

The genes involved in asthma with the treatment of human embryonic stem cell-derived mesenchymal stem cells

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ABSTRACT

Background: Asthma is affecting more than 300 million people worldwide, which represents the most common chronic disease among children. We previously found that mesenchymal stem cells (MSCs) derived from induced pluripotent stem cells (iPSCs) modulated the immune response on Th2-mediated asthma *in vivo* and *in vitro*. This study further evaluated the immunomodulatory effects of MSCs from human embryonic stem cells (hESCs) on asthma.

Methods: Multipotent hESC-MSCs were obtained using a feeder-free method. The hESC-MSCs were analysed for the expression of stem cell surface markers by flow cytometry, their differentiation potentials were analysed using *in vitro* trilineage differentiation methods hESC-MSCs were transplanted into the murine model with ovalbumin (OVA)-induced airway allergic inflammation. The expression levels of allergic related genes were measured by the mRNA PCR arrays.

Results: The hESC-MSCs expressed classical MSC markers and held the capability of differentiation into multiple mesoderm-type cell lineages. hESC-MSCs were able to suppress allergic inflammation by modulating Th2 cells and eosinophils in the mice, and reversed the reduction of regulatory T cells. By using PCR array, 5 mRNAs-chemokine (C-C motif) ligand 11 (*Ccl11*), *Ccl24*, interleukin13 (*Il13*), *Il33* and eosinophil-associated, ribonuclease A family, member 11 (*Ear11*) were identified the most relevant in murine airway allergic inflammation and hESC-MSCs treatment.

Conclusions: The therapeutic effects of hESC-MSCs were identified in the murine model of airway allergic inflammation with key mRNAs involved. This study will provide a better understanding regarding the mechanisms underlying hESC-MSCs therapeutic application in airway allergic inflammation.

1. Introduction

Asthma is a chronic, reversible airway disease that affecting more than 300 million people worldwide and causing substantial medical and financial burdens (Martinez and Vercelli, 2013; Asher and Pearce, 2014). Most allergic asthma have common characters such as high serum immunoglobulin E (IgE) antibodies, increased cellular

infiltration and excessive activation of type 2 T helper (Th2) cells (Wills-Karp, 1999). The amount of mRNA and protein expression in the related cells have also changed with the progress of asthma (Pascoe et al., 2015; Plager et al., 2010). For example, Th2-associated cytokines including IL-4, IL-5 and IL-13 play a key role in asthma occurrence (Steinke and Borish, 2001; Deo et al., 2010) and the increase of programmed cell death 1 (PDCD1) can reduce the activation, proliferation

Abbreviations: APC, allophycocyanin; aMEM, medium eagle alpha modification; ANOVA, one-way analysis of variance; BALF, bronchoalveolar lavage fluid; bFGF, basic fibroblast growth factor; BM-MSC, bone marrow-derived mesenchymal stem cell; Ccl, chemokine (C-C motif) ligand; CD, cluster of differentiation; CGH, comparative genomic hybridization; Ear11, eosinophil-associated, ribonuclease A family, member 11; EGF, epidermal growth factor; ELISA, enzyme-linked immunosorbent assay; FACS, fluorescence activated cell sorting; FBS, fetal bovine serum; H&E, haematoxylin and eosin; hESCs, human embryonic stem cells; hESC-MSCs, human embryonic stem cells derived mesenchymal stem cells; IL, Interleukin; Ig, immunoglobulin; iPSC-MSCs, induced pluripotent stem cells derived mesenchymal stem cells; iPSCs, induced pluripotent stem cells; MSCs, mesenchymal stem cells; NEAA, non-essential amino acid; OVA, ovalbumin; PAS, periodic acid-Schiff; PBS, phosphate buffer; PCR, polymerase chain reaction; PDCD1, programmed cell death 1; PE, phycoerythrin; SNP, single nucleotide polymorphism; Th2, type 2 helper T cell; Treg, T regulatory; WGS, whole genome sequencing

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and the survival of T cells (Davies et al., 2016). Therefore, targeting the genes involved in asthmatic responses and immunomodulation would be helpful for further asthma treatment.

Mesenchymal stem cells (MSCs) show the rosy prospect in clinical therapeutic application owing to their differentiation potential (Fehrer and Lepperdinger, 2005) and immunomodulatory properties. MSCs have been found to be able to modulate the immune response in allergic diseases via both soluble factors and cell–cell contact (Shi et al., 2010; Keating, 2012). MSCs can increase the phagocytosis of neutrophils by inducing Th2 cells secreting IL-17 (Duffy et al., 2011). Not only inhibit the activation and the proliferation of T cells, MSCs also inhibit the differentiation of Th1 and Th17 cells with the promotion of Treg cells (Luz-Crawford et al., 2013a). MSCs can promote the Ig secretion of B cells *in vitro* and *in vivo*. Furthermore, animal studies and clinical trials have demonstrated that MSCs hold great potential in clinical applications for the treatment of immune-related diseases (Goodwin et al., 2011; Xiao et al., 2012).

However, there are several inherent defects such as senescence (Giuliani et al., 2011), quality variation (Galipeau, 2013) and genomic mutation (Wang et al., 2013) exist in MSCs derived from bone marrow or other adult tissues, hindering their regeneration efficient for standard clinical applications (Law and Chaudhuri, 2013). In contrast, according to previous studies, MSCs derived from human embryonic stem cells (hESC-MSCs) or induced pluripotent stem cells (iPSC-MSCs) not only showed similar biological characteristics to bone marrow mesenchymal stem cells (BM-MSCs) including surface markers, multilineage differentiation and immunomodulation, but also had higher proliferation, regenerative capacities and less heterogeneity (Frobel et al., 2014; Lian et al., 2007).

Pluripotent stem cells potentially offered an unlimited source for functional and qualified MSCs generation. However, iPSCs after reprogramming show genetic instability and tumorigenicity *in vivo* (Barrilleaux and Knoepfler, 2011; Tan et al., 2014). To meet the safety standards, it would be necessary for iPSCs to finish some complex and expensive evaluations such as whole genome sequencing (WGS) and comparative genomic hybridization (CGH) single nucleotide polymorphism (SNP) analysis before clinical application. Over the disadvantages of iPSCs, ESCs will be more suitable for clinical large-scale applications.

