



Bone marrow mononuclear cell therapy in experimental allergic asthma: Intratracheal versus intravenous administration

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ARTICLE INFO

Article history:

Accepted 9 November 2012

Keywords:

Collagen
Lung mechanics
Stem cells
Asthma
Growth factor

ABSTRACT

We hypothesized that the route of administration would impact the beneficial effects of bone marrow-derived mononuclear cell (BMDMC) therapy on the remodelling process of asthma. C57BL/6 mice were randomly assigned to two main groups. In the OVA group, mice were sensitized and challenged with ovalbumin, while the control group received saline using the same protocol. Twenty-four hours before the first challenge, control and OVA animals were further randomized into three subgroups to receive saline (SAL), BMDMCs intravenously (2×10^6), or BMDMCs intratracheally (2×10^6). The following changes were induced by BMDMC therapy in OVA mice regardless of administration route: reduction in resistive and viscoelastic pressures, static elastance, eosinophil infiltration, collagen fibre content in airways and lung parenchyma; and reduction in the levels of interleukin (IL)-4, IL-13, transforming growth factor- β and vascular endothelial growth factor. In conclusion, BMDMC modulated inflammatory and remodelling processes regardless of administration route in this experimental model of allergic asthma.

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1. Introduction

Intravenous administration of bone marrow-derived mononuclear cells (BMDMCs) attenuates both inflammatory and remodelling responses in experimental allergic asthma (Abreu et al., 2011a). This improvement was observed despite a very low engraftment rate, possibly as a result of immune response modulation promoted by the administered cells through the release of cytokines and growth factors (Abreu et al., 2011a).

Intravenous infusion is often used in preclinical studies for the delivery of various cell types, including mesenchymal stem cells (MSCs) (Bonfield et al., 2010; Nemeth et al., 2010; Goodwin et al., 2011) and BMDMCs (Abreu et al., 2011a). This is because the intravenous route provides broad biodistribution and easy administration. However, only a small number of cells are delivered to

the damaged area using this route (Schrepfer et al., 2007). Meanwhile, a previous study with cardiosphere-derived cells found that the benefits of cell administration were associated with injection route and with the number of cells delivered with each route at the site of injury (Bonios et al., 2011).

We hypothesize that intratracheal (IT) delivery of BMDMCs may be more effective than intravenous (IV) administration to reduce the inflammatory and remodelling processes and promote airway epithelial repair in experimental allergic asthma. To test this hypothesis, lung histology findings, collagen fibre content in the airway and alveolar septa, levels of cytokines and growth factors in lung tissue, and lung mechanics were analyzed following IT and IV administration of BMDMCs in a murine model of allergic asthma.

2. Materials and methods

This study was approved by the Ethics Committee of the Health Sciences Centre, Federal University of Rio de Janeiro. All animals received humane care in compliance with the “Principles of Laboratory Animal Care” formulated by the National Society for Medical Research and the U.S. National Research Council “Guide for the Care and Use of Laboratory Animals”.

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2.1. Extraction and characterization of bone marrow-derived mononuclear cells

Bone marrow cells were extracted from male C57BL/6 mice (weight 20–25 g, $n = 10$) and administered on the day of collection. Alternatively, BMDMCs were obtained from GFP+ male mice (weight 20–25 g, $n = 5$) and administered to C57BL/6 female mice to evaluate the degree of pulmonary GFP+ cell engraftment. Briefly, bone marrow cells were aspirated from the femur and tibia by flushing the bone marrow cavity with Dulbecco's modified Eagle's medium (DMEM) (Life Technologies, Grand Island, NY, USA). After a homogeneous cell suspension was achieved, cells were centrifuged ($400 \times g$ for 10 min), re-suspended in DMEM and added to Ficoll-Hypaque (Histopaque 1083, Sigma Chemical Co., St. Louis, MO, USA), and again centrifuged and re-suspended in phosphate-buffered saline (PBS). Cells were counted in a Neubauer chamber with Trypan Blue for the evaluation of viability. For the administration of saline or BMDMCs, mice were anaesthetized with sevoflurane, the jugular vein or the trachea of each mouse was dissected, and cells were slowly injected. A small aliquot of mononuclear cells was used for immunophenotypic characterization of the injected cell population. Cell characterization was performed by flow cytometry using antibodies against CD45 (leukocytes), CD34 (haematopoietic precursors), CD3, CD8, and CD4 (T lymphocytes), CD19 (B lymphocytes), CD14 (monocytes), CD11b, CD29 and CD45 (mesenchymal stem cells), all from BD Biosciences, USA.

2.2. Animal preparation and experimental protocol

Thirty-six female C57BL/6 mice (20–25 g) were randomly assigned to two groups. In the OVA group, mice were immunized using an adjuvant-free protocol by intraperitoneal injection of sterile ovalbumin (OVA, 10 μ g OVA in 100 μ l saline) on 7 alternate days. Forty days after the start of sensitization, 20 μ g of OVA in 20 μ l saline was instilled intratracheally. This procedure was performed 3 times with 3-day intervals between applications (Xisto et al., 2005). The control group (C) received saline using the same protocol. The C and OVA groups were further randomized to receive saline solution (0.9% NaCl, 50 μ l, SAL) or BMDMCs (2×10^6 in 50 μ l, CELL), intravenously (through the left jugular vein) or intratracheally, 24 h before the first challenge (Fig. 1).

2.3. Mechanical parameters

Twenty-four hours after the last intratracheal challenge with saline or OVA, animals were sedated (diazepam 1 mg ip), anaesthetized (thiopental sodium 20 mg/kg ip), tracheotomized, paralyzed (vecuronium bromide, 0.005 mg/kg iv), and ventilated with a constant flow ventilator (Samay VR15; Universidad de la Republica, Montevideo, Uruguay) set to the following parameters: frequency 100 breaths/min, tidal volume (V_T) 0.2 mL, and fraction of inspired oxygen (FiO_2) 0.21. The anterior chest wall was surgically removed and a positive end-expiratory pressure of 2 cmH₂O applied. Airflow and tracheal pressure (P_{tr}) were measured (Burburan et al., 2007). Lung mechanics were analyzed by the end-inflation occlusion method (Bates et al., 1988). In an open chest preparation, P_{tr} reflects transpulmonary pressure (P_L). Briefly, after end-inspiratory occlusion, there is an initial rapid decline in P_L (ΔP_1) from the preocclusion value down to an inflection point (P_i), followed by a slow pressure decay (ΔP_2), until a plateau is reached. This plateau corresponds to the elastic recoil pressure of the lung (P_{el}). ΔP_1 selectively reflects the pressure used to overcome airway resistance. ΔP_2 reproduces the pressure spent by stress relaxation, or viscoelastic properties of the lung, as well as a minor contribution of *pendelluft*. Static lung elastance (E_{st}) was determined by dividing P_{el} by V_T . Lung mechanics measurements were obtained

10 times in each animal. All data were analyzed using ANADAT software (RHT-InfoData, Inc., Montreal, Quebec, Canada).

