

Regulatory T cells protect against hypoxia-induced pulmonary arterial hypertension in mice

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Abstract. Pulmonary arterial hypertension (PAH) is a life-threatening disease characterized by the complex proliferation of the pulmonary vascular endothelium and progressive pulmonary vascular remodeling. CD4⁺CD25⁺ regulatory T cells (Tregs) have been the focus of numerous studies into PAH. The present study aimed to investigate the role and mechanisms of Tregs in hypoxia-induced PAH. A total of 60 male mice were divided at random into three groups: Normoxia group, hypoxia control group and Tregs group. Measurements were obtained of the right ventricle systolic pressure (RVSP) and the Fulton's index; in addition, the mRNA and protein expression of pro-inflammatory cytokines including monocyte chemotactic protein 1 (MCP-1), interleukin (IL)-1 β and IL-6, as well as the anti-inflammatory cytokine IL-10 in the lungs were determined by reverse transcription quantitative polymerase chain reaction and western blot analysis *in vivo*. Human pulmonary artery smooth muscle cells (HPASMCs) were cultured under hypoxic condition with or without Tregs for 48 h, and the proliferation rate and cell cycle of HPASMCs were determined. In addition, the protein levels of phosphorylated (p)-Akt and p-extracellular signal-regulated kinase (ERK) were measured in HPASMCs *in vitro*. The results showed that Treg treatment significantly reduced the increased the hypoxia-induced RVSP and Fulton's index, decreased pro-inflammatory cytokine expression as well as enhanced IL-10 levels *in vivo*. Furthermore, Treg treatment significantly reduced HPASMCs proliferation and the expression of cyclin D1, cyclin-dependent kinase (CDK)4, p-Akt and p-ERK, as well as increased p27 expression *in vitro*. In conclusion, the results of the present study indicated that Tregs protected against hypoxia-induced PAH in mice; the

mechanisms of which may proceed via the suppression of the inflammatory response, as Tregs were found to enhance anti-inflammatory cytokine levels, inhibit HPASMCs proliferation and regulate the cell cycle. These results therefore indicated that Tregs may be a potential novel target for the treatment of PAH.

Introduction

Pulmonary arterial hypertension (PAH) is a life-threatening disease, which contributes to the morbidity and mortality of patients with various lung and heart diseases (1). PAH has a multifactorial pathology and the pathogenesis remains to be fully elucidated. It has been reported that a numerous cell types, including endothelial cells, smooth muscle cells as well as inflammatory cells and platelets, may be implicated in the progression of PAH (2).

Vascular smooth muscle cells (VSMCs) are located in the medial wall of blood vessels; under normal conditions, these cells are quiescent and express a differentiated phenotype in order to maintain vascular tone (3). However, under pathological conditions, VSMCs switch to a 'synthetic' phenotype, in which they secrete inflammatory cytokines and contribute to vascular pathogenesis (4). VSMCs proliferation in the pulmonary artery has been considered to be one of the primary causes of pulmonary arterial remodeling (5). Progressive pulmonary arterial remodeling is characteristic of PAH and has an important role in the persistent deterioration involved in PAH, as well as contributes to the difficult reversal of the disease phenotype (6).

The role of the immune system in the progression of PAH has been the focus of numerous studies in recent years (7). However, the immunomodulatory mechanisms which contribute to the pathogenesis of the disease remain to be fully elucidated. CD4⁺CD25⁺ regulatory T cells (Tregs) are a specific subpopulation of T cells, which have been reported to participate in the regulation of the immune response as well as the progression of autoimmune diseases (8). Therefore Treg deficiency or dysfunction may disrupt immune homeostasis and lead to numerous pathological conditions.

At present, few therapies have been developed for the effective treatment of pulmonary arterial structure remodeling and PAH. Tregs have been reported to exert beneficial effects

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on the progression of numerous diseases (9,10), including atherosclerosis (11), abdominal aortic aneurysms (12) and inflammatory bowel disease (13). However, the role of Tregs in the development of PAH remains to be elucidated. The present study aimed to determine whether Tregs affected the development of PAH, and to investigate the underlying mechanisms.

