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Effects of Educated Monocytes with Xenogeneic Mesenchymal Stem Cell–Derived Conditioned Medium in a Mouse Model of Chronic Asthma

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ABSTRACT

Background: This study was conducted to determine the potential of the monocytes educated with rat bone marrow mesenchymal stem cell–derived conditioned medium (MCM) in ameliorating animal model of asthma.

Methods: Chronic asthma was induced in the BALB/c mice using ovalbumin (OVA) sensitization. The monocytes were isolated from blood of mice and supplemented with 50% MCM or negative control media. After 24 h, the cells were designated as “non-educated or educated”. Fourteen weeks after the onset of the study, animals were treated with educated or non-educated monocytes twice with a 1-week interval.

Results: The educated monocytes showed a reduction in the potential production of the respiratory burst and nitric oxide and the secretion of IL-12 and IL-4 compared to non-educated monocytes. Conversely, these monocytes exhibited a significant increase in the production of IL-10 and TGF- β . Also, the levels of CD68+/CD206+ cells significantly increased in the population of educated monocytes. More importantly, the severity of histopathological lesions, NF- κ B p65 mRNA level in lung tissues, total serum IgE and the total cell count, as well as the eosinophil count in the bronchoalveolar lavage fluid, were significantly decreased in OVA-inhaled mice treated with educated monocytes compared to OVA-sensitized group receiving non-educated monocytes. With no advantage in up-regulation of Foxp3 Treg cells, the treatment with educated monocytes reduced the secretion of IL-5 and IL-13 by splenocytes of asthma mice more than splenocytes of the asthma mice treated with non-educated monocytes.

Conclusion: The educated monocytes with MCM may be as a promising strategy for cell-based therapies of asthma.

KEYWORDS

Asthma; conditioned medium; mesenchymal stem cells; monocyte therapy

Introduction

Asthma is a chronic heterogeneous inflammatory disorder of the airways, characterized by persistent airway inflammation and remodeling (Kang et al., 2017). Diverse classes of pharmacological agents, including corticosteroids, β 2-adrenoceptor agonists, methylxanthines and cromolyn, have been accepted to manage asthma. Despite such strategies,

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many patients have exposed a less than perfect response (Agbetile and Green, 2011; Chung et al., 2014). New evidence has suggested that the transplantation of mesenchymal stem cells (MSCs) is beneficial to ameliorate airway inflammation in asthma-like conditions (Kang et al., 2017; Lau et al., 2012).

The MSCs are generally found in the bone marrow and many other tissues, like adipose tissue, umbilical cord blood (Brandau et al., 2010), dermal tissue (Bartsch et al., 2005), and peri-endothelial areas (Crisan et al., 2008). It is obvious that the MSCs have potent immunoregulatory and regenerative properties (Fan et al., 2016; Herrero and Perez-Simon, 2010; Ma et al., 2014). It is suggested that the MSCs can reduce the proliferation of B cells and helper and cytotoxic T lymphocytes via cell cycle arrest in G0/G1 phase (Hesami et al., 2017). The MSCs are able to regulate innate immune cells like natural killer cells, dendritic cells, and especially monocyte/macrophage cells (Fan et al., 2016). The MSCs mediate their positive functions mostly through their paracrine activities, such as the secretion of soluble molecules or the release of extracellular vesicles (Lo Sicco et al., 2017). Similar to the MSCs, many studies have documented that the conditioned media derived from mesenchymal stem cells (MCM) have therapeutic benefits in diverse experimental diseases (Pawitan, 2014). Of note, the isolation and expansion of MSCs are time-consuming procedures (Eggenhofer and Hoogduijn, 2012). On the other hand, the inadequate vitality of the transplanted MSCs, reaching the inflamed or damaged tissues, has limited their potential beneficial effects (Mirzamohammadi et al., 2015).

Undoubtedly, monocytes, and especially monocyte-derived macrophages, possess an essential role in the initiation and resolution of inflammatory conditions (Lo Sicco et al., 2017). Interestingly, evidence has exhibited that the MSCs or MSC-derived conditioned medium can educate monocytes/macrophages toward an alternative anti-inflammatory pathway (Anderson et al., 2013; Cho et al., 2014; Geng et al., 2014; Hu et al., 2016; Hwang et al., 2016; Ionescu et al., 2012; Magatti et al., 2017; Selleri et al., 2016). For example, in vivo infusion of MSCs could alleviate rhabdomyolysis via the activation of M2 anti-inflammatory macrophages (Geng et al., 2014). Also, it has been reported that the education of adipose-derived MSCs by bone marrow-derived macrophages possess therapeutic benefits in inflammatory bowel diseases and sepsis (Anderson et al., 2013). So, the anti-inflammatory monocytes/macrophages can be generated by the isolation of peripheral blood monocytes, followed by the co-cultivation with a universal source of the third-party (allogeneic or xenogeneic) MSC-derived conditioned medium. This strategy may provide a sufficient number of monocytes/macrophages with anti-inflammatory properties within a few days. It is clear that the injected monocytes are preferentially enriched in inflamed tissues (Eggenhofer and Hoogduijn, 2012). However, there is no or limited information on the role of monocytes educated by MCM in ameliorating the inflammatory conditions, such as asthma. Hence, the purpose of this research was to determine whether or not the MCM-educated monocytes/macrophages could attenuate the ovalbumin (OVA)-induced chronic asthma in the mouse model.

