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Immunosuppression does not affect human bone marrow mesenchymal stromal cell efficacy after transplantation in traumatized mice brain

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A B S T R A C T

The need for immunosuppression after allo/xenogenic mesenchymal stromal cell (MSC) transplantation is debated. This study compared the long-term effects of human (h) bone marrow MSC transplant in immunocompetent or immunosuppressed traumatic brain injured (TBI) mice.

C57Bl/6 male mice were subjected to TBI or sham surgery followed 24 h later by an intracerebroventricular infusion of phosphate buffer saline (PBS, control) or hMSC (150,000/5 μl). Immunocompetent and cyclosporin A immunosuppressed (CsA) mice were analyzed for gene expression at 72 h, functional deficits and histological analysis at five weeks.

Gene expression analysis showed the effectiveness of immunosuppression (INFγ reduction in CsA treated groups), with no evidence of early rejection (no changes of MHCII and CD86 in all TBI groups) and selective induction of T-reg (increase of Foxp3) only in the TBI hMSC group. Five weeks after TBI, hMSC had comparable efficacy, with functional recovery (on both sensorimotor and cognitive deficits) and structural protection (contusion volume, vessel rescue effect, gliotic scar reduction, induction of neurogenesis) in immunosuppressed and immunocompetent mice.

Therefore, long-term hMSC efficacy in TBI is not dependent on immunosuppressive treatment. These findings could have important clinical implication since immunosuppression in acute TBI patients may increase their risk of infection and not be tolerated.

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1. Introduction

The adult brain, once considered immunologically privileged, is subject to considerable immune surveillance (Hickey, 2001). The dynamic interaction between resident and recruited immune/inflammatory cell populations and the injured tissue enables the brain to respond to transplanted stem cells. As a consequence, the majority of cell types transplanted to the injured brain suffer poor survival (Capone et al., 2007; Coyne et al., 2006). In the experimental setting, high doses of immunosuppressant are needed, to improve efficacy and graft survival after allogenic- or xenotransplantation (Al Nimer et al., 2004; Cho et al., 2010; Omori et al., 2008; Seminatore et al., 2010). Immunosuppression has potential toxic side effects for the acute brain injured patient (Marik and Flemmer, 2012). Therefore, a primary goal for translational research would be to assess the patient's need for immunosuppression.

Recent findings suggest that mesenchymal stromal cells (MSC) are a good source for transplantation strategies in acute brain injury (Caplan, 2009; Li and Chopp, 2009; Walker et al., 2012b). Data from acute brain injury models, including traumatic brain injury (TBI) (Kim et al., 2010; Li et al., 2011; Zanier et al., 2011), stroke (Nomura et al., 2005; Wakabayashi et al., 2010; Xin et al., 2010) and spinal cord injury (Cizkova et al., 2011; Hu et al., 2010; Yang et al., 2008), show the efficacy of allo- and xeno-transplanted MSC with different paradigms of immunosuppression (Anderson et al., 2011). The promising preclinical data of MSC transplantation in rodent TBI models has led to the launch of a clinical TBI trial with human autologous bone marrow derived stem cells (www.clinicaltrials.gov:

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NCT00254722). However, harvesting patient-specific tissue poses logistic, timing and economic constraints and can introduce differences in cell potency related to the patient's age and disease, possibly limiting their therapeutic potential (Pietilä et al., 2012). There would be clear advantages if allogenic donor MSC could be used for transplantation without the need for immunosuppression. MSC do not appear to retain intrinsic immunogenic properties, do not trigger alloreactivity, suppress proliferation of T-cells *in vitro* (Uccelli et al., 2008) and can survive and differentiate into allogenic or even xenogenic immunocompetent recipient *in vivo* (Atoui and Chiu, 2012). Thus, MSC have been proposed as "universal donor cells". However, this has been challenged. First, although they may retain their immunosuppressive properties *in vitro*, allogenic murine MSC could be immunogenic in immunocompetent animals (Eliopoulos et al., 2005; Nauta et al., 2006). Second, MSC are rejected after xeno-transplantation into the ischemic rodent myocardium and immunosuppression is needed to improve their efficacy and survival in the ischemic heart (Grinnemo et al., 2004, 2006). Third, transplantation of MSC into the non-injured adult rodent brain can induce an inflammatory response leading to rapid and complete rejection of the transplanted cells, preventing plastic effects (Coyne et al., 2007, 2006). Consequently, the immunological impunity of MSC *in vivo* is not fully supported, and a dedicated study is needed to assess whether long-term efficacy of MSC in traumatized mice brain is dependent or not on immunosuppression.