Materials and methods

Animals. A total of 60 male C57BL/6 J mice (10 weeks old) were purchased from Beijing University Animal Research Center (Beijing, China). The mice were randomly divided into three groups, with 20 mice in each, as follows: Normoxia, hypoxia control and Tregs. Mice in the normoxia group were maintained in air and exposed to a normoxic environment. Mice in the hypoxia control and Tregs groups were exposed to hypoxic conditions (10% O₂), as maintained using a litre ventilated chamber (volume, 500 l; Flufrance apparatus, Cachan, France) for four weeks. All animals were kept in the same room and had access to standard mouse feed and water, the hypoxic group were kept in a hypoxic chamber. All animal procedures were reviewed and approved by the Animal Care and Use Committee of Shandong University (Jinan, China).

Isolation and adoptive transfer study. Ten C57BL/6 J (six weeks old) wild-type male mice, also obtained from Beijing University Animal Research Centre, served as Treg cells donors. These mice were housed in a pathogen-free animal care facility at a constant temperature (24°C) and a 12-h light/dark cycle, with free access to water. Mice were euthanized by ketamine-xylazine (75 and 3 mg/kg, respectively; Sigma-Aldrich, St. Louis, MO, USA) injection and then immersed in 75% ethanol (Annjet, Shandong, China) for 10 minutes and spleens were then isolated from the mice under aseptic conditions. Spleens were then gently mechanically disrupted and passed through a cell strainer. phosphate-buffered saline (PBS; Sigma-Aldrich) was then added to make a suspension up to 10 ml and the suspension was centrifuged at 800 x g for 5 min at 4°C. The supernatant was then discarded and the pellet was resuspended in 1 ml PBS. Purified Tregs were then isolated using a CD4⁺CD25⁺ Regulatory T cell Isolation kit (Miltenyi Biotec, Bergisch Gladbach, Germany). The average purity of Tregs was >97%, as determined by fluorescence-activated cell sorting analysis (BD FACSCalibur; BD Biosciences, Franklin Lakes, NJ, USA). Cells were suspended in a total volume of 200 µl PBS for injection, as previously described (9,10,14). One day before exposure to hypoxic conditions, the mice in the control and Tregs groups were injected with PBS or Tregs (1x10⁶ cells), respectively, into the tail vein.

Hemodynamic measurements. Mice were anesthetized via intraperitoneal injection of ketamine (6 mg/100 g) and xylazine (1 mg/100 g). Mice were then placed in a supine position and the trachea was cannulated. A 26-gauge needle (Sigma-Aldrich) was passed percutaneously into the thorax via a subxyphoid approach and the right ventricular systolic pressure (RVSP) was measured and recorded using a miniature pressure transducer (MPCU-200; Millar, Houston, TX, USA) digitized by a data acquisition system (Hitachi, Tokyo, Japan). During

surgery, the mice inhaled room air spontaneously and their heart rate was maintained between 300 and 600 beats/min. Following measurement of RVSP, the right ventricle (RV) was isolated from the left ventricle (LV) and the septum (S) and each were weighed in order to calculate the Fulton's index [RV/(LV+S)].

Lipid profile. Mice were starved overnight and blood samples were collected prior to sacrifice. Serum levels of total cholesterol (TC), triglyceride (TG), low density lipoprotein cholesterol (LDL-C) and high density lipoprotein cholesterol (HDL-C) were determined using an enzymatic assay.