2.4. Lung histology

Laparotomy was performed immediately after determination of lung mechanics and heparin (1000 IU) was injected into the vena cava. The trachea was clamped at end expiration and the abdominal aorta and vena cava were sectioned, producing massive haemorrhage and rapid terminal bleeding. The left lung of each animal was then removed, flash-frozen by immersion in liquid nitrogen, fixed with Carnoy solution, and embedded in paraffin. Four-micrometre-thick slices were cut and stained with haematoxylin–eosin.

Lung histology analysis was performed with an integrating eyepiece with a coherent system consisting of a grid with 100 points and 50 lines (known length) coupled to a conventional light microscope (Olympus BX51, Olympus Latin America Inc., Brazil). The volume fraction of collapsed and normal pulmonary areas, magnitude of bronchoconstriction, and number of mononuclear (MN) and polymorphonuclear cells (PMN, neutrophils and eosinophils) in lung tissue were determined by the point-counting technique (Weibel, 1990; Hsia et al., 2010) across 10 random, non-coincident microscopic fields (Xisto et al., 2005; Burburan et al., 2007).

Collagen (Picrosirius-polarization method) and elastic fibres (Weigert's resorcin fuchsin method with oxidation) were quantified in airways and alveolar septa using Image-Pro Plus 6.0 (Xisto et al., 2005; Antunes et al., 2009, 2010).

2.5. Transmission electron microscopy

Three 2 mm $\times$ 2 mm $\times$ 2 mm fragments were cut from three different segments of the right lung and fixed [2.5% glutaraldehyde and phosphate buffer 0.1 M (pH = 7.4)] for electron microscopy analysis (JEOL 1010 Transmission Electron Microscope, Tokyo, Japan). In each electron microscopy image (50/animal), the following structural changes were analyzed: (a) shedding surface epithelium, (b) airway oedema, (c) eosinophil and neutrophil infiltration, (d) subepithelial fibrosis, (e) smooth muscle hypertrophy, (f) myofibroblast hyperplasia, (g) mucous cell hyperplasia and (i) multinucleated cells (Antunes et al., 2010; Abreu et al., 2011a). Pathologic findings were graded on a five-point semi-quantitative severity-based scoring system, where 0 = normal lung parenchyma, 1 = changes in 1–25%, 2 = changes in 26–50%, 3 = changes in 51–75%, and 4 = changes in 76–100% of examined tissue. Analysis was performed by two blinded pathologists.

2.6. Confocal microscopy

Fluorescent images of the basement membrane were obtained using a confocal microscope (Leica Microsystems Ltd., Heidelberg, Germany). Tissue sections were pretreated with PBS for 30 min and incubated overnight at room temperature in a humidified chamber with a mouse antibody against type V collagen (1:50), followed by double staining with fluorescein and rhodamine (rhodamine-conjugated goat anti-mouse IgG-R, dilution 1:40, Santa Cruz Biotechnology, Santa Cruz, CA). For recipients of GFP marrow transplants, 1 week after BMDMC administration, frozen sections were treated with 4',6-diamidino-2-phenylindole dihydrochloride (DAPI)-supplemented mounting medium (Vectashield, Vector Labs, Burlingame, CA), cover-slipped and examined for GFP expression by confocal microscopy. Background autofluorescence was determined through examination of 10 simultaneously prepared negative control sections that were stained only with DAPI. Images were processed and reconstructed using NIH Image software and contrast and colour levels were adjusted in Adobe Photoshop 7.0. The number of GFP+ cells per tissue area was determined by the

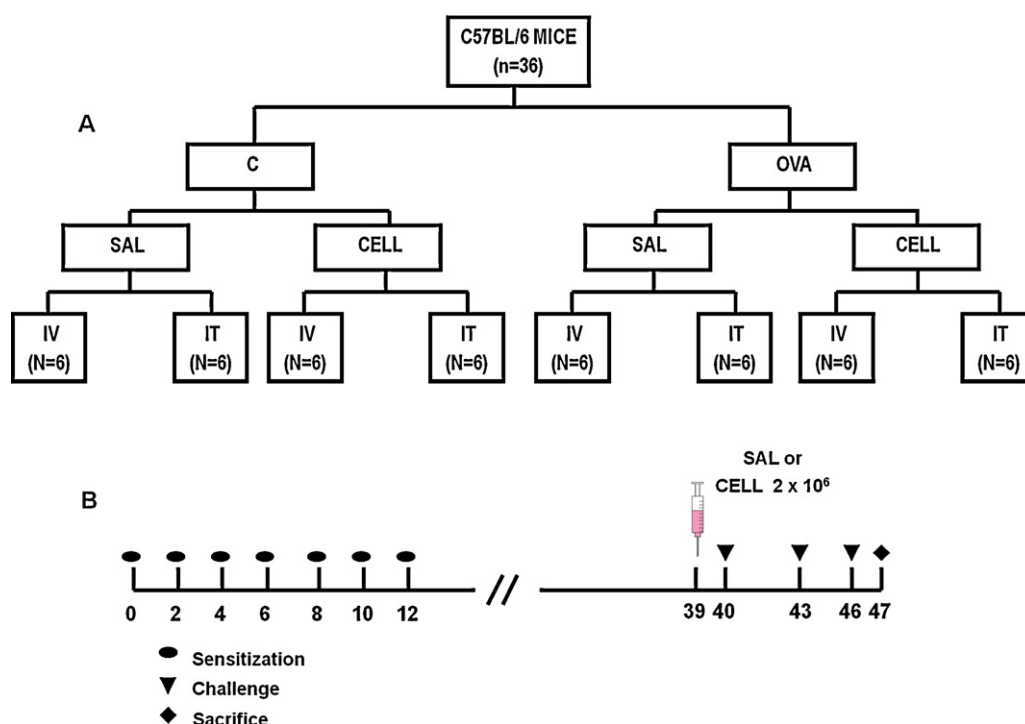


Fig. 1. Schematic flow chart (A) and timeline (B) of study design. C: Mice sensitized and challenged with saline; OVA: mice sensitized and challenged with ovalbumin; SAL: mice treated with saline; CELL: mice treated with BMDMCs (2×10^6) 24 h before the first challenge; IV: intravenous injection; IT: intratracheal instillation. All data were analyzed on day 47.

point-counting technique (Weibel, 1990; Araujo et al., 2010) across 10 random, non-coincident microscopic fields.

