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Original article

Bone marrow mesenchymal stem cells and their conditioned media could potentially ameliorate ovalbumin-induced asthmatic changes



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ABSTRACT

Background: The major feature of asthma is governed by chronic airway inflammation. This investigation was proposed to achieve the suitable candidate for ameliorating long-term chronic asthmatic changes of respiratory tract.

Methods: 36 rats were classified into healthy (C) and ovalbumin (OVA)-sensitized animals (S). To sensitize, the rats were exposed to OVA over a course of 32 ± 1 days. One day after sensitization, equal six different groups were subjected to experimental procedure ($n=6$); Rats only received intratracheally 50 ml PBS (CPT and SPT groups), 50 μ l conditioned medium (CM) (CST and SST groups) and 50 μ l PBS containing 2×10^6 rat bone marrow-derived mesenchymal stem cells (rBMMSCs) (CCT and SCT groups). Two weeks after treatment, tracheal responsiveness, immunologic responses and recruitment of rBMMSCs into the lung as well as pathological changes were evaluated.

Results: A high degree of tracheal responsiveness, total white blood cell and percentages of eosinophil and neutrophil was significantly recorded in all sensitized groups rather than of controls ($p < 0.001$ to $p < 0.05$). Of interest, all above-mentioned parameters decreased significantly in SST and notably SCT groups as compared to S group ($p < 0.001$ to $p < 0.05$). The results revealed decrease number of blood $CD3^+CD4^+$ and concurrent increase in $CD3^+CD8^+$ in all sensitized rats as compared to control ($p < 0.001$ to $p < 0.05$). Noticeably, no significant modulatory effects of either cell or CM administration were achieved on the $CD3^+CD4^+$ and $CD3^+CD8^+$ populations in non-asthmatic rats. Moreover, the number of $CD3^+CD4^+$ in SST and SCT groups tended to increase, which coincided with a decreased manner of $CD3^+CD8^+$ populations as compared with S group ($p < 0.001$ to $p < 0.05$). However, the $CD3^+CD4^+$ cells in SCT rats were significantly higher than the group SST ($p < 0.01$) whereas $CD3^+CD8^+$ cells diminished simultaneously ($p < 0.001$). Real-time PCR analysis further showed that both CM and particularly MSCs changed the expression of interleukin (IL)-4 and IL-10 in the asthmatic groups to the near level of control rats ($p < 0.001$ to $p < 0.05$). Histopathological analysis revealed a profound reduction of lungs injuries in asthmatic rats when received CM and peculiarly mesenchymal stem cells ($p < 0.01$ to $p < 0.05$).

Conclusion: Our study shed light on the superior effects of rBMMSCs, rather than CM, in attenuating of chronic asthmatic changes in the rat model.

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1. Introduction

Asthma is conceived as one of the most common chronic respiratory worldwide disorders with a very high socioeconomic burden, characterized by reversible airflow obstruction,

hyper-responsiveness, aberrant tissue remodeling, and mainly airway inflammation [1–5]. In accordance to a plethora of studies, many inflammatory cells originated from different lineages, such as lymphocytes, eosinophils, neutrophils, mast cells and macrophages insidiously enter to asthmatic niche and trigger a sequential stepwise of airway inflammation via secretion of various mediators causing recurrent episodes of wheezing, breathlessness, chest tightness, and coughing [6,7]. There is increasing interest in the role of lymphocytes during asthmatic

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changes, for example, pathological injuries are worsened with the number of bronchial CD3⁺CD8⁺ and CD3⁺CD4⁺ cells changed in the pulmonary tissue [8]. Based on facts, asthma is identical by the increased ratio of Th2/Th1. Activated Th2 cells, encoding interleukin (IL) –4, and IL –5 actively govern, eosinophilic infiltration and accumulation, which result in degradation of elastin fibers [9]. Th2 cytokines mainly, IL-4 and IL-13 initiated asthma pathophysiology by the interaction with different resident lung cells, such as fibroblasts, epithelial cells, smooth muscle cells. Although some data provided that CD8 positive could produce also Th2-like cytokines [10]. Otherwise, anti-inflammatory cytokines, such as IL-10, secreted by T regulatory cells, regulate asthmatic inflammation via downregulation of the Th2-driven inflammatory response [11]. Therefore, the modulation of pro- and anti-inflammatory cytokines was found to be essential for a suitable resolution of lung inflammation [12].

Asthma per se cannot be cured by any current drugs and convenient medications commonly used to alleviate or suppress related manifestations, bringing a lot of complications. Moreover, once the drug is discontinued, the disease symptoms are most likely to relapse [13–15]. Therefore, exploiting up-to-date promising approaches are essential not only to sedate asthma-related symptoms, but also to accelerate the regeneration rate of chronic changes [16–18]. Since consecutive events of airway inflammation happened during asthma, thereby the main solution for the treatment of this disorder is focused to control the intensity and course of afore-mentioned process [14,20]. In line with this statement, various potent Stem Cells, peculiarly mesenchymal stem cells (MSCs) are touted as a population of multi-potent cells, which can be pooled from all tissues constituting approximately in values less than 0.01% of total harvested cells [21]. Possessing an inherent plastic adherence, self-renewability and fibroblast-like appearance, they are easily trans-differentiate into a variety of mesenchymal lineages by means of controlling niche [22]. Owning immune-modulatory characteristics, it makes MSCs suitable to be used broadly for treatment of various inflammatory diseases such as asthma [23,24].

Since, the stem cell-secreted growth factors are responsible for some of the therapeutic effects of stem cells inside the body, it seems logical that the paracrine functions of MSCs driven by secretome could be monitored in the supernatant Conditioned Medium (CM) [25,26]. Limiting factors related to stem cell administration; notably, total costs and long-time of cellular expansion, immunologic reactions and tumorigenicity make paracrine soluble factors more suitable for the regeneration of target tissue [27]. Due to specific anatomical and histological characteristics, it makes the lungs good candidate as typified organs in which Bone marrow MSCs (BMMSCs) and CM can be delivered easily either by local or systemic administration [28].

During last decades, there is a controversy relating the mutual role of MSCs on the inflammation procedure [17,29]. For instance, Abreu et al. showed a superior effect by remarked reduction in inflammatory response in chronic asthmatic murine regardless of stem cell administration route [17]. Conversely, Trzil and colleagues already affirmed that systemic route of adipose-derived MSCs administration could not elicit immune-compromisation in the feline model of chronic allergic asthma [29]. Interestingly, in our previous study we showed that systemic administration of BMMSCs, but not CM reduced airway inflammation and pathological changes in OVA-challenged asthmatic changes in rat model [30].

To our knowledge, the most of previously published data investigated different mechanisms in the murine model of asthma. Also, there is no empirical research to decipher both juxtacrine and paracrine therapeutic effects of locally injected bone marrow-derived MSCs monoculture, CM, and MSCs thereof separately on immunologic response by illumination of CD4⁺ and CD8⁺ cells

percent in a rat model, resembling to asthma pathophysiology in human.

To attain the proper candidate for ameliorating long-term chronic asthmatic changes and to decipher underlying mechanisms of the inflammation in allergic asthma pathophysiology, we used both cellular and CM fractions intratracheally in ovalbumin sensitized rats.

2. Method and material

2.1. Animal ethics

All phases of our study were affirmed by the published guideline of The Care and Use of Laboratory Animals (NIH Publication No. 85-23, revised 1996) and confirmed by the Animal Care Committee of Tabriz University of Medical Sciences (No: TBZMED.REC.1394.386).

2.2. Cell isolation and expansion procedure

Rat bone marrow-derived mesenchymal stem cells (rBMMSCs) were isolated and expanded as described already by the different set of experiments [31]. Shortly, rats were sacrificed by the application of an overdose combination of xylazine and ketamine solution followed by complete excision of femurs. After cutting the epiphysis, medullary components immediately flushed out by PBS solution containing 2% fetal bovine serum (FBS; Gibco). Then, bone marrow mononuclear cells were harvested by Ficoll (Sigma) gradient centrifugation at 400g for 20 min. After centrifugation, mononuclear cells in the interphase between Ficoll and PBS were collected and washed twice by PBS. The mononuclear cells fraction was resuspended in Dulbecco's modified Eagle's medium low glucose (DMEM/LG; Gibco) containing 20% FBS, 100 U/ml penicillin and 100 mg/ml streptomycin (Biosera). Finally, initial density of 1×10^5 cells was seeded in each square centimeter of 6-well plates (SPL). The exhausted medium was further replaced every 4 days and passaged at 70–80% confluency. After passage one, cells were transferred to 75 cm² T flask (SPL). Cells of passage three were subjected to subsequent analyses.