Materials and methods

Reagents

The May-Grünwald-Giemsa stain was purchased from Merck (Darmstadt, Germany). The monoclonal antibodies used in the flow cytometric analysis were obtained from Becton

Dickinson (USA). The cytokine assays by the enzyme-linked immunosorbent assay (ELISA) kits were obtained from Peprotech (UK). The culture media, fetal calf serum (FBS), and the other chemicals were procured from Sigma-Aldrich (USA).

Animals

Male inbred BALB/C mice (6–8 weeks old) and male Wistar rats (6–8 weeks old) were obtained from the Pasteur Institute of Iran. The animals were maintained in regular cages under the constant temperature condition (22–24°C) in a 12 h light/dark cycle and received food and water ad libitum. The research performed under the supervision of the ethics committee of the research on laboratory animals of Urmia University, Urmia, Iran (No. 1343/TD.T/3). Animal care and the general protocols for animal management were observed in compliance with the regulations of the Ministry of Health and Medical Education of I.R. Iran, approved by the Medical Ethics Committee of the University for Animal Studies (Abtahi Froushani et al., 2014).

Asthma induction and experimental design

Forty male BALB/c mice were entered into this survey. Ten animals were not sensitized and served as the control group, while others were challenged and sensitized three times by an intravenous injection of 100 µl of the suspension, containing 400 µg OVA, 1,000 µl PBS, and 400 mg Al(OH)₃ gel on days 0, 7, 14, and 21. From days 27 to 31, three times per week for a period of 12 weeks, they were sensitized in a closed environment, filled with OVA aerosol (20 ng OVA in 50 µl PBS) for 30 min every day. In 14 weeks after the beginning of the study, the animals were randomly allocated in four equal groups ($n = 8$) as follows: (1) Asthma group (the mice received placebo twice and with a 7-day interval); (2) Asthma group + non-educated monocytes (the mice twice (with a 7-day interval) treated with the intravenous administration of 10^5 untreated monocytes in a 100 µl volume); (3) Asthma group + educated monocytes (the animals twice (with a 7-day interval) treated with the intravenous injection of 10^5 monocytes, educated with MCM) (Supplementary file 1).

Isolation of MSCs and conditioned medium preparation

The bone marrow–derived MSCs were isolated on the basis of their ability to adhere to the culture plates, as described elsewhere (Baghaban Eslaminejad et al., 2008; Shushtari and Abtahi Froushani, 2017). In brief, the bone marrow was flushed out from the tibias and femurs of the sacrificed Wistar rats. After two items of washings, the cells were cultured in 75-cm² tissue culture flasks at the concentrations of $0.3\text{--}0.4 \times 10^6$ cells/cm² in a DMEM medium supplemented with 15% FBS in a humidified incubator with 5% CO₂ at 37°C. The non-adherent cells were carefully discarded on the fourth day, and the adherent ones were fed twice per week. Upon 80% confluence, the MSCs were detached by trypsin/EDTA and then counted and re-plated in a ratio of 1:3 (about 1.5×10^6 cells/75-cm² flask). The cell passage was performed up to the third-generation passage.

MCM was prepared according to the previously reported methodology (Akram et al., 2013). Briefly, the MSCs (about 5×10^6 cells/75-cm² flask) were incubated in a serum-free

DMEM medium without FBS for 24 h. The supernatants were collected and centrifuged for 10 min at 300 g to remove cellular debris. Afterward, the collected supernatants from each individual rat were mixed and filtered through a 0.2- μ m filter in order and stored at -80°C prior to testing.

Negative control media were simply produced by filtering and storage of the serum-free DMEM medium at -80°C in the same condition with MCM.

Monocyte isolation and education

The heparinized blood sample was isolated from the deeply anesthetized mice by cardiac puncture and in RPMI-1640 medium (1:1) on a Ficoll-Hypaque gradient. After centrifugation at 400 g for 30 min, the mononuclear cell layer was re-suspended in DMEM and cultured in six-well plates (9×10^6 cells/well) at 37°C for 2 h. This method supported monocyte adherence to the plate. The non-adherent cells (mostly lymphocytes) were discarded by extensive washing with PBS. Then, the adherent monocytes were cultured in DMEM medium supplemented with 10% FBS and 50% conditioned media or negative control media. After 24 h, the cells were harvested, washed three times, and designated as “non-educated or educated”, and applied for in vivo and in vitro experiments.

The data of flow cytometry revealed that the sub-culture 3 of the third generation of the adherent cells, isolated from the bone marrow was positive for the MSC markers of rat (CD29 and CD90), but negative for the marker of the hematopoietic cells (CD 45) (Figure 1).

Evaluation of the in vitro cytokine profile of monocytes

The cells were stimulated with Lipopolysaccharide (LPS) (10 pg/mL) for 24 h and the cell-free supernatants were collected and checked for the IL-10, IL-4, TGF- β , and IL-12 levels by the sandwich ELISA kits, according to the manufacturer's instructions.

Evaluation of the respiratory burst in monocytes

The cells were incubated for 20 min with 100 ng/ml 12-O-tetradecanoyl phorbol-13-acetate (TPA) and 0.1% Nitro blue tetrazolium chloride (NBT). The unexploited NBT dye was removed by rinsing and the reduced dye was extracted in KOH (2 M) and DMSO and quantitated at 520 nm (Shushtari and Abtahi Froushani, 2017).

