



Effects of bone marrow-derived mononuclear cells on airway and lung parenchyma remodeling in a murine model of chronic allergic inflammation

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ABSTRACT

We hypothesized that bone marrow-derived mononuclear cells (BMDMC) would attenuate the remodeling process in a chronic allergic inflammation model. C57BL/6 mice were assigned to two groups. In OVA, mice were sensitized and repeatedly challenged with ovalbumin. Control mice (C) received saline under the same protocol. C and OVA were further randomized to receive BMDMC (2×10^6) or saline intravenously 24 h before the first challenge. BMDMC therapy reduced eosinophil infiltration, smooth muscle-specific actin expression, subepithelial fibrosis, and myocyte hypertrophy and hyperplasia, thus causing a decrease in airway hyperresponsiveness and lung mechanical parameters. BMDMC from green fluorescent protein (GFP)-transgenic mice transplanted into GFP-negative mice yielded lower engraftment in OVA. BMDMC increased insulin-like growth factor expression, but reduced interleukin-5, transforming growth factor- β , platelet-derived growth factor, and vascular endothelial growth factor mRNA expression. In conclusion, in the present chronic allergic inflammation model, BMDMC therapy was an effective pre-treatment protocol that potentiated airway epithelial cell repair and prevented inflammatory and remodeling processes.

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1. Introduction

Recently, new attention has been directed to the long-term changes in asthmatic airways as indicated by the accelerated rate of lung function decline despite therapy with inhaled corticosteroids. These structural changes in the airway wall, termed airway remodeling, are now thought to be a key component of the pathophysiology of asthma. Airway remodeling is characterized by: subepithelial fibrosis, mucous metaplasia, wall thickening, smooth muscle cell hypertrophy and hyperplasia, myofibroblast hyperplasia, vascular proliferation, and changes in the extracellular matrix, such as deposition of collagen fiber and elastic fiber fragmentation (Holgate et al., 2004; Xisto et al., 2005). Therefore, a therapy that hastens the repair process and attenuates both inflammatory and remodeling responses is thought to be important to improve asthma management.

Bone marrow-derived stem cells are potent modulators of immune responses, promoting the proliferation and re-epithelialization of lung cells. Several studies have shown beneficial effects of cell-based therapy in respiratory diseases (Rojas et al., 2005; Gupta et al., 2007; Zhao et al., 2008; Zhen et al., 2008) resulting from cell plasticity (Krause, 2002; Herzog et al., 2003) and/or the release of paracrine factors (Rojas et al., 2005; Ortiz et al., 2007). So far, however, no study has analyzed the effects of bone marrow-derived stem cells in experimental allergic asthma.

We tested the hypothesis that pre-treatment with bone marrow-derived mononuclear cells (BMDMC) may prevent inflammatory and remodeling processes while improving lung function in a murine model of chronic allergic inflammation. For this purpose, lung mechanics and histology, elastic and collagen fiber content in airways and alveolar septa, the amount of smooth muscle-specific actin present in distal airways and alveolar duct walls, and the expression of inflammatory cytokines and growth factors were analyzed.

2. Material and methods

This study was approved by the Ethics Committee of the Health Sciences Centre, Federal University of Rio de Janeiro. All animals

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received humane care in compliance with the “Principles of Laboratory Animal Care” formulated by the National Society for Medical Research and the “Guide for the Care and Use of Laboratory Animals” prepared by the National Academy of Sciences, USA.

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

Bone marrow cells were extracted from male C57BL/6 (20–25 g, $n=13$) mice and administered on the day of collection. BMDMC from GFP+ male mice (20–22 g, $n=3$) were administered to 16 C57BL/6 female mice to evaluate the degree of pulmonary GFP+ cell engraftment. Briefly, under anesthesia with ketamine (25 mg/kg) and xylazine (2 mg/kg) iv, 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 supplemented with phosphate-buffered saline (PBS). Cells were counted in a Neubauer chamber with Trypan Blue for evaluation of viability. For the administration of saline or BMDMC, mice were anesthetized with sevoflurane, the jugular vein of each mouse was dissected, and cells were slowly injected. A small aliquot of the mononuclear cells was used for immunophenotypic characterization of the injected cell population. Cell characterization was performed by flow cytometry using antibodies CD45 (leukocyte), CD34 (hematopoietic precursors), CD3, CD8, and CD4 (T lymphocyte), CD14 (monocytes and macrophages), CD11b, CD29 and CD45- (non-hematopoietic precursors), all from BD Biosciences, USA.

2.2. Animal preparation and experimental protocol

Eighty female C57BL/6 mice (20–25 g) were used. Lung mechanics and histology as well as molecular biology were analyzed in 24 female C57BL/6 mice ($n=6$ /group). Of the remaining 56 female C57BL/6 mice, 20 ($n=5$ /group) were used to compute airway hyper-

responsiveness, 20 to evaluate total and differential cell count in bronchoalveolar lavage fluid (BALF, $n=5$ /group) and 16 to evaluate the degree of pulmonary GFP+ cell engraftment ($n=4$ /group). All females 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 of OVA in 100 μ l) on 7 alternate days. Forty days after the beginning of sensitization, 20 μ g of OVA in 20 μ l saline was intratracheally instilled. 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. C and OVA groups were further randomized to receive saline solution (0.9% NaCl, 50 μ l, SAL) or BMDMC (2×10^6 in 50 μ l, CELL) administration through the left jugular vein 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), anesthetized (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) with the following parameters: frequency of 100 breaths/min, tidal volume (V_T) of 0.2 ml, and fraction of inspired oxygen of 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 was 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 fast drop 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 the airway resistance. ΔP_2 reproduces the pressure spent by stress relaxation, or viscoelastic properties of the lung, together with a small contribution of *pendelluft*. Static lung elastance (E_{st}) was determined by dividing P_{el} by V_T . Lung mechanics measurements

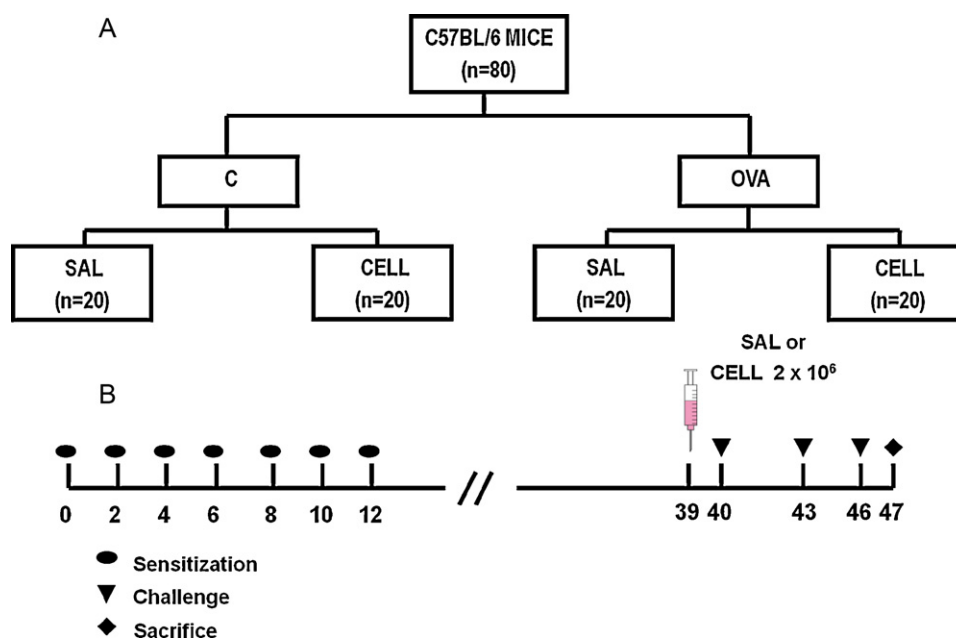


Fig. 1. Schematic flow chart (A) and timeline (B) of the study design. C: mice sensitized and challenged with saline; OVA: mice sensitized and challenged with ovalbumin; SAL: mice treated with intravenous injection of saline; CELL: mice treated with BMDMC (2×10^6) 24 h before the first challenge. All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