2.3. Immunophenotyping characterization of isolated rBMMSCs

To further affirm the stemness characteristics in expanded cells, rBMMSCs at passage 3 were assessed by BD FACS Calibur™ flow cytometry system (FACS Calibur, BD) [32]. To characterize, a panel of antibodies directed against to stem cells, including FITC-conjugated anti-CD133, CD44 and CD34 as well as PE-conjugated anti-CD31 was performed (all from eBioscience). In addition, matched isotype control antibodies were also used to normalize background staining. Briefly, the cells were trypsinized by 0.025% Trypsin-EDTA solution, washed by PBS, blocked by 1% bovine serum albumin (Sigma) for 15 min. Thereafter, recommended antibody concentration for each above-mentioned marker added into 100 µl of PBS containing 5×10^5 cells, incubated for 30 min at RT and then washed by PBS. Cells were, at the end, subjected to flow cytometric system apparatus and taken output data were analyzed by Flow Jo software ver.7.6.1.

2.4. Vital staining of rBMMSCs via cell tracker

To monitor the recruitment and homing properties of rBMMSCs into lung parenchyma in sensitized rats, the cells were trypsinized and labeled with 2 µM Cell Tracker™ CM-Dil for 20 min at 37 °C (Catalog No. C-7000; Molecular Probes). A final volume of 50 µl PBS containing 2×10^6 CM-Dil pre-labeled cells per rat was gently administrated by intratracheal route one day after sensitization.

2.5. Conditioned media (CM) preparation

To examine the possible paracrine activity of rBMMSCs in the alleviation of ovalbumin-induced manifestations, rBMMSCs-derived CM was prepared. The supernatant of 70–80% confluent cells was discarded, washed three times with PBS and incubated with DMEM/LG devoid of FBS for 72 h [33]. Next, the supernatant was collected, centrifuged at 400g for 5 min, filtered by 0.20- μ m-pore syringe filter, and further concentrated approximately 50-fold by centrifuge filter tubes with molecular weight cut-offs at 4 kDa (Catalog No. 003099.125, Eppendroft). The protein content of concentrated CM was measured by using bicinchoninic acid assay (BCA assay, iNtRon, Korea). Our preliminary analysis revealed that total protein content of concentrated CM reached to 500 μ g/ml. An equal volume of CM, containing similar protein concentration was used in this study. 50 μ l/per case of concentrated CM was intratracheally injected one day after sensitization [17].

2.6. Protocol for animal sensitization

Forty mature male Wistar rats, weighing 200–250 g, were enlisted in the current study. The animals were kept in standard PVC cages under 12:12 light/dark cycle at $20 \pm 2^\circ\text{C}$ without limitation to provided rat chow and water. After adaptation, four rats were blindly selected for extraction and characterization of rBMMSCs prior to sensitization procedure. The rest of thirty six rats were randomly subclassified into two main groups; healthy (C) and sensitized (S) rats which further were divided into 3 subgroups. The non-sensitized and sensitized rats were designated as follows;

1. Rats only received 50 μ l PBS (CPT and SPT groups)
2. Rats received 50 μ l CM (CST and SST groups)
3. Rats received 50 μ l PBS containing 2×10^6 rBMMSCs (CCT and SCT groups)

In the sensitized groups, rats were exposed with ovalbumin (OVA) over a course of 32 ± 1 days according to previously published guidelines [34]. Shortly, rats were intraperitoneally administrated by 1 mg OVA (Sigma-Aldrich) and 200 mg aluminum hydroxide, which pre-dissolved in 1 ml saline on first and 8th days. By 14th day, the sensitized rats were entered to aerosolized condition of 4% ovalbumin creating by a nebulizer (CX3, Omron Co., Netherland), for 18 ± 1 consecutive days, lasting 5 min per day. The recent procedure was induced in a closed box with dimensions $30 \times 20 \times 20 \text{ cm}^3$ (Fig. 1). In healthy subjects, saline was administered instead of ovalbumin with the same protocol [35]. One-day post sensitization, the tracheas of sensitized rats were exposed by ventral neck dissection and cell suspension or relevant CM was injected slowly through a trans-tracheal puncture [36]. Notably, a great body of studies showed that the respiratory responses originated by ovalbumin induced sensitization last over period of two weeks [37,38]. The measurements done in this study were performed two weeks after treatment.

2.7. Tissue preparation for tracheal response assessment

Two weeks after administration, a combination of Ketamine (75 mg/kg) and Xylazine (3 mg/kg) were intraperitoneally injected to anesthetize rats [39]. Shortly, their trachea was slowly excised post-tracheostomy and chopped longitudinally into two equal pieces with size of 5 mm. Each slice was horizontally mounted between two stainless steel hook. The lower hook was attached to the base of the organ bath filled with 20 ml of Krebs solution and enriched by continuous 95% O_2 and 5% CO_2 gas mixture at 37°C (Schuler organ bath type 809, Germany) and the upper hook connected to an isometric transducer (MLT02020, AD Instruments, Spain) [40]. To achieve a steady spontaneous tone level, a passive tension of 1 g was also applied for at least 60 min prior to drug additions. During this period, the exhausted Krebs solution was regularly replenished every 15 min. Any changes in isometric force were calculated by a transducer coupled to a Power Lab (ML-750, 4 channel recorder, March-Hugstetten, Germany), amplified by an amplifier, and output data were interpreted [41].

2.8. The assessment of tracheal responsiveness to methacholine administration

In each assay, a dose-dependent response curve of methacholine induced contraction of the tracheal piece was drawn. In short, aggregative concentrations (10^{-7} to 10^{-3} mM) of methacholine were added consecutively to the bath chamber every 3 min and the elicited response by each additional dose were registered after the contraction of the tracheal smooth muscle had plateaued. To plot the curve, the percentage of contraction caused by each methacholine concentration in proportion to the maximum contraction acquired by its final concentration was charted against log concentration of methacholine. Thereafter, EC_{50} as the effective concentration of drug generating 50% of the peak response was indicated in each trial using methacholine response curves. The contractility response to 10 μ M methacholine was also described as the intensity of tracheal contraction [42].

2.9. Total and differential white cells count in blood

Before broncho-alveolar lavage in all rats, a volume of 5 ml blood samples was collected via the inferior vena cava were diluted 1 in 20 with Thoma solution and counted by using Neubauer's chamber. Then, the prepared smears were stained by Wright-Giemsa solution and the percentage of each leukocyte type calculated by using a light microscope [20].

2.10. The value of $\text{CD3}^+\text{CD4}^+$ and $\text{CD3}^+\text{CD8}^+$ populations in circulatory system

To characterize whether the administration of BMMSCs or CM by the intratracheal approach could alter the percentage of circulating CD4^+ or CD8^+ cells, samples were submitted to flow cytometry assay. A ratio of 1:1 of blood and PBS was prepared and overlaid on Ficoll (Sigma) solution, centrifuged at 400g for 30 min at 4°C . By three-step consequential PBS wash, the harvested

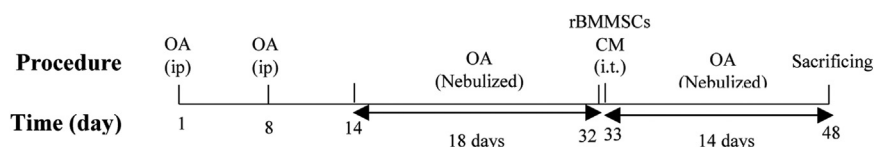


Fig. 1. Flowchart and timeline of the study design. OA; ovalbumin, ip; intraperitoneally.

mononuclear cells were characterized through a panel of antibodies against T lymphocyte sub-population, including FITC-conjugated mouse anti-rat CD3, PE-conjugated mouse anti-rat CD4 and CD8 (all from eBioscience).