Measurement of nitric oxide by monocytes

The culture medium of monocytes was removed and the cells were stimulated with LPS (10 pg/ml) for 24 h. After this period, the nitrite accumulation in the culture media was measured as an indicator of nitric oxide (NO) by using the Griess method, as previously explained (Shushtari and Abtahi Froushani, 2017).

Bronchoalveolar lavage fluid (BALF) and serum collection

At the end of the challenge, the heparinized blood samples were collected under ether anesthesia by cardiac puncture, and the serum samples were isolated by centrifugation

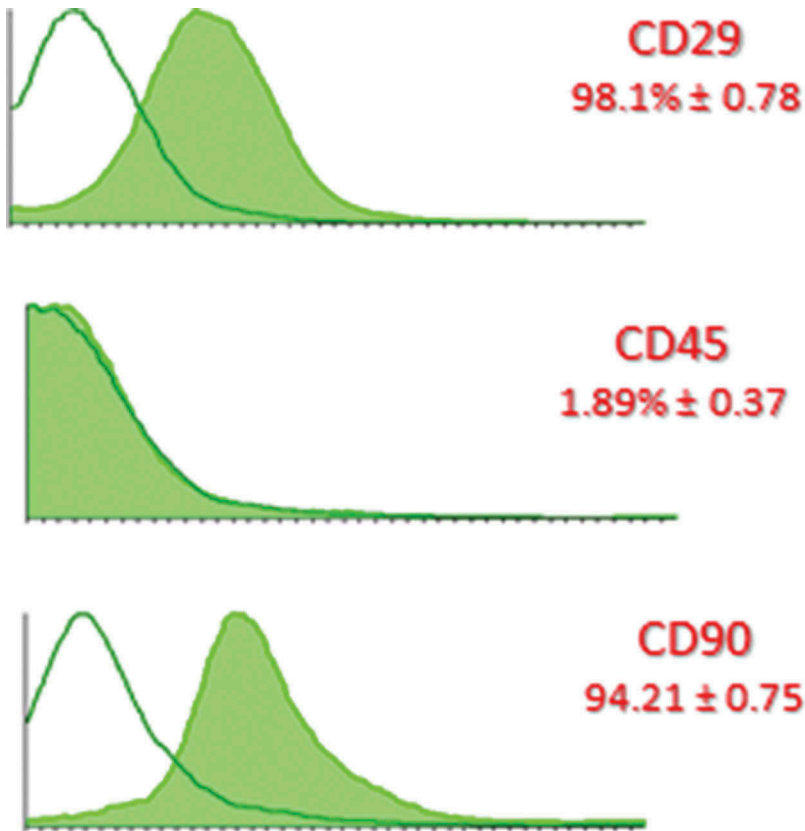


Figure 1. Flow cytometry analysis of MSCs for surface markers. The positivity of the given marker is given by the rightward shift against the background. MSCs expressed CD29 and CD90 but not CD45. The experiments were performed in triplicate sets. The results of the immunophenotypic characterization of isolated MSCs were presented as Mean $\pm$ SD.

and used to estimate the IgE production using the ELISA kit, according to the manufacturer's instructions.

Simultaneously, ice-cold PBS (0.5 mL) was instilled twice into the lungs, and the BALF was aspirated. Total cells were counted using a hemocytometer and at least, 200 cells were counted per slide.

Splenocytes isolation and cytokines assay

Splenocytes were aseptically isolated from mice at sacrifice time. In brief, single-cell suspensions of splenocytes were prepared in RPMI 1640 medium supplemented with 10% FBS. Red blood cells (RBCs) were deleted by RBC lysis buffer. Next, cell suspensions (2×10^6 cells/ml) were cultured in 24-well plates and stimulated with 50 μ l OVA solution (200 μ g/ml). The culture supernatants were removed after 72 h and used for ELISA experiments according to the manufacturer's instructions.

Histopathological examination

The histopathologic examination was done on those mice, which were not subjected to BALF (three mice in each group). The lower lobe of the right lung was dissected for detection of NF- κ B p65 and I κ B α mRNA expression. The other parts of lung tissues were fixed in 4% neutral buffered formalin. The lung tissues were dissected from the middle zone of the left lung and fixed in 4% neutral-buffered formalin. The lung tissues were then sectioned, embedded in paraffin, and cut at 5 μ m. The tissue sections were stained with H&E to recognize peribronchial and perivascular inflammation, and scored on the subjective scales of 0–3, as described elsewhere (Kang et al., 2017; Li et al., 2016); 0: no inflammation was observed; 1: inflammatory cells were occasionally observed; 2: most bronchi were surrounded by a thin layer of inflammatory cells (1–5 cells); 3: most bronchi or vessels were surrounded by a thick layer of inflammatory cells (more than five cells). Five randomly selected tissue sections for each animal were examined by two researchers, blinded to the treatment group.

Flow cytometric analysis

The MSCs were examined for the surface expression of a minimal panel to characterize MSCs at passage 3. In brief, the MSCs (5×10^5 in 100 μ l PBS/0.5% BSA/2 mmol/EDTA) were stained with 10 μ l of the fluorescently labeled monoclonal antibody (anti-rat CD29 (Integrin b chain; Ha2/5; FITC), CD45-FITC, and CD90-PCY5 (Thy-1/Thy-1.1-FITC)) and maintained in the dark at 4°C for 30 min. The stained cells were, then, washed three times with wash buffer (PBS, containing 2% BSA) and re-suspended in PBS.