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

2.4. Airway responsiveness

Twenty-four hours after the last challenge, airway responsiveness was measured. One dose of saline (Baseline) was administered followed by increasing doses of methacholine (Sigma Chemical Co., Saint Louis, MI, USA) (3, 10, 30, and 100 µg/kg) via a syringe catheter tied into the jugular vein. Data were stored at 30 s, 1, 3, and 5 min after methacholine injection. Airflow and *Ptr* were measured after achieving the maximal increase in *Ptr* that followed each intravenous infusion of methacholine (Xisto et al., 2005). Respiratory system resistance was obtained using the equation of motion of respiratory system: $Ptr(t) = E \cdot V(t) + R \cdot \dot{V}(t)$, where t is time. Resistance was expressed as a percentage of baseline measurements.

2.5. Lung histology

A laparotomy was done immediately after the determination of lung mechanics and heparin (1000 IU) was intravenously injected in the vena cava. The trachea was clamped at end-expiration, and the abdominal aorta and vena cava were sectioned, yielding a massive hemorrhage that quickly killed the animals. Right lung was then removed, fixed in 3% buffered formaldehyde and paraffin embedded. Four-µm-thick slices were cut and stained with hematoxylin–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). Volume fraction of collapsed and normal pulmonary areas, the magnitude of bronchoconstriction, and the number of mononuclear and polymorphonuclear cells in pulmonary 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 fibers (Weigert's resorcin fuchsin method with oxidation) were quantified in airways and alveolar septa (Xisto et al., 2005; Antunes et al., 2009, 2010).

2.6. Transmission electron microscopy

Three 2 mm × 2 mm × 2 mm slices were cut from three different segments of the left lung and fixed [2.5% glutaraldehyde and phosphate buffer 0.1 M (pH 7.4)] for electron microscopy (JEOL 1010 Transmission Electron Microscope, Tokyo, Japan) analysis. For each electron microscopy image (20/animal), the following structural changes were analyzed: a) epithelial detachment, b) airway edema, c) eosinophil infiltration, d) neutrophil infiltration, e) degenerative changes in ciliated airway epithelial cells, f) subepithelial fibrosis, g) elastic fiber fragmentation, h) smooth muscle hypertrophy, i) myofibroblast hyperplasia, j) mucous cell hyperplasia, and l) multinucleated cells (Antunes et al., 2010). The pathologic findings were graded according to a 5-point semi-quantitative severity-based scoring system as: 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.

2.7. Confocal microscopy

Fluorescent images of 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, 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 by using NIH Image software and contrast and color levels were adjusted with Adobe Photoshop 7.0. The number of GFP+ cells per tissue area was determined by the point-counting technique (Weibel, 1990; Araújo et al., 2010) across 10 random, non-coincident microscopic fields.

2.8. Evaluation of BALF

Lungs were lavaged via a tracheal tube with PBS solution (1 ml) containing EDTA (10 mM). Total leukocyte numbers were measured in Neubauer chambers under light microscopy after diluting the samples in Türk solution (2% acetic acid). Differential cell counts were performed in cytospin smears stained by the May–Grünwald–Giemsa method.

2.9. Expression of growth factors and IL-5

Quantitative real-time reverse transcription (RT) polymerase chain reaction (PCR) was performed to measure the expression of interleukin (IL)-5, transforming growth factor (TGF)-β, platelet derived growth factor (PDGF), vascular endothelial growth factor (VEGF) and insulin-like growth factor (IGF) genes. Central slices of left lungs were cut, collected in cryotubes, quick-frozen by immersion in liquid nitrogen, and stored at −80 °C. Total RNA was extracted from the frozen tissues, using the Trizol reagent (Invitrogen, Carlsbad, CA) according to the manufacturer's recommendations. RNA concentration was measured by spectrophotometry in Nanodrop® ND-1000. First-strand cDNA was synthesized from total RNA using M-MLV Reverse Transcriptase Kit (Invitrogen, Carlsbad, CA). PCR primers for target gene were purchased (Invitrogen, Carlsbad, CA). The following primers were used: IL-5 [5'-AAC TGT CCG TGG GGG TAC T-3' (forward) and 5'-CTC GTC CTC CGT CTC TCC T-3' (reverse)], VEGF [5'-CCA CGA CAG AAG GAG AGC A-3' (forward) and 5'-AAT CGG ACG GCA GTA GCT T-3' (reverse)], TGF-β [5'-ATA CGC CTG AGT GGC TGT C-3' (forward) and 5'-GCC CTG TAT TCC GTC TCCT-3' (reverse)], PDGF [5'-TCG AAG TCA GAT CCA CAG CA-3' (forward) and 5'-CTT GTC TCC AAG GCA TCC TC-3' (reverse)], IGF [5'-CTG GTG GAT GCT CTT CAG TTC-3' (forward) and 5'-CCA GTC TCC TCA GAT CAC AGC-3' (reverse)]. Relative mRNA levels were measured with a SYBR green detection system using ABI 7500 Real-Time PCR (Applied Biosystems, Foster City, CA). All samples were measured in triplicate. The relative expression of each gene was calculated as a ratio compared with the reference gene, glyceraldehyde-3-phosphate dehydrogenase (GAPDH) [5'-AAC TTT GGC ATT GTG GAA GG-3' (forward) and 5'-GTC TTC TGG GTG GCA GTG AT-3' (reverse)] and expressed as fold change relative to C-SAL.

2.10. Statistical analysis

Normality of data was tested using the Kolmogorov–Smirnov test with Lilliefors' correction, while the Levene median test was used to evaluate the homogeneity of variances. If both condi-

tions were satisfied, two-way ANOVA followed by Tukey's test when required was used to compare differences among the groups. Nonparametric data were analyzed using two-way ANOVA on ranks followed by Tukey's test. Parametric data were expressed as means $\pm$ SEM, while non-parametric data were expressed as medians (interquartile range). All tests were performed using the SigmaStat 3.1 statistical software package (Jandel Corporation, San Raphael, CA, USA), and statistical significance was established as $p < 0.05$.

3. Results

The pool of intravenously injected BMDMC was characterized by flow cytometry showing the following subpopulations: total lymphocyte (lower SSC/ CD45+/CD11b–/ CD29–/ CD34– = 29.7%), T lymphocyte (lower SSC/ CD45+/ CD3+/ CD34– = 5.4%), T helper lymphocyte (CD3+/ CD4+/ CD8– = 2.4%), T cytotoxic lymphocyte (CD3+/CD4–/ CD8+ = 2.3%), monocytes (CD45+/ CD29+/ CD11b+ low/ CD34–/CD3– = 4.9%), neutrophils (CD45+/ CD11b+/ CD34–/ CD29–/ CD14–/ CD34–/ CD3–), hematopoietic progenitors (CD34+/CD45+ = 0.3%), and other progenitors cells (CD45– = 3.8%) (data presented in Fig. S.1 in the Supplementary Material).