Cell culture. Human peripheral blood mononuclear cells (PBMCs) were isolated from eight healthy volunteers (male:female, 5:3; aged 20-45). This study was approved by the ethics committee of Qilu Hospital, Shandong University and written informed consent was obtained from each patient for the use of their blood samples. Tregs were isolated from PBMCs using a CD4⁺CD25⁺ Regulatory T cell Isolation kit (Miltenyi Biotec) according to the manufacturer's instructions. Human pulmonary artery smooth muscle cells (HPASMCs) were purchased from the American Type Culture Collection (Manassas, VA, USA) and cultured in Dulbecco's modified Eagle's medium (ScienCell Research Laboratories, Carlsbad, CA, USA) containing 10% fetal bovine serum (FBS; Gibco-BRL, Carlsbad, CA, USA) at 37°C in a 5% CO₂ and 95% air atmosphere. Cells at up to passage 4 were used for the subsequent experiments. HPASMCs were kept under hypoxic conditions (1% O₂, 5% CO₂) in a cell culture without Tregs (control group) or with Tregs (5x10⁵/well; Tregs group) in the presence of mouse monoclonal anti-CD3 antibody (50 ng/ml; #ab8671) at 37°C for 48 h, as previously described (15). At last, floating T cells were discarded, HASMCs were collected.

Measurement of HPASMC proliferation. The proliferation of HPASMCs was determined using an MTT assay. Briefly, HPASMCs were seeded into 96-well plates at a density of 5,000 cells/well. Following exposure to hypoxic conditions without or with Tregs treatment, HPASMCs were incubated with 10 µl MTT (5 mg/ml)/well reagent for 4 h at 37°C. The supernatant was carefully removed and 75 µl/well dimethyl-sulfoxide (DMSO) was added to dissolve the formazan crystals. Samples were then analyzed at 570 nm using a Varioskan Flash multifunction plate reader (Thermo Scientific, Waltham, MO, USA).

For cell counting, HPASMCs were seeded into a six-well plate (5,000 cells/well) and then treated as described above. Cells were then washed with PBS, harvested with trypsin, and counted using a hemacytometer (Beckman Coulter, Inc., Fullerton, CA, USA).

Reverse transcription quantitative polymerase chain reaction (RT-qPCR). The lungs of the mice were isolated following sacrifice and HPASMCs were harvested, as described above. RNA was then prepared using TRIzol[®] reagent (Invitrogen Life Technologies, Carlsbad, CA, USA). RNA concentrations were determined using standard spectrophotometric techniques (Varioskan Flash; Thermo Fisher Scientific, St. Louis, MO, USA) and the mRNA expression was analyzed. For the

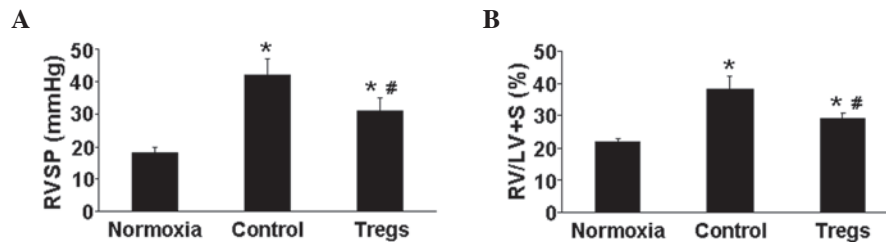


Figure 1. Tregs reduce the increased (A) RVSP and (B) Fulton's index in mice following hypoxia. *P<0.05 vs. the normoxia group; #P<0.05 vs. the control group. Tregs, CD4⁺CD25⁺ regulatory T cells; RVSP, right ventricular systolic pressure; RV/LV+S, weight of the right ventricle/left ventricle +septum (Fulton's index).

in vivo experiments, qPCR was performed using the following primers (GenePharma, Shanghai, China): forkhead/winged helix transcription factor (Foxp3) sense, 5'-CCCATCCCC AGGAGTCTTG-3' and antisense, 5'-ACCATGACTAGGGG ACTGTA-3'; monocyte chemotactic protein 1 (MCP-1) sense, 5'-CAGCCAGATGCAGTTAACGC-3' and antisense, 5'-GCC TACTCATTTGGGATCATCTTG-3'; interleukin (IL)-1 β sense, 5'-GCAACTGTTCTCTGAAGTCAACT-3' and antisense, 5'-ATCTTTTGGGGTCCGTCAACT-3'; IL-6 sense, 5'-AGT CACAGAAGGAGTGGCTAAG-3' and antisense, 5'-GAG GAATGTCCACAACTGATA-3'; IL-10 sense, 5'-GCTCTT ACTGACTGGCATGAG-3' and antisense, 5'-CGCAGCTCT AGGAGCATGTG-3'; and β -actin sense, 5'-CACTGTGCC CATCTACGA-3' and antisense, 5'-GTAGTCTGTTCAGGTC CCG-3'.