Cells were incubated with recommended concentration of antibodies according to manufacturer's instruction. Ultimately, cells were immune-phenotyped by flow cytometric apparatus (FACS Calibur, BD) and thereby final output data were analyzed by Flow Jo software ver.7.6.1.

2.11. Collecting of lung lavage fluid (LLF) and total and differential leukocyte count

After taking the trachea, the lavage was gently initiated by using 1 ml of normal saline with 10 replicates via a catheter connected into the remaining part of a trachea. A part of collected fluid was stained with the equal volume of Turk solution (1:1 dilution) and then total leukocytes were calculated microscopically by hemocytometer slide. Turk solution consisted of 1 ml of Glacial Acetic Acid, 1 ml of Gentian Violet Solution 1% and 100 ml distilled water.

For differential leukocyte count, the rest of taken fluid samples were centrifuged at 2500g for 10 min at 4 °C. The supernatant was removed, a thin smear of cells was spread and stained with Wright-Giemsa stain solution. Finally, the rates of differential leukocytes were determined using a light microscope [43].

2.12. Tracking of migrated cell by immunofluorescence assay

To confirm the presence of injected cells into the lung parenchyma at the end stage of experimental procedure, pulmonary tissues were embedded in Tissue Freezing Media, snap-frozen and sectioned at 5 µm using with Cryostat apparatus [32]. Then, the sections were thawed at RT, washed twice with PBS (5 min each) and stained with 4', 6-diamidino-2-phenylindol (DAPI, 1 µg/ml, Catalog# D9542, Sigma-Aldrich) for nuclear counterstaining.

2.13. Detecting viability of cells after injection

The survival rate of rBMSCs after administration was determined. Briefly, we prepared samples from rats after injection and digested by 0.1% Collagenase solution (Sigma) for 30 min at 37 °C. The single cell suspensions were stained with 7-Amino-Actinomycin D (7AAD) for 20 min and the survival rate was determined by the percent of 7-AAD negative cells via flow cytometry analysis.

2.14. Total RNA isolation and real-time PCR assay

The left lung of each animal was quickly removed and frozen in liquid nitrogen. Total RNA was isolated from lung homogenate using YTA total RNA extraction mini kit (Cat No.YT9065, Taiwan). RNA concentration and purity were determined by a Nanodrop 1000 spectrophotometer (Thermo scientific, USA). Reverse transcription was performed by cDNA Synthesis Kit (Cat No.YT4500, Taiwan). Each cDNA was used as a template for separate assay for

mRNA quantitative real-time PCR by using SYBR Green master mix (Cat No.YT2551, Taiwan). The primer sequences are outlined in Table 1. Real-time PCR reactions were accomplished on a Rotor-Gene 6000 instrument (Corbett Life Science, Australia). House-keeping GAPDH (glyceraldehyde-3-phosphate dehydrogenase) gene was used to normalize the amount of PCR products for mRNA extracted samples. We used $2^{-\Delta\Delta CT}$ formula to ascertain relative-quantitative expression levels of IL-4 and IL-10 mRNAs. The results were expressed as fold change versus the relevant controls [44].

2.15. Pathological evaluation

The right lung of each rat was removed and then fixed in 10% buffer formalin solution (37%, Merck, Germany). Tissue specimens were processed through Passage method by increasing concentrations of ethanol (up to 100%). The processed specimens were then cleared by xylol solution, and embedded in paraffin. We prepared serial 4-µm thick sections by a microtome. All sections were carefully handled on glass slides, stained by hematoxylin-eosin (H&E) solution.

The pathological changes were identified under a light microscope in the lungs of all experimental groups in terms of hyperemia, leukocyte infiltration, epithelialization, emphysema and atelectasis varied greatly from none to scattered changes. We nominated grades according to the following manner; 0 = absence of pathologic changes; 1 = patchy changes; 2 = local changes; 3 = scattered changes.

2.16. Statistical analysis

All quantitative data were analyzed using one-way analysis of variance (ANOVA) with Tukey-Kramer post-hoc test and presented as mean ± SEM. The data of pathological changes were analyzed using Kruskal-Wallis test with the post-hoc Mann-Whitney test. We considered statistical significance at $p < 0.05$.

3. Results

3.1. Phenotypic verification of MSCs by flow cytometry

Immune-phenotypically assessment of rBMSCs after third passage confirmed the stemness character of isolated cells. Moreover, histograms showed cells positively stained with CD133 and CD44 (Fig. 2).

3.2. An improvement in tracheal responsiveness was identified in sensitized rats received rBMSCs and CM

Based on our analysis, the concentration-response curve to methacholine was shifted to leftward in all of the sensitized groups in comparison with the control group (CPT). Of interest, the plotted curve of SCT and SST groups showed rightward shift in comparison with the SPT group. A more rightward shift was evident in the curve of SCT group rather than SST group. Additionally, a similar

Table 1
The design of primers for mRNAs.

Gene name	Accession number	Primer sequence	Product length
IL-4	NM_201270.1	Forward: 5'-TAC GGC AAC AAG GAA CAC CAC-3' Reverse: 5'-CAG ACC GCT GAC ACC TCT AC-3'	157bp
IL-10	NM_012854.2	Forward: 5'-CAC TGC TAT GTT GCC TGC TC-3' Reverse: 5'-AAC CCA AGT AAC CCT TAA AGT CC-3'	225bp
GAPDH	NM_017008.4	Forward: 5'-CAA GTT CAA CGG CAC AGT CAA G-3' Reverse: 5'-ATA CTC AGC ACC AGC ATC ACC -3'	121bp

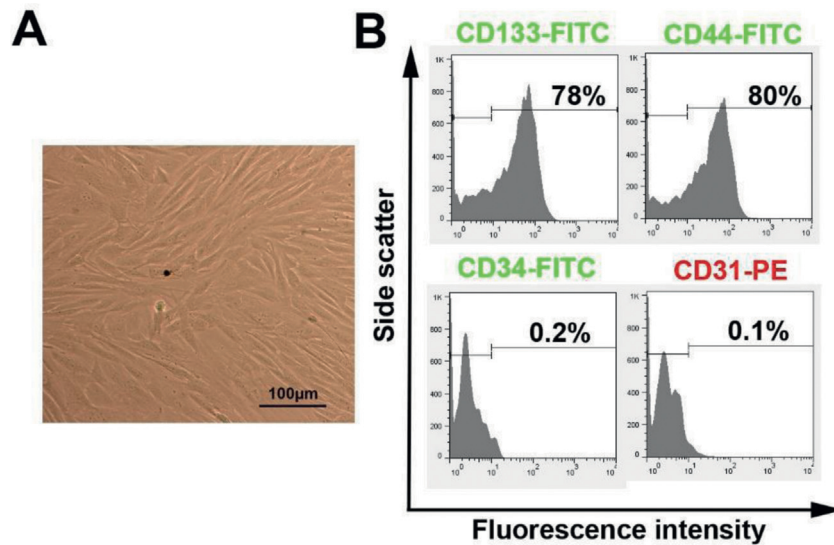


Fig. 2. Phase contrast micrographs of the monolayer confluent of rBMSCs at third passage (A). Isolated rBMSCs phenotypic characterization (B). The expression of both positive and negative surface markers of rBMSCs was calculated using flow cytometry analysis. Histograms display cells stained positively with CD133 and CD44.

pattern of curves was seen between CCT, CST and CPT groups (Fig. 3).

The mean value of EC₅₀ in the tracheal pieces of SPT ($0.6 \pm 0.01 \mu\text{M}$), SST ($0.64 \pm 0.01 \mu\text{M}$) and SCT ($0.78 \pm 0.01 \mu\text{M}$) groups were significantly lower than the group CPT ($2.95 \pm 0.1 \mu\text{M}$, $p < 0.001$). However, the mean value of EC₅₀ in the tracheal pieces of SCT and SST group has shown significant improvement in comparison with group SPT ($p < 0.001$ for SCT group, and $p < 0.05$ for SST). Mean value of EC₅₀ in the tracheal pieces of SCT group was significantly higher than of SST group ($p < 0.001$, Fig. 4).