Est (33%), $\Delta P1$ (15%), and $\Delta P2$ (49%) were higher in the OVA-SAL than in the C-SAL group. BMDMC significantly inhibited Est, $\Delta P1$, and $\Delta P2$ alterations (Fig. 2A, B). Furthermore, the increase in airway resistance evoked by methacholine was significantly augmented the OVA-SAL group; however, these changes were minimized in OVA animals receiving BMDMC (Fig. 2C).

In the OVA-SAL group, morphometric examination of lungs demonstrated a significant increase in the fraction area of alveolar collapse, the number of mononuclear and polymorphonuclear cells, collagen fiber content in airways and alveolar septa and in the volume proportion of smooth muscle-specific actin in terminal bronchioles and alveolar ducts (Table 1). In Fig. 3, a reduction in the central airway diameter and inflammatory cell infiltration can be observed. BMDMC therapy minimized the fraction area of alveolar collapse and polymorphonuclear cells, and the volume proportion of smooth muscle-specific actin in alveolar ducts, but prevented changes in airway contraction, amount of collagen fiber in airway and alveolar septa, and in volume proportion of smooth muscle specific actin in terminal bronchiole. Elastic fiber content in airway and alveolar septa was similar in all groups (Table 1). Additionally, central airway diameter was observed to be less constricted and presented a reduction in inflammation in OVA-CELL group (Fig. 3).

Fig. 4 depicts typical electron microscopy findings of airway and lung parenchyma in each group. OVA-SAL animals showed airway epithelial cell detachment from the basement membrane, degenerative changes in ciliated airway epithelial cells, eosinophil and neutrophil infiltration, myofibroblast and mucous cell hyperplasia, subepithelial fibrosis, and smooth muscle hypertrophy (Table 2). BMDMC therapy attenuated all these ultrastructural changes and promoted the appearance of undifferentiated cells, without a defined phenotype, and multinucleated cells (Table 2).

Confocal microscopy showed basement membrane rupture associated with increased inflammatory process in the OVA-SAL group, which was prevented with the use of BMDMC (Fig. 5).

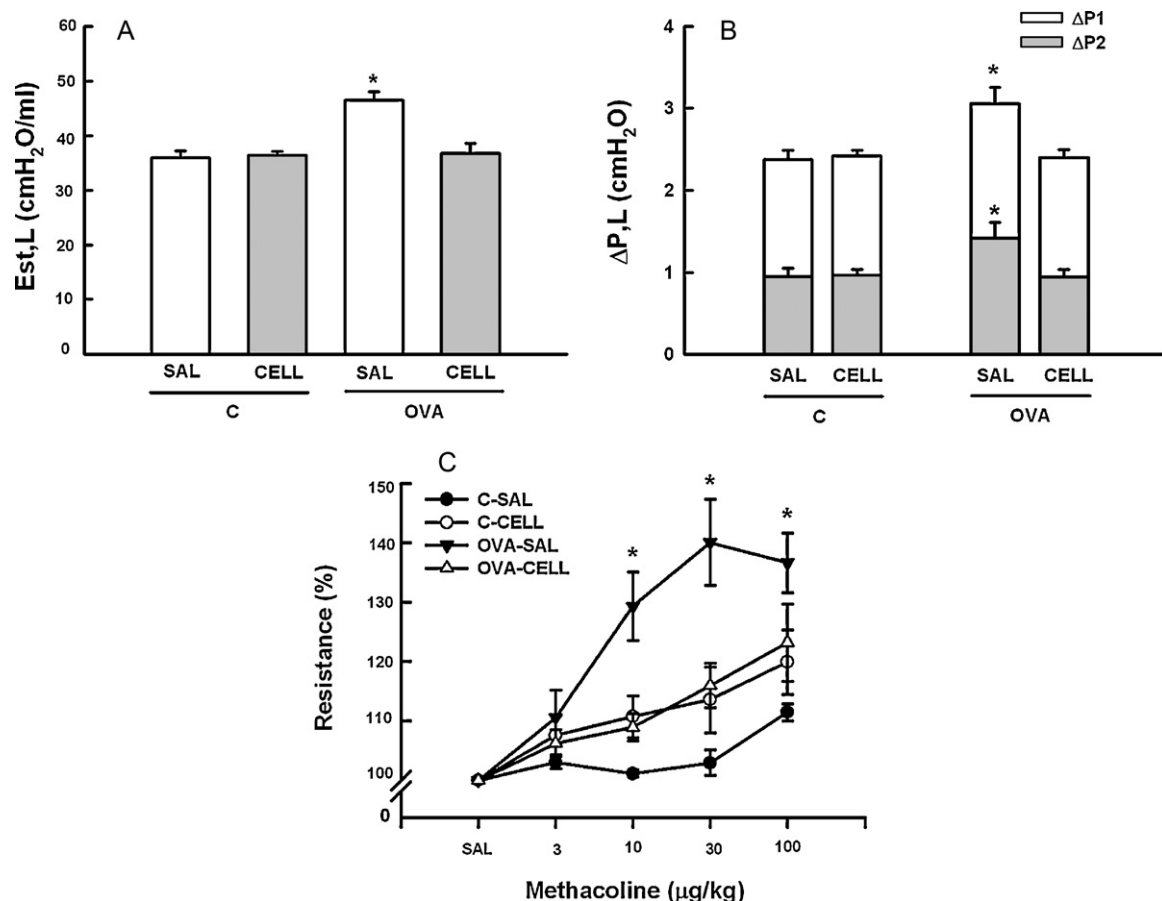


Fig. 2. Lung static elastance (Est) (Panel A); resistive ($\Delta P1$, gray bar), viscoelastic ($\Delta P2$, white bar) and total pressures ($\Delta P_{tot} = \Delta P1 + \Delta P2$) (Panel B); and airway hyperresponsiveness (Panel C). C: mice sensitized and challenged with saline; OVA: mice sensitized and challenged with ovalbumin; SAL: mice treated with intravenous injection of saline (white bars); CELL: mice treated with BMDMC (2×10^6) 24 h before the first challenge. *Significantly different from C-SAL ($p < 0.05$).

Table 1
Lung morphometry.