Additionally, we have demonstrated that the treatment of iPSC-MSCs significantly suppressed the pathology and permitted a re-balance in the immune response in a mouse model of airway allergic inflammation (Sun et al., 2012). Therefore, this study will continue investigating the role of ESC-MSCs treatment in murine airway allergic inflammation model. In this study, we developed an efficient method for generating hESC-MSCs, the effects of hESC-MSC administration were then investigated in a mouse airway allergic inflammation model. By using PCR array, the genes involved in the process of hESC-MSCs immunomodulation were identified.

2. Materials and methods

2.1. Cell culture

H9 human embryonic stem cells (hESCs) obtained from WiCell (Madison, Wisconsin) were cultured as previously described (Lian et al., 2007; Sagar et al., 2014). When the cells reached 60% confluence in the Matrigel-coated 6-well-plate, the hESCs culture medium was replaced by fresh MSCs differentiation medium comprised of Medium Eagle Alpha Modification (aMEM) medium (Gibco, Gaithersburg, MD), 10% fetal bovine serum (FBS) (Gibco), 50 μ mol/L-ascorbate-2-phosphate (Gibco), 100 IU/mL penicillin (Gibco), 100 μ g/mL streptomycin (Gibco). After 12 days culture in the MSCs differentiation medium that was changed every day, the cells were harvested with Accutase™ (Innovative Cell Technologies, San Diego, CA) and seeded onto 0.1% gelatin-coated 6-well-plate with MSCs differentiation medium. The cells

were defined as hESC-MSCs passage 0 (P0). When the cells reached 80–90% confluence, they would be trypsinized and seeded onto non-coated T25 flasks containing MSCs growth medium comprised of Dulbecco's modified eagle medium (DMEM)/High Glucose (Gibco), 10 % FBS (Gibco), 100 μ M Nonessential amino acids (NEAA) (Gibco), 10 ng/mL Human basic fibroblast growth factor (bFGF) (Gibco), 1 ng/mL Human epidermal growth factor (EGF) (Gibco), 55 μ M 2-mercaptoethanol (Gibco), 100 IU/mL penicillin (Gibco), 100 μ g/mL streptomycin (Gibco) (defined as hESC-MSCs P2). These MSC-like cells were continued culturing with MSCs growth medium in feeder-free condition and passaged at 1:4 splits every 3 days when the clones reach an optimal density. The differentiation of hESCs has been performed three times to evaluate the robustness of the protocol.

2.2. Flow cytometry analysis of cell surface marker

Flow cytometry analysis of hESC-MSCs surface antigen expressions was carried out with P4 and P8 hESC-MSCs. The cells were harvested and resuspended to 5×10^5 cells in 100 μ L FACS buffer. The cells were incubated with phycoerythrin (PE)-conjugated mouse anti-human Cluster of differentiation (CD)73, CD90, CD166, PerCP-Cy5.5-conjugated mouse anti-human CD44, CD146 and allophycocyanin (APC)-conjugated mouse anti-human CD45, CD105 monoclonal antibodies on ice for 30 min respectively (BD Biosciences, San Jose, NJ). All antibodies were diluted 1:20 for incubation according to the manufacturer's instructions. Then, 2.5×10^5 cells in 400 μ L FACS buffer were analysed using the BD FACSCanto flow cytometer (BD Biosciences, NJ). All data were analysed using FlowJo v10.0.7 software (Tree Star, Ashland, OR).

2.3. Multilineage differentiation of hESC-MSCs

The differentiation potential of hESC-MSCs was assessed using the OriCell™ Adipogenesis, Osteogenesis and Chondrogenesis Kit (Cyagen Biosciences, Shanghai, China). All procedures were carried out according to manufacturer's instruction. Oil Red O staining analysis, Alizarin Red staining analysis and Alcian Blue staining analysis were used to validate hESC-MSCs adipogenic differentiation, osteogenic differentiation and chondrogenic differentiation, respectively.

2.4. Animals

Female BALB/c mice (4–6 weeks) were purchased from the Guangdong Medical Laboratory Animal Centre (Guangzhou, China). All procedures were performed in accordance with the Guidelines for Animal Experiments and using Committee (Approval No. SCXK 2013-0002) approved by The University Animal Ethics Committee of Sun Yat-sen University Institutional Animal Care.

2.5. Mouse airway allergic inflammation model and the transplantation of hESC-MSCs

The mouse airway allergic inflammation model was established by sensitization and challenge with ovalbumin (OVA, grade V, Sigma, St. Louis, MO) according to previous descriptions with minor modification (Sun et al., 2012). Briefly, 40 μ g OVA/mouse for the sensitization and 5% OVA in phosphate-buffered saline (PBS) for the challenge were employed to induce allergic airway inflammation in the mouse. The hESC-MSCs (P7 or P8) were suspended in sterile PBS at a density of 5×10^6 cells per ml and 0.2 ml of the cell suspension was intravenously injected via the tail vein on day 20 before the challenge (Supplementary Fig. 1). An equal volume of PBS was injected as the control. Mice were divided into control, model and treatment three groups: Control group (PBS/PBS/PBS) that mice were sensitized and challenged with PBS, then injected with PBS on day 20 ($n = 19$); Model group (OVA/OVA/PBS) that mice were sensitized and challenged with OVA, then treated with PBS on day 20 ($n = 17$); hESC-MSCs treatment group (OVA/OVA/