The contractility response of the tracheal pieces to methacholine in all of sensitized groups was significantly higher than CPT group ($p < 0.001$ for SPT group, $p < 0.01$ for SST group and $p < 0.05$ for SCT). The contractility response in SCT and SST groups were significantly lower than group SPT ($p < 0.001$ for SCT group, and $p < 0.05$ for SST). Moreover, the contractility response of the group SST was significantly greater than of group SCT ($p < 0.05$, Fig. 5).

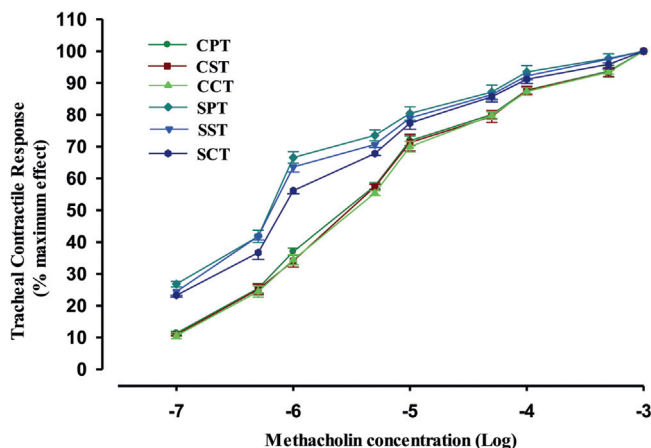


Fig. 3. Cumulative log concentration-response curves of methacholine-induced contraction of the isolated trachea in CPT (control group), CST (healthy animals received CM), CCT (healthy animals received rBMSCs), SPT (Sensitized animals), SST (Sensitized animals received CM), SCT (Sensitized animals received rBMSCs) (for each group, $n = 6$).

3.3. A prominent repopulation capacity of MSC-received rats WBCs were evident rather than CM-administrated subjects in peripheral blood

The mean values of total WBC in all of sensitized groups were increased significantly in comparison with the group CPT (98.7%; for SPT, 83.9%; for SST, 59.4%; for SCT and, $p < 0.001$ for all). The mean value of total WBC in SCT and SST groups decreased significantly compared to group SPT (19.7%; $p < 0.001$ for SCT group, and 7.4%; $p < 0.05$ for SST). Moreover, the mean value of total WBC in SCT group was very significantly lower than SST group ($p < 0.01$, Table 2).

3.4. WBC subtypes efficiently returned to near-normal levels in cell treated subjects in peripheral blood

We here reported that the percentage of eosinophil in all sensitized groups was significantly higher than the group CPT (346%; for SPT, 240%; for SST, 128.8%; for SCT and $p < 0.001$ for all). There was a significant decrease in eosinophil percentage of SCT and SST groups in comparison with SPT group (48.6%; $p < 0.001$ for SCT group and 23.6%; $p < 0.05$ for SST). Moreover, the percentage of eosinophil in SCT group was very significantly lower than SST group ($p < 0.01$).

The percentage of neutrophil in all sensitized groups was significantly higher than the group CPT (75.4%; $p < 0.001$ for SPT group, 49.9%; $p < 0.01$ for SST group and 27.5%; $p < 0.05$ for SCT). There was a significant decrease in neutrophil percentage of SCT and SST groups in comparison with SPT group (27.3%; $p < 0.01$ for SCT group, and 14.5%; $p < 0.05$ for SST). Moreover, the percentage of eosinophil in SCT group was significantly lower than SST group ($p < 0.05$).

The lymphocyte percentage in all sensitized groups were significantly lower than the group CPT (23.4%; $p < 0.001$ for SPT group, 14.9%; $p < 0.001$ for SST group and 7.5%; $p < 0.05$ for SCT). However, there was a significant increase in lymphocyte count of SCT and SST groups in comparison with SPT group (20.7%; $p < 0.001$ for SCT group, and 11.1%; $p < 0.05$ for SST). Moreover, the percentage of lymphocyte in SCT group was significantly lower than SST group ($p < 0.05$).

The percentage of monocyte in all sensitized groups was significantly lower than the group CPT (53.7%; $p < 0.001$ for SPT

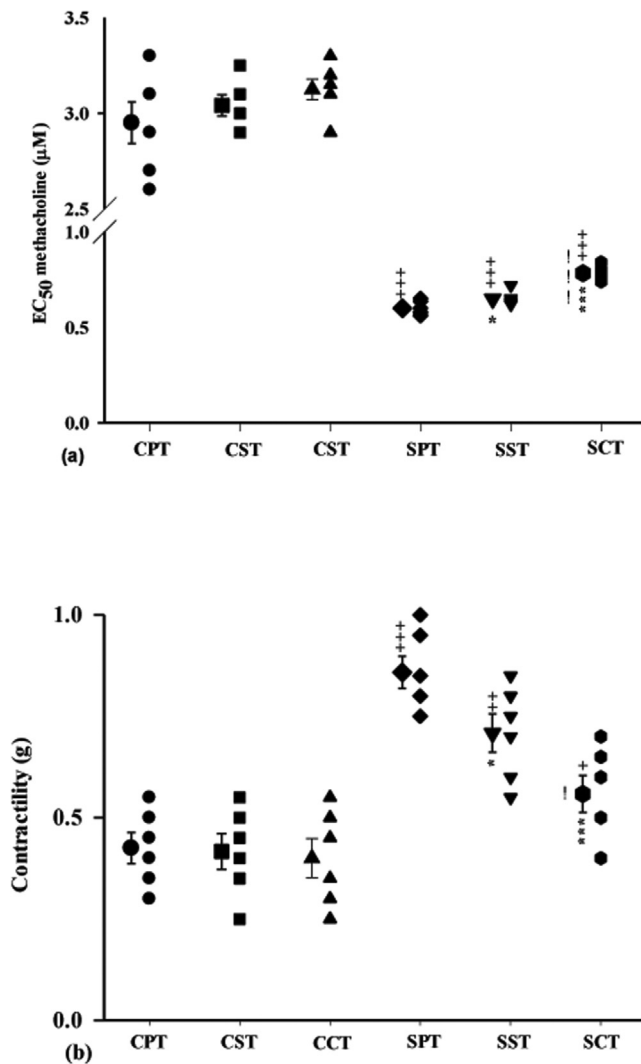


Fig. 4. Individual values and means (Big symbols with bars) of tracheal response to methacholine (a) and tracheal contractility response to 10 μ M Methacholine (b) in CPT (control group), CST (healthy animals received CM), CCT (healthy animals received rBMMSCs), SPT (Sensitized animals), SST (Sensitized animals received CM), SCT (Sensitized animals received rBMMSCs) (for each group, $n=6$). Statistical differences between control and different groups: +; $p < 0.05$, ++; $p < 0.01$ and +++; $p < 0.001$. Statistical differences between SST and SCT vs SPT group: *; $p < 0.05$ and ***; $p < 0.001$. Statistical differences between SST and SCT groups: !; $p < 0.05$.

group, 49.2%; $p < 0.001$ for SST group and 26.9%; $p < 0.05$ for SCT). There was a significant increase in monocyte count of SCT group in comparison with SPT and SST group (57.8%; $p < 0.01$ for SPT group, 43.6%; $p < 0.5$ for SST). Moreover, there was a non-significant increase in monocyte percentage of SST group in comparison with SPT group (Table 2).

3.5. Total leukocyte count was decreased by MSCs and derived-CM in lung lavage fluid

Corroborating to our results, the mean values of total leukocyte in LLF in all sensitized groups were significantly higher than that of group CPT (175%; for SPT, 155%; for SST, 124.4%; for SCT and $p < 0.001$ for all). A superior effect in total leukocyte count was in SCT and SST groups compared to group SPT (18.5%; $p < 0.001$ for SCT group, and 7.4%; $p < 0.05$ for SST). Moreover, the mean value of total leukocyte in SCT group was very significantly lower than SST group ($p < 0.01$, Table 3).

