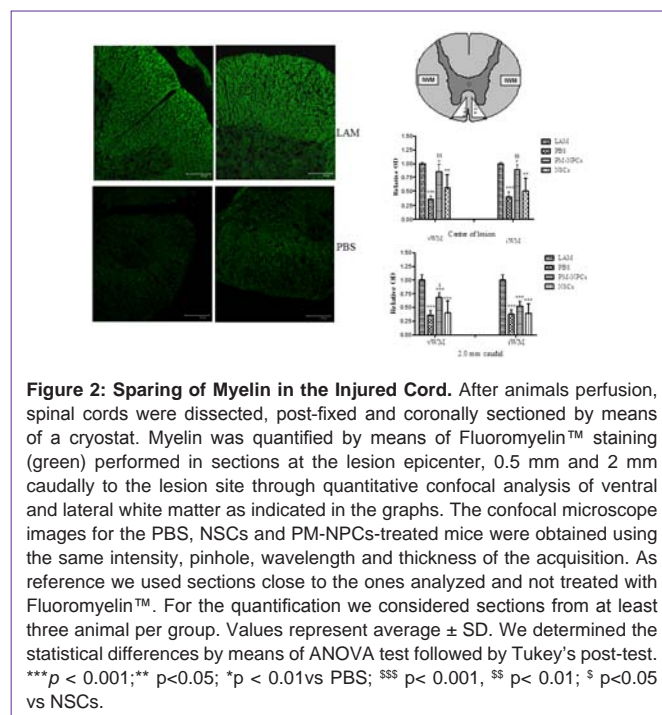
None of the transplanted PM-NPCs have differentiated in GFAP-positive cells. That is quite different from in vitro differentiation, where about 40-50% of the cells became GFAP-positive cells. Also not transplanted PM-NPCs showed positivity for oligodendrocyte markers (data not shown).

Most engrafted PM-NPCs accumulated at the edges of the lesion (data not shown), where formed clusters since the early days of their administration, then the clusters dispersed along the lesion edges and transplanted cells differentiate assuming gradually the asymmetric

cellular conformation of neurons. Most PM-NPCs show dendritic like processes that are fully immunostained by the specific antibody to TUJ1. This is particularly evident at days 30 after lesioning (Figure 3).

### PM-NPCs-Mediated chemokine and growth factor expression in the injured cord

The marked and progressive PM-NPCs-mediated recovery of function suggested possible additional effects by these cells possibly mediated via anti-inflammatory agents and neurotrophic factors. We evaluated quantitatively the mRNA levels of six different factors (Tables 2 and 3), namely nerve growth factor (NGF); brain derived



**Figure 2: Sparing of Myelin in the Injured Cord.** After animals perfusion, spinal cords were dissected, post-fixed and coronally sectioned by means of a cryostat. Myelin was quantified by means of FluoroMyelin™ staining (green) performed in sections at the lesion epicenter, 0.5 mm and 2 mm caudally to the lesion site through quantitative confocal analysis of ventral and lateral white matter as indicated in the graphs. The confocal microscope images for the PBS, NSCs and PM-NPCs-treated mice were obtained using the same intensity, pinhole, wavelength and thickness of the acquisition. As reference we used sections close to the ones analyzed and not treated with FluoroMyelin™. For the quantification we considered sections from at least three animal per group. Values represent average  $\pm$  SD. We determined the statistical differences by means of ANOVA test followed by Tukey's post-test. \*\*\*  $p < 0.001$ ; \*\*  $p < 0.05$ ; \*  $p < 0.01$  vs PBS; \$\$\$  $p < 0.001$ , \$\$  $p < 0.01$ , \$  $p < 0.05$  vs NSCs.

**Table 3: Growth Factors mRNA expression levels 2 and 7 days after lesioning and i.v. treatment with PM-NPCs, PBS, NSCs.** Expression of growth factors mRNA levels was evaluated by real time RT-PCR. Each group was composed of six mice, and we performed three quantitative (Q)-RT-PCRs in each experiment, we run a duplicate per each sample and a parallel PCR for the gene of interest and the reference gene Glyceraldehyde-3-Phosphate Dehydrogenase. Values represent average  $\pm$  SD.

