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Stem cells from human-exfoliated deciduous teeth reduce tissue-infiltrating inflammatory cells improving clinical signs in experimental autoimmune encephalomyelitis

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ABSTRACT

Stem cells from human exfoliated deciduous teeth (SHED) have great therapeutic potential and here, by the first time, we evaluated their immunomodulatory effect on experimental model of autoimmune encephalomyelitis (EAE). Specifically, we investigated the effect of SHED administration on clinical signs and cellular patterns in EAE model using Foxp3 GFP + transgenic mice (C57Bl/6-Foxp3GFP). The results showed that SHED infusion ameliorated EAE clinical score with reduced number of infiltrating IFN-γ^+CD8$^+$, IL-4$^+$CD8$^+$, IFN-γ^+CD4$^+$ and IL-4$^+$CD4$^+$ T cells into the central nervous system (CNS). In addition, we observed that SHED promoted a significant increase in CD4$^+$FOXP3+ T cells population in the spleen of EAE-affected animals. Taken together, our results provide strong evidence that SHED can modulate peripherally the CD4$^+$ T cell responses suggesting that SHED would be explored as part of cellular therapy in autoimmune diseases associated with CNS.

1. Introduction

Experimental Autoimmune encephalomyelitis (EAE) is characterized by an autoimmune response against central nervous system (CNS) resident proteins such as the presence of infiltrating inflammatory cell, myelin defragmentation and neuronal degeneration [1]. Considering its similar clinical and cellular manifestation with multiple sclerosis (MS), EAE model can be useful for study and interpretation of molecular pattern in MS. In MS patients, accumulative lesions can be found in the spinal cord, optic nerve, or brainstem/cerebellum [2]; [3]. In addition, distinct clinical courses are also observed in MS patients: i) secondary-progressive, ii) primary-progressive, iii) progressive-relapsing and iv) relapsing–remitting (RRMS) type, which corresponds to 80% of the diagnosed MS patients. Sequelae and residual deficit upon recovery are usually seen, while relapses followed by complete recovery are rare [3–6].

Then, it is speculated that effective therapeutic strategies could improve these variable clinical conditions of MS [5,7,8]. The use of stem cells (SC) for the treatment of MS patients has been currently proposed such as an innovative therapeutic strategy [9–12]. In fact, it was observed that distinct SC subtypes can ameliorate clinical signs of EAE, for instance: i) human embryonic stem cell-derived mesenchymal stroma cells can reduce the clinical score and prevent demyelination in EAE [11]; ii) human bone marrow-derived mesenchymal stem cells induced Th2 cell polarization and improved EAE [13] (Bai et al., 2009); iii) human endometrial-derived mesenchymal stem cells reduced Th17 cells in CNS [14]; iv) human adipose-derived stem cells also improved EAE clinical signs [15]. However, studies using stromal stem cells from human exfoliated deciduous teeth (SHED) for treatment and improvement of EAE have not been reported.

SHED expresses Nestin, the marker for ectodermal and neuronal lineages, which allows them to differentiate into mesenchymal lineages (i.e. bone, adipose, muscle and cartilage) and nervous-like cells [16]. Additionally, we have previously demonstrated that

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SHED can induce a shift in the immune profile of monocyte-derived dendritic cells, which resulted in an increased frequency of regulatory T cells *in vitro* [17], suggesting that SHED could be applied specifically for treatment of autoimmune associated diseases. In this regard, we aimed to investigate the therapeutic effect of SHED in EAE model using Foxp3 GFP + transgenic mice (C57Bl/6-Foxp3GFP). The results showed that SHED infusion ameliorates EAE clinical signs and cellular pattern with reduced frequency of infiltrating inflammatory cells into the central nervous system (CNS), beyond a significant increase in $CD4^{+}FOXP3+$ T cells in the spleen.

2. Materials and methods

2.1. Experimental design

SHED were injected intraperitoneally (i.p.) one day before immunization (EAE induction) and two days post-immunization (p.i) in C57Bl/6-Foxp3+ Green fluorescent protein (GFP) mice (EAE-SHED group; n = 4). Additionally, a group of animals were maintained without treatment (EAE group; n = 4). On day 14 (peak of disease), the all animals were euthanized. Total splenocytes were evaluated by flow cytometry for FOXP3 expression (mean fluorescence intensity) and CD4 and CD8 cells markers. IFN-γ and IL-4-producing T cells were also evaluated in suspension cells of CNS.

2.2. Stem cells isolation, culture and characterization

Cellular isolation, culture and characterization were performed as previously described in Silva et al. (2014) [17]. SHED were obtained from healthy volunteers after written consent and approval by the Institutional Review Board at Dentistry School/University of São Paulo (USP) (number 129/10). The pulp extraction process was performed by tearing and culturing until adhesion and release of the cells to the culture dish. The cells (passage P7-P10) were cultivated with basal medium, consisting of F12 medium (Gibco, USA) supplemented with 15% serum Hyclone (Thermo, USA), antibiotic-antimycotic solution (100 U/ml penicillin, 100 mg/ml streptomycin, and 25 mg/ml amphotericin; Gibco), 2.5 mM L-glutamine and nonessential amino acids (Gibco).

