Int J Clin Exp Pathol 2016;9(1):61-72
www.ijcep.com /ISSN:1936-2625/IJCEP0017918

Original Article

Human umbilical cord derived mesenchymal stem cells suppress over-proliferation of pulmonary artery smooth muscle cells in a rat model of pulmonary hypertension

Junfeng Liu[1,2], Zhibo Han[3], Zhongchao Han[3], Zhixu He[1]

[1]Laboratory of Tissue Engineering and Stem Cell, Guiyang Medical College, Guiyang, Guizhou, P. R. China; [2]Department of Pediatrics, The General Hospital of Huabei Oil field Company, Renqiu, Hebei, P. R. China; [3]National Engineering Research Center of Cell Products, AmCellGene Co. Ltd, Tianjin, P. R. China

Received October 15, 2015; Accepted November 28, 2015; Epub January 1, 2016; Published January 15, 2016

Abstract: Inflammation and over-proliferation of pulmonary artery smooth muscle cells (PASMCs) have been considered as the major pathological features of pulmonary hypertension (PH). Even though transplantation of mesenchymal stem cells (MSCs) could improve PH in many experimental animal models, the exact mechanism of MSCs to suppress inflammation associated over-proliferation of PASMCs is not completely clear. Here we aimed to determine that MSCs suppress over-proliferation of PASMCs in PH via TNF-α/CaN/NFAT pathway. By transplantation of Human umbilical cord derived MSCs (UC-MSCs) into monocrotaline (MCT) induced PH rats, and establishing a co-culture system in vitro consist of MSCs, activated T cells, PASMCs, we assessed the therapeutic effects of MSCs on PH and the changes of correlate factors in TNF-α/CaN/NFAT pathway. The results indicated that transplantation of MSCs could improve the hemodynamics and histology in the progression of PH induced by MCT. Furthermore, in the co-culture system in vitro, MSCs could suppress the TNF-α production of T cells, and then reduce the intracellular calcium level in PASMCs and the expression of CaN and NFATc2, suppress the CaN activity and NFATc2 activation, and could finally achieve the suppressive effect on proliferation of PASMCs. Similar regulatory effects with MSCs could be observed with the presence of infliximab in vitro, and recombinant TNF-α could still be able to promote the proliferation of PASMCs via CaN/NFAT pathway, even under the suppressive effect of MSCs. Taken together, our findings have suggested that MSCs suppress inflammatory associated over-proliferation of PASMCs and remodeling via TNF-α/CaN/NFAT pathway in PH.

Keywords: Mesenchymal stem cell, pulmonary hypertension, model, pathway, calcineurin, nuclear factor of activated T-cells, tumor necrosis factor-α

Introduction

Pulmonary hypertension (PH) is a serious lung disease with high mortality. The major functional and structural alterations of this disorder were increased pulmonary vascular resistance and pulmonary vascular remodeling. Progressively increased pulmonary arterial pressure would result in the overload of the right ventricle and heart failure eventually. Even though the clinical symptoms of PH could be relieved partly by targeted medicine such as bosentan and Sildenafil, long-term outcomes of this disorder are still not satisfying. Therefore more effective therapeutic protocols for this disease are of urgent demand.

Along with the advancement in studies on mechanisms of PH, inflammation and over-proliferation of pulmonary artery smooth muscle cells (PASMCs), as the major pathological features of PH, have attracted more attention, and may be a promising target for the treatment of PH. In many animal models and PH patients, inflammatory infiltration, even tertiary lymphoid follicle composed of T cells, B cells and less dendritic cells were found around or near the remodeled pulmonary vessels [1-4]. Enormous cytokines and chemokines such as interleukin (IL)-6, tumor necrosis factor (TNF)-α, produced by these inflammatory cells could be found, and the level of certain cytokines acted as predictive factors of outcome in PH patients [5]. It is yet uncertain whether inflammation initiates pulmonary vascular remodeling, or is just a bystander. But it has at least been explicited that the perivascular accumulation of inflam-

matory cells is essential to hypoxia-induced pulmonary vascular remodeling [6].

Over-proliferation of PASMCs also plays a crucial role in vascular remodeling. Activation of calcineurin (CaN)-nuclear factor of activated T-cells (NFAT), as the critical pathway of SMC proliferation, could be observed in many PH animal model and patients [7, 8], and may be therapeutical target for PH [8]. Stimulated by some cytokines for cell-surface receptors coupled to phospholipase C, calcium mobilization and influx through specific channels on SMC membrane increased evidently. High levels of calcium in cytoplasm could active CaN-NFAT, and then initiated the proliferation of SMCs [9]. TNF-α could increase the levels of cytosolic ionized calcium by influx of external calcium and release from endosomal stores in endothelial cells [10], but whether the same effect of TNF-α could be observed in SMCs is uncertain. TNF-α could promote the proliferation of SMCs [11], even though the exact mechanism is obscure, we speculate that activation of CaN-NFAT induced by increased cytoplasm calcium may be involved in this process partly. TNF-α, produced by inflammatory cells around pulmonary vessels in PH, could activate CaN-NFAT by increasing cytoplasm calcium of SMCs, thus initiating the over-proliferation of SMCs and pulmonary vascular remodeling.

Mesenchymal stem cells (MSCs), as primitive cells with the potential of multi-lineage differentiation and self-renewal, could evidently decrease the production of inflammatory cytokines, such as TNF-α and IFN-γ, when co-cultured with activated CD4+ T cells [12]. And this immunosuppressive effect of MSCs is highly related to their ability of immuno-regulatory cytokines and PGE2 secretion [12]. The immunosuppressive effect of MSCs has attracted much attention for their potential application in immune disorders, such as autoimmune diseases and graft-versus-host disease [13, 14]. Furthermore, it has also been confirmed that administration of MSCs could relieve PH in experimental animal model [15, 16]. However, to what extent of the therapeutic effects was accounted by immunosuppression of MSCs is still unknown. Nor is the mechanism through which MSCs suppress the over-proliferation of PASMCs.

In the present study, by using a rat model with monocrotaline (MCT) induced PH and co-cultured umbilical cord derived MSCs (UC-MSCs) with PASMCs and concanavalin A (ConA) activated T cells, we assess the suppressing effects of UC-MSCs on over-proliferation of PASMCs. The correlative factors on TNF-α/CaN/NFAT pathway, including the levels of TNF-α on lung tissue and supernatants of co-cultured cells, the levels of cytoplasm calcium in PASMCs, the expression and activation of CaN, NFAT, were also evaluated. This helps better understand the mechanism of MSCs on over-proliferation of PASMCs involved in PH.