In order to determine the anti-inflammatory phenotype in monocytes, the cells were stained by marker expression, the cells were labeled with FITC anti-mouse CD68 and APC anti-mouse CD206, as described elsewhere (Hu et al., 2016).

At the end of the study, a single-cell suspension of splenocytes (1×10^6 cells/ml) was prepared and stained with anti-mouse CD4-FITC, anti-mouse CD25-PE, and anti-mouse Foxp3-cyanine 5 as described previously (Abtahi Froushani et al., 2014).

The stained cells (MSCs, monocytes, and splenocytes) were analyzed immediately on a DAKO flow cytometer (Partec, Germany).

Detection of NF- κ B p65 and I κ B α mRNA expression in lung

Total tissue RNA was extracted from the right lung tissue samples by a commercial kit according to the manufacturer's instructions. The cDNA synthesized and amplified through quantitative real-time reverse transcription PCR analysis. The following primer sets were used: NF- κ B p65, PCR forward primer: 5'-TGCAGGCTCCTGTGCGAGTG-3' and reverse primer: 5'-TCCGGTGGCGATCGTCTGTGT-3'; I κ B α forward primer: 5'-CGTGTCTGCACCTAGCCTCTATC-3' and I κ B α reverse 5'-GCGAAACCAGGTCAGGATTC-3' and the β -actin forward primer: 5'-GCAGGAGTACGATGAGTCCG-3' and reverse 5'-ACGCAGCTCAGTAACAGT CC-3'. The PCR program included 95°C for 30 s and 40 PCR cycles (95°C for 5 s and 60°C for 30 s). The NF- κ B p65 mRNA expression was normalized to β -actin.

Statistical analysis

The values were presented as means $\pm$ SD. The one-way ANOVA, followed by Dennett's test, was performed to uncover the significant differences between the parametric data. The non-parametric values (i.e., histopathological scores) were analyzed by the Mann-Whitney U-test. The critical level of significance was set at $p < 0.05$.

Results

Characterization of MSCs

The flow cytometric data revealed that the sub-culture 3 of the third generation of the adherent cells, isolated from the bone marrow was positive for the MSC markers of rat (CD29 and CD90), but negative for the marker of the hematopoietic cells (CD 45) (Figure 1).

In vitro monocyte evaluation

The education of monocytes with MCM showed to regress the potential of the respiratory burst and NO production by monocytes compared with non-educated monocytes (Figure 2A and B). Moreover, a significant decrease in pro-Th1 (IL-12) and pro-Th2 (IL-4) cytokines and a significant increase in anti-inflammatory IL-10 and TGF- β production were witnessed in the monocytes/macrophages, educated with MCM, compared to non-educated monocytes/macrophages (Figure 2C). The flow cytometry demonstrated that the MCM treatment significantly increased the levels of CD68⁺/CD206⁺ cells when monocytes/macrophages were cultured with MCM compared to non-educated monocytes/macrophages ($46.9\% \pm 2.1$ vs. $23.7\% \pm 2.98$, $p < 0.001$, Figure 2D).

Evaluation of cell therapy

The hallmark of OVA-induced asthma is a significant increase in OVA-specific IgE in serum and eosinophil infiltration in BALF (Barnes, 2015; Postma et al., 2015).

Ova-specific IgE

The in vivo analysis after cell therapy of asthma mice indicated that the total serum IgE concentrations in the OVA-inhaled mice, treated with educated monocytes/macrophages, were markedly lower than those OVA-sensitized, which received non-educated monocytes, or placebo (Figure 3A). The non-educated monocyte therapy decreased the IgE concentrations further than the OVA-inhaled mice without treatment, although there was no statistical difference (Figure 3A).

Assessment of BALF

On the other hand, the BALF was isolated and the cells in the BALF were counted, as presented in Figure 3B. As expected, few total cells and eosinophils were recognized in the PBS control group. It was realized that the number of infiltrating cells was increased after