3.6. A drop in eosinophil population and uptrend retrieval pattern of lymphocytes diagnosed in MSC- and CM-treated asthmatic subjects in LLF

The percentages of eosinophil, neutrophil, basophil in all sensitized groups were significantly higher than the group CPT (respectively 192%, 80.5% and 75% for SPT group, 109.2%, 70%; and 67% for SST group, 60.3%, 43.2% and 58.5% for SCT and $p < 0.001$ to $p < 0.05$).

There was a significant decrease in eosinophil and neutrophil percentages of SCT group in comparison with SPT and SST groups (respectively 45.2% and 20.67% for SPT, 23.4% and 15.78% for SST and $p < 0.001$ to $p < 0.05$). The percentage eosinophil in SST group was significantly decreased compared to SPT group (28.5%; $p < 0.01$). However, the neutrophil and basophil percentages in SST group were non-significantly decreased compared to SPT group.

The percentage lymphocyte and monocyte in all sensitized groups was significantly lower than the group CPT (respectively 23.4% and 60.3% for SPT group, 15.6% and 41.6% for SST group, 9.1% and 25% for SCT and $p < 0.001$ to $p < 0.05$).

There was a significant increase in lymphocyte and monocyte percentage of SCT group in comparison with SPT and SST groups (respectively 18.7% and 89.2% for SPT group, 7.7% and 28.4% for SST group and $p < 0.001$ to $p < 0.05$). The percentage of lymphocyte and monocyte in SST group were significantly higher than the group SPT (respectively 10.2%, 47.31% and $p < 0.001$ to $p < 0.05$, Table 3).

3.7. Administration of MSCs and CM could repopulate the CD4 and CD8 positive cells in blood of asthmatic rats

We showed that no significant contributory effects of either MSCs administration or CM injection were attained on the population of $CD3^+CD4^+$ and $CD3^+CD8^+$ cells in non-sensitized rats. By contrast, the percentages of $CD3^+CD4^+$ lymphocytes significantly decreased in all sensitized groups in comparison with the group CPT ($p < 0.001$) while $CD3^+CD8^+$ lymphocytes increased ($p < 0.001$ to $p < 0.05$). The levels of $CD3^+CD4^+$ and $CD3^+CD8^+$ cells in SCT and SST groups showed drastic difference in comparison with SPT group. In detail, the percentages of $CD3^+CD4^+$ increased significantly ($p < 0.001$ to $p < 0.05$) whereas $CD3^+CD8^+$ cells diminished simultaneously ($p < 0.001$ to $p < 0.05$). However, the $CD3^+CD4^+$ cells population in SCT group was significantly higher than the group SST ($p < 0.01$) whereas $CD3^+CD8^+$ cells diminished simultaneously ($p < 0.001$, Fig. 6).

3.8. Pre-labeled cells appropriately recruited into pulmonary parenchyma

Here it was successfully approved the existence of pre-labeled MSCs either in non-asthmatic, and notably asthmatic rats received intratracheally rBMMSCs. It seems that MSCs could potentially recruit into normal and sensitized lung tissue (Fig. 7).

3.9. Detecting viability of injected cells into pulmonary parenchyma

The flow cytometric analysis revealed near to 95% of injected cells were alive after injection in current experiment (Fig. 8).

3.10. MSCs and CM efficiently modulated the IL-4 and IL-10 mRNA expression levels in asthmatic pulmonary tissue

Quantitative Transcript Analysis of the interleukins in asthmatic lungs showed a marked expression in the levels of IL-4 with decrease of IL-10 as compared to healthy rats ($p < 0.001$ to

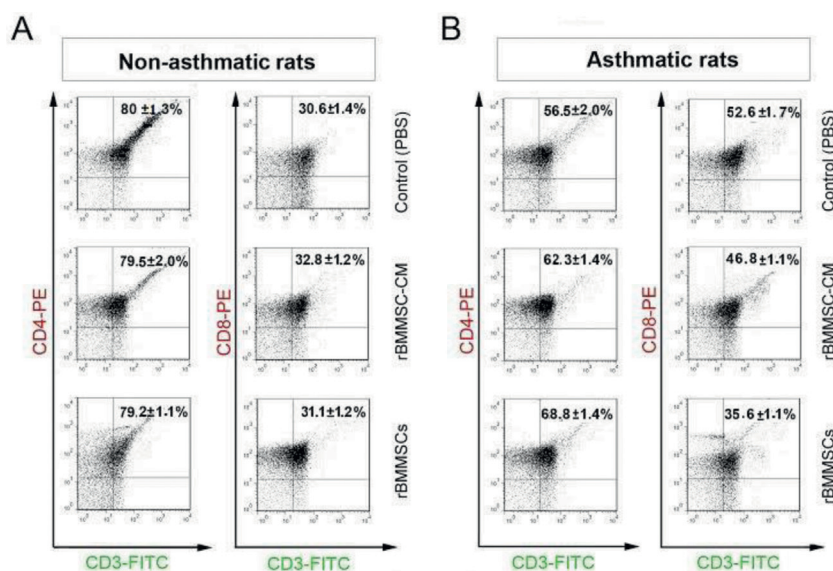


Fig. 5. Flow cytometric analysis of rat peripheral MNCs in different groups (A and B). The cells were stained with a panel of antibodies, including CD3-CD4 and CD3-CD8. The percentage of MNCs expressing each marker is expressed as mean $\pm$ SEM.

Table 2

Total and differential percentage of white blood cell (WBC) count in CPT (control group), CST (healthy animals received CM), CCT (healthy animals received rBMSCs), SPT (Sensitized animals), SST (Sensitized animals received CM), SCT (Sensitized animals received rBMSCs) (for each group, n=6).

	CPT	CST	CCT	SPT	SST	SCT
Total WBC (Number/mm ³)	6500 $\pm$ 165.33	6400 $\pm$ 160.73	6350 $\pm$ 159.69	12916.67 $\pm$ 312.43	11958.34 $\pm$ 290.52	10366.67 $\pm$ 257.77
Eosinophil (%)	2.84 $\pm$ 0.3	3 $\pm$ 0.36	3.16 $\pm$ 0.4	12.67 $\pm$ 1.11	9.67 $\pm$ 0.67	6.5 $\pm$ 0.61
Neutrophil (%)	16.34 $\pm$ 1.43	17 $\pm$ 1.41	17.34 $\pm$ 1.25	28.67 $\pm$ 1.45	24.5 $\pm$ 1.17	20.84 $\pm$ 1.07
Lymphocyte (%)	70.34 $\pm$ 1.64	69.17 $\pm$ 1.93	69.34 $\pm$ 1.62	53.84 $\pm$ 1.74	59.84 $\pm$ 1.55	65 $\pm$ 1.67
Monocyte (%)	10.5 $\pm$ 0.92	10.84 $\pm$ 0.79	10 $\pm$ 0.85	4.86 $\pm$ 0.6	5.34 $\pm$ 0.61	7.67 $\pm$ 0.61

Statistical differences between control and different groups: +; $p < 0.05$, ++; $p < 0.01$, +++; $p < 0.001$. Statistical differences between SST and SCT vs SPT group: *; $p < 0.05$, **; $p < 0.01$. Statistical differences between SST and SCT groups: !; $p < 0.05$, !!; $p < 0.01$.

$p < 0.05$). Noticeably, rBMSCs and CM administration attributed to expression of IL-10, whereas a downward trend was evident in the content of IL-4 transcripts in asthmatic rats as compared to parallel asthmatic control ($p < 0.001$ to $p < 0.05$). These changes were prominent in asthmatic rats received cells than that of CM subjects ($p < 0.01$) (Fig. 9).

3.11. Pathological injuries were reduced in the presence of CM and notably MSCs

Pathological changes observed in the pulmonary environments of all sensitized groups were significantly evident in comparison with the CPT group, unless the rate of leukocyte infiltration in SCT

Table 3

Total and differential percentage of white blood cell (WBC) count in lung lavage fluid of CPT (control group), CST (healthy animals received CM), CCT (healthy animals received rBMSCs), SPT (Sensitized animals), SST (Sensitized animals received CM), SCT (Sensitized animals received rBMSCs) (for each group, n=6).