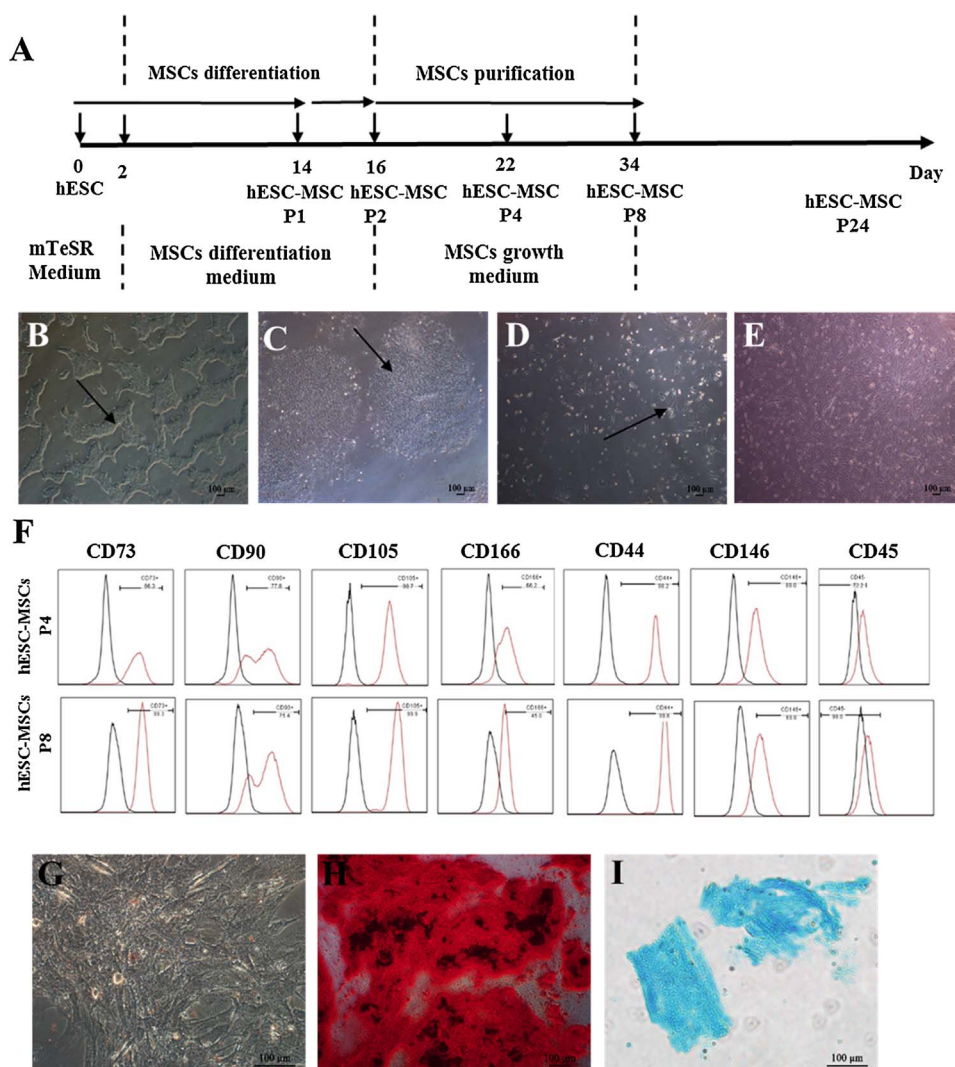


Fig. 1. Derivation of MSCs from hESCs. **A**, Schematic illustration of differentiating hESCs into MSCs; hESC colony (arrowhead) with smooth margin (**B**); Within 2–3 days, hESCs (arrowhead) began forming epithelial foci (arrow) (**C**); during the period of MSCs enrichment, the entire culture presented an epithelial phenotype mixed with various forms of cells (**D**); The entire culture presented MSCs like cells (**E**); FACS surface antigen profiling of CD73, CD90, CD105, CD166, CD44, CD146, CD45 on P4 and P8 hESC-MSCs (**F**); G–I, multi-lineages differentiation potential validation of hESC-MSCs; Oil Red O staining for adipogenesis (**G**); Alizarin red staining for osteogenesis (**H**) and Alcian blue staining for chondrogenesis (**I**). Abbreviations: hESC, human embryonic stem cells; MSCs, mesenchymal stem cells; hESC-MSCs, embryonic stem cells derived mesenchymal stem cells; CD, Cluster of differentiation. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

hESC-MSC) that mice were sensitized and challenged with OVA, then treated with hESC-MSCs on day 20 ($n = 19$).

2.6. Histology analysis and inflammation scoring

Four hours after the last challenge, the left lung tissues along main stem bronchus collected from mice were fixed in 4% neutral formalin for 24 h. After the routine tissue process, the paraffin-embedded lung tissues were sectioned at 15 consecutive fields with a thickness of 3 μ m. Haematoxylin and eosin (H&E) and periodic acid–Schiff (PAS, Baso Diagnostics Inc., Zhuhai, Guangdong, China) staining were used to stain the sections. Goblet cell (PAS positive cell) counts and inflammation scores in the lungs were then evaluated as previously described (Sun et al., 2012).

2.7. Bronchoalveolar lavage fluid

Bronchoalveolar lavage fluid (BALF) was collected by pressing 1 ml PBS into the lung through trachea 4 h after the last challenge. The collected BALF was centrifuged at 700 g for 8 min at 4 °C and the cell pellet was then resuspended in fresh PBS. For the differential cell counts, Diff-Quick staining (Baso Diagnostics Inc., Zhuhai, Guangdong, China) was used to evaluate for eosinophils, macrophages, neutrophils, and lymphocytes which spun on a glass slide. The levels of IL-4, IL-5 and IL-13 in the supernatant were measured by enzyme-linked immunosorbent assay (ELISA) analysis following the manufacturer's

instructions (R&D Systems, Minneapolis, MN). If not measured at once, the BALF collected from the mice was kept frozen at -80 °C for future measurement.

2.8. Treg cells measurement

Splenocytes were isolated from the mice of all the three groups 4 h after the last challenge using Percoll solution (Sigma, St. Louis, MO). After staining with the Mouse Regulatory T cell staining kit (eBioscience, San Diego, CA) including FITC-anti-CD4 (0.125 μ g/test), APC-anti-CD25 (0.06 μ g/test), PE-anti-FOXP3 (0.5 μ g/test) antibodies, the splenocytes were analysed with BD FACSCanto flow cytometer to see whether hMSC-ESC treatment could promote Treg cells.

2.9. OVA-specific immunoglobulin (Ig) analysis

The sera of the sacrificed mice were obtained from the collected blood using centrifugation at 600 g for 10 min. OVA-specific IgE, IgG1 and IgG2a levels in serum were then measured using commercially available ELISA kits according to manufacturer's instructions. If not measured at once, the serum collected from the supernatant were kept frozen at -80 °C for future measurement.