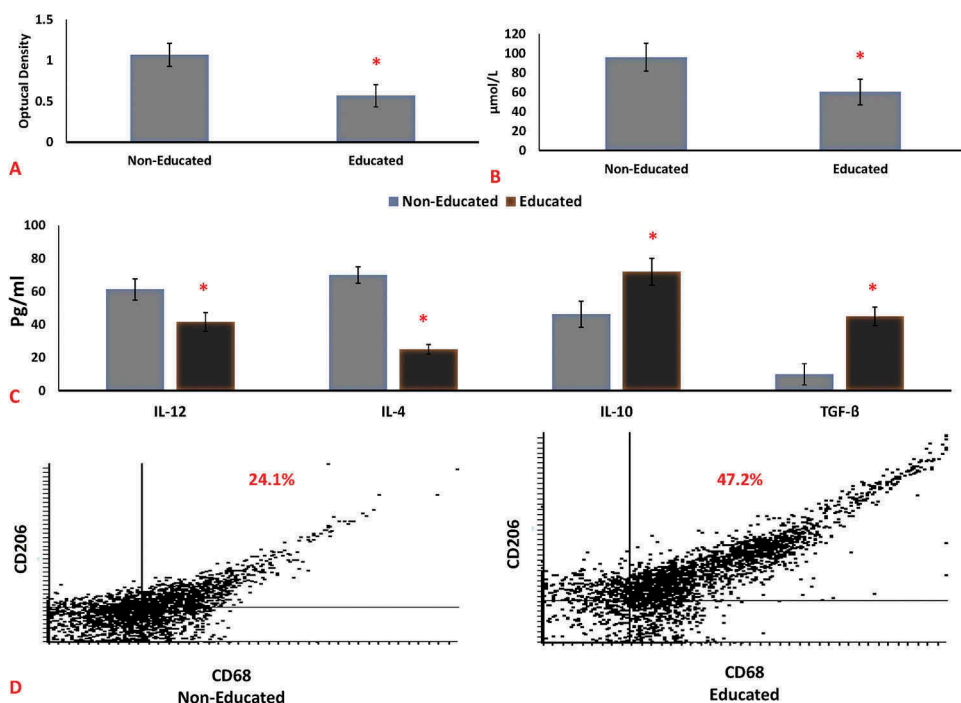


Figure 2. Modulation of monocytes activity after co-culture with MCM. Monocytes were cultured alone or cocultured with 50% of MCM. After 24 h, the cells were harvested and designated as “non-educated or educated” and applied for further experiments. (A) Evaluation of the respiratory burst of monocytes after challenge with TPA. (B) The potential of nitric oxide production by monocytes. (C) Effects of the conditioned medium of mesenchymal stem cells on cytokine production by monocytes. (D) Flow cytometry analysis of monocytes to detect the percentage of CD206⁺CD68⁺ macrophages. The experiments were carried out in five sets. Data were presented as mean ± SD (*P < 0.05 vs. non-educated monocytes).

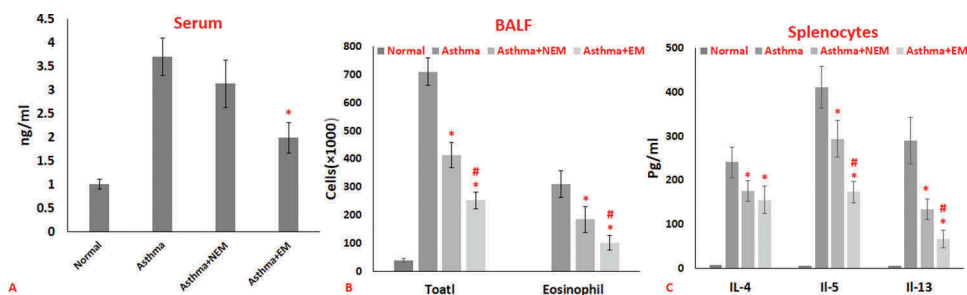


Figure 3. Assessment monocyte therapy in OVA-sensitized mice. (A) Evaluation of OVA-specific IgE. (B) Bronchoalveolar lavage fluid cell count. (C) Effects of drug treatment on OVA-specific cytokine production by splenocytes. Each group had 10 animals. The experiments were performed in triplicate sets. Values were presented mean ± SD (*P < 0.05 versus asthma mice received placebo; #P < 0.05 vs. asthma mice treated with non-educated monocytes). (EM: Educated monocytes; NEM: Non-educated monocytes.)

the OVA challenge in the OVA-sensitized mice. Interestingly, the total cell count and the eosinophil count in the BALF of the OVA-sensitized mice, treated with either educated or non-educated monocytes/macrophages, significantly decreased compared to the positive

control mice (Figure 3B). It is worth indicating that the total cell count and the eosinophil count were significantly decreased in the OVA-sensitized mice receiving educated monocytes/macrophages more than the OVA-sensitized mice receiving monocytes/macrophages without treatment (Figure 3B).

Ex vivo evaluation of the splenocytes

The ex vivo evaluation of the OVA-stimulated splenocytes disclosed a meaningful decrease in the production of Th2 cytokines (IL-4, IL-5, and IL-13) in cells from the cell-treated OVA-inhaled mice compared to the splenocytes of the asthma mice without treatment (Figure 3C). Of note, upon the antigen-specific re-stimulation, the asthma mice treated with educated monocytes/macrophages suppressed the levels of IL-5 and IL-13 more than those asthma mice treated with non-educated monocytes/macrophages (Figure 3C). The IL-4 concentration was at comparable levels between the OVA-sensitized mice receiving monocytes/macrophages without treatment or treated with the educated monocytes/macrophages (Figure 3C).

To further investigate the potential of monocyte therapy, we assessed the level of CD4⁺CD25⁺Foxp3⁺Treg cells in the splenocytes of mice. The evaluation method of the Treg population is shown in Figure 4A and B. Attained data showed that the treatment with educated monocytes/macrophages in mice sensitized with OVA significantly up-regulated Treg frequency compared to the vehicle-treated OVA-sensitized mice (Figure 4C). Treg cell frequency in splenocytes of OVA-sensitized mice was also observed an increasing trend in corresponding therapy with non-educated monocytes/macrophages, although there was no statistical difference (Figure 4C).