2.7. Enzyme-linked immunosorbent assay (ELISA)

Levels of interleukin (IL)-4, IL-13, transforming growth factor (TGF)- β and vascular endothelial growth factor (VEGF) in lung tissue 24 h after the last challenge were evaluated by ELISA using matched antibody pairs from PreproTech and R&D Systems (Minneapolis, MN, USA), according to manufacturer instructions. Results are expressed in pg/ml.

2.8. Statistical analysis

Data were tested for normality using the Kolmogorov–Smirnov test with Lilliefors correction and the homogeneity of variances was assessed with the Levene median test. If both conditions were satisfied, two-way ANOVA, followed by Tukey's test when required, was used for the comparison of differences among the groups. Non-parametric data were analyzed using ANOVA on ranks followed by Tukey's test. Parametric data were expressed as mean $\pm$ SEM, while non-parametric data were expressed as median (interquartile range). All tests were performed using the SigmaPlot 11 software package (SYSTAT, Chicago, IL, USA), and statistical significance was established as $p < 0.05$.

3. Results

The pool of injected BMDMCs showed the following sub-populations: total lymphocyte (lower SSC, CD45+/CD11b-/CD29-/CD34- = 9.50%), T lymphocyte (lower SSC/CD45+/CD3+/CD34- = 5.4%), T helper lymphocyte (CD3+/CD4+/CD8- = 1.7%), T cytotoxic lymphocyte (CD3+/CD4-/CD8+ = 7.8%), B lymphocytes (CD19+ = 7.65%), monocytes (CD45+/CD29+/CD11b+ low/CD34-/CD3- = 9.58%), haematopoietic progenitors

(CD34+/CD45+ = 1.5%) and mesenchymal stem cells (CD34-/CD45-/CD11b- = 3.8%).

Because parameters of lung mechanics were similar regardless of administration route in all control groups (C-SAL-IV and C-SAL-IT, C-CELL-IV and C-CELL-IT) (data not shown), only the overall results for C-SAL and C-CELL are presented. The OVA-SAL groups, both IV and IT, had higher E_{st} (26% and 29%), ΔP_1 (15% and 11%), and ΔP_2 (49 and 64%) compared to C-SAL, respectively. E_{st} , ΔP_1 , and ΔP_2 were lower in OVA-CELL than OVA-SAL regardless of the route of administration (Fig. 2).

Lung morphometric examination demonstrated that the fraction area of alveolar collapse (Figs. 3A and 4A), the number of mononuclear cells and PMN in lung tissue (Fig. 3B), contraction index (Fig. 3C and 4B), and collagen fibre content in the airway and alveolar septa (Fig. 5) were higher in the OVA-SAL group than in the C-SAL group. BMDMC therapy reduced the fraction area of alveolar collapse (Fig. 3A and 4A) and PMN infiltration (Fig. 3B). It also prevented changes in airway diameter (Fig. 3C and 4B) and in the amount of collagen fibre in the airway and alveolar septa (Fig. 5).

Electron microscopy showed degenerative changes in ciliated airway epithelial cells, inflammatory infiltration, myofibroblast and mucous cell hyperplasia, subepithelial fibrosis with increased thickness of basement membrane and smooth muscle hypertrophy in OVA-SAL-IT and OVA-SAL-IV animals (Table 1, Fig. 6). Both IT and IV BMDMC instillation attenuated these ultrastructural changes. Also, both IT and IV instillation of BMDMC promoted Clara cell proliferation and appearance of multinucleated cells and of undifferentiated cells without a defined phenotype (Table 1, Fig. 6).

In a separate set of experiments, BMDMCs isolated from GFP+ mice were used to compare the level of engraftment between administration routes 1 week after cell administration. GFP+ cells were detected in both OVA groups, but intratracheal instillation led to higher pulmonary engraftment (4%) compared with intravenous injection (1%). GFP+ cells were not detected in control lungs.

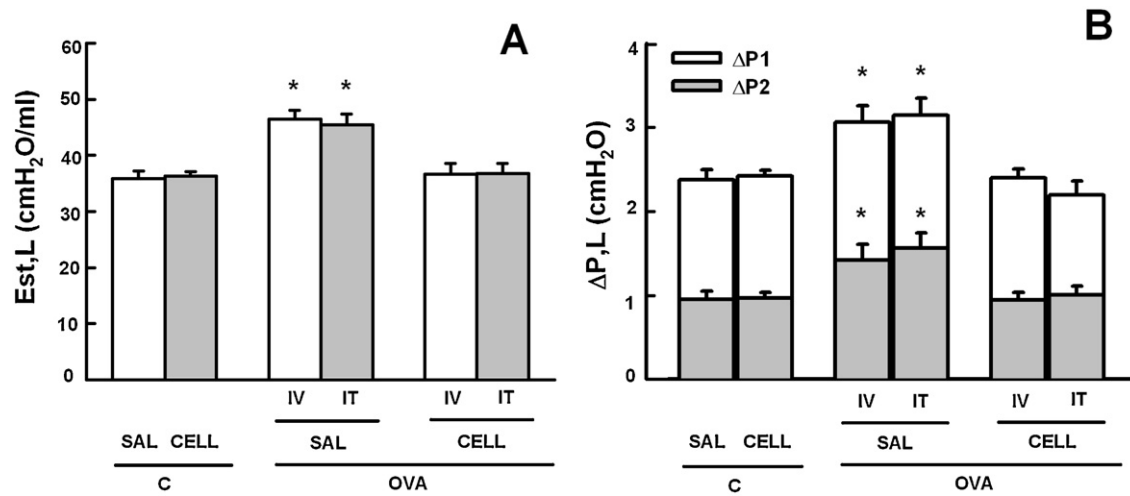


Fig. 2. Lung static elastance (E_{st}) (Panel A); resistive (P_1 , grey bar), viscoelastic (P_2 , white bar) and total pressures ($\Delta P_{tot} = \Delta P_1 + \Delta P_2$) (Panel B). C: mice sensitized and challenged with saline; OVA: mice sensitized and challenged with ovalbumin; SAL: mice treated with saline; CELL: mice treated with BMDMCs (2×10^6) 24 h before the first challenge. IV: intravenous injection; IT: intratracheal instillation. *Significantly different from C-SAL ($p < 0.05$). **Significantly different from respective OVA-SAL ($p < 0.05$).