Materials and methods

Human UC-MSCs and experimental animals

All studies were approved by the Institutional Review Board of Guiyang Medical College, and the donors have written informed consent. UC-MSCs were isolated and identified according to the protocol described previously [17].

Female Sprague-Dawley (SD) rats with body weight of approximately 200 grams were housed in specific pathogen-free (SPF) units of the Laboratory Animals Center at Tianjin Blood Diseases Hospital. All animal studies were approved by the Institutional Animal Care and Use Committee of Guiyang Medical College.

Establishment of PH rat model and examination of hemodynamics and pathology

Twenty-four rats were randomly divided into model group, MSCs transplantation group and control group, 8 rats in each group. Rats were given a single subcutaneous injection of MCT (60 mg/kg, sigma system, USA) to induce PH [18]. Phosphate buffered saline (PBS) was used as controls. Five days after injection of MCT, 10^6 MSCs were transplanted into the rats via caudal vein for one time. To observe the distribution of transplanted MSCs in lung, additional 2 rats received transplantion of MSCs prelabeled with CM-DiI (Invitrogen, USA) under the same protocol.

At days 21, the right ventricular systolic pressure (RVSP) and mean aortic pressure (MAoP) of rats were detected [19]. Following that, peripheral blood sample was collected from right external jugular vein into EDTA-containing tubes. After centrifugation, plasma was collected and stored at -80°C. Rats were sacrificed by decapitation afterwards, the lung tissues were

Table 1. Primers for RT-PCR

Rat GAPDH forward	5'-CCATTCTTCCACCTTTGATGCT-3'
Rat GAPDH reverse	5'-TGTTGCTGTAGCCATATTCATTGT-3'
Rat CaN forward	5'-CAGAGGGTGCTTCGATTCTC-3'
Rat CaN reverse	5'-CCCCTAAGAAGAGGTAGCGA-3'
Rat NFATc2 forward	5'-CAGCAGATTTGGGAGATGGAAG-3'
Rat NFATc2 reverse	5'-GACTGGGTGGTAAGTAAAGTGC-3'

removed, and fixed in 10% paraformaldehyde or embedded in OCT medium at -80°C. The examination of lung pathology and medial wall thickness of pulmonary arteriole (WT) was performed according to previous study [19].

Immunohistochemistry staining for TNF-α in lung tissue

Lung tissue sections were deparaffinized and rehydrated. After sequential incubation with 0.3% Triton X-100 and 3% hydrogen peroxide, the sections were incubated overnight at 4°C with goat polyclonal primary antibody against TNF-α (1:400, Santa Cruz Biotecnology, USA). After incubated with the biotinylated rabbit anti-goat secondary antibody (1:100, Boster, China) for 30 minutes, the immunoreactivity was detected by a 3-amino-9-ethylcarbazole peroxidase substrate kit (Boster, China).

Isolation of rat PASMCs and T cells

SD rat was sacrificed by decapitation, the pulmonary arteries and spleen were harvested, and PASMCs were cultured in DMEM/F-12 medium supplemented with 10% FBS and 100 U/ml penicillin-streptomycin by tissue explant method. Identification of PASMCs was performed by immunofluorescent staining for α-smooth muscle actin (1:100, Proteintech, USA). T cells were isolated from spleens by non-adherence to nylon wool and frozen at -80°C for further study.

Co-culture of PASMCs, T cells and MSCs

Cells were cultured in DMEM/F-12 medium supplemented with 10% FBS and 100 U/ml penicillin-streptomycin. Five groups were divided as: Group A (PASMCs alone), Group B (PASMCs + T cells), Group C (PASMCs + T cells + MSCs), Group D (PASMCs + T cells + infliximab) and Group E (PASMCs + T cells + MSCs + TNF-α). 10^4 PASMCs were seeded into 24-well plate and cultured for 24 h. The supernatant was discarded, and then 10^5 T cells suspending in 700 μl medium were added in. T cells were stimulated by ConA (10 μg/ml, Sigma, USA). 10^4 MSCs were resuspended with 300 μl medium in the upper chamber of a modified Boyden chamber (3 mm pore size), and placed in the 24-well culture plate. Furthermore, infliximab (Essex Pharma, Germany), as TNF-α monoclonal antibody, at concentration of 1 μg/ml, and recombined rat TNF-α (Prospect Biosystems, USA) at concentration of 10 ng/ml were added respectively.

Proliferation of PASMCs

After 3 days of culture, the supernatant was removed, and the PASMCs were washed 3 times with PBS. 300 μl new medium containing 15 μl 3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-etrazolium, inner salt (MTS, Promega, USA) were added and cells were incubated for 4 hours. The supernatant was collected and the optical density (OD) was read at 490 nm by microplate reader.

TNF-α level in plasma and supernatant of co-cultured cells

The plasma and co-cultured supernatant levels of TNF-α were measured by enzyme-linked immunosorbeny assay (ELISA) technique using the kit from Peprotech Company according to the supplier's instruction.