For the *in vitro* experiment, the sequences of primers were as follows: Cyclin D1 sense, 5'-CTC CTCTCCGGAGCATTT TGATA3' and antisense, 5'-TTAAAGACAGTTTTTGGG TAATCT3'; cyclin-dependent kinase (CDK)4 sense, 5'-ATG GCTACCTCTCGATATGAGCCA-3' and antisense, 5'-TCA CTCCGGATTACCTTCATCCTT-3'; p27 sense, 5'-CTTGGA GAAGCACTGCCGAGA-3' and antisense, 5'-CCCTGGACA CTGCTCCGCTA-3'; and β -actin sense, 5'-CATGTACGT TGCTATCCAGGC-3' and antisense, 5'-CTCCTTAATGTC ACGCAGAT-3'. Amplification, detection and data analysis were performed using the iCycler Real-Time PCR system (Bio-Rad Laboratories, Inc., Hercules, CA, USA). Relative expression of genes was calculated using the $2^{-\Delta\Delta C_t}$ method. Each sample was analyzed in triplicate, and expression was normalized to that of β -actin.

Western blot analysis. Total proteins were extracted from the lung of the mice or HPASMCs. Protein samples were separated using a 10-12% SDS-polyacrylamide gel (Beyotime, Jiangsu, China) and electrophoretically transferred onto a nitrocellulose membranes (Millipore, Billerica, MA, USA). Following blocking with 5% non-fat milk for 2 h at room temperature, the membranes were washed three times with Tris-buffered saline with Tween 20 (TBS-T; Boster, Hubei, China) for 10 min and then incubated with primary antibodies for rabbit polyclonal anti-cyclin D1 (1:1,000; #2922; Cell Signaling Technology, Danvers, MA, USA), rabbit polyclonal anti-CDK4 (1:500; #ab7955; Abcam, Cambridge, MA, USA), rabbit polyclonal anti-p27 (1:500; #ab7961; Abcam), rabbit monoclonal anti-Akt (1:1,000; #4685; Cell Signaling Technology, rabbit monoclonal p-Akt (1:1,000; #13038; Cell Signaling Technology), rabbit polyclonal anti-extracellular

signal-regulated kinase (ERK; #9102; Cell Signaling Technology), rabbit monoclonal anti-p-ERK (1:1,000; #4376; Cell Signaling Technology) and rabbit polyclonal anti- β -actin (1:1,000; #4967; Cell Signaling Technology) at 4°C overnight. Following washing three times in TBS-T, the membranes were incubated with a horseradish peroxidase-conjugated secondary antibody (Jackson ImmunoResearch, West Grove, PA, USA). The bands were detected using an enhanced chemiluminescence method (Millipore) and analyzed using Image-Pro Plus 6.0 (MediaCybernetics, Rockville, MD, USA).

Statistical analysis. SPSS 13.0 (SPSS Inc., Chicago, IL, USA) was used to analyze the data. All statistical comparisons were tested using an unpaired Student's t-test or one-way analysis of variance. Values are presented as the mean \pm standard error and P<0.05 was considered to indicate a statistically significant difference between values.

Results

Body weight and lipid profile studies. Following four weeks of treatment, only one mouse succumbed in the control group and no adverse effects were observed in each group during the experiment. No significant differences were observed in the body weight and serum levels of TC, TG, LDL-C and HDL-C among the three groups (data not shown). These results suggested that Tregs had no effect on the above parameters in mice.