NF-κB extraction in the lung

The mRNA level of NF-κBp65 in lung tissues was significantly down-regulated in OVA-sensitized mice receiving cell therapy compared to vehicle-treated OVA-sensitized mice (Figure 5). It should be noted that the mRNA level of NF-κB p65 in OVA-inhaled mice

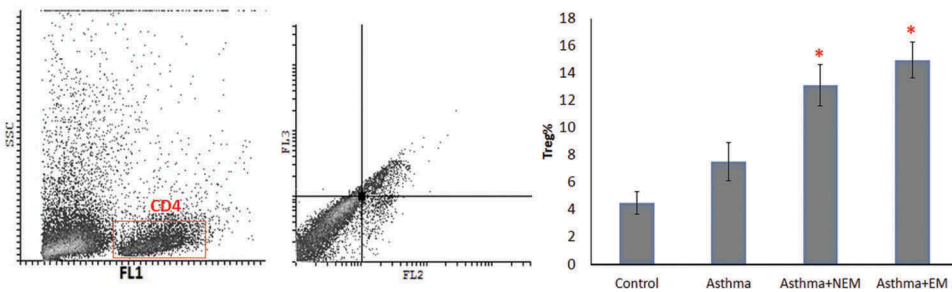


Figure 4. Comparison of regulatory T-cells spleen by flow cytometry. Representative density plots and histogram documented the regions and gates for immune phenotypic analysis. (A) CD4 lymphocytes were gated on a side scatter versus FL-1 density plot. (B) Then, the CD25⁺ FoxP3⁺ cells were analyzed on CD4⁺ gated cells. Each group had 10 animals. The experiments were performed in triplicate sets. The values were presented as mean $\pm$ SD (*P < 0.05 vs. asthma mice treated with non-educated monocytes). (EM: Educated monocytes; NEM: Non-educated monocytes; FL1, CD4⁺; FL2, CD25⁺; FL3, FoxP3⁺.)

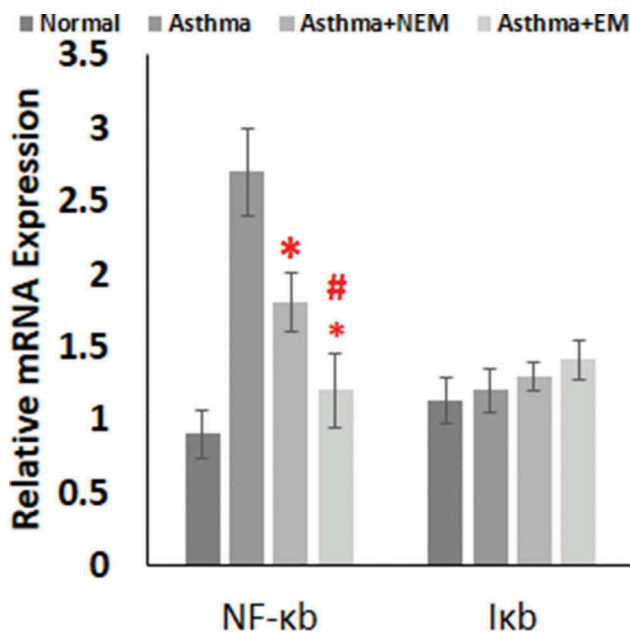


Figure 5. Relative mRNA expression in lung tissues. Each group had 10 animals. The experiments were carried out in triplicate sets. The results were shown as mean $\pm$ SD (* $P < 0.05$ vs. asthma mice received placebo; # $P < 0.05$ v. asthma mice treated with non-educated monocytes). (EM: Educated monocytes; NEM: Non-educated monocytes.)

treated with educated monocytes/macrophages was markedly lower than those of OVA-sensitized receiving non-educated monocytes (Figure 5). Nevertheless, the mRNA level of Ikb α showed no significant difference between the groups (Figure 5).

Histopathology

The representative fields in Figure 6 depicted the histopathological characters used for scoring the sections. The blind analyses specified that the degree of infiltration, localized primarily around the airway walls (bronchi and bronchiole), was significantly reduced in both of the cell-treated mouse groups compared to the PBS-treated OVA-sensitized mice (Figure 7). However, in the OVA-sensitized mice treated with the educated monocytes/macrophages, the lesions were significantly alleviated over the OVA-sensitized mice receiving monocytes/macrophages without any treatment (Figure 7).

Discussion

Cell therapy with MSCs has some limitation. For example, the efficacy of implanted MSCs is highly dependent on the environmental inflammatory conditions (Kim and Cho, 2015). Due to these possible limitations, we used MCM for the education of monocytes. Intravenous injection of monocytes is a valuable procedure for cell transplantation (Wagner et al., 2014). Previous documents showed that intravenously injected of monocytes can augment the collateral vessel growth in a rabbit model of arteriogenesis