	CPT	CST	CCT	SPT	SST	SCT
Total WBC	40.84 $\pm$ 2.38	40 $\pm$ 2.88	39.17 $\pm$ 3	112.5 $\pm$ 2.14	104.17 $\pm$ 2.38	91.67 $\pm$ 2.47 +++ +
Eosinophil (%)	7.17 $\pm$ 0.79	7 $\pm$ 1.06	6.5 $\pm$ 0.84	21 $\pm$ 0.85	15 $\pm$ 1.18	11.5 $\pm$ 0.76
Neutrophil (%)	11.17 $\pm$ 0.94	11 $\pm$ 1.06	11.34 $\pm$ 1.05	20.17 $\pm$ 0.94	19 $\pm$ 1.06	16 $\pm$ 0.7
Lymphocyte (%)	64 $\pm$ 2.28	63.67 $\pm$ 2.09	64.34 $\pm$ 2.39	49 $\pm$ 1.29	54 $\pm$ 1.31	58.17 $\pm$ 1.19
Monocyte (%)	16 $\pm$ 1.06	16.5 $\pm$ 0.99	16.34 $\pm$ 0.95	6.34 $\pm$ 0.66	9.34 $\pm$ 0.66	12 $\pm$ 0.89
Basophil (%)	2 $\pm$ 0.36	1.67 $\pm$ 0.33	1.84 $\pm$ 0.3	3.5 $\pm$ 0.42	3.34 $\pm$ 0.34	3.17 $\pm$ 0.3

Statistical differences between control and different groups: +; $p < 0.05$, ++; $p < 0.01$, +++; $p < 0.001$. Statistical differences between SST and SCT vs SPT group: *; $p < 0.05$. Statistical differences between SST and SCT groups: !; $p < 0.05$.

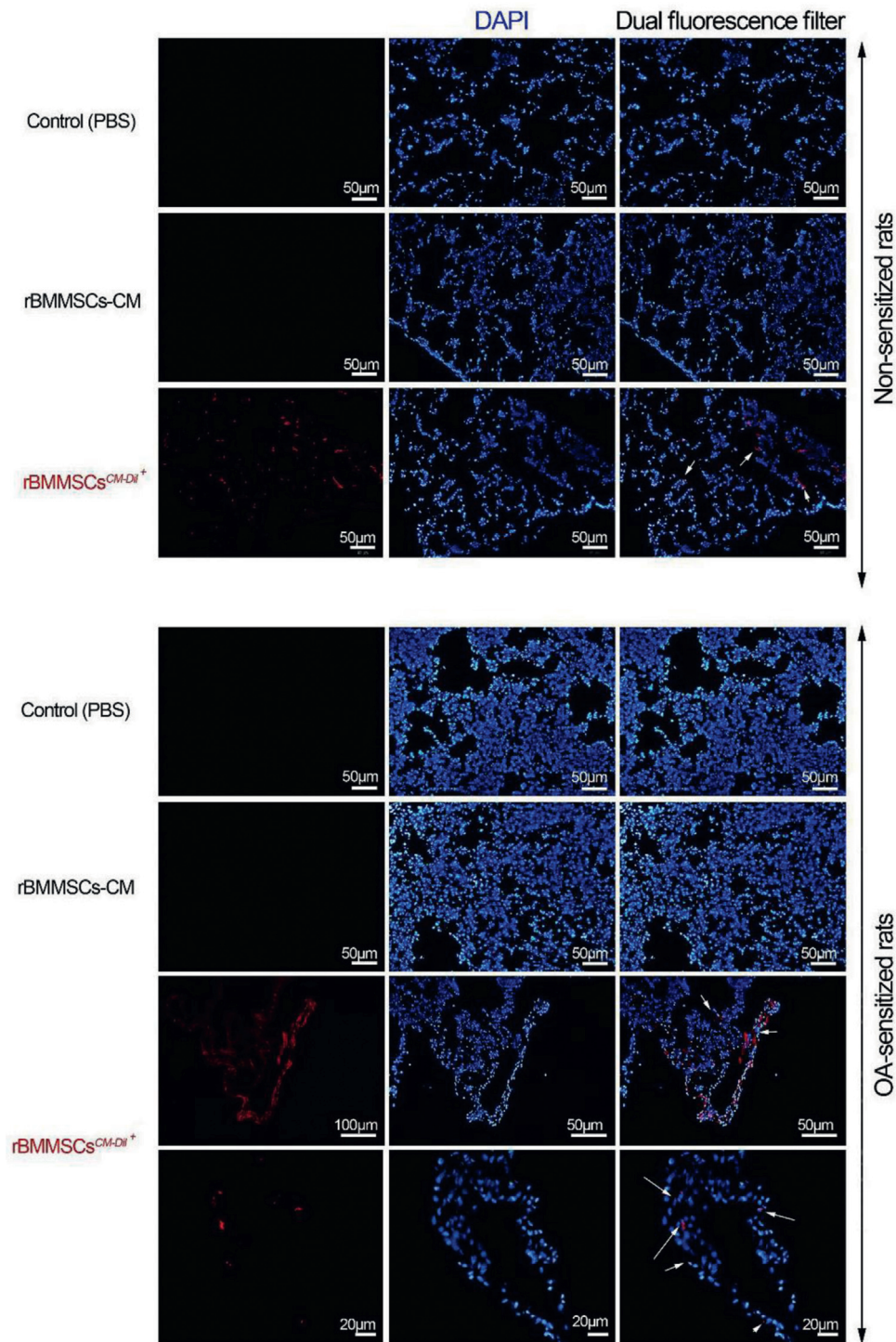


Fig. 6. Representative fluorescent image of lung parenchyma obtained from non-sensitized and sensitized rats receiving rBMSCs-CM or rBMSCs and compared to control groups. As seen, the MSCs are labeled with CM-Dil and administrated intratracheally.

group ($p < 0.001$ to $p < 0.05$). There was a significant decrease in the pathological changes measured in asthmatic rats received cells and CM in comparison with control asthmatic group ($p < 0.01$ to $p < 0.05$) while no obvious significant changes were obtained in term of atelectasis for SST group. Calling attention, we also notified that MSCs could efficiently ameliorate the asthmatic associate pathological changes more than CM alone ($p < 0.01$ to $p < 0.05$). Although, we did not see any significant differences in the rate of atelectasis of lungs between SST and SCT groups (Table 4).

4. Discussion

In this experiment, both juxtacrine and paracrine therapeutic effects of rat bone marrow-derived MSCs monoculture and relevant CM were evaluated intratracheally in a rat model of asthma. The presumable modulation on tracheal responsiveness and inflammatory status chiefly focused on total WBC count of blood, LLF, pro- and anti-inflammatory cytokines and pathological changes as well, generated by cellular component or supernatant

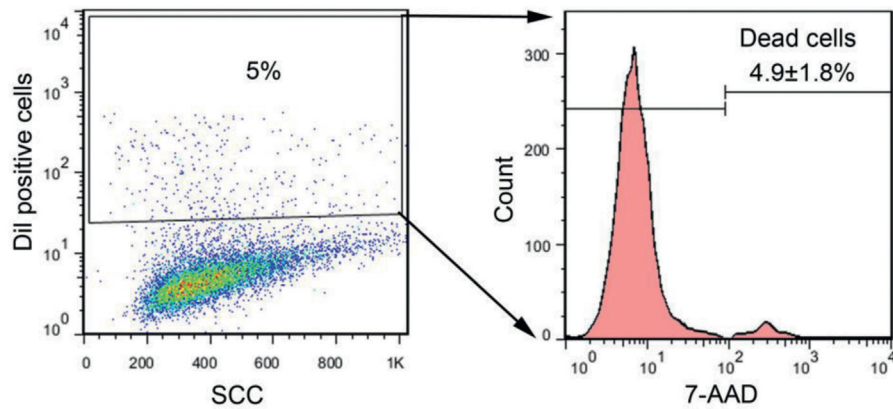


Fig. 7. Flow cytometric analysis of rBMSCs after administration into pulmonary tissue. Near to 95% of injected cells were alive after transplantation.

secretome, were further investigated. In addition, the percentages of circulating $CD3^+CD4^+$ and $CD3^+CD8^+$ cells were also notified in

response to rBMSCs and CM administration. Our goal, in selecting a cellular fraction and CM phase, was to attain the suitable candidate for attenuating long-term chronic asthmatic changes of respiratory apparatus and to scrutinize underlying mechanisms of the inflammation in allergic asthma pathophysiology.