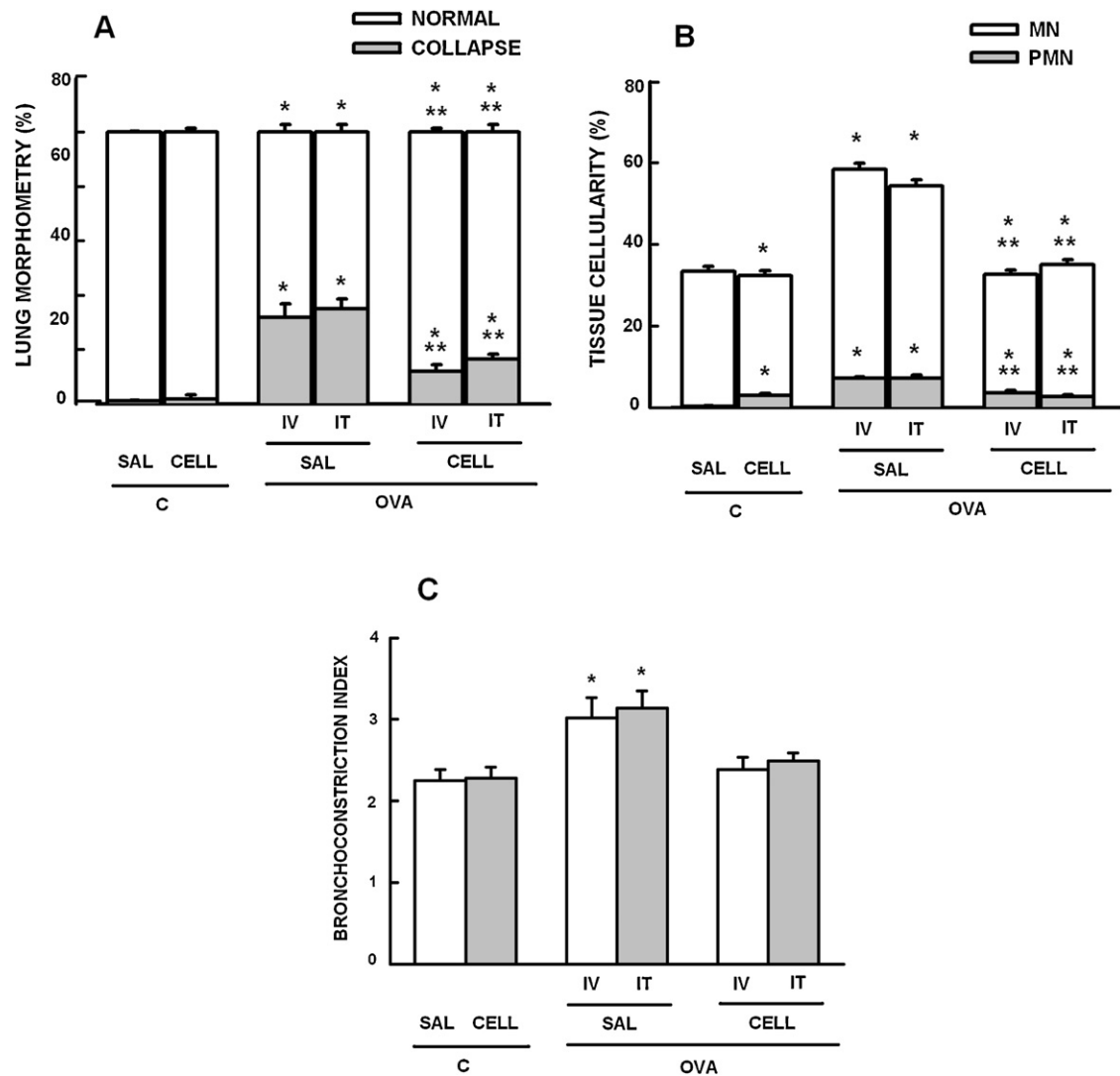


Fig. 3. Fraction area of normal (white bar) and collapsed alveoli (grey bar, Panel A), mononuclear (MN, white bar) and polymorphonuclear (PMN, grey bar) cells (Panel B), bronchoconstriction index (Panel C). All values were computed in 10 random, non-coincident fields per mouse. C: mice sensitized and challenged with saline; OVA: mice sensitized and challenged with ovalbumin; SAL: mice treated with saline; BMDMC: mice treated with BMDMCs (2×10^6) 24 h before the last challenge. IV: intravenous injection; IT: intratracheal instillation. *Significantly different from respective C-SAL ($p < 0.05$). **Significantly different from respective OVA-SAL ($p < 0.05$).