To answer these questions we intracerebroventricularly (icv) transplanted hMSC isolated from bone marrow in immunosuppressed and immunocompetent traumatic brain injured mice. The study was designed to determine whether immunosuppression with cyclosporine A (CsA) affects the efficacy of hMSC transplanted into the traumatically injured mouse brain.

2. Materials and methods

2.1. Isolation and culture of hMSC

The local institutional review board approved the study and informed consent was obtained from healthy donors. hMSC were isolated from bone marrow of healthy donors and expanded *ex vivo* as previously described (Salvadè et al., 2010). Cells were cultured in Dulbecco's Modified Eagle's Medium (DMEM, Lonza Basel, Switzerland) with 5% freshly thawed Platelet Lysate (PL), 2 mM L-glutamine (LiStarFish, Milano, Italy) and 1% penicillin/streptomycin (Sigma–Aldrich, St. Louis, MO, USA). hMSC used were between passage (P) 3 and 5. Additional details on phenotypic characterization, multilineage differentiation and proliferation assay are in online Supplementary Materials.

2.2. Phenotypic characterization of hMSC

Expanded hMSC were characterized by the following monoclonal antibodies, according to the manufacturer's instructions: phycoerythrin (PE)-labeled anti-CD14, anti-CD90, anti-CD105 (eBioscience, San Diego, CA, USA); PE-labeled anti-CD45, anti-CD73, anti-MHCII (Becton Dickinson (BD), Franklin Lakes, NJ, USA); fluorescein isothiocyanate (FITC)-labeled anti-CD34 (IQ product, Groningen, The Netherlands); FITC-labeled anti-MHC class I (BD). Samples acquired by FACScalibur (BD) were analyzed with CellQuest Software (BD).

2.3. Multilineage differentiation

The osteogenic and adipogenic differentiating ability of hMSC was determined at P3 (Gatto et al., 2012) and, evaluated respectively after induction conditions, by Alizarin Red (Sigma–Aldrich) and Oil Red O (Sigma–Aldrich) staining.

2.4. Proliferation assay

Peripheral blood mononuclear cells (PBMC) were stimulated with 5 μg/mL of phytohemagglutinin (PHA) (Irvine Scientific, Santa Ana, CA, USA) and co-cultured with different doses of irradiated (35 Gy) hMSC in the 96-well plates; 48 h after co-culture, cells were pulsed for 16 h with [^{3}H]-thymidine at 1 μCi/well (Perkin Elmer, Waltham, MA, USA) then harvested. [^{3}H]-thymidine incorporation was measured using a Multipurpose Scintillation Counter (Beckman Coulter, Brea, CA, USA).

2.5. Animals

Procedures involving animals and their care were conducted in conformity with the institutional guidelines at the IRCCS – Institute for Pharmacological Research "Mario Negri" in compliance with national (Decreto Legge nr 116/92, Gazzetta Ufficiale, supplement 40, February 18, 1992; Circolare nr 8, Gazzetta Ufficiale, July 14, 1994) and international laws and policies (EEC Council Directive 86/609, OJL 358, 1, Dec. 12, 1987; Guide for the Care and Use of Laboratory Animals, U.S. National Research Council (Eighth Edition) 2011). Male C57Bl/6 mice (20–24 g, Harlan Laboratories, Italy) were housed in a specific pathogen-free vivarium (room temperature 21 ± 1 °C, 12 h light–dark cycle, free access to food and water). All efforts were made to minimize animal suffering and to reduce the number of animals used.