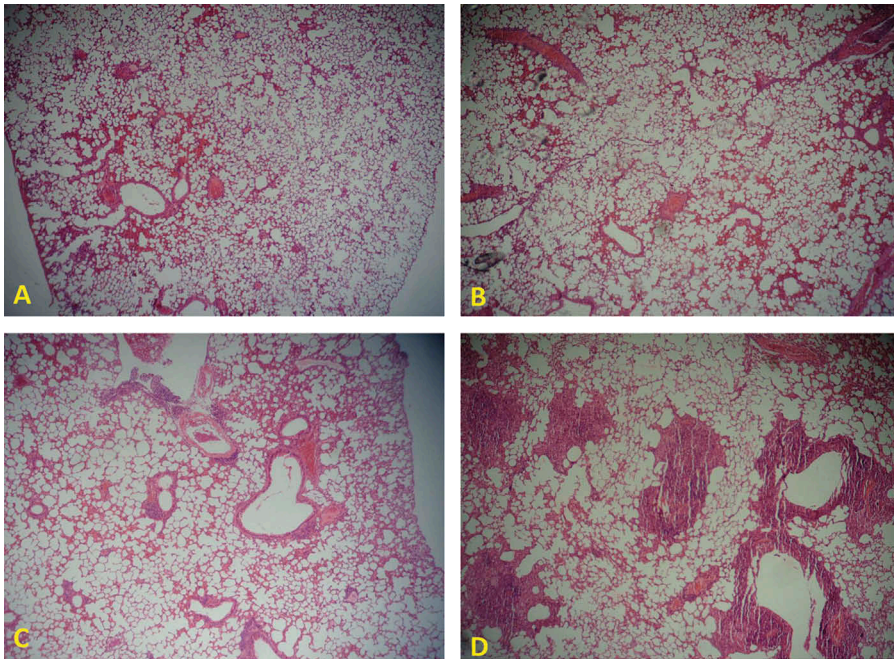


Figure 6. Representative sections depict scoring system used for histopathological examinations (X400). (A) Grade 0: no inflammation was observed; (B) Grade 1: inflammatory cells were occasionally observed; (C) Grade 2: most bronchi were surrounded by a thin layer of inflammatory cells; (D) Grade 3: most bronchi or vessels were surrounded by a thick layer of inflammatory cells.

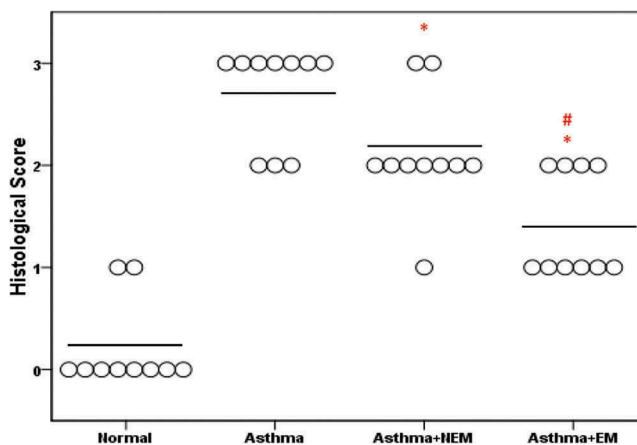


Figure 7. Results of blind analysis of histopathological sections. The sections were scored, as described in material and methods and Figure 6. Each point is the value of an individual mouse. The line drawn across each group indicates the mean (* $P < 0.05$ vs. asthma mice received placebo; # $P < 0.05$ vs. asthma mice treated with non-educated monocytes). (EM: Educated monocytes; NEM: Non-educated monocytes.)

(Herold et al., 2004). Also, the perfusion of bone marrow-derived monocytes into BALB/c mice 24 h after femoral artery ligation can augment the arteriogenesis in a murine model of femoral artery ligation (Herold et al., 2004; Wagner et al., 2014). Interestingly, it has

been reported that the intravenous injection of monocytes can transmigrate across the intact blood–brain barrier and into the CNS (Wu et al., 2006). Accordingly, we used the intravenous route for monocyte therapy.

OVA-induced asthma in mice is an animal model of human bronchial asthma, including various inflammatory cells like mastocytes, eosinophilic granulocytes, and T lymphocytes (Nials and Uddin, 2008). During the inflammation, monocytes/macrophages are recruited to the site of damage. Nowadays, it is clear that macrophages, like alveolar macrophages, may undergo reprogramming resulting in the emergence of a variety of different functional phenotypes (Cho et al., 2014; Mansouri Motlagh et al., 2015). Classically activated macrophages or M1 macrophages possess potent antimicrobial and inflammatory properties and may contribute to protection against invading pathogens, whereas alternatively activated macrophages or M2 macrophages possess less pro-inflammatory mediators, and have a pivotal role in the resolution of inflammation via secretion of trophic factor and higher phagocytic activity (Jiang and Zhu, 2016; Mansouri Motlagh et al., 2015; Pannell et al., 2016). Recent research suggests that M2 macrophages should be classified into alternatively activated cells (M2a), type II alternatively activated cells (M2b), or acquired deactivated cells (M2c), and M2d cells (Jiang and Zhu, 2016). Among M2 subtypes, M2c cell subtype possesses the highest impact on the termination and resolution of the inflammatory condition like asthma (Lu et al., 2013; Tang et al., 2017; Verreck et al., 2004). Interestingly, it has been shown that the MSCs (similar to the method used in this research for the education of monocytes) and regulatory T lymphocyte via production of IL-10 and indoleamine 2,3-dioxygenase, can promote the formation of M2c macrophages (Jiang and Zhu, 2016). A very recent study indicated that a single intravenous injection (10^6 cells) of both M2a (induced by IL-4 and IL-13) and M2c (induced by IL-10 and TGF- β) could suppress lung inflammation and injury in acute lung injury induced by LPS. Albeit, M2c macrophages more effectively could reduce lung injury and fibrosis than M2a macrophages (Tang et al., 2017). Our results indicate that twice intravenous injection of monocytes/macrophages educated with xenogeneic MCM (10^6 cells) can significantly regress the lung inflammation induced by OVA-sensitization.

The co-expression of CD68 and CD206 is the well-known marker for M2 macrophages gating in flow cytometric analysis (Hu et al., 2016). CD68 is a glycoprotein that binds to low-density lipoprotein and expressed on monocytes/macrophages (Holness and Simmons, 1993). CD206 (mannose receptor) has an important function in the promotion of higher phagocytosis and clearance capacities of M2 macrophages than M1 cells (Chang et al., 2015; Mansouri Motlagh et al., 2015). In this regard, the obtained data has demonstrated that the levels of CD68⁺/CD206⁺ cells were significantly increased in the MCM treated monocytes compared to non-educated ones.