		C-SAL	C-CELL	OVA-SAL	OVA-CELL
Normal (%)		99.0 ± 0.72	98.0 ± 2.60	68.2 ± 11.1*	88.1 ± 5.91 *#
Collapse (%)		1.00 ± 0.72	1.80 ± 2.69	31.8 ± 11.1*	11.8 ± 5.91*#
MN (%)		33.13 ± 2.90	29.35 ± 3.20	51.2 ± 3.06*	29.03 ± 1.61
PMN (%)		0.30 ± 0.32	3.00 ± 1.06*	7.20 ± 0.96*	3.64 ± 1.63*#
Contraction index		2.28 ± 0.32	2.25 ± 0.32	3.02 ± 0.51*	2.39 ± 0.31
Collagen fiber (μm ² /μm)	Airways	1.04 ± 0.05	1.02 ± 0.05	3.30 ± 0.40*	1.06 ± 0.15
	Alveolar septa	0.014 ± 0.03	0.014 ± 0.03	0.070 ± 0.007*	0.011 ± 0.002
Elastic fiber (μm ² /μm)	Airways	1.47 ± 0.17	1.35 ± 0.28	1.40 ± 0.15	1.39 ± 0.20
	Alveolar septa	0.26 ± 0.04	0.25 ± 0.05	0.27 ± 0.02	0.26 ± 0.01
Actin (%)	Terminal bronchiole	3.11 ± 0.36	2.98 ± 0.36	6.70 ± 0.80*	4.08 ± 0.42
	Alveolar duct	0.23 ± 0.17	1.05 ± 0.19	2.08 ± 0.17*	1.87 ± 0.21*#

The fraction of area of normal and collapsed alveoli, mononuclear (MN) and polymorphonuclear (PMN) cells, contraction index, collagen and elastic fibers in airways and alveolar septa, volume proportion of smooth muscle-specific actin in terminal bronchiole and alveolar duct. All values were computed in ten random, non-coincident fields per mice. C: mice sensitized and challenged with saline; OVA: mice sensitized and challenged with ovalbumin; SAL: mice treated with intravenous injection of saline; CELL: mice treated with BMDMC (2×10^6) 24 h before the first challenge. *Significantly different from C-SAL ($p < 0.05$). #Significantly different from OVA-SAL ($p < 0.05$).

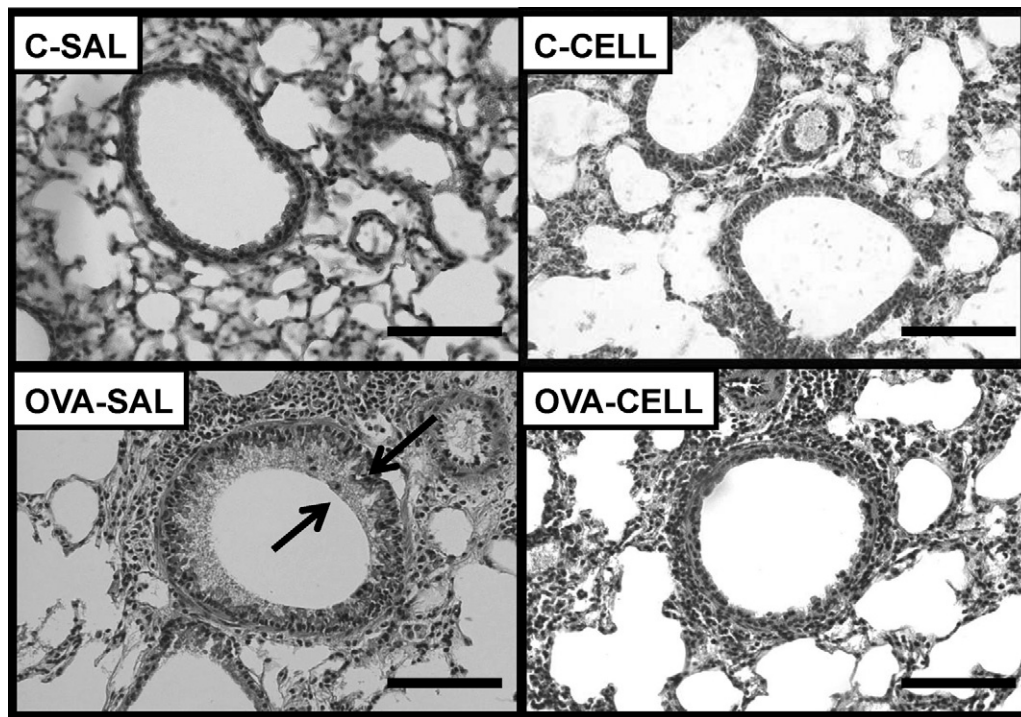


Fig. 3. Representative photomicrographs of airways stained with hematoxylin–eosin. Note the reduction in central airway diameter as well as increased cell infiltration (black arrows) in the OVA-SAL group. C: Mice sensitized and challenged with saline; OVA: mice sensitized and challenged with ovalbumin; SAL: mice treated with intravenous injection of saline; CELL: mice treated with BMDMC (2×10^6) 24 h before the first challenge. Original magnification: $\times 400$. Bars = 100 μm.

Table 2
Semi-quantitative analysis of electron microscopy.

	C-SAL	C-CELL	OVA-SAL	OVA-CELL
Epithelial detachment	0 (0–0)	0 (0–0)	2 (2–3)*	1 (0–1)*#
Airway edema	0 (0–0)	0 (0–0)	3 (2–4)*	1 (0–2)*#
Eosinophil infiltration	0 (0–0)	0 (0–0)	3 (2–4)*	1 (1–2)*#
Neutrophil infiltration	0 (0–0)	1 (0–1)*	2 (1–3)*	1 (0–2)*#
Degeneration of ciliated airway epithelial cells	0 (0–0)	0 (0–0)	3 (3–4)*	1 (1–2)*#
Subepithelial fibrosis	0 (0–0)	0 (0–0)	3 (3–4)*	1 (1–2)*#
Elastic fiber fragmentation	0 (0–0)	0 (0–0)	2 (2–3)*	0 (0–1)
Smooth muscle hypertrophy	0 (0–0)	0 (0–0)	3 (2–4)*	1 (1–2)*#
Myofibroblast hyperplasia	0 (0–0)	0 (0–0)	3 (3–4)*	1 (1–2)*#
Mucous cells hyperplasia	0 (0–0)	0 (0–0)	3 (2–4)*	1 (0–2)*#
Multinucleated cells	0 (0–0)	1 (0–1)*	0 (0–0)	2 (1–3)*§

Pathologic findings were graded according to a 5-point semi-quantitative severity-based scoring system: 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 intravenous injection of saline; CELL: mice treated with BMDMC (2×10^6) 24 h before the first challenge. *Significantly different from C-SAL ($p < 0.05$). §Significantly different from C-CELL ($p < 0.05$). #Significantly different from OVA-SAL ($p < 0.05$).