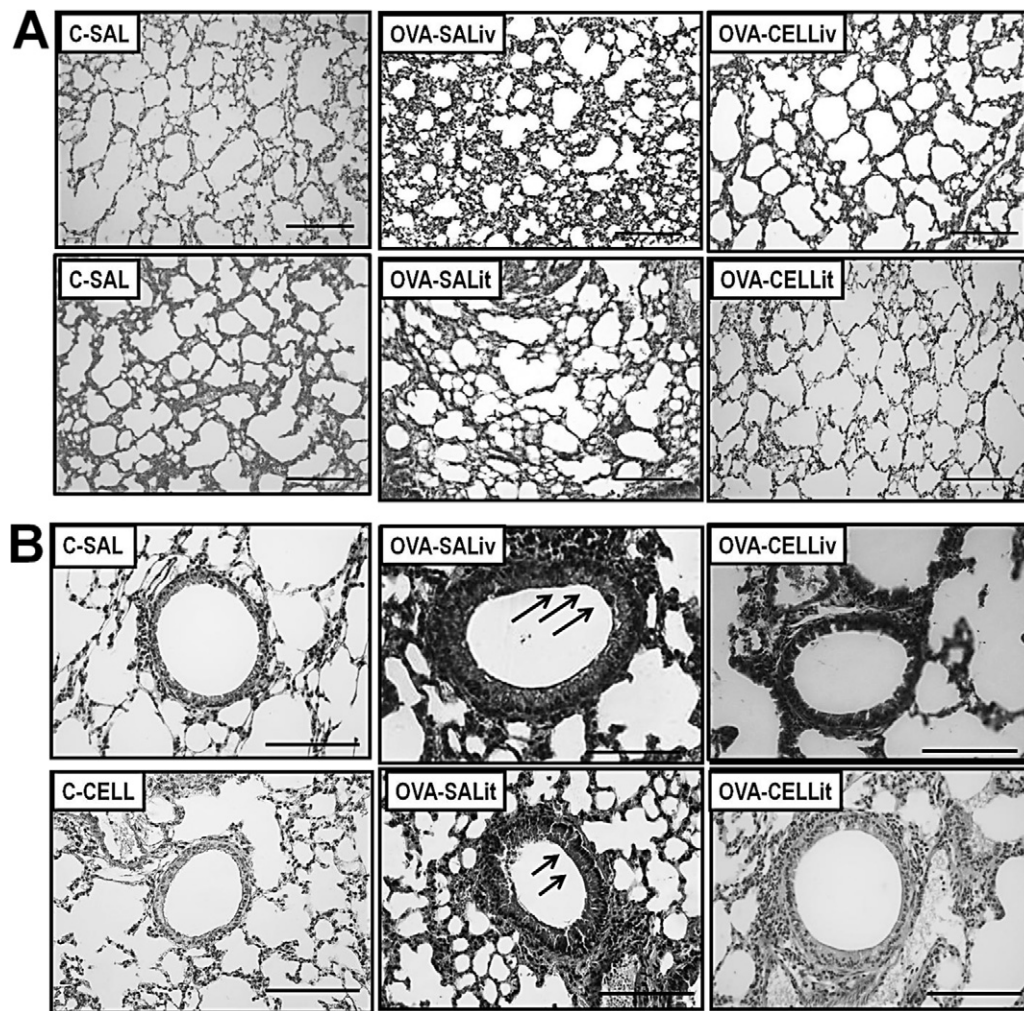


Fig. 4. Representative photomicrographs of lung parenchyma (upper panels) and airways (lower panels), haematoxylin–eosin stain. Collapsed areas in the lung parenchyma and a reduction in central airway diameter, as well as increased cell infiltration (arrows), are visible in OVA-SAL animals. C: mice sensitized and challenged with saline; OVA: mice sensitized and challenged with ovalbumin; SAL: mice treated with saline; CELL: mice treated with BMDMCs (2×10^6) 24 h before the first challenge. IV: intravenous injection; IT: intratracheal instillation. Original magnification: $\times 200$ and $\times 400$ (upper and lower panels, respectively). Bars = 100 μm .

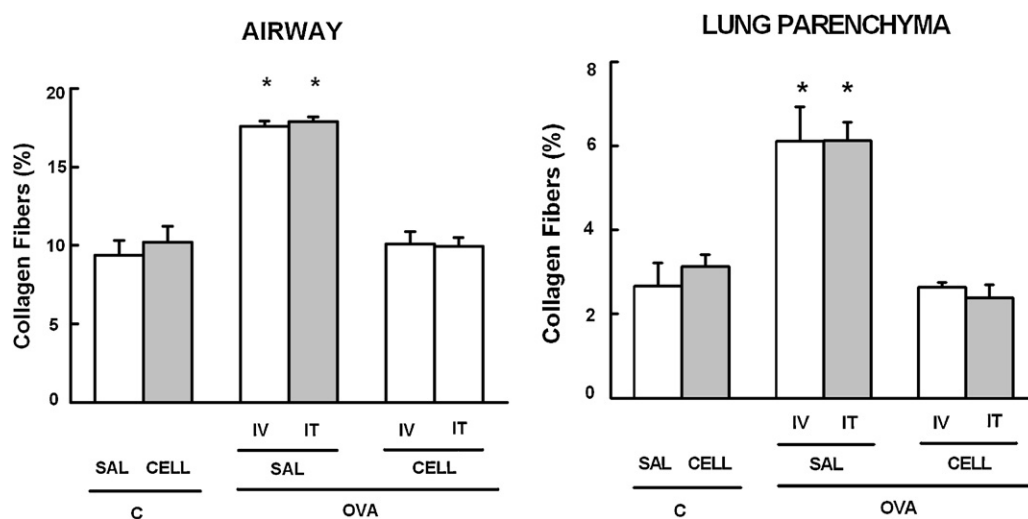


Fig. 5. Collagen fibre content in the airways (Panel A) and lung parenchyma (Panel B). C: mice sensitized and challenged with saline; OVA: mice sensitized and challenged with ovalbumin; SAL: mice treated with saline; CELL: mice treated with BMDMCs (2×10^6) 24 h before the first challenge. IV: intravenous injection; IT: intratracheal instillation. *Significantly different from C-SAL ($p < 0.05$). **Significantly different from respective OVA-SAL ($p < 0.05$).

Table 1
Semi-quantitative electron microscopy analysis.

Groups	C				OVA			
	SAL		CELL		SAL		CELL	
	IV	IT	IV	IT	IV	IT	IV	IT
Shedding of surface epithelium	0 (0-0)	0 (0-0)	0 (0-0)	1 (0-1)*	2 (2-3)*	2 (1-4)*	1 (0-1)*.#	1 (0-2)*.#
Airway oedema	0 (0-0)	0 (0-0)	0 (0-0)	0 (0-0)	2 (2-3)*	3 (2-4)*	1 (1-2)*.#	1 (0-1)*.#
Eosinophil infiltration	0 (0-0)	0 (0-0)	0 (0-0)	0 (0-0)	3 (3-4)*	3 (2-4)*	1 (1-2)*.#	1 (1-2)*.#
Neutrophil infiltration	0 (0-0)	0 (0-0)	1 (0-1)*	1 (0-1)*	2 (1-3)*	2 (1-4)*	1 (0-1)*.#	1 (1-2)*.#
Disarrangement of ciliated epithelial cells	0 (0-0)	0 (0-0)	0 (0-0)	0 (0-0)	2 (1-3)*	2 (2-3)*	1 (1-2)*.#	1 (1-2)*.#
Subepithelial fibrosis	0 (0-0)	0 (0-0)	0 (0-0)	0 (0-0)	2 (2-3)*	2 (2-3)*	1 (1-2)*.#	1 (1-2)*.#
Elastic fibre fragmentation	0 (0-0)	0 (0-0)	0 (0-0)	0 (0-0)	2 (2-3)*	2 (2-3)*	1 (0-2)*.#	1 (0-1)*.#
Smooth muscle hypertrophy	0 (0-0)	0 (0-0)	0 (0-0)	0 (0-0)	2 (2-3)*	3 (2-4)*	1 (0-1)*.#	1 (1-2)*.#
Myofibroblast hyperplasia	0 (0-0)	0 (0-0)	0 (0-0)	0 (0-0)	3 (3-4)*	3 (3-4)*	1 (1-2)*.#	1 (1-2)*.#
Clara cell hyperplasia	0 (0-0)	0 (0-0)	1 (0-1)*	1 (0-1)*	0 (0-0)	0 (0-0)	1 (1-2)*.#	2 (1-3)*.#
Multinucleated cells	0 (0-0)	0 (0-0)	1 (0-1)*	1 (0-1)*	0 (0-0)	0 (0-0)	2 (2-4)*.#	2 (1-3)*.#

Pathologic findings were graded as: 0 = normal lung parenchyma, 1 = changes in 1–25%, 2 = 26–50%, 3 = 51–75%, and 4 = 76–100% of the examined tissue. C: mice sensitized and challenged with saline; OVA: mice sensitized and challenged with ovalbumin; SAL: mice treated with saline; BMDMC: mice treated with BMDMCs (2×10^6) 24 h before the last challenge. IV: intravenous injection; IT: intratracheal instillation.