It is clear that the overexpression of reactive oxygen species (ROS) and nitrogen species (such as NO) can participate in the pathogenesis of inflammatory conditions (Mansouri Motlagh et al., 2015). The gained data revealed that the supernatants of the MSCs profoundly suppressed the production of potentially harmful ROS and NO by educated cells.

IL-12, IL-4, IL-10, and TGF- β are the most polarizing cytokine that produced by antigen presenting cells and affect the T cells (Muranski and Restifo, 2013). Obtained data showed that the education of monocytes with MCM led to a significant decrease in pro-Th1 (IL-12) and pro-Th2 (IL-4) cytokines and a significant increase in anti-inflammatory

IL-10 and TGF- β production compared to cytokine profiles generated by non-educated monocytes. Previous data indicated that M2 macrophages, especially M2c macrophage, release a large amount of IL-10 and TGF- β to promote regulatory T cell and terminate Th2 inflammation (Jiang and Zhu, 2016; Tang et al., 2017). Our work also showed that the educated monocytes produce a significantly higher level of anti-inflammatory IL-10 and TGF- β compared to non-educated monocytes.

It is clear that the cytokine milieu induced by infiltrating OVA-specific CD4⁺ T lymphocytes into the lung has a pivotal role in determining the kind and extent of the histopathology of asthma (Barnes, 2015; Postma et al., 2015). Multiple documents indicated that OVA-induced asthma in mice and bronchial asthma mediated by excessively polarized Th2 cytokine milieu like IL-4, IL-5, and IL-13 (Busse et al., 2004; Nials and Uddin, 2008). Th2 cytokines participate in IgE production, eosinophil recruitment and activation, neutrophil and monocyte invasion to tissues, fibrosis and excess mucus production (Neamati et al., 2013). For example, IL-5 is a major regulator of eosinophil accumulation in the lung with asthma. The IL-5 also regulate eosinophil behavior at every stage from maturation to survival (Greenfeder et al., 2001). Allergen-specific IgE plays a pivotal role in initiation and propagation of asthma via sensitization and activation of mast cells and basophils in allergen re-exposure (Hirose et al., 2017; Kubo, 2017). Interestingly, the cell therapy with educated or non-educated monocytes/macrophage could suppress the levels Th2 cytokines (IL-4, IL-5, and IL-13) in splenocytes and eosinophils count in the BALF of OVA-inhaled mice. However, the levels of IL-5 and IL-13 in splenocytes, IgE levels and eosinophil count in the BALF of OVA-inhaled mice receiving educated monocytes/macrophage were significantly lower than the levels of these cytokines in OVA-inhaled mice received non-educated cells. Moreover, it has been shown that FoxP3⁺Treg cells possess a crucial role in limiting or terminating the pathology induced by Th2 or Th1 cells (Raedler et al., 2015; Zhu et al., 2017). In this regard, it has been shown that the percentage of the CD4⁺CD25⁺Foxp3⁺ Treg lymphocytes was significantly lower in the mice with bronchial asthma than that in the normal control group (Zhu et al., 2017). Our data suggest that the cell therapy with educated monocytes caused a significant up-regulation of regulatory T cells in splenocytes population probably via secretion a large amount of IL-10 and TGF- β .

NF- κ B is a well-known potent inflammatory transcription factor that controls transcription of IL-1, IL-6, IL-4, IL-5, IL-13, and IL-17. On the other hand, these cytokines can potentiate the activation of the NF- κ B pathway (Li et al., 2016). Previous documents report that the levels of NF- κ B were upregulated in the lung tissues of the asthmatic individuals (Lai et al., 2013) and the intranasal administration of NF- κ B decoy oligodeoxynucleotides could suppress the allergic response in OVA-sensitized mice (Wee et al., 2017). Based on our data, the educated monocytes/macrophages could significantly down-regulate the mRNA level of NF- κ B in OVA-inhaled mice compared to other groups. I κ B α inhibits NF- κ B by masking the nuclear localization signals and keeping them sequestered in an inactive form in the cytoplasm (Li et al., 2016). Nevertheless, the mRNA level of I κ B α showed no significant difference between groups.

In conclusion, similar to some previous research (Geng et al., 2014; Hu et al., 2016; Magatti et al., 2017; Anderson et al., 2013), our results indicated the co-culture of monocytes with conditioned media of MSCs could polarize the cells toward M2 anti-inflammatory phenotype. Notably, this study demonstrated for the first time that showed

the monocytes primed by conditioned media of xenogeneic MSCs can suppress OVA-induced asthma in mice efficiently, primarily through suppressing the Th2 pathway and Treg induction. The most important advantages of educated monocytes are the reduction in the levels of OVA-specific IgE production, mRNA level of NF- κ B, and IL-5 and IL-13 cytokines more than the reduction of these factors by non-educated monocytes. It is likely that the further reduction of these factors by educated monocytes led to the more significant beneficial effects (includes the further reduction in the degree of histopathological damage and infiltration of eosinophils into the BALF) compared to treatment with non-educated monocytes. However, other mechanisms may also be involved in the benefits of this therapy, and these remain to be clarified. Altogether, this approach may be as a promising strategy for cell-based therapies of asthma.

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