2.6. Study design and blinding of in vivo studies

A) Immunosuppression and early rejection were evaluated on a total of 48 mice equally divided into six experimental groups: 1. sham operated mice given phosphate buffered saline, 24 h after surgery (SHAM PBS); 2. sham operated mice given hMSC (SHAM hMSC). 3. TBI mice given PBS (TBI PBS); 4. TBI mice given hMSC (TBI hMSC). 5. TBI mice given PBS and CsA (TBI PBS CsA) 6. TBI mice given hMSC and CsA (TBI hMSC CsA). Mice ($n = 8$) were euthanized 3 days post-surgery for real time reverse transcription (RT)-PCR analysis.
B) hMSC protection on brain function and structure in immunocompetent and immunosuppressed mice was evaluated on 72 mice. First, to exclude confounding factors related to any direct neuro-protective/toxic effects of CsA we assessed the effects of our CsA immunosuppressive protocol on anatomical and functional damage one week after TBI (two groups of mice were used: TBI PBS and TBI PBS CsA, $n = 6$). Since there was no difference in anatomical or functional damage between the two groups (see Results and Fig. S2), immunocompetent TBI mice (TBI PBS) were considered the appropriate control for all further experiments.
C) Long term effects were evaluated on 60 mice divided into five equal experimental groups (1. SHAM PBS, 2. SHAM hMSC, 3. TBI PBS, 4. TBI hMSC, 5. TBI hMSC CsA). Mice ($n = 12$) were used for behavioral analysis up to five weeks post-injury. After euthanasia, brains were processed and contusion volume ($n = 12$), hMSC distribution ($n = 12$), vessel density ($n = 8$), gliotic scar ($n = 8$) and endogenous neurogenesis ($n = 8$) were quantified.

Mice were assigned to surgery and treatment groups with surgery and treatment distributed equally across cages and days. Investigators who did behavioral and *post mortem* analysis were blinded to the treatment allocation. Fig. 1 illustrates the experimental design.

2.7. Experimental brain injury

Anesthetized mice (sodium pentobarbital 65 mg/kg, intraperitoneal ip), were placed in a stereotaxic frame, and craniectomy was followed by induction of controlled cortical impact (CCI) brain injury as previously described (Zanier et al., 2011). Our injury model uses a 3 mm rigid impactor driven by a pneumatic piston, rigidly mounted at 20° from the vertical plane and applied perpendicularly to the exposed dura mater over the left parieto-temporal cortex at a velocity of 5 m/s and 1 mm depth. The craniotomy was then covered with a cranioplasty and the scalp sutured. Body temperature was maintained at 37 °C during all surgical procedures. Sham-injured mice received identical anesthesia without brain injury.

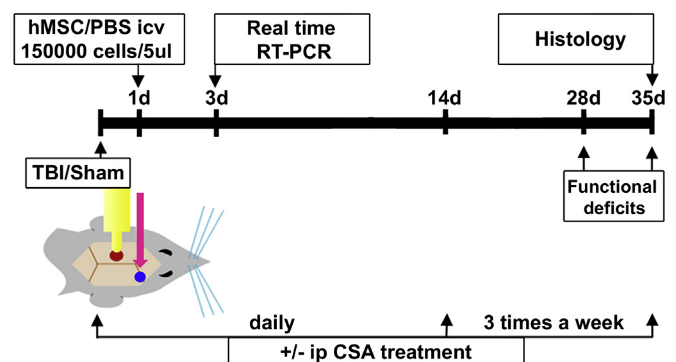

Fig. 1. Experimental design. hMSC or PBS (control) were infused icv in the contralateral ventricle 24 h after TBI or sham surgery. To assess the need for immunosuppression TBI mice transplanted with hMSC or PBS were given immunosuppressive treatment with cyclosporin A (CsA, 10 mg/kg ip, daily for the first 15 days, then three times/week) or no treatment. Behavioral tests, histology and real time RT-PCR analysis were done at the time points indicated.

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