2.10. The mRNA PCR arrays

Right lung tissues in mice from control, model and hESC-MSC

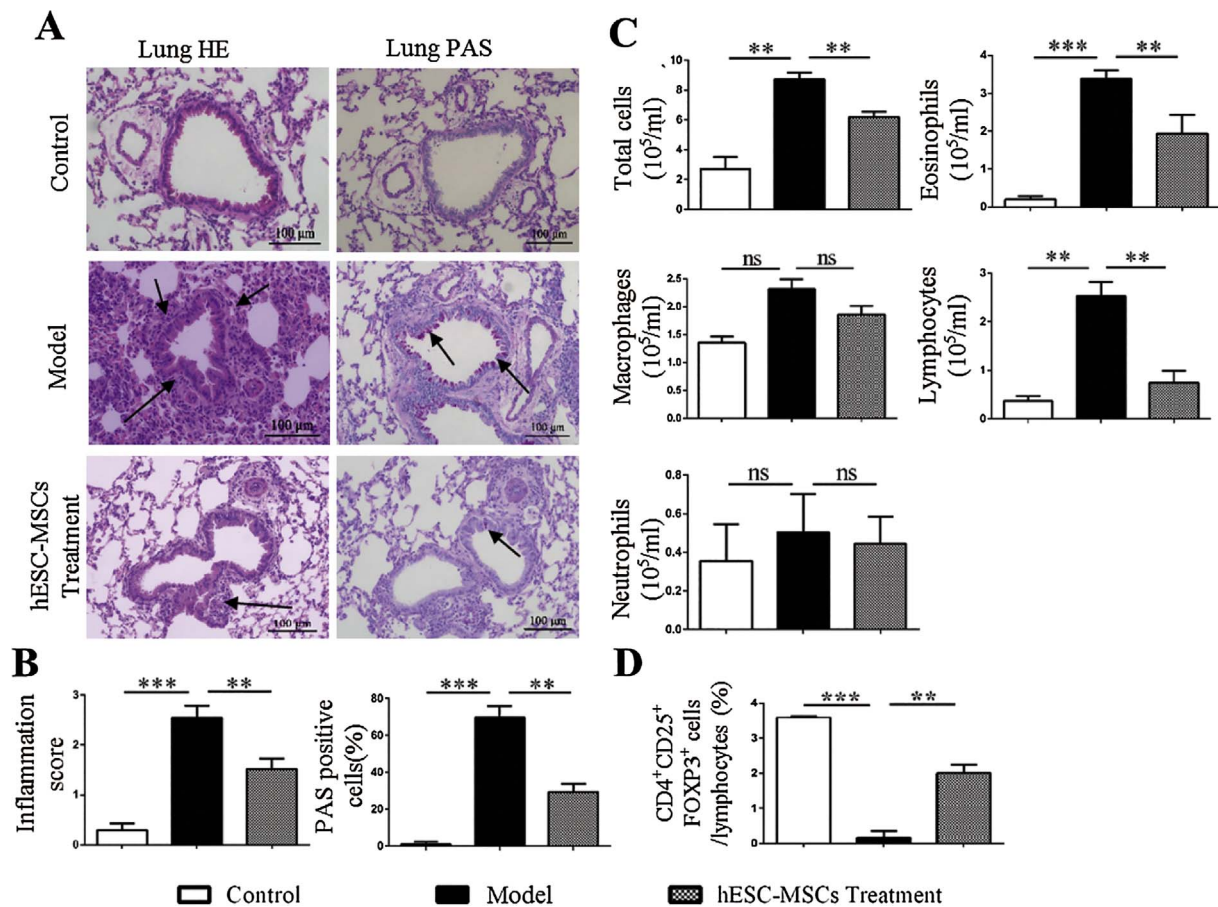


Fig. 2. Administration of hESC-MSCs attenuates OVA-induced airway allergic inflammation in the lung, and upregulates Treg cells. **A.** Representative photomicrographs of H&E and PAS stained lung sections from each group, inflammatory infiltrates (lung H&E, shown by arrows) and mucus accumulation at the luminal surface of the bronchi (lung PAS staining, shown by arrows) were significantly increased in model group (original magnification: $\times 200$); **B.** Statistical analysis for inflammation score and mucus hypersecretion as quantified by H&E and PAS scores respectively; **C.** Statistical analysis of total cells, macrophages, eosinophils, lymphocytes and neutrophils counts in the collected BALF from each group; **D.** Statistical analysis of CD4⁺CD25⁺FOXP3⁺ Treg cell percentages in the lymphocytes from spleen with different treatments. All data were presented in Mean $\pm$ SEM. *, $p < .05$; **, $p < .01$; ***, $p < .001$. Abbreviations: H&E, hematoxylin and eosin; PAS, Periodic Acid-Schiff; hESC-MSCs, embryonic stem cells derived mesenchymal stem cells.

treatment groups ($n = 6$ for each group) were collected for the mRNA PCR arrays. Total RNA was isolated with Trizol reagent (Invitrogen, Paisley, UK) and cDNA was synthesised with a cDNA synthesis kit (Exiqon, Vedbaek, Denmark). The synthesised cDNA from all groups were evaluated by a custom-made mRNA PCR Array (SA Biosciences, Frederick, MD, USA) including 11 different pathways or catalogues which related to allergy. All allergic airway inflammation or asthma-related genes used in the PCR array were summarised mainly according to our previous study (Tang et al., 2016). The threshold for up- and down-regulation of the genes change between different groups was set as fold change ≥ 1.5 , with p value $\leq .05$. These differentially expressed mRNAs were submitted to hierarchical clustering with the Cluster Treeview software from Stanford University (Palo Alto, CA, USA).

2.11. Statistical analysis

The experimental data were presented as mean $\pm$ SEM. All statistical analysis was performed with the SPSS software. One-way analysis of variance (ANOVA) followed by post-hoc Tukey (for equal homogeneity) or Dunnett T3 (for unequal homogeneity) test was used for multiple comparisons between different groups ($p < .05$ was considered statistically significant).