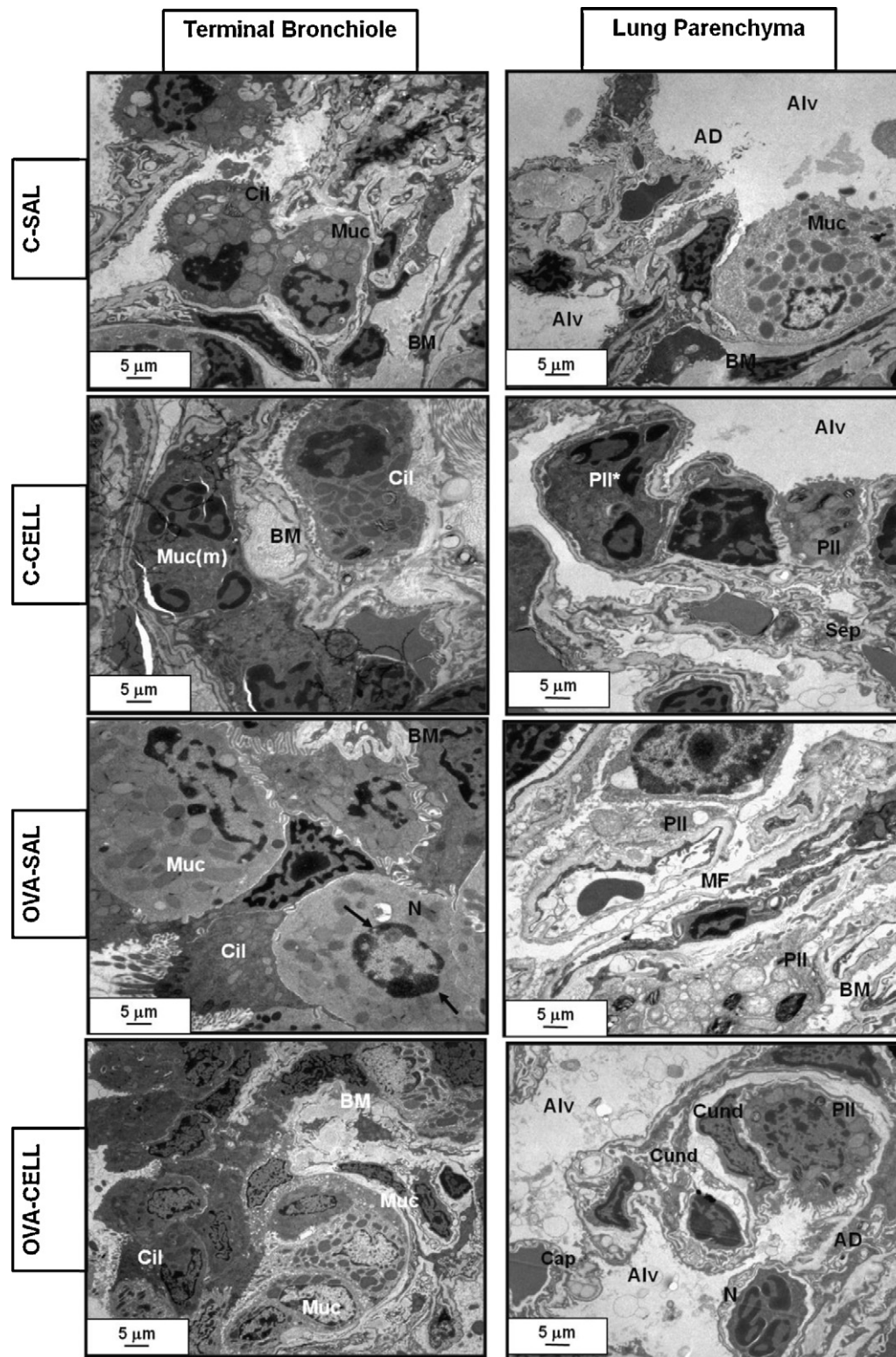


Fig. 4. Electron microscopy of terminal bronchiole (left panels) and lung parenchyma (right panels). C: mice sensitized and challenged with saline; OVA: mice sensitized and challenged with ovalbumin; SAL: saline; CELL: bone marrow-derived mononuclear cell therapy. Basal membrane (BM), mucous cells (Muc), multinucleated mucous cells [Muc(m)] ciliated cells (Cil), alveolar ducts (AD), alveolar lumen (Alv), undifferentiated cells (Cund), alveolar septa (Sep), pneumocytes type II (PII), multinucleated pneumocytes type II (PII*), capillary (Cap), apoptosis (arrows), MF (myofibroblast), neutrophils (N).

A very small number of GFP+ cells was detected in the OVA-CELL group (<1%), vs. none in C-CELL (Fig. 6). The number of total cells and eosinophils in BALF was higher in OVA-SAL (Fig. 7A, B). BMDMC yielded an increase in the number of total cells in the C-CELL group resulting from an increased monocyte count (Fig. 7A, C).

In the OVA-CELL group, the number of total cells and eosinophils was lower than in OVA-SAL.

IL-5, TGF- β , PDGF, VEGF, and IGF mRNA expressions in lung tissue were higher in OVA-SAL (Fig. 8A–E). BMDMC administration promoted an increase in IGF mRNA expression in the C-CELL and

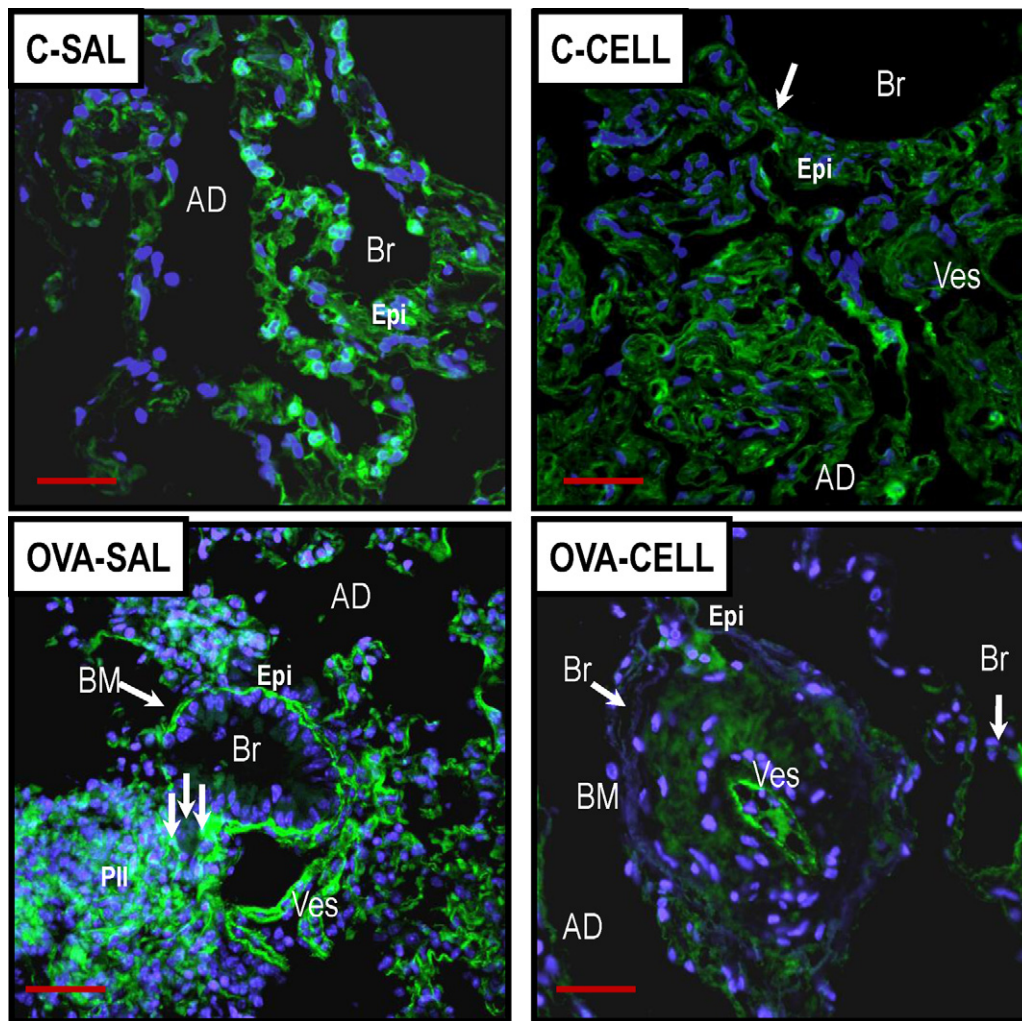


Fig. 5. Representative photomicrographs of airways and lung parenchyma using confocal microscopy. Note, in green, rupture of basement membrane (arrows) in the OVA-SAL group. Basement membrane (BM), epithelial cells (Epi), terminal bronchiole (Br), alveolar duct (AD), vessel (Ves), and type II pneumocyte (PII). C: Mice sensitized and challenged with saline; OVA: mice sensitized and challenged with ovalbumin; SAL: mice treated with intravenous injection of saline; CELL: mice treated with BMDMC (2×10^6) 24 h before the first challenge. Original magnification: $\times 400$. Bars = 100 μm . (For interpretation of the references to color in this figure legend, the reader is referred to the web version of the article.)