Concentration of intracellular calcium

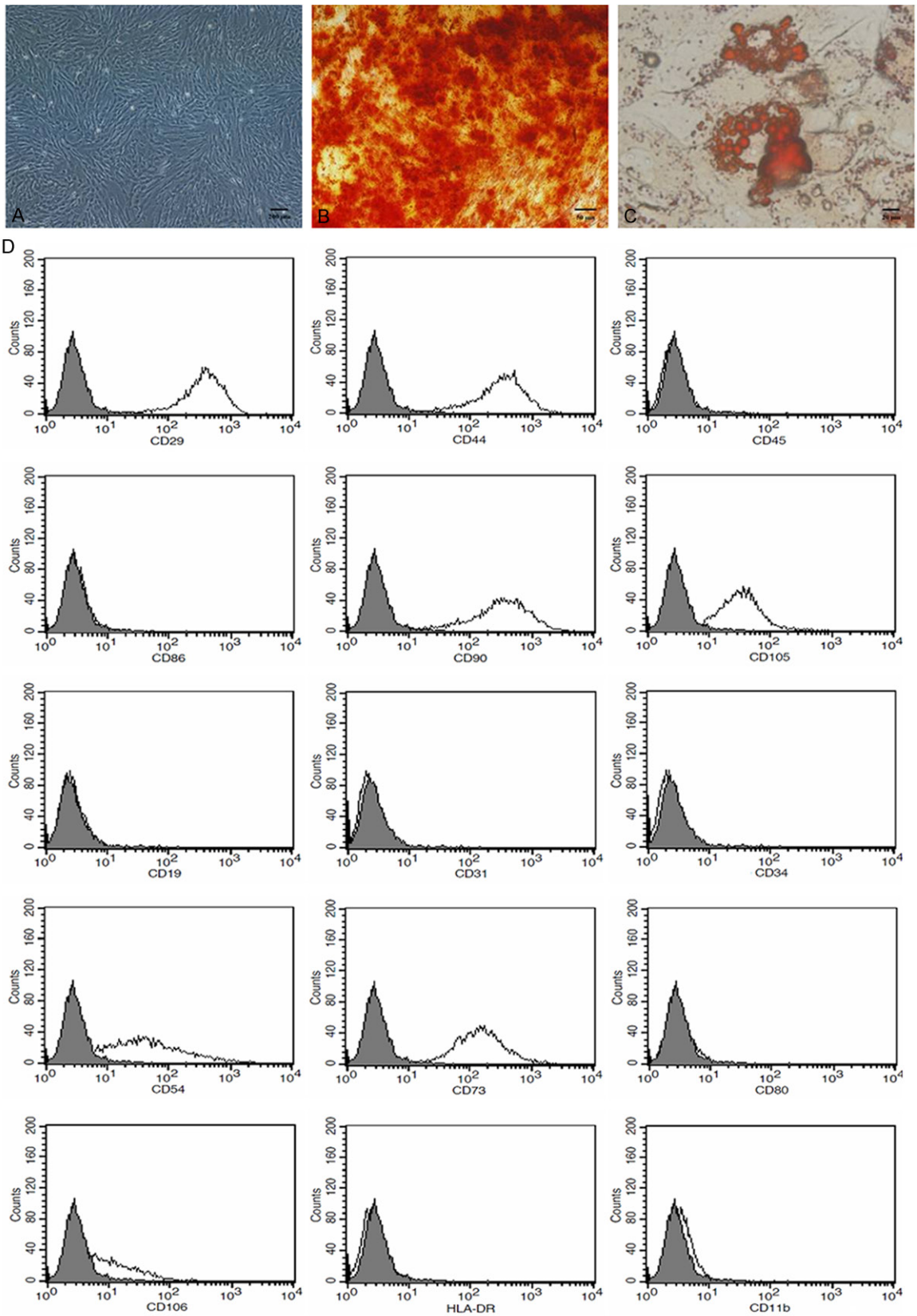
The intracellular free calcium concentration of PASMCs was examined according the previous study [20].

RNA isolation, reverse transcription and real time PCR

Total RNA of PASMCs was extracted by E.Z.N.A. Total RNA Kit I (OMEGA, USA), and then reverse transcribed to cDNA by MLV RT kit (Invitrogen, USA). Real-time polymerase chain reaction (PCR) analyses for CaN and NFATc2 were performed by Platinum® SYBR® Green qPCR SuperMix-UDG w/ROX (Invitrogen, USA) with Applied Biosystems 7300 Real-Time PCR System. The primers were listed in **Table 1**.

CaN activity

PASMCs were treated in 1 ml lysate buffer, after repeated freeze/thaw 3 times, cell homogenate was centrifuged, and the supernatant


A
B
C
D
Counts
CD29
CD44
CD45
CD86
CD90
CD105
CD19
CD31
CD34
CD54
CD73
CD80
CD106
HLA-DR
CD11b

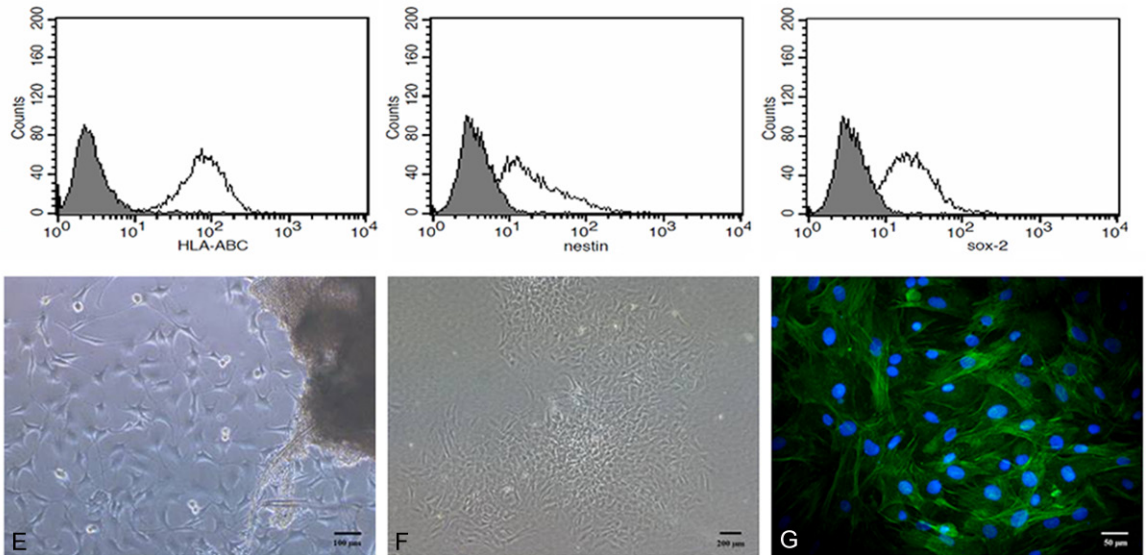


Figure 1. Identification of MSCs and PASMCs. A: MSCs Passage 3 cultured in vitro. B: Osteogenic differentiation. C: Adipogenic differentiation. D: Phenotype analysis of MSCs. E: PASMCs cultured for 5 days by tissue explant method. F: PASMCs Passage 3 cultured in vitro. G: Immunofluorescent staining of PASMCs for α-smooth muscle actin.

was collected and used for measurement of protein and calcineurin phosphatease activity using calcineurin assay kit (Nanjing Jiancheng Company, China).