* Significantly different from respective (IV or IT) C-SAL ($p < 0.05$).
Significantly different from respective (IV or IT) OVA-SAL ($p < 0.05$).

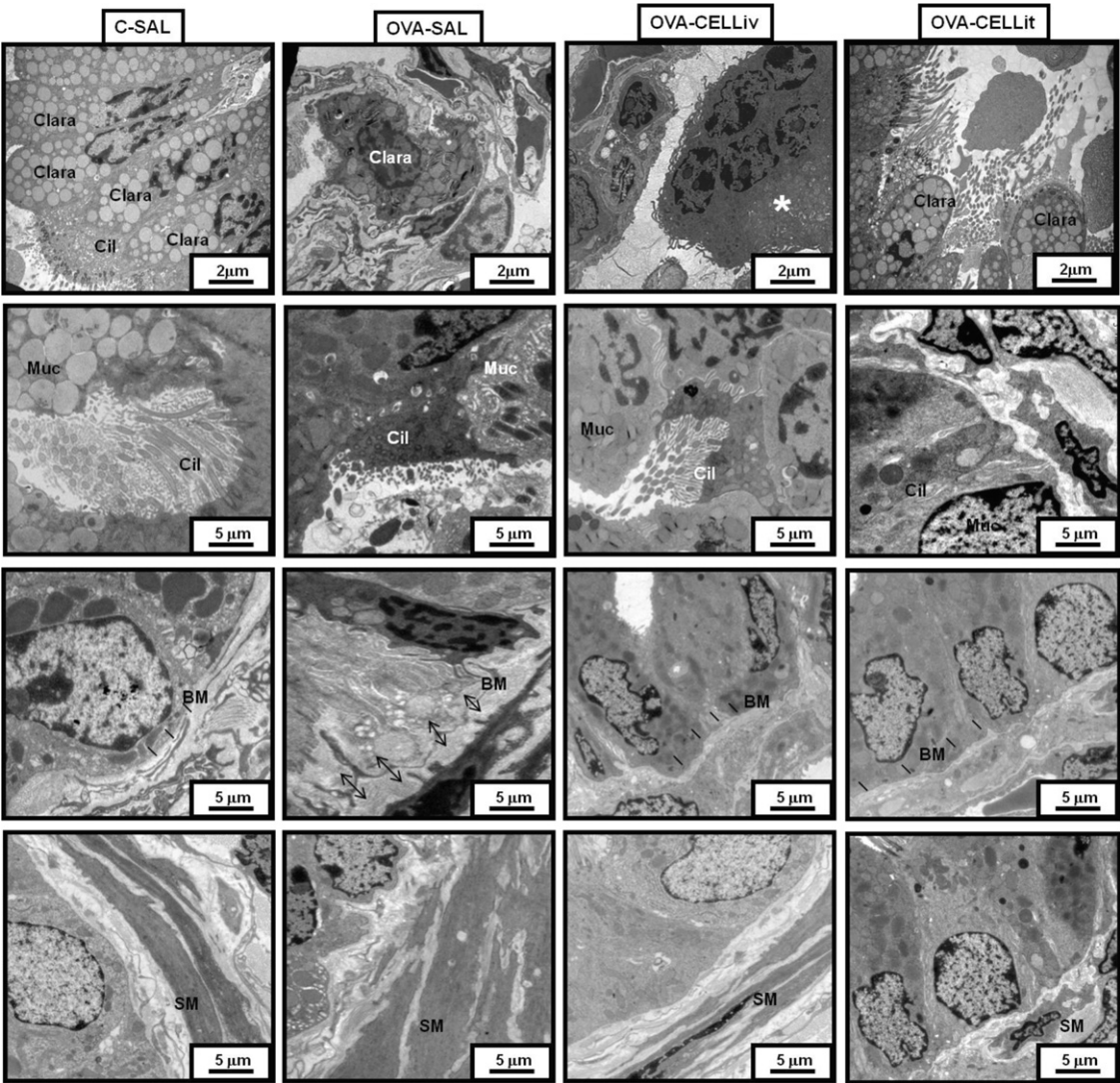


Fig. 6. Electron microscopy of lung parenchyma. C: mice sensitized and challenged with saline; OVA: mice sensitized and challenged with ovalbumin; SAL: mice treated with saline; CELL: mice treated with BMDMCs (2×10^6) 24 h before the first challenge. IV: intravenous injection; IT: intratracheal instillation. Clara cells (Clara), mucous cells (Muc), ciliated cells (Cil), multinucleated cells (*), basal membrane (BM), basal membrane thickness (arrows) and smooth muscle hypertrophy (SM).