On behalf of numerous common features of allergic asthma observed previously in rats and human, an allergic asthma induction was created by ovalbumin exposure in the present study [45]. A significant increase in the tracheal responsiveness and contractility rate was seen in response to methacholine coincided with a simultaneous increase of circulatory total WBC, particularly in the percentages of eosinophil and neutrophil that followed by a remarkable decrement in lymphocyte and monocytes counts in all sensitized groups as compared with parallel controls. Additionally, leukocyte infiltration, epithelialization, atelectasis, emphysema and hyperemia were induced in asthmatic rats. These changes per se confirmed the effectiveness of sensitization procedure in rats submitted to our study [20,46,47]. In one study on OVA-induced asthmatic mice, Abreu and co-workers showed that therapeutic effect of bone marrow mononuclear cells diminished in sensitized animals as compared with healthy animals MSCs. Nonetheless, MSCs of healthy rats were used [36]. By virtue of anatomical and histological features, lungs are unique and suitable organs candidate in which BMSCs and CM can be easily delivered by local or systemic administration [28]. In set of experiments, it was reported that both administration routes appropriately introduced transplanted stem cells to lung, but homing efficiency in systemic approach was less than local administration. Although, intravenous injection of MSC is the most widely used, but on the other hand, local introduction of MSCs may be more therapeutic as a vast number of cells easily participate in healing and repair processes [28,48,49].

Our data further revealed that the direct injection of rat MSCs and relevant CM could improve the contractility response and tracheal responsiveness to methacholine in sensitized rats. Simultaneously, the total and differential WBC percentage both in the blood and lung lavage fluid reached to near-normal values as compared to control. Regarding on different cell panels typified here, the decrement of $CD3^+CD8^+$ cells was synchronized with an increase in $CD3^+CD4^+$ population either in cell- or CM-treated rats. We also determined that most of histopathological features analyzed in our study returned to near-normal state in treatment asthmatic groups, especially in cell-treated rats. Calling attention, neither MSCs nor MSC-CM exerted a significant change on the measured values of saline-challenged rats, which was consistent to the data of many previous experiments [17,19]. In agreement with our data, local and systemic injection of MSCs and CM palliated the

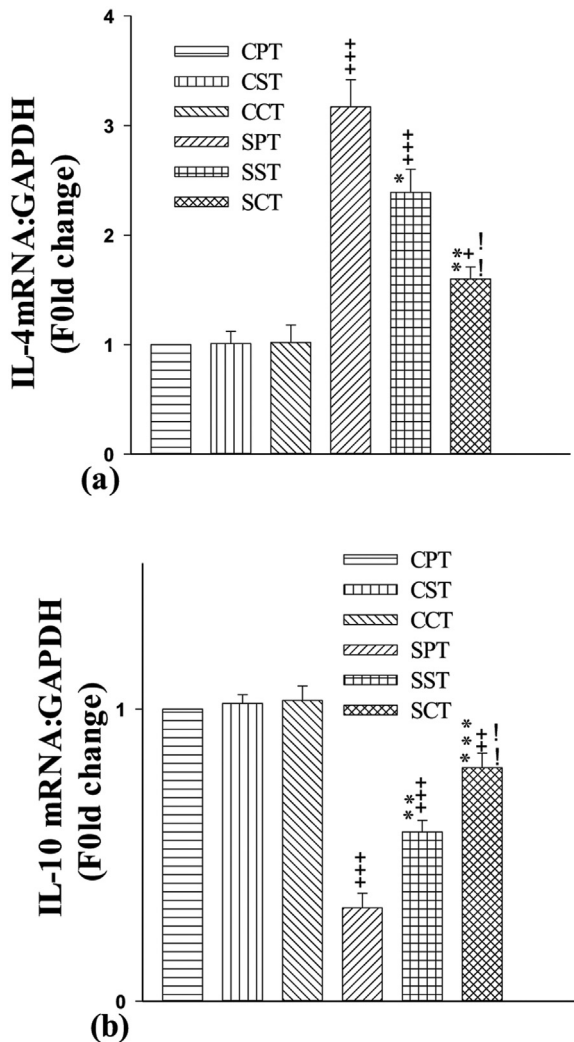


Fig. 8. Expression levels of IL-4 (a) and IL-10 (b) mRNA in the lung tissue of CPT (control group), CST (healthy animals received CM), CCT (healthy animals received rBMSCs), SPT (Sensitized animals), SST (Sensitized animals received CM), SCT (Sensitized animals received rBMSCs) (for each group, n=6). Statistical differences between control and different groups: +; $p < 0.05$, ++; $p < 0.01$ and +++; $p < 0.001$. Statistical differences between SST and SCT vs SPT group: *; $p < 0.05$ and **; $p < 0.01$. Statistical differences between SST and SCT groups: !!; $p < 0.01$.