2 DAYS	CTRL	LAM	PBS	PM-NPCs	NSCs
LIF	1.00 $\pm$ 0.39	0.82 $\pm$ 0.19	40.48 $\pm$ 15.59 (p<0.001 vs CTRL; p<0.001 vs LAM)	15.73 $\pm$ 5.39 (p<0.001 vs CTRL; p<0.001 vs LAM; p<0.001 vs PBS)	81.00 $\pm$ 20.84 (p<0.001 vs CTRL; p<0.001 vs LAM; p<0.001 vs PBS; p<0.001 vs PM-NPCs)
BDNF	1.00 $\pm$ 0.21	0.79 $\pm$ 0.13	1.26 $\pm$ 0.31 (p<0.05 vs CTRL; p<0.05 vs LAM)	2.20 $\pm$ 0.50 (p<0.001 vs CTRL; p<0.001 vs LAM; p<0.001 vs PBS)	4.35 $\pm$ 3.54
NGF	1.00 $\pm$ 0.26	0.54 $\pm$ 0.89	26.06 $\pm$ 10.87 (p<0.001 vs CTRL; p<0.001 vs LAM)	18.42 $\pm$ 9.92 (p<0.001 vs CTRL; p<0.001 vs LAM; p<0.01 vs PBS)	23.55 $\pm$ 15.24 (p<0.001 vs CTRL; p<0.001 vs LAM)
7 DAYS	CTRL	LAM	PBS	PM-NPCs	NSCs
LIF	1.00 $\pm$ 0.32	0.29 $\pm$ 0.05 (p<0.01 vs CTRL)	1.17 $\pm$ 0.47 (p<0.01 vs LAM)	4.66 $\pm$ 2.00 (p<0.001 vs CTRL; p<0.001 vs LAM; p<0.001 vs PBS)	1.27 $\pm$ 0.24 (p<0.001 vs LAM; p<0.001 vs PM-NPCs)
BDNF	1.00 $\pm$ 0.30	0.51 $\pm$ 0.11	0.83 $\pm$ 0.24 (p<0.05 vs LAM)	0.63 $\pm$ 0.11	0.75 $\pm$ 0.32
NGF	1.00 $\pm$ 0.14	0.44 $\pm$ 0.04 (p<0.01 vs CTRL)	0.85 $\pm$ 0.38 (p<0.05 vs LAM)	2.08 $\pm$ 0.63 (p<0.001 vs CTRL; p<0.001 vs LAM; p<0.01 vs PBS)	0.91 $\pm$ 0.76 (p<0.05 vs LAM; p<0.01 vs PM-NPCs)

growth factor (BDNF), tumor necrosis factor  $\alpha$  (TNF $\alpha$ ); interleukin-6 (IL-6); macrophage inflammatory protein 2 (MIP-2) and leukemia inhibitory factor (LIF). The comparison was made between the animals treated with PBS, NSCs or PM-NPCs. As additional controls, mRNA levels of the same chemokines were assayed in the T9 region of the spinal cord of both naïve uninjured and laminectomized animals. As previously described in rats by Gorio and co-workers [7,18] the

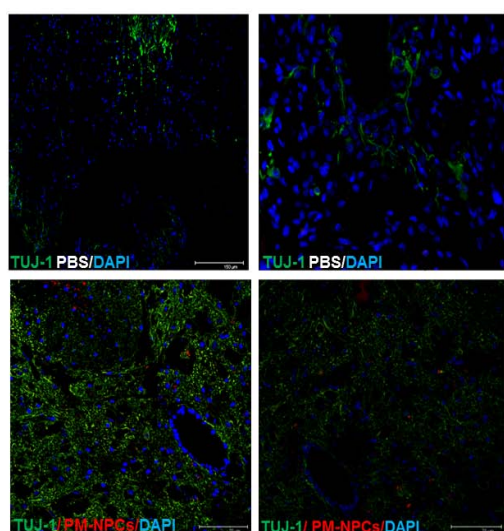
expression of these chemokines was affected by the injury and by the cellular treatments. We observed that injury-increased MIP 2 expression is very efficiently diminished by PM-NPCs at 48 hours and 1 week after lesioning; differently NSCs failed to counteract the expression of this chemokine (Table 2). Also the increased expression of IL-6, TNF $\alpha$  is reduced by PM-NPCs at both earlier and later times. NSC were able to counteract the expression of IL-6 only at 7 days and did not affect TNF-alpha expression (Table 2). NGF and LIF were reduced by PM-NPCs treatment at the earlier time, but resulted significantly increased at 1 week compared both to saline and NSCs treated mice. BDNF was increased by PM-NPCs in lesioned mice only at 48 hours (Table 3).

#### Inflammatory cell migration to the lesion site

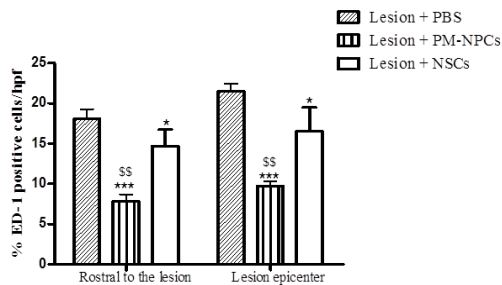
The administration of PM-NPCs significantly reduced the invasion of the lesioned spinal cord by macrophages. This was investigated by monitoring the number of ED-1-positive cells (macrophages) both in the lesion epicenter and rostrally one week after lesioning and cell treatment. The quantification of macrophages tissue invasion show that transplanted PM-NPCs are able to strongly reduce the amount ED-1-positive cells (Figure 4; Materials and Methods for details). The effect was far less evident when animals were transplanted with adult NSCs.