NFATc2 activation

PASMCs and serial 5 μm cryosections were fixed in 4% paraformaldehyde for 10 minutes. After sequentially incubated with 0.3% Triton X-100 and 1% bovine serum albumin (BSA), the sections were incubated overnight at 4°C with mouse monoclonal antibody against rat NFATc2 (1:100, Novus Biologicals, USA). And then, the sections were incubated for 2 hours with fluorescein isothiocyanate (FITC)-conjugated goat anti-mouse immunoglobulin G (1:100, Proteintech, USA). After 10 seconds incubation with DAPI (1 μg/ml), the sections were assessed by confocal laser scanning microscope.

Statistical analysis

Data were presented as mean ± SD, and SPSS software (version 17.0) was used for statistical analysis. Differences were compared using One-Way ANOVA tests. *P* value < 0.05 was considered as statistically significant.

Results

Identification of MSCs and PASMCs

Human UC-MSCs have shown typical fibroblastic shape (**Figure 1A**), and identified by the ability of osteogenic (**Figure 1B**) and adipogenic (**Figure 1C**) differentiation, and specific phenotype (**Figure 1D**).

Rat PASMCs have shown typical shape of "hill and valley" when cultured in vitro (**Figure 1F**) and identified by immunofluorescent staining for α-smooth muscle actin (**Figure 1G**).

MSCs improve the hemodynamic and histological abnormality in PH rats

Two days after transplantation of CM-DiI labeled MSCs, a few scattered distributed cells that were CM-DiI positive were found in the lung (**Figure 2A**). But more cells were found in the spleen (**Figure 2B**).

Twenty-one days after subcutaneous injection of MCT, The RVSP in the model group increased significantly compared to the control group (**Figure 2D**), and the MAoP decreased (**Figure 2E**). Histological examination also indicated that, medial hypertrophy of pulmonary muscular arterioles was evident (**Figure 2C**), and the WT increased (**Figure 2F**). By transplantation of MSCs, the hemodynamic and histological abnormality could be improved evidently.

MSCs inhibit TNF-α and NFATc2 ativationin MCT induced PH

In the model group, the levels of TNF-α in plasma and lung tissue were all increased (**Figure**

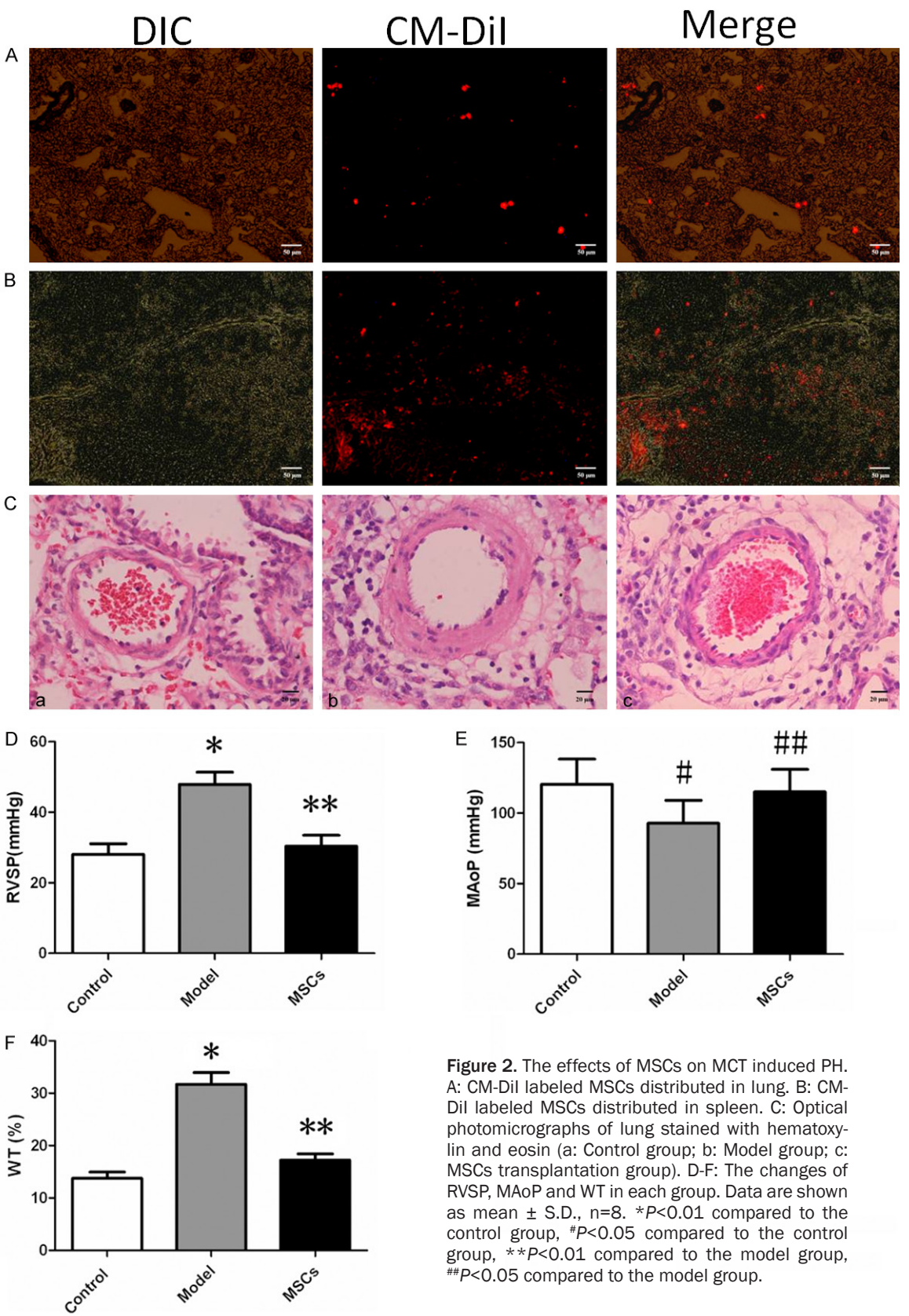


Figure 2. The effects of MSCs on MCT induced PH. A: CM-DiI labeled MSCs distributed in lung. B: CM-DiI labeled MSCs distributed in spleen. C: Optical photomicrographs of lung stained with hematoxylin and eosin (a: Control group; b: Model group; c: MSCs transplantation group). D-F: The changes of RVSP, MAoP and WT in each group. Data are shown as mean ± S.D., n=8. $^{*}P<0.01$ compared to the control group, $^{\#}P<0.05$ compared to the control group, $^{**}P<0.01$ compared to the model group, $^{\#\#}P<0.05$ compared to the model group.