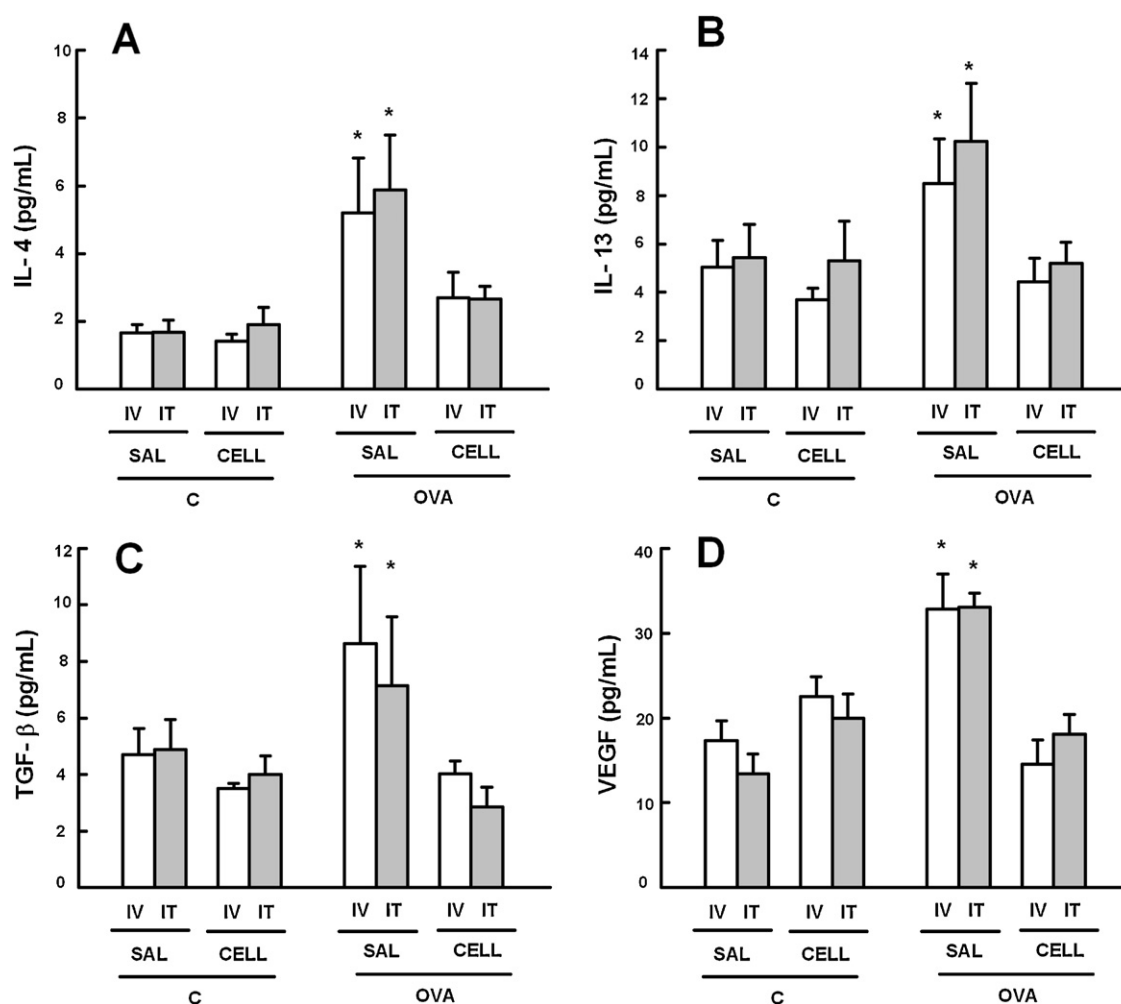


Fig. 7. Levels of interleukin (IL)-4, IL-13, transforming growth factor (TGF)- β in mouse lung tissue. C: mice sensitized and challenged with saline; OVA: mice sensitized and challenged with ovalbumin; SAL: mice treated with saline; CELL: mice treated with BMDMCs (2×10^6) 24 h before the first challenge. IV: intravenous injection; IT: intratracheal instillation. Values are mean $\pm$ SEM of 6 animals in each group. *Significantly different from C-SAL ($p < 0.05$). **Significantly different from respective OVA-SAL ($p < 0.05$).

Levels of IL-4, IL-13, TGF- β and VEGF in lung tissue were higher in the OVA-SAL group than in the C-SAL group. Intravenous and intratracheal BMDMC administration yielded similar reductions in the levels of these cytokines and growth factors (Fig. 7).

4. Discussion

In the murine model of allergic asthma used in the present study, early BMDMC therapy through the intravenous and intratracheal routes resulted in similar benefits, reducing alveolar collapse, bronchoconstriction, eosinophil infiltration, collagen fibre content in the airway and alveolar septa, airway oedema, and myofibroblast hypertrophy and hyperplasia, as well as improving airway epithelial repair and lung mechanics. Since the higher BMDMC pulmonary engraftment observed with intratracheal instillation compared to intravenous injection did not potentiate the beneficial effects of BMDMC therapy, these beneficial changes may be attributed to the ability of BMDMCs to modulate IL-4, IL-13, TGF- β and VEGF levels in lung tissue from a distant site.

In the present study, we used a model of allergic inflammation previously described by our group in BALB/c mice (Xisto et al., 2005; Burburan et al., 2007; Antunes et al., 2009). Nevertheless, C57BL/6 mice were used, because they serve as a background strain for GFP mice (Abreu et al., 2011a) and exhibit inflammatory (eosinophilia

and Th2 pro-inflammatory cytokine increase) and ultrastructural changes in the airway and lung parenchyma which closely mirror human disease compared to other strains, even in the absence of alum adjuvant (Yu et al., 2006; Antunes et al., 2009; Allen et al., 2012). A recent study demonstrated that NLRP3 inflammasome activation is essential in alum-free models of allergic asthma as it leads to IL-1 production, a critical factor for the induction of Th2 inflammatory allergic response (Besnard et al., 2011). Even though the use of alum adjuvant during the immunization phase of the OVA model has been demonstrated to enhance the cardinal features of allergic airway disease, this practice has been called into question, since it is an artificial method of asthma induction with major differences in relation to the pathogenesis of allergic disease in humans.

Several recent studies have investigated the intravenous administration of mesenchymal stem cells in experimental models of asthma, focusing on the beneficial effects of these cells on lung remodelling and inflammation (Bonfield et al., 2010; Firinci et al., 2011; Goodwin et al., 2011). However, MSC pose a series of disadvantages, such as culture conditions detrimental to cell transplantation and risk of contamination and immunologic reactions. In light of these limitations, our group evaluated the effects of intravenous BMDMC administration in a model of allergic asthma (Abreu et al., 2011a). BMDMCs can be administered easily and

safely on the day of harvesting. They also express several genes involved in inflammatory response and chemotaxis (Ohnishi et al., 2007), and are less costly than MSCs. Additionally, further studies should investigate whether the nature of BMDMCs as a heterogeneous mix of progenitor and immune cells could induce beneficial effects, with each cellular type playing a specific role. In that scenario, the action and interaction between each cell type present in the bone marrow mononuclear fraction would be essential to achievement and potentiation of the functional effects of cell therapy (Mathieu et al., 2009; Lu et al., 2011). The present analysis is not sufficient to distinguish which cell fraction in the BMDMC sample gave rise to the therapeutic effects observed. Determination of which specific cell types are responsible for these features will require future experiments, such as transplant studies using cell sorters, a comparative study of bone marrow cell populations and *in vitro* functional bioassays of BMDMCs.

Although intravenous administration of BMDMCs has been effective as a pre-treatment protocol for asthma, reducing inflammation and remodelling and yielding better lung function (Abreu et al., 2011a), we investigated whether intratracheal instillation of BMDMCs, a more direct route to the lungs, would be more effective, delivering a higher number of cells (Bonios et al., 2011). This would translate in clinical practice into bronchoscopic delivery of these cells, a procedure that can be performed safely in asthmatic patients following allergen challenge (Elston et al., 2004; Busse et al., 2005). In order to identify homing of bone marrow cells in lung parenchyma, GFP-positive cells derived from male mice (a reliable marker of engrafted cells) were quantified. GFP-positive cells were observed in the OVA-CELL groups, but not in C-CELL lungs, suggesting that tissue damage is necessary to attract and retain these cells even when they are intratracheally administered. As stated elsewhere, the inflammatory process plays an essential role in cell recruitment to the injured area (Herzog et al., 2006). Nevertheless, the source of signals responsible for mobilization and homing of endogenous and exogenous stem cells remains unclear. Stem cell recruitment varies according to the degree (Herzog et al., 2006) and type of tissue damage (Abe et al., 2004).