3. Results

3.1. Derivation of MSCs from hESCs

We designed a two-stage protocol for MSCs generation from hESCs (Fig. 1A). The first stage called MSCs differentiation, hESCs has grown on the Matrigel-coated dish were incubated with MSCs differentiation medium for 14 days during this stage. Within 2–3 days, the hESCs gradually showed morphology alteration starting with the edge of the colonies. Those cells exhibited fibroblastic cellular morphology and increased cytoplasm, they also accompanied with abundant cell death at the early stage. With the extension of incubation time, the cells presented an increasing of the epithelial phenotype similar to that of BM-MSCs in the dish, cell death was decreased at the same time (Fig. 1B–D). On day 16, MSCs differentiation medium was replaced by MSCs growth medium, the culture presented MSCs like cells were defined as passage 1 (P1) hESC-MSCs (Fig. 1E). These hESC-MSCs were maintained in continuous culture for purification at 1:4 splits every 3 days till P8. They were able to be cultured for more than 24 passages, P4–P8 hESC-MSCs were used for the following experiments,

3.2. hESC-MSCs characteristics

The surface markers of hESC-MSCs were assessed using FACS analysis. Both P4 and P8 hESC-MSCs were assessed. According to flow cytometry analysis, hESC-MSCs from both passages could be defined

qualitatively similar to that of BM-MSCs. Vast majority of the cells strongly expressed the classical MSC markers CD73 (> 96.3%), CD90 (> 75.4%), CD105 (> 99.7%), they also positive for other known MSC markers CD166 (> 45.0%), CD44 (> 98.2%), CD146 (> 89.0%), whereas negative for haematopoietic stem cell marker CD45 (< 8.0%) (Fig. 1F). Interestingly, P8 hESC-MSCs expressed higher percentages of MSC markers such as CD73, CD105 and lower negative MSC markers CD45 compared with those of P4 hESC-MSCs. Which probably because P8 hESC-MSCs were much more purified than P4 cells.

When incubated in standard adipogenic medium, the adherent hESC-MSCs were remarkably adipogenic, the differentiated monolayer lipid-containing MSCs were generated within 4 weeks (Fig. 1G). The adhered cells were also able to generate monolayer of mineralizing osteoblasts (Fig. 1H) and micro-mass culture cartilages (Fig. 1I). The hESC-MSCs met the standard criteria of MSCs (Dominici et al., 2006), they expressed classical MSC markers and showed the capability of differentiation into multiple mesoderm-type cell lineages *in vitro*, indicating therefore that we have successfully developed a protocol to derive MSCs from hESCs.

3.3. hESC-MSCs reduced airway inflammation

OVA-induced asthmatic model mice were used to evaluate the effects of the systemic hESC-MSCs administration on allergic inflammation. hESC-MSC treatment showed a significant inhibition of peribronchial and perivessel inflammation (H&E staining), mucus secretion from hyperplastic goblet cells (PAS staining) in the lungs (Fig. 2A). The inflammation scores (HE staining) and PAS positive cells counts (PAS staining) in the hESC-MSC treatment group were ~2-fold decreased compared to those in the model group (Fig. 2B, $p < .01$). The eosinophils, lymphocytes number and the total inflammatory cell numbers were reduced in the hESC-MSC treatment group compared with those in model group, as which demonstrated in the BALF. Whereas more macrophages numbers were observed in control and hESC-MSC treatment groups, and no significant difference of neutrophil numbers was observed among all groups (Fig. 2C & Supplementary Fig. 2, $p < .01$). In addition, splenocytes were isolated from each group of the mice to examine if hESC-MSC treatment could promote Treg cells. The percentages of CD4⁺CD25⁺FOXP3⁺ Treg cells in lymphocytes were significantly decreased in the model group, while hESC-MSC treatment promoted the percentages of Treg cells even in the presence of OVA challenge according to the flow cytometry results (Fig. 2D & Supplementary Fig. 3, $p < .01$). It was reported that eosinophils were acting as professional antigen-presenting cells in murine models of airway inflammation (Wang et al., 2007). hESC-MSCs in this study, therefore, might suppress airway allergic inflammation by modulating eosinophils together with lymphocytes.

Excessive activation of Th2 cells was found in allergic airways diseases and always accompanied by the rise of Th2-associated cytokines including IL-4 and IL-13 (Wills-Karp, 1999). In this study, hESC-MSC treatment significantly decreased these Th2 cytokine levels in the BALF compared to those in the model group (Fig. 3A, $p < .01$). Th2-specific immunoglobulin concentrations in the sera were also found to be different between the model group and hESC-MSC treatment group. Serum OVA-specific IgE secretion level and the ratio of serum OVA-specific IgG1/IgG2a in the hESC-MSC treatment group were significantly lower than those in model group, especially serum OVA-specific IgE (Fig. 3B, $p < .001$). IL-4 secreted by Th2 cells can promote the synthesis of IgE, which was reduced following the decrease of IL-4 after the hESC-MSCs treatment. It should be noticed that the levels of the protective immune factor of IgG2a increased after the induction of asthma and increased higher while no significant change of IgG1 can be observed after the hESC-MSC treatment (Fig. 3B). The results confirmed the hESC-MSC immunomodulatory effects in airway allergic inflammation by the down-regulating Th2 response. Taken together, these data indicated that MSCs derived from hESCs were capable of alleviating murine

airway allergic inflammation, similarly to our previous study (Sun et al., 2012; Tang et al., 2016).

3.4. Aberrant mRNAs in the model and hESC-MSC treated mice

Protein-coding gene PCR array with 84 genes that covered most of the immunomodulatory factors and important proteins involved in allergic airway inflammation was performed to identify aberrant mRNAs in the model and hESC-MSC treated mice. A total of 22 genes were differentially expressed after the induction of airway allergic inflammation, with 7 mRNAs upregulated and 15 mRNAs down-regulated in model group compared with those in control group. After hESC-MSC treatment, 47 genes were observed differentially expressed, with 42 mRNAs upregulated and 5 mRNAs down-regulated in the hESC-MSCs treatment group compared with those in model group (Table 1). The differentially expressed genes were divided into 6 subgroups based on the comparisons between different groups. For instance, the *Tlr2*, *Ccl22*, *Ccl11*, *Ccl24*, *Il13*, *Il33* and *Ear11* were upregulated in the model group compared with the control group; *Tlr2* and *Ccl22* continued increasing to higher levels, and *Ccl11*, *Ccl24*, *Il13*, *Il33* and *Ear11* levels were decreased after the hESC-MSC treatment. Similar divisions were performed for all aberrant mRNAs.

The key mRNAs that presented reverse variation tendencies between model group and hESC-MSC treatment group should have more significance in exploring MSC-mediated airway allergic inflammation modulation. Therefore, hierarchical clustering was performed to analyse the differentially expressed genes. Systematic variations were found in the control, model and hESC-MSC treatment groups (Fig. 4A). The results further suggested that hESC-MSC treatment were able to alter the immune states in murine allergic airway inflammation model. We found that 5 mRNAs including *Ccl11*, *Ccl24*, *Il13*, *Il33* and *Ear11* expressed significant changes compared with the other genes. These 5 mRNAs showed the same tendency that increased after OVA induction and decreased after the hESC-MSC treatment. Further analysis demonstrated that these mRNA levels increase from 1.98 fold (*Ccl11*) to 135.69 fold (*Ear11*) after model induction, hESC-MSCs treatment was able to reduce 16% (*Il33*) to 62% (*Il13*) of their expression levels (Fig. 4B). The results indicated that these 5 mRNAs might be the potential targets of MSC immunomodulation in allergic airway inflammation.