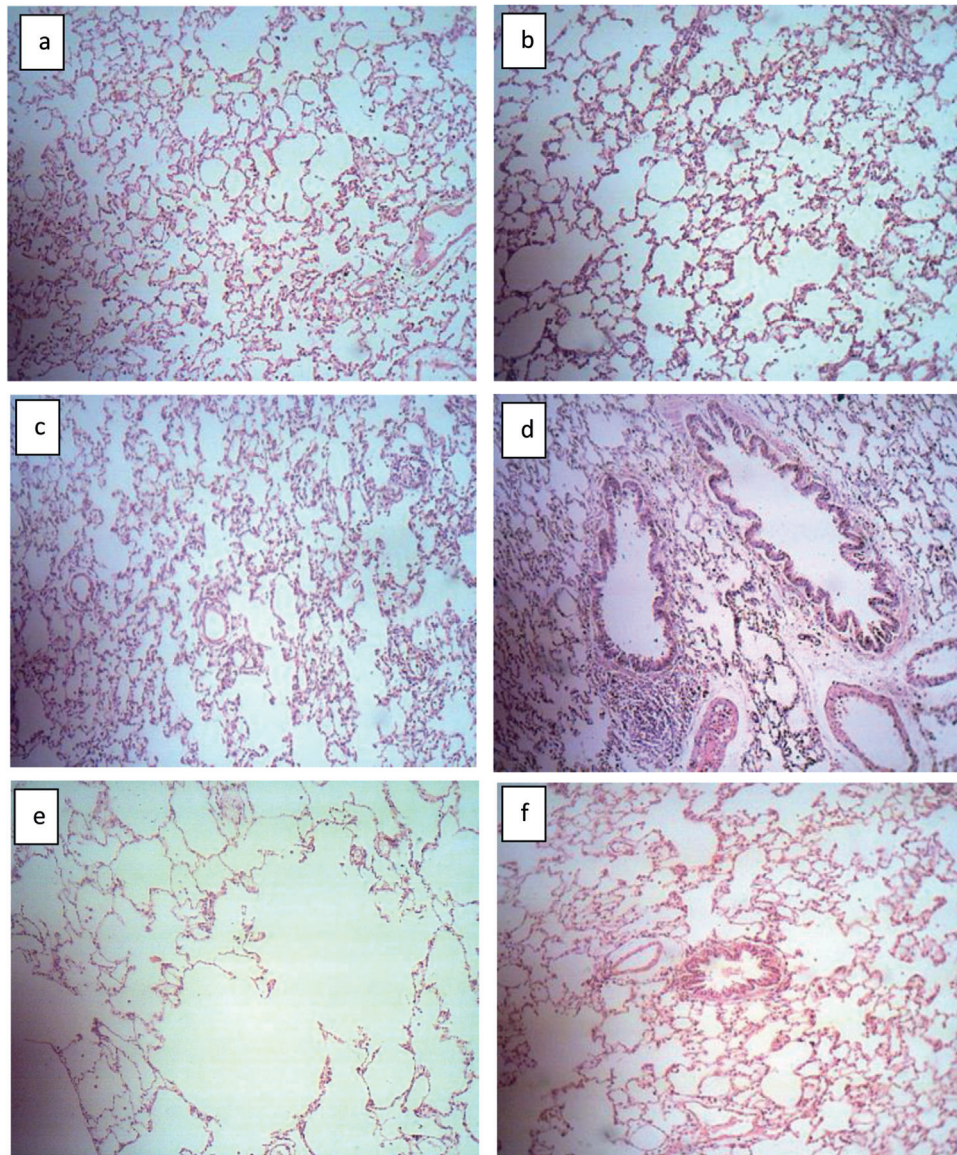


Fig. 9. Photographs of a lung specimen in different groups (magnification for each group; 8×10). Normal lung tissue in control group (CPT, a), healthy animals received CM (CST, b) and healthy animals received rBMMSCs (CCT, c). Cell infiltration and hyperemia in sensitized group (SPT, d), alveolar emphysema in sensitized animals received CM (SST, e) and cell infiltration and alveolar emphysema in sensitized animals received rBMMSCs (SCT, f).

Table 4

Pathological finding scores in lung of control group (CPT), healthy animals received CM (CST), healthy animals received rBMMSCs (CCT), sensitized animals (SPT), sensitized animals received CM (SST), and sensitized animals received rBMMSCs (SCT).

Pathological findings	Scores in groups					
	Median (Minimum-Maximum)					
	CPT	CST	CCT	SPT	SST	SCT
Emphysema	0 (0–1)	0 (0–1)	0 (0–1)	3 (2–3) +++	2 (2–3) ++ *	1 (1–2) + ** !
Atelectasis	0 (0–1)	0 (0–1)	0 (0–1)	1.5 (1–2) +++	1 (0–2) +	1 (0–1) + *
Hyperemia	0 (0–1)	0 (0–1)	0 (0–1)	2.5 (2–3) +++	2 (1–2) +++ *	1 (1–2) + ** !
Epithelialization	0 (0–1)	0 (0–1)	0 (0–1)	3 (2–3) +++	2 (1–2) ++ *	1 (1–2) +++ !
Leukocyte infiltration	0 (0–1)	0 (0–0)	0 (0–0)	2 (1–2) +++	1 (0–1) + *	0 (0–1) ** !

The pathological changes were scored in the following manner: 0 = absence of pathologic changes; 1 = patchy changes; 2 = local changes; 3 = scattered changes. Statistical differences between control and different groups: +; $p < 0.05$, ++; $p < 0.01$, +++; $p < 0.001$. Statistical differences between SST and SCT vs S group: *; $p < 0.05$, **; $p < 0.01$, Statistical differences between SST and SCT groups: !; $p < 0.05$, !!; $p < 0.01$.

lung injury, airway inflammation rate and histopathological changes during respiratory disorders [17,50–56]. We also postulated that the detection of active cytokines could be useful in the prediction of asthmatic lungs regeneration induced by MSCs and CM. Consistent with previous experiments, we confirmed the potential dynamic of pro-and anti-inflammatory cytokines, IL-4 and IL-10, in advancing the status of asthma [11,57]. The irregularity between CD4⁺ Th1 and Th2 immune responses has been recognized as the major contributing factor to the pathogenesis of asthma [58]. For instance, IL-4 and IL-13, produced by Th2 lymphocytes, are crucial cytokines involved in the pathogenesis of allergic inflammation [57]. Otherwise, anti-inflammatory cytokines, such as IL-10, secreted by T regulatory cells, regulate asthmatic inflammation via down-regulation of the Th2-driven inflammatory responses [11]. Therefore, tightly regulation of pro-and anti-inflammatory cytokines balance is essential for modulation of lung inflammation [12]. Considering therapeutic impact of CM and peculiarly MSCs, we did emphasize that an efficient suppression of asthma yielded by increase of IL-10 and reduction of IL-4 levels.

Similar to work conducted by Sutsko and co-workers, the therapeutic effects originating from direct injection of rBMSCs on the air way inflammations were more substantial than CM emerged [59], but a plethora of experiment showed that therapeutic effect of direct administration of CM are most similar to BMSCs [51,52]. The logic explanation for this discrepancy could be possibly related to animal species studied, sensitization method, the source, dosage, route and time of cell and CM administration [30,60,61]. Furthermore, MSCs release their secretion “spontaneously” or after induction by the local conditions [62]. It seems that one of the possible reasons for higher effects of rBMSCs in compared with CM on the parameters measured during this study is the variety in type and amount secreted agents. Therefore, injection with high volume and/or repeated doses could be more therapeutic. Another possible reason for higher effects of BMSCs in compared with CM on the parameters measured is presumably be due to the fact that the factors within the CM are adsorbed by the surrounding damaged tissue, because the CM contains only a finite amount of factors whereas the presence of the stem cells provides a source for the long-term secretion of soluble factors [59].

Some authorities also acclaimed that distinct post-transplantation period must be considered to track MSCs in specific tissues [50,63]. In one study, human and mouse adipose stem cells were successfully recruited three-weeks post-transplantation [63]. Similar to work by Ghorbani et al. we here approved the existence of pre-labeled cells in sensitized lungs after 2 weeks [50]. Due to distributed vascular bed formed in the pulmonary parenchyma, we however affirmed the presences of pre-labeled cells even in normal non-asthmatic rats. Contrary, Abreu and other authorities indicated MSCs homing only in injured lung tissue [18,50]. By virtue of animal strain and species, injection route, and recovery time after cell injection, stem cells type and the number of injected cells, grade and type of damaged tissue, and volume and type of cell tracker used controversial results have been obtained until yet [17,29,64–66].

In a great body of literature, a wide number of cells ranging from 1×10^6 to 5×10^7 has been used in different trials [67–69] whenever a systemic injection of a higher number of MSCs from different species not only regenerated pulmonary tissue but also increased side effects and mortality rate as the occurrence of embolism [66]. Fortunately, a well-tolerated situation without any mortality or adverse effects was obtained in designated experimental groups.

The precise molecular mechanisms by which MSCs augment their therapeutic effects aren't completely elucidated [70].

Nevertheless, it is thought that MSCs exert promising effects in the injured site by both trans-differentiation into host tissue cell types and paracrine effects by secreting soluble factors, such as cytokines [62]. One of the major characteristics of inflammation is ischemic condition that is thought to be an essential problem for MSCs survival in the site of injury [71]. Inasmuch the survival and differentiation rate of MSCs at sites of injury and inflammation is partly poor, it is proposed that the basic mechanism of their beneficial effects is paracrine effects [71–73]. It is thought the paracrine effects of MSC could be partially managed by the released exosomes into surrounding microenvironments, participating in reciprocal cell–cell interaction. Both stimulatory and inhibitory effects of MSC-derived exosome have been documented in different niches [26]. For instance, Mokarizade et al. investigated the co-culture of isolated exosomes originated from MSCs with murine splenic mononuclear cells. They found that the addition of exosomes could inhibit auto-reactive lymphocyte reactivity while carrying a set of anti-inflammatory cytokines such as IL-10 and TGF- β [74]. In different experiments, exosomes from human or murine MSCs mitigated allergic airway hyperresponsiveness and lungs inflammation [75]. In line with the modulatory effects of MSCs on immune cells, it is shown that these cells are able to inhibited T cell proliferation and maturation of antigen presenting cells in vitro conditions via juxtacrine and paracrine manner [76].

The most limitation related to the present study is that the percent of CD4 subpopulation, including T helper 1, 2 and T regulatory was not investigated.

5. Conclusion

The results of this study showed anti-inflammatory therapeutic effects of intratracheal administration of rBMSCs and MSC-CM on experimental sensitized rats. However, the regenerative responses observed post local administrations of rBMSCs were higher than MSC-CM.

Conflict of interest

All authors declare there is no conflict of interest exists.

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