After culture, the cells were harvested by treatment with trypsin (Gibco), washed and suspended in phosphate-buffered saline (PBS); approximately 1×10^5 cells were incubated on ice for 20 min with conjugated monoclonal antibodies (1:100) against CD73, CD90, CD105, CD45, CD34, (BD Biosciences, USA). The acquisition was done in a FACSCanto II flow cytometer (BD Biosciences) and the analysis performed using the FlowJo software, Ver.7.2.4 (Tree Star, Ashland, OR, USA). SHED adipogenic and osteogenic differentiation were evaluated, as described by Pittenger et al. (1999) [18]. SHED were cultivated until total confluence and, then, induced to differentiation by the Mesenchymal Stem Cell Adipogenesis Kit and Mesenchymal Stem Cell Osteogenesis Kit respectively (Chemicon, USA), according to the manufacturer instructions. The medium was replaced every three to four days over a period of 21 days. Adipogenesis was determined by staining with oil red O, to verify neutral lipids accumulation in fat vacuoles, and osteogenesis was detected by accumulation of calcium compounds (by Alizarin Red staining). SHED were positive for CD73, CD90 and CD105 and negative for CD45 and CD34 markers; and were capable of differentiating into mesodermal lineages (Fig. 1).

2.3. Animals

Around 6-8-week-old C57Bl/6-Foxp3GFP mice (20-25 g) were used in this study. Mice were housed at four mice (per group) per cage, with light/dark cycle of 12:12-hs and water and chow *ad libitum*. All animals were bred and experiments were performed at the Animal Care Facility of the Institute of Biomedical Sciences, University of Sao Paulo (USP), São Paulo, Brazil. All experiments were performed in accordance with the guidelines of the Committee on Animal Research of USP; with approval of the Committee Animal Research of IPEN/USP (number 81/11).

2.4. EAE induction and SHED treatment

For EAE induction, mice were subcutaneously injected with 200 μl (on the tail base) of the Freund's Adjuvant, Complete (CFA; v/v) (Sigma, USA) emulsion containing 1 mg/ml of *M. tuberculosis* H37RA (Difco, USA) and 200 μg of myelin oligodendrocyte glycoprotein $(MOG)_{35-55}$ (Proteimax, BRA). Mice also received two intraperitoneal doses of 200 ng of pertussis toxin (Sigma), before immunization and 48 hs later. All animals were scored daily, according to: 0, no disease; 1, limp tail; 2, weak/partially paralyzed hind legs; 3, completely paralyzed hind legs; 4, complete hind and partial front leg paralysis; and 5, total paralysis (moribund) [14]. Animals were divided into two groups: i) the treated group (EAE-SHED) was intraperitoneally treated with 1×10^6 SHED in 200 μl (PBS) one day before and two days post EAE induction; and ii) the control group (EAE) received PBS one day before and two days post EAE induction. All animals were euthanized on day 14 post EAE.

2.5. Isolation of CNS mononuclear cells

Brain and spinal cords were harvested, macerated and maintained in 4 ml of DMEM (Gibco, USA) supplemented with collagenase D (250 μg/ml; Roche, USA) and incubated at 37 °C with CO_2 5%. 45 min later, the reaction was stopped with EDTA (2 mM) and suspensions were centrifuged (5 min, 450*g*, 4 °C; Eppendorf, GER). Cells were then suspended in percoll 37% (GE Healthcare, UK) and gently laid over percoll 70% in 15 ml tubes. The tubes were centrifuged (20 min, 950*g*, 24 °C) without brakes. Then, the interface containing mononuclear cells was collected, washed and centrifuged (5 min, 450*g*). Cellular suspensions were then suspended in PBS with bovine serum albumins (BSA) 0,2% for cell staining (Gibco).

2.6. In vitro stimulation of CNS-Infiltrating cells

Infiltrating mononuclear cells were seeded (5×10^5 cells/well) in 96 flat-bottom plates and stimulated with MOG_{35-55} peptide (50 μg/ml) with Brefeldin A (1:1000; Biolegend, USA) overnight, followed by PMA (50 ng/ml) and ionomycin (1 μg/ml) for 4 hs. Cells were then stained with anti-mouse CD4-APC or CD8-PerCP (Biolegend) for 30 min at 4 °C. For intracellular staining, cells were fixed and permeabilized with Cytofix/Cytoperm kit (BD Biosciences, USA) according to manufacturer protocol and incubated with anti-IFN-γ-FITC and anti-IL-4-PE (Biolegend). Cells were washed, and acquired by BD Accuri 6 (BD Biosciences, USA) equipment, and analyzed using C6 Software (BD Biosciences, USA).

2.7. Isolation and characterization of splenocytes

On day 14 post EAE, the spleen was mechanically dissociated and homogenized in 5 ml of sterile DMEM (Gibco) to form a cell suspension. This suspension was centrifuged (Eppendorf) at 450 g for 5 min, resuspended in 9 mL of sterile ammonium chloride to lyse erythrocytes and centrifuged again. Total splenocytes were stained with CD4-APC and CD8-PE antibodies (Biolegend), as previously described. Cells were washed, fixed in paraformaldehyde 1%, acquired, counted and analyzed using flow cytometry (BD