4. Discussion

In the present study, we have derived multipotent MSCs from hESCs, which was demonstrated to be able to alleviate OVA-induced airway allergic inflammation. MSCs were successfully derived from hESCs simply in two stages without additional flow cytometric cell sorting, which requires technical challenging and may cause cell damage. The hESC-MSCs were able to suppress allergic inflammation by modulating eosinophils, Th2 cells and Treg cells *in vivo*. During hESC-MSC immunomodulation, 47 protein-coding mRNAs were identified differentially expressed. Hierarchical clustering analysis confirmed that 5 mRNAs including *Ccl11*, *Ccl24*, *Il13*, *Il33* and *Ear11* were significantly upregulated after model induction and down-regulated by hESC-MSC treatment. These mRNAs might be the potential targets of allergic reaction and MSC-mediated immunomodulation.

MSC immunomodulation was regarded as a novel therapeutic approach for a variety of immune-related diseases due to their anti-inflammatory and immune-privileged potential (Wang et al., 2012; Wang et al., 2016). However, MSC therapy has shown contradictory results, often with poor clinical outcomes (Kim and Cho, 2016). Probably because of the inconsistent characterization of MSCs from different donors. Unlike MSCs isolated from adult tissues, MSCs derived from pluripotent stem cells have a higher proliferative rate, being more consistent (Lin et al., 2012). As a result, MSCs derived from pluripotent stem cells can be homogeneous proliferated on a large scale for

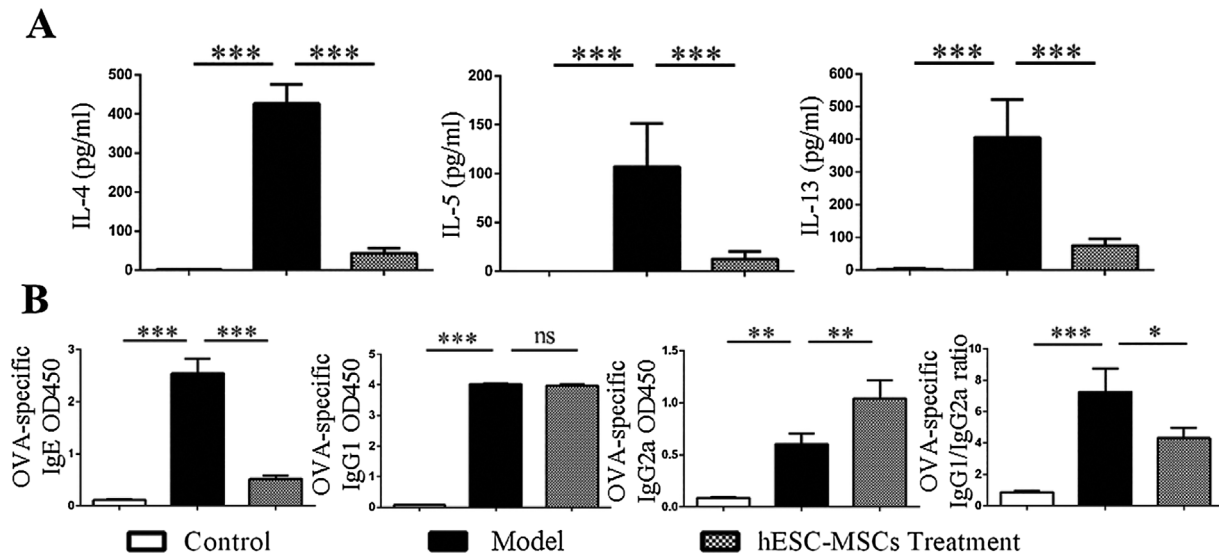


Fig. 3. Systemic administration of hESC-MSCs inhibited the production of inflammatory cytokines in the BALF and decreased Th2 related Ig levels in serum. Statistical analysis of IL-4, IL-5 and IL-13 levels in BALF (A); statistical analysis of OVA-specific IgE and the OVA-specific IgG1/IgG2a ratio in sera (B); the cytokines levels and Ig were measured using enzyme-linked immunosorbent assay, data were presented in mean $\pm$ SEM; *, $p < .05$; **, $p < .01$; ***, $p < .001$. Abbreviations: BALF, bronchoalveolar lavage fluid; IL: interleukin; Ig, Immunoglobulin; OD, optical density; hESC-MSCs, embryonic stem cells derived mesenchymal stem cells.

Table 1

PCR array results of inflammatory-related genes differentially expressed (fold change > 1.5) after asthma induction and after hESC-MSCs treatment.

Classify	M/C – hESC-MSCs/M $\uparrow$	M/C – hESC-MSCs/M $\downarrow$	M/C $\uparrow$ hESC-MSCs/M $\uparrow$	M/C $\uparrow$ hESC-MSCs/M $\downarrow$	M/C $\downarrow$ hESC-MSCs/M $\uparrow$	M/C $\downarrow$ hESC-MSCs/M $\downarrow$
Toll-Like receptors	<i>Tlr1, Tlr4, Tlr6, Tlr7, Tlr9</i>		<i>Tlr2</i>		<i>Tlr3, Tlr5</i>	
NFkB Signaling	<i>Rel</i>					
MAPK	<i>Mapk1, Mapk3, Mapk9, Mapk14</i>					
Janus Kinase Activity	<i>Jak3</i>					
STAT Family	<i>Stat1, Stat4, Stat5b, Stat6</i>					
TGF β Superfamily Receptors					<i>Acvr2a</i>	
Notch Pathway	<i>Notch1</i>					
Transcription Factors	<i>Foxp3, Rorc</i>				<i>Gata3</i>	
Th2 Cytokines and Related Genes	<i>Il10, Il12b, Il21, Ifng</i>		<i>Ccl22,</i>	<i>Ccl11, Ccl24, Il13, Il33</i>	<i>Il12a, Tslp</i>	
Mast Cells, IgE and Eosinophils	<i>Cpa3,</i>				<i>Cma1, Kit</i>	
Inflammatory Response Cytokine Receptors	<i>Gsk3b</i>					
Eosinophils				<i>Ear11</i>	<i>Itga4</i>	
Asthma	<i>Ltb4r1</i>				<i>Adam33, Adrb2</i>	
TH1 Cytokines and Related Genes					<i>Tbx21</i>	
Others					<i>Postn, Irs1, B2m</i>	

M/C: Model/Control; hESC-MSCs/M: hESC-MSCs treatment/Model; –: No obvious change; $\uparrow$: Raise, $p < .05$; $\downarrow$: Decline, $p < .05$. hESC-MSCs, human embryonic stem cells-derived cells-derived mesenchymal stem cells.