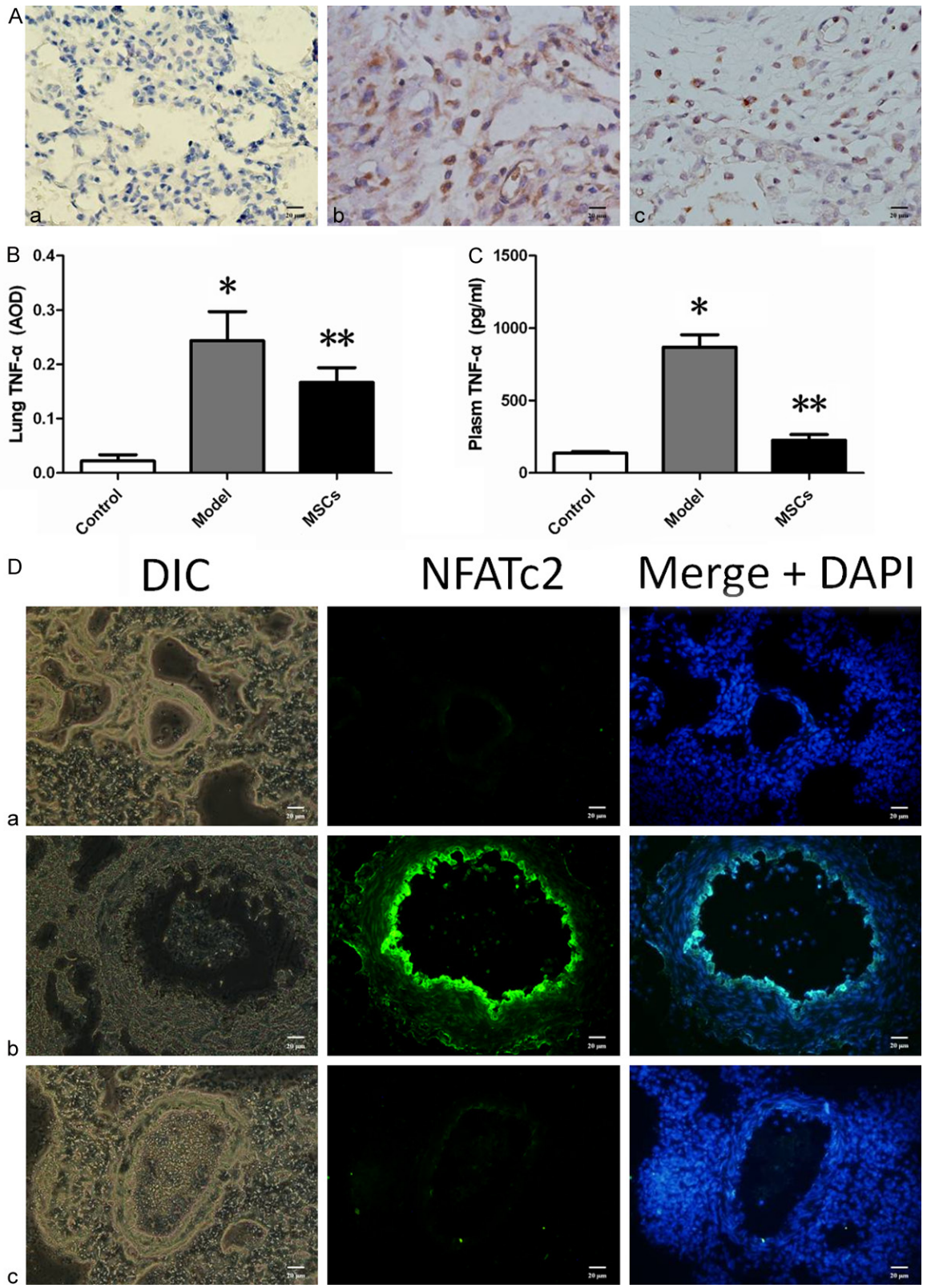


Figure 3. TNF-α level and activation of NFATc2. A: Immunohistochemistry stain of lung for TNF-α in the control group (a), model group (b) and MSCs transplantation group (c). B: The levels of TNF-α in lung. C: The levels of TNF-α in plasma. Data are shown as mean ± S.D., n=8. **P*<0.01 compared to the control group, ***P*<0.01 compared to the model group. D: The activation of NFATc2 in pulmonary artery in the control group (a), model group (b) and MSCs transplantation group (c).

3A-C). Meanwhile, high expression of NFATc2 could be observed in pulmonary arterioles, which were mostly distributed in the nucleus, thus indicating the activation of NFATc2 (**Figure 3D**). By transplantation of MSCs, the levels of TNF-α decreased significantly, and the activation of NFATc2 also be inhibited.

MSCs suppressed TNF-α and the proliferation of PASMCs in co-culture system

In the co-culture system, ConA stimulated T cells could produce high level of TNF-α, and the proliferation potential of PASMCs co-cultured with them increased evidently compared to that of PASMCs cultured alone. But these effects could be inhibited effectively by MSCs or infliximab. However, even under the inhibition of MSCs, the presence of TNF-α still had the ability to stimulate the proliferation of PASMCs (**Figure 4A**, **4B**).

MSCs decreased intracellular level of calcium in PASMCs and down-regulated the expression of CaN and NFATc2

When co-cultured with ConA stimulated T cells, the intracellular free calcium concentration of PASMCs increased evidently compared to that in PASMCs cultured alone. Moreover, the expression of CaN and NFATc2 were up-regulated. But these effects could be suppressed significantly by MSCs or infliximab. However, TNF-α could still increase the intracellular free calcium concentration of PASMCs, and up-regulate the expression of CaN and NFATc2, even under the suppressive effect of MSCs (**Figure 4C-E**).

MSCs suppressed CaN activity and NFATc2 activation in PASMCs

When co-cultured with ConA stimulated T cells, the intracellular CaN activity in PASMCs was up-regulated evidently, and majority of NFATc2 was translocated to nucleus, which means the activation of NFATc2. MSCs or infliximab could all suppress the intracellular CaN activity and the activation of NFATc2 effectively. But TNF-α could still up-regulate the intracellular CaN activity in PASMCs and activate NFATc2 even under the suppressive effect of MSCs (**Figure 4F**, **4G**).