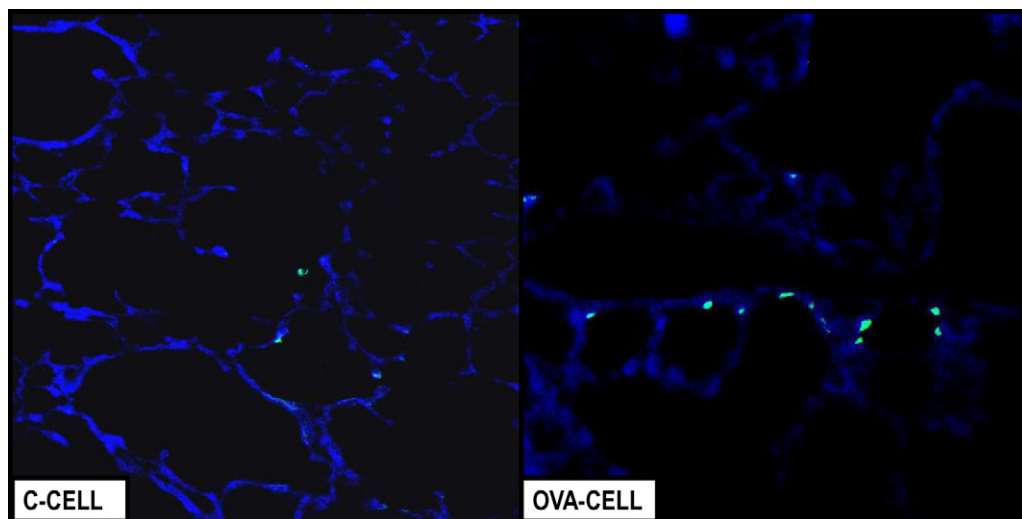


Fig. 6. Confocal microscopy of lung parenchyma in C and OVA C57BL/6 female mice treated from BMDMC from GFP male mice. C: mice sensitized and challenged with saline; OVA: mice sensitized and challenged with ovalbumin; CELL: mice treated with BMDMC (2×10^6) 24 h before the first challenge. Note that few GFP+ cells (green) were present in the lung parenchyma. Original magnification: $\times 200$. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of the article.)

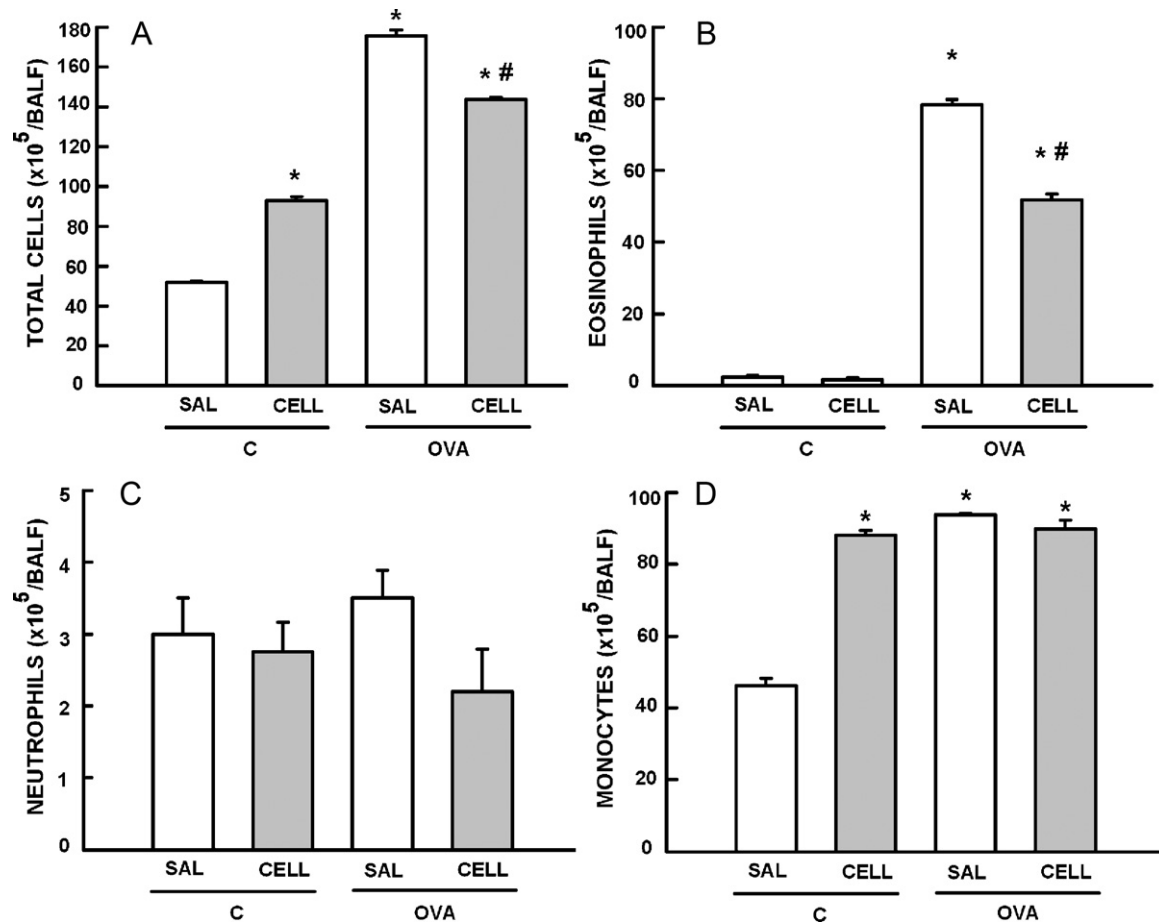


Fig. 7. Number of total cells (Panel A), eosinophils (Panel B), neutrophils (Panel C), and monocytes (Panel D) in bronchoalveolar lavage fluid. C: mice sensitized and challenged with saline; OVA: mice sensitized and challenged with ovalbumin; SAL: mice treated with intravenous injection of saline; CELL: mice treated with BMDMC (2×10^6) 24 h before the first challenge. *Significantly different from C-SAL ($p < 0.05$). #Significantly different from OVA-SAL ($p < 0.05$).

OVA-CELL groups (Fig. 8E), as well as a reduction in IL-5, TGF- β , PDGF, and VEGF expressions in OVA-CELL (Fig. 8A–D).

4. Discussion

In the present murine model of chronic allergic inflammation, early intravenous BMDMC therapy led to: 1) a significant reduction in lung static elastance, resistive and viscoelastic pressures, airway hyperresponsiveness, fraction area of alveolar collapse, bronchoconstriction index, number of cells in lung tissue and BALF, collagen fiber content, and volume proportion of smooth muscle-specific actin; and 2) repair of airway epithelium, reducing detachment of basement membrane, subepithelial fibrosis and myofibroblast hypertrophy and hyperplasia. These results were achieved despite a small degree of pulmonary engraftment, suggesting a prominent role of paracrine effects.