Lung accumulation of intravenously injected stem cells is proportional to the presence of adhesion molecules on the cell surface and to the size of the cell. Most cells in the bone marrow fraction do not express major adhesion molecules, such as vascular cell adhesion molecule-1 (VCAM-1), when binding to pulmonary vascular endothelium. BMDMCs are also smaller compared to other cell types, such as MSCs (Fischer et al., 2009). Therefore, BMDMCs pass easily through the pulmonary capillaries and into the systemic circulation when injected intravenously, reaching distal organs rather than remaining in the lung tissue (Lassance et al., 2009). We expected that intratracheal instillation would promote a more marked pulmonary engraftment than that actually observed in the present study. However, cell trapping in the lungs was only evaluated 1 week after BMDMC administration, which may explain the low degree of GFP-positive cell pulmonary engraftment: during these 7 days, a portion of the administered cells may have been lost due to immune rejection by the host, since our population of BMDMCs constitutes an allotransplant (Ortiz et al., 2003). Simultaneously, just as these cells can pass from the intravascular space to the lungs, so can they pass from the lung tissue to the intravascular space, reaching the systemic circulation and being distributed throughout the body, reducing even further the number of GFP-positive cells in the lung parenchyma.

Even though intratracheal instillation yielded a higher number of cells trapped in the lung parenchyma, suggesting that this route of administration could maximize cell delivery to the lung and directly reach the injury site, both administration routes led to a decrease in collapsed areas and cell infiltration in the airway and lung parenchyma, as well as a reduction in collagen fibre

content, improving lung mechanics. Therefore, the beneficial effects of BMDMC therapy observed in the present study may be associated with the ability of BMDMCs to modulate cytokine and growth factor synthesis without being present at the site of injury (Abreu et al., 2011b; Goodwin et al., 2011; Ratajczak et al., 2011). In control animals, injection of BMDMCs led to an increase in PMN levels in lung tissue, with no functional effects. This increment may be associated with the presence of immune cells in the BMDMC pool or recruitment of these cells by chemoattraction (Araujo et al., 2010; Protá et al., 2010; Abreu et al., 2011a,b; Maron-Gutierrez et al., 2011; Cruz et al., 2012).

Complete regeneration of the airway epithelium is a complex phenomenon that encompasses both epithelial wound repair and differentiation (Knight et al., 2010). Regeneration implies two components: epithelial stem/progenitor cells and factors able to regulate this process. In asthma, the ability to restore the epithelial barrier may fail after repeated injury leads to airway remodelling (Volckaert et al., 2011). Therefore, administration of BMDMCs may potentiate airway epithelial cell repair. In this study, we observed that BMDMCs, regardless of administration route, appeared to repair airway ciliated epithelial cells associated with several features of the regenerative process, such as proliferation of Clara cells (airway progenitor cells) and the presence of multinucleated and undifferentiated cells in lung parenchyma (Table 1). It has been demonstrated that, after airway epithelial cell injury, Clara cells are stimulated to undergo a transient epithelial-to-mesenchymal transition (EMT) to initiate the repair process, promoting restoration and function of the airway epithelium (Morimoto and Yatera, 2002). However, the precise mechanisms underlying cell restoration remain unclear. BMDMC-derived soluble factors may be the main mechanism involved in the effective impact of BMDMC therapy on airway function and histology in asthma. Both OVA groups in our study exhibited high levels of IL-4 and IL-13, important mediators in the pathophysiology of asthmatic airway disease (Barlow et al., 2012). Fibrocytes stimulated with IL-4 and IL-13 produce high levels of collagen and non-collagen components of the extracellular matrix (Bellini et al., 2011), and the balance between levels of these cytokines is related to recruitment of eosinophils to the lung parenchyma (Rothenberg et al., 2011). Therefore, the reduction in IL-4 and IL-13 promoted by BMDMC therapy may be associated with a decrease in the number of PMNs and collagen fibre content. Similarly, both BMDMC administration routes were able to reduce TGF- β and VEGF levels, contributing to airway repair and curtailing the remodelling process. In this context, TGF- β , the major mediator of EMT (Alipio et al., 2011), may impair airway epithelial sheet migration over matrix-coated plates due to enhancement of cell adhesion (Spurzem et al., 1993). It may also play a key role in bronchial angiogenesis and vascular remodelling in asthma via VEGF, an important angiogenic molecule (Willems-Widyastuti et al., 2011). In this line, a recent study has reported that VEGF receptor inhibition led to a significant reduction in inflammation and remodelling in experimental asthma (Lee et al., 2006). Future studies should be conducted to address the role of pathways involved in chemokine and growth factor production in the context of BMDMC therapy.

Our study has some limitations: (1) BMDMCs were injected 24 h before the first ovalbumin challenge, before the remodelling process was established. Thus, more studies should be performed to assess whether these routes of administration could promote similar effects in a remodelled airway; (2) we cannot ascertain whether the role of cytokines and growth factors is related to engraftment. To clarify this issue, specific gene-deficient animals should be used; (3) even though the amount of GFP was quantified in lung tissue, we did not analyze whether these engrafted cells transdifferentiated into any type of lung cell; and (4) we were unable to ascertain the role of MSCs in our bone marrow fraction, even though they

accounted for approximately 4% of cells in this fraction (a proportion higher than the average reported in the recent literature).

In conclusion, bone marrow-derived mononuclear cells were effective as a pre-treatment protocol in the murine model of allergic asthma used herein, leading to a reduction in inflammatory and remodelling processes and improving airway epithelial repair and lung mechanics regardless of administration route. These improvements were not affected by the higher pulmonary engraftment observed after intratracheal instillation compared to intravenous administration, suggesting an important role of BMDMCs in modulating immune response.

Funding

This study was supported by Centres of Excellence Program (PRONEX-FAPERJ), Brazilian Council for Scientific and Technological Development (CNPq), Rio de Janeiro State Research Foundation (FAPERJ), Coordination for the Improvement of Higher Education Personnel (CAPES), São Paulo State Research Foundation (FAPESP), and INCT-INOVAR.

Acknowledgements

The authors would like to express their gratitude to Mr. Andre Benedito da Silva for animal care, Mr. Bruno Paredes for his help with flow cytometry analysis, Mrs. Ana Lucia Neves da Silva for her help with microscopy, and Mrs. Moira Elizabeth Schöttler and Ms. Claudia Buchweitz for their assistance in editing the manuscript.

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