Discussion

In the present study, by using a rat model of PH, we have demonstrated that, transplantation of human UC-MSCs decreased the TNF-α level in the lung and inhibited the activation of CaN-NFAT in pulmonary arterioles, which in turn suppressed the over-proliferation of pulmonary arterioles SMCs and the vascular remodeling, thus the hemodynamic and histology were improved in the progression of MCT induced PH. To further illuminate the mechanism which MSCs suppressed the over-proliferation of PASMCs in PH, a co-culture system consisted of MSCs, T cells and PASMCs was established. Through the coculture system, we have demonstrated that, MSCs suppressed the production of TNF-α in T cells, reduced the intracellular calcium level in PASMCs, down-regulated the expression of CaN and NFATc2, and suppressed the CaN activity and NFATc2 activation, which finally led to the suppressive effect to proliferation of PASMCs.

As a subset of stromal stem cells, MSCs can be found in almost all tissues, and share the same biologic characteristics, including self-renewing and the potential of multi-lineage differentiation. Although they were first isolated from bone marrow [21], MSCs have been reported to be isolated from umbilical cord [17], placenta [22], cord blood [23] and adipose tissue [24] so far. Among these MSCs with diverse origins, bone marrow derived MSCs have been preferred in many clinical trials and animal experiments. However, the particular superiority of UC-MSCs in terms of less pain, non-invasiveness, harmless to donors, faster growth rate and greater expansion capability [17, 25] seems to warrant promising potential of their future application, despite of the fact that these cells have been rarely studied in clinical trials. Hence, in the present study, UC-MSCs were studied as a potential treatment of PH. The results have shown that, the transplantation of UC-MSCs significantly improve the hemodynamic and histological parameters in the progression of MCT induced PH. Similar therapeutic effects of UC-MSCs to PH have been described in previous studies [15, 16], while the exact mechanism have not been elucidated.

The hypothesis that exogenous transplantation of MSCs could differentiate into lung tissue types during repair has been questioned, because the issue of niche for MSCs to reside during injury may be an obstacle to their engraftment or differentiation [26]. And MSCs

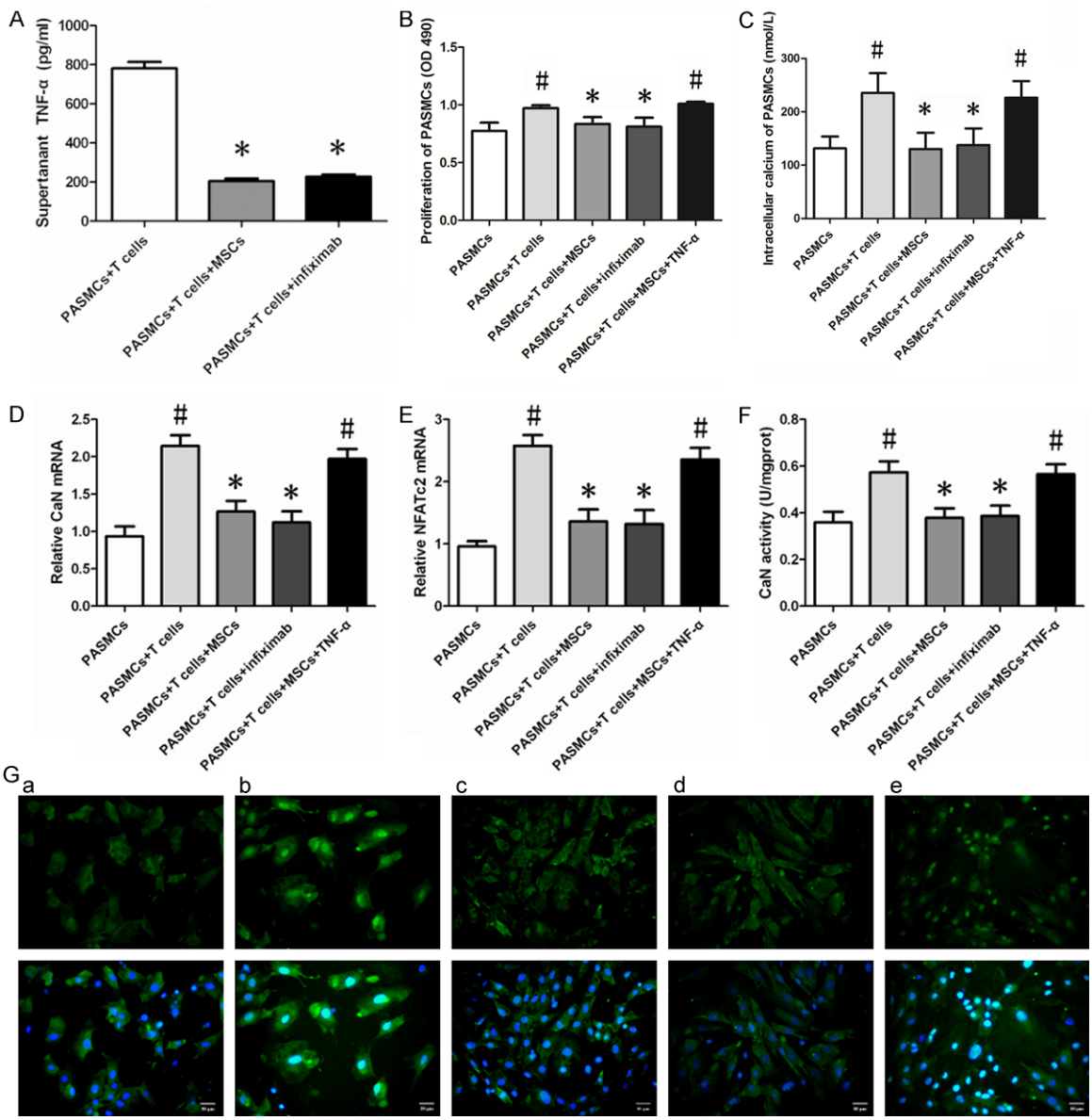


Figure 4. The changes of TNF-α/CaN/NFAT in the co-culture system: A: The TNF-α level; B: The proliferation potential of PASMCs; C: The intracellular calcium level of PASMCs; D, E: The expression of CaN and NFATc2 in PASMCs; F: The CaN activity in PASMCs. Data are shown as mean ± S.D., n=8. **P*<0.01 compared to (PASMCs + T Cells); #*P*<0.01 compared to (PASMCs). G: The activation of NFATc2 in PASMCs in the co-culture system (a: PASMCs; b: PASMCs + T Cells; c: PASMCs + T Cells + MSCs; d: PASMCs + T Cells + infliximab; e: PASMCs + T Cells + MSCs + TNF-α. Merge with DAPI).