We developed a model of chronic allergic inflammation in C57BL/6 mice. Although this model has been described in BALB/c mice (Xisto et al., 2005; Burburan et al., 2007; Antunes et al., 2009, 2010), C57BL/6 mice were used since they serve as a background strain for GFP mice. Indeed, C57BL/6 remodeled differently from BALB/c mice considering the same experimental chronic allergic inflammation model (Antunes et al., 2009); however, the ultra-structural changes of airway and lung parenchyma, although less intense, were similar to those observed in BALB/c mice.

Previous experimental studies have investigated the restorative capacity of bone marrow mesenchymal stem cells (MSC) in respira-

tory diseases (Rojas et al., 2005; Gupta et al., 2007; Ortiz et al., 2007; Zhao et al., 2008; Zhen et al., 2008). However, MSC present disadvantages, such as culture conditions, which are detrimental for cell transplantation, and risk of contamination and immunologic reactions. Based on these limitations, we chose BMDMC, which we can be easily and safely administered on the day of harvesting, express several genes involved in inflammatory response and chemotaxis (Ohnishi et al., 2007), and present lower cost compared to MSC. Therefore, BMDMC therapy is applicable in clinical settings. To our knowledge, this is the first study investigating the effects of BMDMC therapy on lung function and the remodeling process in experimental chronic allergic inflammation. BMDMC were administered 24 h before the first ovalbumin challenge protocol, since the inflammatory process, characterized in the present model by an increase in eosinophils in lung tissue, is essential for cell recruitment to the injured area (Herzog et al., 2006). The possibility of antigen mismatch was considered taking into account the administration of male BMDMC in females. However, pilot studies demonstrated that similar results were obtained independently of the sex of BMDMC donors. GFP-positive cells were used to identify homing of bone marrow cells in lung parenchyma. A very small number was observed in the OVA-CELL group, but not in C-CELL lungs, suggesting that tissue injury is necessary to attract and retain these cells.

Several studies have shown that the airway epithelium of asthmatic patients has lower regenerative ability since these patients have a small number of circulating progenitor cells (Holgate, 2008). Therefore, the administration of BMDMC may potentiate airway

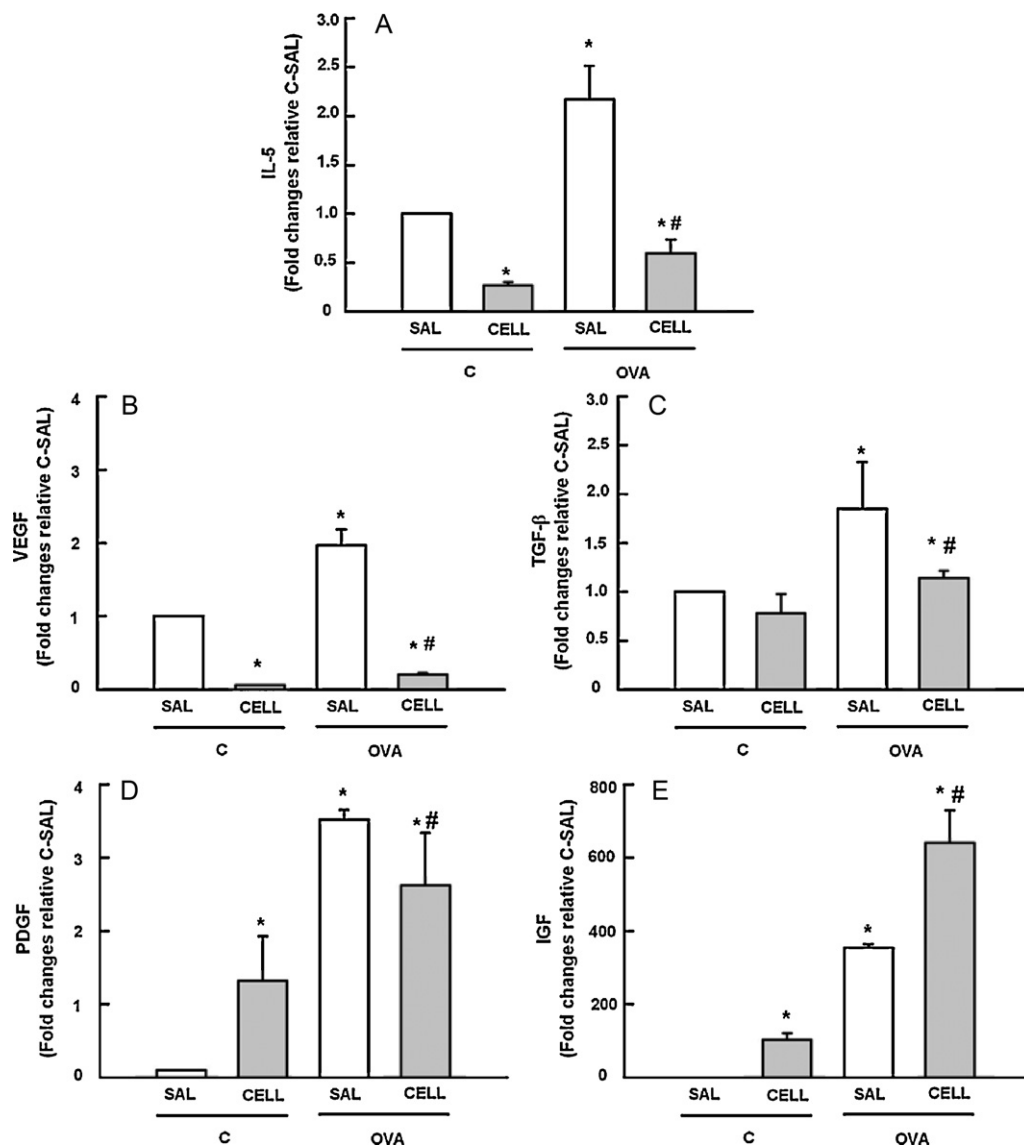


Fig. 8. Real-time polymerase chain reaction analysis of IL-5: interleukin-5 (Panel A), VEGF: vascular endothelial growth factor (Panel B), TGF-β: transforming growth factor-beta (Panel C), PDGF: platelet derived growth factor (Panel D), and IGF: insulin growth factor (Panel E) mRNA expressions of mouse lung tissue. C: mice sensitized and challenged with saline; OVA: mice sensitized and challenged with ovalbumin; SAL: mice treated with intravenous injection of saline; CELL: mice treated with BMDMC (2×10^6) 24 h before the first challenge. *Significantly different from C-SAL ($p < 0.05$). #Significantly different from OVA-SAL ($p < 0.05$).

epithelial cell repair as well as reduce remodeling and inflammatory processes through paracrine effects. Ultrastructural analysis using electron and confocal microscopy showed repair of ciliated airway epithelial cells associated with a reduction in subepithelial fibrosis and smooth muscle cell hypertrophy and hyperplasia. We also observed the presence of many multinucleated and undifferentiated cells in lung parenchyma, suggesting that these cells can be an important source for the repair of damaged area. These multinucleated cells could be macrophages that fused with BMDMC or with injured epithelial cells (Krause, 2008). Additionally, it has been described that macrophages behave *in vitro* as stem cell attractors. Once at the site of injury, the ability of precursor cells to reconstitute the damaged tissue depends on the signals generated in situ by the macrophages (Lolmede et al., 2009). However, in accordance with previous reports in other lung diseases, the beneficial effects of cell therapy in the present study seem to have been mainly related to paracrine effects modulating immune responses through the release of cytokines and growth factors (Krause et al., 2001; Ortiz et al., 2003; Abe et al., 2004; Yamada et al., 2004; Araújo et al.,