## Discussion

Considering their characteristic abilities to self-renew and differentiate into any cell type in the body, the therapeutic promise of stem cells may justify the intense efforts on investigating their application in cell therapy approaches [4,7,9,22-25]. In this study it is shown that the treatment of spinal cord injury with PM-NPCs attenuates secondary degeneration and enhances significantly the amount of tissue sparing and myelin preservation leading to a marked and long lasting improvement in motor function recovery. This is associated with a progressive and profound differentiation



**Figure 3: PM-NPCs beta-tubulin III Trans-Differentiation in Lesioned Cords.** PKH-labelled PM-NPCs (red) are present throughout the edges of lesion site at 30 days after their i.v. administration to spinal cord injured mice. Cord sections (10  $\mu$ m) from PBS or PM-NPCs treated animals were immunostained for  $\beta$ -tubulin III (green). Several PM-NPCs are present among the surviving neural profiles, most of PKH26-labeled cells are positive for  $\beta$ -tubulin III. All labeled cells have one nucleus. Nuclei are stained in blue (DAPI). Bars correspond to 150  $\mu$ m in upper panels and 75  $\mu$ m in lower panels.



**Figure 4: Tissue infiltration by macrophages.** Coronal cryostat section of cord lesion epicenter and cord 0.4 mm rostral to the lesion were stained for ED1, and the number of positive cells was quantitatively evaluated as described in materials and methods. Results are expressed as the number of ED1 positive cells per hpf. For the quantification we considered sections from at least six animal per group. Values represent average  $\pm$  SD. \*\*\* $p < 0.00$ , \* $p < 0.05$  vs PBS; SS  $p < 0.01$  vs NSCs.

of transplanted PM-NPCs into neuronal-like cells with cellular dendritic-like processes positive for  $\beta$ -tubulin III immunoreactivity. Differently the effects of adult NSCs administration produced a minor improvement in hind limb recovery of function that is accompanied by a rather early death as described previously [7].

The enhanced recovery of function must be ascribed to the presence of the live transplanted PM-NPCs in the cord, since the transplantation of killed-PM-NPCs yielded a recovery comparable to that obtained with PBS administration. The marked reduction of macrophage infiltration in the lesion site was particularly significant in PM-NPCs-treated animals, and this is suggestive of a strong effect on the cellular components of the inflammatory reaction triggered by SCI. The reduced cellular component of the inflammatory reaction promoted by PM-NPCs may be the basis of the observed myelin sparing. A similar sparing effect was previously reported by us when describing the effects of two agents EPO and reparixin, capable of improving recovery of function after such injury through these mechanisms [4,18,26]. The creation of a favorable environment is likely the major action of PM-NPCs, and this has certainly improved their rate of survival and the extent of differentiation.

We also showed here that i.v. administration of NSCs into injured animals promote an acute and rapid neuroprotective effect that did not lead to further improvement in the recovery at later time, probably because most transplanted cells had died. This is in agreement with what was previously reported also with both NSCs and embryonic stem cells transplant in which most cells died or were phagocytized by macrophages within 10-20 days after their accumulation at the lesion site in the injured spinal cord [7,9,22]. Differently from others neural precursors cells, PM-NPCs are able to differentiate in the lesion environment suggesting that the cellular differentiation process involving PM-NPCs in the lesioned spinal cords leads to a diminution of reactive gliosis in the areas of the cord where the transplanted cells are accumulated and to their almost total neuronal-like differentiation. This is supported by the progressive morphological changes and by the expression of the neuronal marker  $\beta$ -tubulin III. Due to the relative small number of surviving transplanted cells compared to the huge neural tissue loss caused by SCI, the potential replacement of such loss by the transplanted cells, as suggested by the successful trans-differentiation in  $\beta$ -tubulin III neuronal phenotype,

cannot be the predominant mechanism responsible for the recovery promotion. Thus we suggest that PM-NPCs pre-activation of HIF pathway and erythropoietin paracrine release, in addition to make these cells more resistant to the lesion unfavorable conditions, allow them to change the local environment that may become more favorable to regeneration of important pathways for locomotor spinal function such as catecholaminergic and serotonergic innervation of the lumbar and sacral cord. The enormous literature of the last decade has described through several different experimental paradigms how the supplementation of erythropoietin promotes neuroprotection and recovery of function in neurodegenerative conditions [27].

## Conclusions

The development of neuroprotective and restorative therapies remains a major unfulfilled medical need. In this regard, a stem cell-based therapy provides a promising therapeutic approach for preventing further neuronal damage through the attenuation of secondary degeneration and promoting repair and recovery of function in SCI [28]. In conclusion, our study suggests that PM-NPCs could affect host hind-limb functional recovery after SCI by means of multiple mechanisms just summarized in the three points above. We showed that PM-NPCs survive and differentiate in an unfavorable environment such as the site of spinal cord injury. These abilities could derive from their post-mortem origin and the consequent metabolic and genetic adaptation with activation of hypoxia induced factor and erythropoietin signaling [6,29]. The excellent survival of PM-NPCs in the early 2-3 weeks post-injury is likely crucial for the creation of a healthier local milieu that favors neuronal differentiation and regeneration across the lesion of descending and ascending pathways (manuscript in preparation). Thus, it is possible that the early survival of PM-NPCs has laid the basis for their neuronal differentiation and the creation of a more favorable environment that underlies the recovery of function.

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