conditioned media could also substantially attenuate pulmonary injury that was induced by hypoxia [27]. Therefore the hypothesis that MSCs plays protective effects via paracrine mechanisms predominantly seems more acceptable. In previous study, we have already demonstrated that MSCs co-cultured with activated CD4+ T cells decrease the production of inflammatory cytokines evidently, and the immunosuppressive activity of MSCs is highly related to their ability for the secretion of immunoregulatory cytokines and PGE2 [12]. The present study in vivo has also demonstrated that exogenous transplantation of MSCs decrease the TNF-α level by its immunosuppressive activity, and on this background the medial hypertrophy of pulmonary muscular arterioles and romodeling were prevented. Meanwhile, as a crucial regulatory factor of SMCs proliferation, the activation of NFATc2

was suppressed significantly. Because more MSCs were found in spleen but not in lung, this phenomenon may be able to partly confirm the hypothesis that MSC regulates the T cell response in an extrapulmonary site [25]. But whether the spleen, which as the reservoir of T cells, is one of the most important regulatory sites, is uncertain in the present study.

To further elucidate how the immunosuppressive activity of MSCs affect the activation, proliferation of PASMCs and remodeling, a co-culture system in vitro consist of PASMCs, T cells and MSCs was established. By assessing factors associated with the proliferation of PASMCs in TNF-α/CaN/NFAT pathway, we could better understand the suppressive mechanism of MSCs on over-proliferation of PASMCs involved in PH. The results indicated that the TNF-α production of T cells increased significantly under the stimulation of ConA. High levels of TNF-α could increase calcium influx and intracellular calcium concentration in PASMCs, and then up-regulated the expression of CaN/NFAT, increased the CaN activity, promoted the activation of NFATc2, thus initiating and accelerating the proliferation of PASMCs. Because of the superior immunosuppressive activity of MSCs, TNF-α production of T cells decreased significantly. The following factors associated with the proliferation of PASMCs mentioned above were all down-regulated. Thus the over-proliferation of PASMCs was suppressed effectively.

To confirm it is the inhibitory action of MSCs to TNF-α, but not to other inflammatory cytokines, which plays a pivotal role in the regulation of proliferation of PASMCs, infliximab and recombined human TNF-α were added in the co-culture system. Similar regulating effects with MSCs could be observed with presence of infliximab, thus demonstrating the important role of TNF-α during proliferation of PASMCs. In addition, TNF-α could still increase the intracellular calcium level in PASMCs, up-regulate the expression of CaN and NFATc2, increase activity of CaN, and promote the activation of NFATc2, even under the suppressive effect of MSCs. Given all these results, in the regulating process of MSCs to inflammation associated proliferation of PASMCs, the significance of regulation by TNF-α could be confirmed.

Taken together, this study confirms for the first time that human UC-MSCs regulate the over-proliferation of PASMCs in PH, and the regulating effect was accomplished by immunosuppressive activity of MSCs, especially for the suppression of TNF-α, and the following inhibitory action for CaN/NFAT pathway of PASMCs. The long-term outcome of this regulating effects and potential side effects need to be confirmed in future study.

Acknowledgements

This study was supported by the National Natural Science Foundation of China (30560159, 30960412, and 31360285).

Disclosure of conflict of interest

None.

Address correspondence to: Dr. Zhixu He, Laboratory of Tissue Engineering and Stem Cell, Guiyang Medical College, Guiyang 550004, P. R. China. Tel: +86-851-6908118; Fax: +86-851-6908118; E-mail: hzx@gmc.edu.cn; Dr. Zhongchao Han, National Engineering Research Center of Cell Products, AmCellGene Co. Ltd, Tianjin 300457, P. R. China. Tel: +86-22-66211206; Fax: +86-22-66211206; E-mail: hanzhongchao@hotmail.com

References

[1] Hall S, Brogan P, Haworth SG, Klein N. Contribution of inflammation to the pathology of idiopathic pulmonary arterial hypertension in children. Thorax 2009; 64: 778-783.

[2] Pinto RF, Higuchi Mde L, Aiello VD. Decreased numbers of T-lymphocytes and predominance of recently recruited macrophages in the walls of peripheral pulmonary arteries from 26 patients with pulmonary hypertension secondary to congenital cardiac shunts. Cardiovasc Pathol 2004; 13: 268-275.

[3] Heath D, Edwards JE. The pathology of hypertensive pulmonary vascular disease; a description of six grades of structural changes in the pulmonary arteries with special reference to congenital cardiac septal defects. Circulation 1958; 18: 533-547.

[4] Perros F, Dorfmüller P, Montani D, Hammad H, Waelput W, Girerd B, Raymond N, Mercier O, Mussot S, Cohen-Kaminsky S, Humbert M, Lambrecht BN. Pulmonary lymphoid neogenesis in idiopathic pulmonary arterial hypertension. Am J Resp Crit Care Med 2012; 185: 311-321.