Gene description: *Acvr2a*, Activin A receptor, type II A; *Adam33*, A disintegrin and metalloproteinase domain 33; *Adrb2*, Adrenergic receptor, beta 2; *B2m*, beta-2-microglobulin; *Ccl*, Chemokine (C-C Motif) ligand; *Cma1*, Chymase 1, mast cell; *Cpa3*, Carboxypeptidase A3 (mast cell); *Foxp3*, Forkhead box P3; *Tlr*, Toll-like receptor; *Ifng*, Interferon gamma; *Il*, Interleukin; *Irs1*, Insulin receptor substrate 1; *Itga4*, Integrin alpha 4; *Gsk3b*, Glycogen synthase kinase 3 beta; *Jak*, Janus kinase; *Ltb4r1*, Leukotriene B4 receptor 1; *Postn*, Periostin osteoblast specific factor; *Rel*, V-rel avian reticuloendotheliosis viral oncogene homolog; *Rorc*, RAR-related orphan receptor C; *Stat*, Signal transducer and activator of transcription; *Mapk*, Mitogen-activated protein kinase; *Tslp*, Thymic stromal lymphopoietin; *Tbx21*, T-box 21.

facilitating rapid scale-up clinical application. In this study and our previous studies, we found that both hESC-MSCs and iPSC-MSCs could be used to treat the mouse with allergic airway inflammation, they were able to significantly inhibit murine airway inflammatory infiltration, decrease Th2 cytokines (IL 4, IL 5, IL 13), OVA-specific IgE levels in BALF/serum and increase IgG2a levels in serum. What's more, hESC-MSC treatment significantly increased the ratios of the CD4⁺CD25⁺FOXP3⁺ Treg cells, which were decreased after OVA challenge. The results were similar with previous study that the Treg cells were significantly increased in murine model of chronic asthma with the treatment of mice compact bone-mesenchymal stem cells (Ogular et al., 2014). The immunomodulatory effects of MSCs have been linked to increased Treg cells and related cytokines, MSCs are able to actively generate functional Treg cells through the differentiation process of Th1 and Th17 cells (Luz-Crawford et al., 2013b). Moreover, a

conditional microenvironment containing Treg cells plays an important role in the function and behaviour of MSCs (Lim et al., 2016). The interaction between MSCs and Th2 cells lead to decreased IL-4 secretion, which can promote the synthesis of IgE. IL-5 can promote the development of eosinophils, including the growth, maturation and the function. With the activation, eosinophils can present the antigen from the infectious agent to CD4⁺ cells; eosinophils can induce the chemokine to promote the recruitment of Th2 cells; eosinophils can damage the epithelial cells of lungs by the secretion of an eosinophil-derived neurotoxin, cationic proteins (eosinophil peroxidase) and major basic protein (Lambrecht and Hammad, 2015). The decrease of IgG1/IgG2a demonstrated that MSC regulates the balance of Th1/Th2 in the treatment of allergic airway inflammation. MSCs derived from pluripotent stem cells might be evaluated further as a better cell source for therapeutic application in airway allergic inflammation.