2010). Even though BMDMC therapy led to an increased number of total cells in the BALF of controls, which may be attributed to the percentage of donor hematopoietic cells in BMDMC (Ratajczak et al., 2004), it significantly reduced the number of total cells and eosinophils in OVA-CELL. We found that BMDMC therapy caused a reduction in the expression of IL-5, which plays an important role in the remodeling process of asthma. Therefore, this reduction can be associated with the decrease in the number of eosinophils in the BALF and in TGF-β expression. Consequently, there was a reduction in myofibroblast activation (Tanaka et al., 2004), diminishing collagen fiber content and volume proportion of smooth muscle-specific actin. Additionally, the decrease in PDGF and VEGF expressions after cell therapy contributed to a reduction in subepithelial fibrosis and smooth muscle layer thickening, as these growth factors are involved in fibroblast, muscle, and endothelial cell activation and proliferation (Klagsbrun and D'Amore, 1991; Saunders et al., 2009). In this line, a recent study has reported that VEGF receptor inhibition led to a significant reduction in the inflammatory and remodeling processes in experimental asthma. Conversely,

BMDMC yielded an increase in IGF expression, which may be associated with the effect of these cells on the re-epithelization process (Yamashita et al., 2005) previously observed in experimental models of idiopathic fibrosis. Thus, the paracrine effects of BMDMC acted on airway and lung parenchyma remodeling resulting in an improvement in lung mechanics and reducing airway responsiveness.

Our study has some limitations: 1) BMDMC were injected 24 h before the first ovalbumin challenge, before the remodeling process was established. Thus, more studies should be performed to analyze BMDMC effects after the last challenge; 2) despite the widespread speculation that MSC constitute a unique cell type distinct from fibroblasts (Horwitz et al., 2007), some investigators have used fibroblast therapy as control (Mei et al., 2007; Xu et al., 2007). Since the use of fibroblasts did not result in beneficial effects (Sun et al., 2009), in the present study we administered saline as control instead of fibroblasts; 3) 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; and 4) even though the amount of GFP was quantified in lung tissue, we did not analyze whether these engrafted cells are transdifferentiated into any type of lung cell.

In conclusion, in the present murine model of chronic allergic inflammation, bone marrow-derived mononuclear cell therapy was an effective pre-treatment protocol, potentiating airway epithelial cell repair and reducing inflammatory and remodeling processes. These results were achieved despite a small degree of pulmonary engraftment, suggesting a role of paracrine effects. Additional experiments are necessary to analyze the effects of BMDMC after the remodeling process has been established.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at [doi:10.1016/j.resp.2010.10.006](https://doi.org/10.1016/j.resp.2010.10.006).

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**Effects of bone marrow-derived mononuclear cells on airway and lung
parenchyma remodeling in a murine model of chronic allergic
inflammation**

Supplementary material

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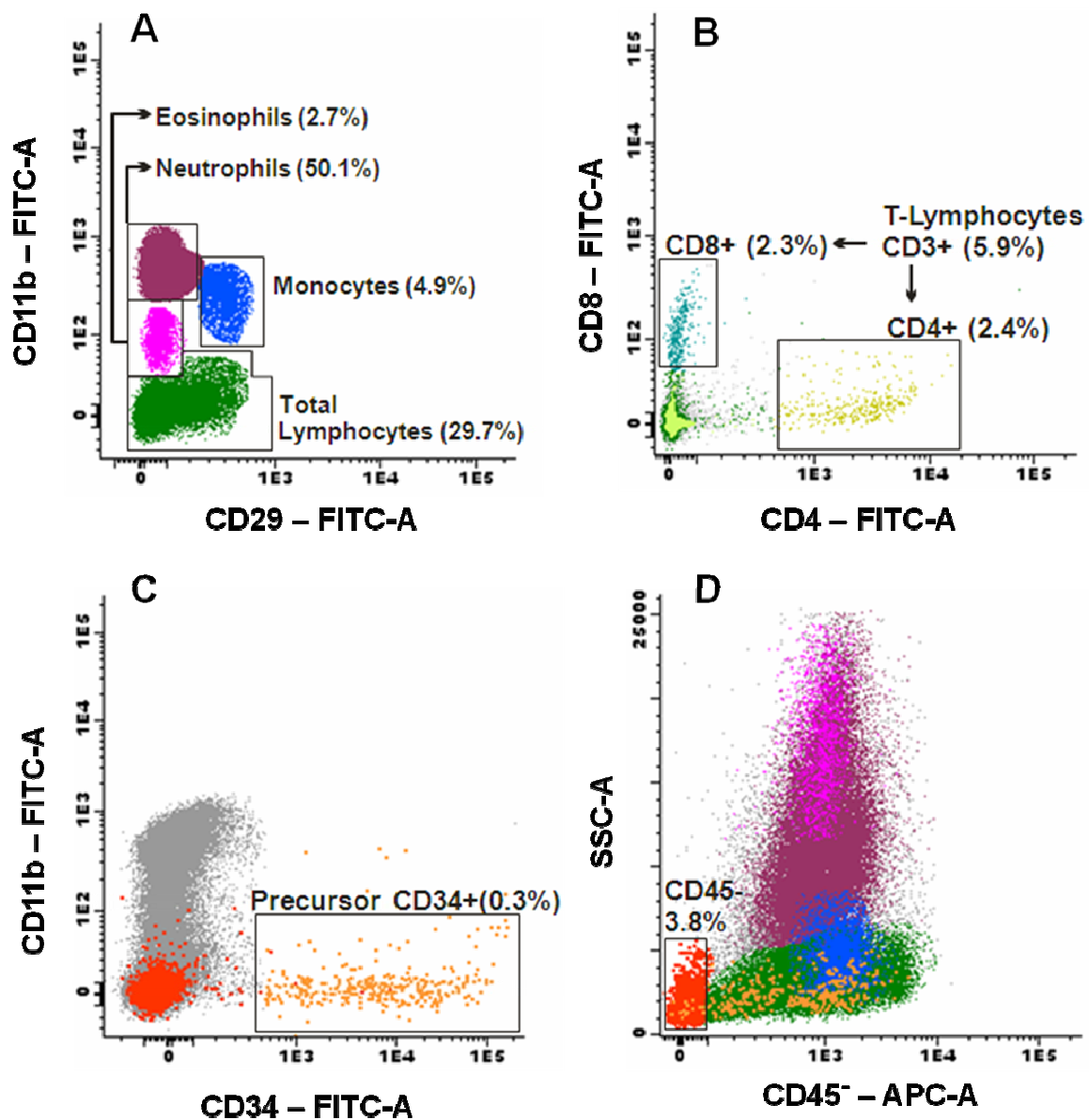


Fig. S.1: Flow cytometry to analyze the phenotype of intravenously injected BMDMC. Immunological cells [eosinophils – pink; neutrophils – purple; monocytes – blue; total lymphocytes – green (Panel A) and T lymphocytes – green (CD8⁺) and yellow (CD4⁺) (Panel B). Hematopoietic precursors – orange (Panel C). Non-hematopoietic precursors – red (Panel D)].