[5] Soon E, Holmes AM, Treacy CM, Doughty NJ, Southgate L, Machado RD, Trembath RC, Jennings S, Barker L, Nicklin P, Walker C, Budd

DC, Pepke-Zaba J, Morrell NW. Elevated levels of inflammatory cytokines predict survival in idiopathic and familial pulmonary arterial hypertension. Circulation 2010; 122: 920-927.
[6] Frid MG, Brunetti JA, Burke DL, Carpenter TC, Davie NJ, Reeves JT, Roedersheimer MT, van Rooijen N, Stenmark KR. Hypoxia-induced pulmonary vascular remodeling requires recruitment of circulating mesenchymal precursors of a monocyte/macrophage lineage. Am J Pathol 2006; 168: 659-669.
[7] de Frutos S, Spangler R, Alò D, Bosc LV. NFATc3 mediates chronic hypoxia-induced pulmonary arterial remodeling with alpha-actin up-regulation. J Biol Chem 2007; 282: 15081-15089.
[8] Bonnet S, Rochefort G, Sutendra G, Archer SL, Haromy A, Webster L, Hashimoto K, Bonnet SN, Michelakis ED. The nuclear factor of activated T cells in pulmonary arterial hypertension can be therapeutically targeted. Proc Natl Acad Sci U S A 2007; 104: 11418-11423.
[9] Said SI, Hamidi SA, Gonzalez Bosc L. Asthma and pulmonary arterial hypertension: do they share a key mechanism of pathogenesis? Eur Respir J 2010; 35: 730-734.
[10] Rowlands DJ, Islam MN, Das SR, Huertas A, Quadri SK, Horiuchi K, Inamdar N, Emin MT, Lindert J, Ten VS, Bhattacharya S, Bhattacharya J. Activation of TNFR1 ectodomain shedding by mitochondrial Ca2+ determines the severity of inflammation in mouse lung microvessels. J Clin Invest 2011; 121: 1986-1999.
[11] Lee SJ, Kim WJ, Moon SK. TNF-alpha regulates vascular smooth muscle cell responses in genetic hypertension. Int Immunopharmacol 2009; 9: 837-843.
[12] Yang ZX, Han ZB, Ji YR, Wang YW, Liang L, Chi Y, Yang SG, Li LN, Luo WF, Li JP, Chen DD, Du WJ, Cao XC, Zhuo GS, Wang T, Han ZC. CD106 identifies a subpopulation of mesenchymal stem cells with unique immunomodulatory properties. PLoS One 2013; 8: e59354.
[13] Le Blanc K, Rasmusson I, Sundberg B, Gotherstrom C, Hassan M, Ringdén O. Treatment of severe acute graft-versus-host disease with third party haploidentical mesenchymal stem cells. Lancet 2004; 363: 1439-1441.
[14] Zappia E, Casazza S, Pedemonte E, Benvenuto F, Bonanni I, Gerdoni E, Giunti D, Ceravolo A, Cazzanti F, Frassoni F, Mancardi G, Uccelli A. Mesenchymal stem cells ameliorate experimental autoimmune encephalomyelitis inducing T-cell anergy. Blood 2005; 106: 1755-1761.
[15] Baber SR, Deng W, Master RG, Bunnell BA, Taylor BK, Murthy SN, Hyman AL, Kadowitz PJ. Intratracheal mesenchymal stem cell administration attenuates monocrotaline-induced pulmonary hypertension and endothelial dysfunction. Am J Physiol Heart Circ Physiol 2007; 292: H1120-1128.
[16] Luan Y, Zhang X, Kong F, Cheng GH, Qi TG, Zhang ZH. Mesenchymal stem cell prevention of vascular remodeling in high flow-induced pulmonary hypertension through a paracrine mechanism. Int Immunopharmacol 2012; 14: 432-437.
[17] Lu LL, Liu YJ, Yang SG, Zhao QJ, Wang X, Gong W, Han ZB, Xu ZS, Lu YX, Liu D, Chen ZZ, Han ZC. Isolation and characterization of human umbilical cord mesenchymal stem cells with hematopoiesis-supportive function and other potentials. Haematologica 2006; 91: 1017-1026.
[18] Matsuda Y, Hoshikawa Y, Ameshima S, Suzuki S, Okada Y, Tabata T, Sugawara T, Matsumura Y, Kondo T. Effects of poroxisome proliferator-activated receptor gamma ligands on Monocrotaline-induced pulmonary hypertension in rats. Nihon Kokyuki Gakkai Zasshi 2005; 43: 283-288.
[19] Liu JF, Du ZD, Chen Z, Han ZC, He ZX. Granulocyte colony-stimulating factor attenuates monocrotaline-induced pulmonary hypertension by upregulating endothelial progenitor cells via the nitric oxide system. Exp Ther Med 2013; 6: 1402-1408.
[20] Huang J, Li LS, Yang DL, Gong QH, Deng J, Huang XN. Inhibitory effect of ginsenoside Rg1 on vascular smooth muscle cell proliferation induced by PDGF-BB is involved in nitric oxide formation. Evid Based Complement Alternat Med 2012; 2012: 314395.
[21] Pittenger MF, Mackay AM, Beck SC, Jaiswal RK, Douglas R, Mosca JD, Moorman MA, Simonetti DW, Craig S, Marshak DR. Multilineage potential of adult human mesenchymal stem cells. Science 1999; 284: 143-147.
[22] Fukuchi Y, Nakajima H, Sugiyama D, Hirose I, Kitamura T, Tsuji K. Human placenta-derived cells have mesenchymal stem/progenitor cell potential. Stem Cells 2004; 22: 649-658.
[23] Lee OK, Kuo TK, Chen WM, Lee KD, Hsieh SL, Chen TH. Isolation of multipotent mesenchymal stem cells from umbilical cord blood. Blood 2004; 103: 1669-1675.
[24] Zuk PA, Zhu M, Mizuno H, Huang J, Futrell JW, Katz AJ, Benhaim P, Lorenz HP, Hedrick MH. Multilineage cells from human adipose tissue: implications for cell-based therapies. Tissue Eng 2001; 7: 211-228.
[25] Baksh D, Yao R, Tuan RS. Comparison of proliferative and multilineage differentiation potential of human mesenchymal stem cells derived from umbilical cord and bone marrow. Stem Cells 2007; 25: 1384-1392.

[26] Jun D, Garat C, West J, Thorn N, Chow K, Cleaver T, Sullivan T, Torchia EC, Childs C, Shade T, Tadjali M, Lara A, Nozik-Grayck E, Malkoski S, Sorrentino B, Meyrick B, Klemm D, Rojas M, Wagner DH Jr, Majka SM. The pathology of bleomycin-induced fibrosis is associated with loss of resident lung mesenchymal stem cells that regulate effector T-cell proliferation. Stem Cells 2011; 29: 725-735.

[27] Aslam M, Baveja R, Liang OD, Fernandez-Gonzalez A, Lee C, Mitsialis SA, Kourembanas S. Bone marrow stromal cells attenuate lung injury in a murine model of neonatal chronic lung disease. Am J Respir Crit Care Med 2009; 180: 1122-1130.