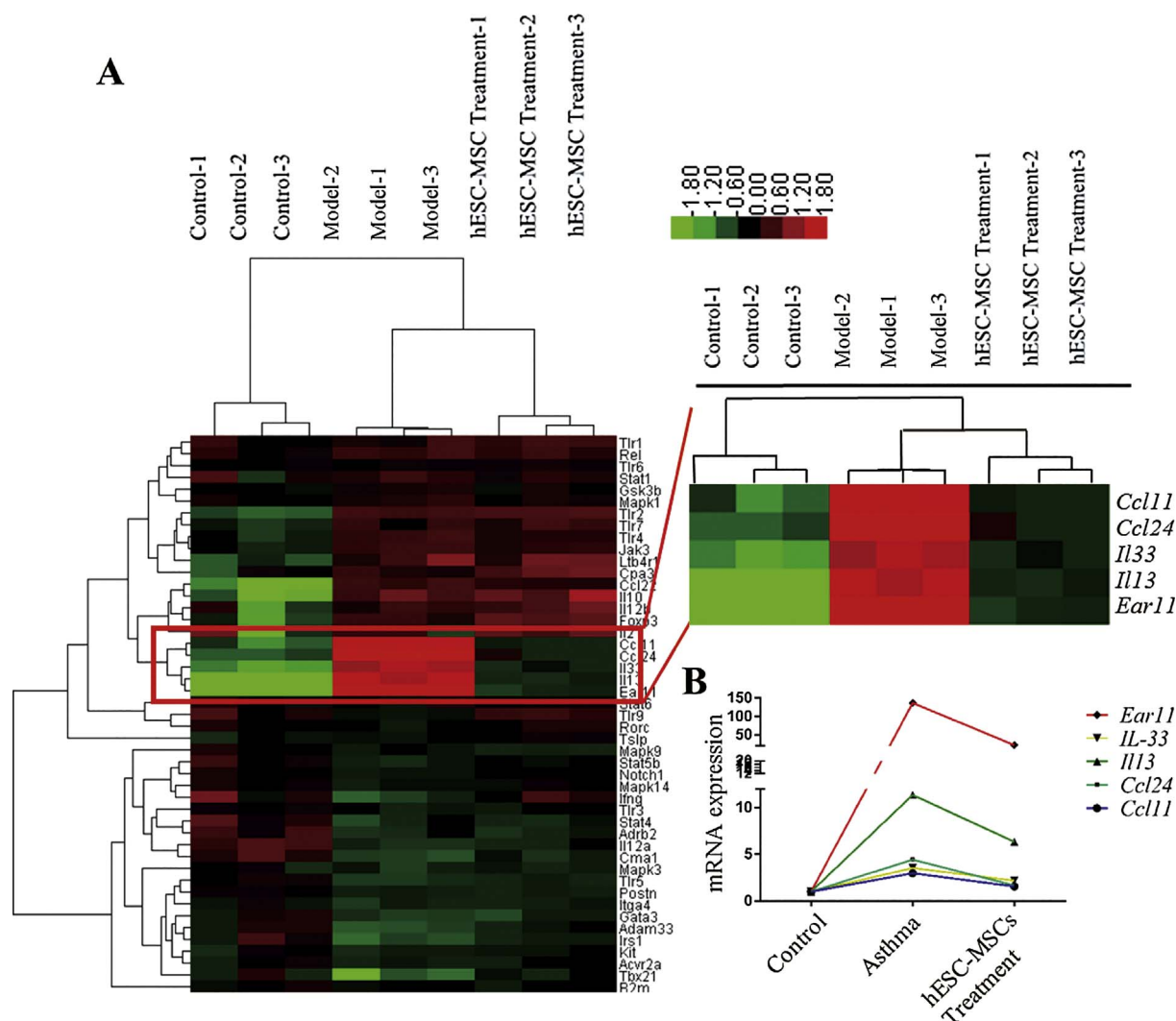


Fig. 4. The mRNAs expression profile in asthma with the treatment of hESC-MSCs. **A.** Hierarchical clustering analysis of all the aberrant protein-coding genes in the PCR array that were differentially expressed either between asthma and control groups or between hESC-MSC treatment and model groups (≥ 1.5 -fold; $p < .05$). **B.** A detailed expression change of 5 mRNAs in the red box. Abbreviations: mRNA, messenger RNA; qRT-PCR, quantitative real-time PCR; hESC-MSCs, embryonic stem cells derived mesenchymal stem cells. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

Theoretically, iPSCs from autologous cells should be immune-tolerated by the recipient. However, issues like frequent changes in the copy numbers during reprogramming, epigenetic memory after differentiation, and tumorigenicity *in vivo* became obstructive of the iPSCs application. Moreover, previous studies reported that recipient mice could be induced immune rejection after the transplantation of autologous iPSCs (Scheiner et al., 2014). Safety standards for clinical use of iPSCs still have not reached a consensus (Li and Belmonte, 2016; Gao et al., 2016a). Rather than “clinical grade” iPSCs, the “clinical grade” ESCs that have been cultivated without direct exposure to animal tissues had been developed in previous studies (Desai et al., 2013). Therefore, in spite of the ethical issues, ESCs as a source of MSCs overcame some of the disadvantages of iPSCs. The ESC-MSCs were more suitable for clinical large-scale applications.

The hESC-MSCs generated in this study alleviated murine airway allergic inflammation which induced by OVA. A total of 47 differentially expressed genes were identified after the transplantation of hESC-MSCs in the mouse with allergic airway inflammation. Differentially expressed genes in model vs. control groups and the hESC-MSC treatment vs. model groups were classified into six patterns. Hierarchical clustering analysis found that four Th2 related genes (*Ccl11*, *Ccl24*, *Il13*, *Il33*) and 1 eosinophils-associated ribonuclease A gene (*Ear11*) were

significantly upregulated after the induction of allergic airway inflammation, and down-regulated after the hESC-MSC treatment.

It is known that many cells of the innate and adaptive immune systems act together with airway epithelial cells to cause airway allergic inflammation (Holtzman et al., 2014). Previous studies demonstrated that MSCs exerted the immunomodulation via the secretion of soluble molecules including prostaglandin E2 (PGE2) and transforming growth factor- β (TGF- β). Moreover, MSCs could decrease the survival and proliferation of activated T cells, increase the proportion of regulatory T (Treg) cells by direct intercellular contact or inductive formation of regulatory (Gao et al., 2016b). Therefore, we further detected the mechanisms of MSC-based immunomodulation in airway allergic inflammation by PCR array. A custom made mRNA array including 84 protein-coding genes of involved in allergic airway inflammatory pathways and catalogues was designed. *Ccl11* and *Ccl24* selectively recruit eosinophils by inducing their chemotaxis (White et al., 1997); *Il-13* which secreted by Th2 has effects on immune cells in inducing allergic inflammation (Wynn, 2003); *Il-33* induces helper T cells, mast cells, eosinophils and basophils to produce type 2 cytokines (Baekkevold et al., 2003). All 5 identified protein-coding genes, therefore, were implicated in allergic responses. Which means they are the most relevant in the process of hESC-MSCs immunomodulation in

murine airway allergic inflammation model. However, there are still some questions need to answer in the coming research. The differences between species often might led to limited representation of the human cases, while it has been found that genomes among species are much more similar than originally thought, which provides a genetic as well as anatomic and physiological basis for using animals to model human diseases and predict human responses to drugs and other therapies (Uhl and Warner, 2015). Of course, the specific gene expression profiles of airway allergic inflammation and stem cell treatment in human should be investigated in the future to confirm our findings. Secondly, there is still not any solid connection of how the MSCs will affect the Th2 cells related genes and proteins. When knowing the mechanism of how to trigger these change we might be able to use some other methods to stimulate it to bypass the other side effect of heterogenic cell therapy.

5. Conclusion

In conclusion, this study has successfully derived multipotent MSCs from hESCs simply in two stages. The hESC-MSCs were able to relieve the allergic airway inflammation in model mouse, they modulated the inflammatory cells including eosinophils and Th2 cells in airway allergic inflammation. Further PCR array analysis identified key mRNAs involved in the process of hESC-MSCs immunomodulation. This study will provide a better understanding regarding the mechanisms underlying hESC-MSCs therapeutic application in airway allergic inflammation.

Authors' contribution

L.Y.D.: collection and/or assembly of data, initial manuscript writing and primary data analysis; X.L.F., H. Z., S.B.F.: manuscript writing and data analysis. M.X.D., C.L.L., Z.L.Q., Y.Q.P., H.Y.Z.: collection and/or assembly of data; Q.L.F.: concept and design, data analysis, manuscript writing and final approval of the manuscript. All authors read and approved the manuscript.

Conflict of interest

The authors declared that they have no conflict of interest.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at <https://doi.org/10.1016/j.molimm.2018.01.013>.

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