Adoptive transfer of Tregs enhances Foxp3 expression. Foxp3 is a specific marker for Tregs lineage, which is exclusively expressed in Tregs and is a requisite factor for the maturation and function of Tregs; in addition, deficiency of Foxp3 may result in Treg dysfunction (16). In the present study, Foxp3 mRNA expression in the lungs of the mice was measured in order to assess whether the intravenously injected Tregs were successful in infiltrating the lung tissues. The results showed that Foxp3 mRNA was significantly increased in mice treated with Tregs compared with that of the control group (data not shown). These results confirmed that the exogenous Tregs were present in the lung tissues.

Adoptive transfer of Tregs improves hypoxia-induced pulmonary hypertension and vascular remodeling. The hemodynamic parameters of mice were measured prior to euthanasia. Compared with that of the normoxia group, hypoxia-exposed mice demonstrated a significant increase in RVSP; however, following

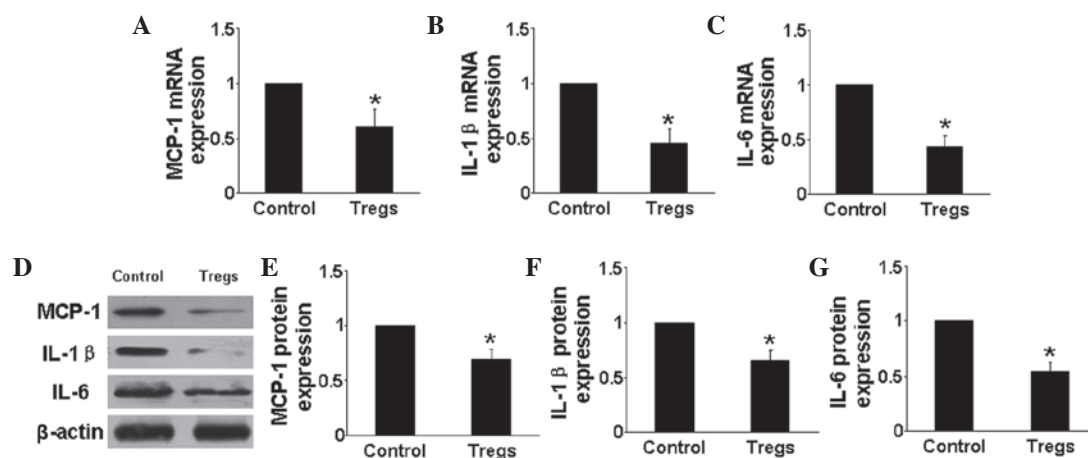


Figure 2. Tregs reduce pro-inflammatory cytokine expression *in vivo*. mRNA expression of (A) MCP-1, (B) IL-1 β and (C) IL-6 was assessed using reverse transcription quantitative polymerase chain reaction. (D) Protein expression of (E) MCP-1, (F) IL-1 β and (G) IL-6 was assessed using western blot and quantitative analysis. * $P < 0.05$ vs. the control group. Tregs, CD4⁺CD25⁺ regulatory T cells; MCP-1, monocyte chemoattractant protein 1; IL, interleukin.

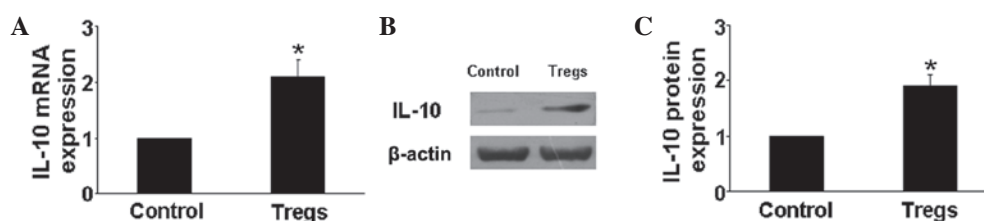


Figure 3. Tregs enhance anti-inflammatory cytokine IL-10 expression *in vivo*. (A) mRNA and (B and C) protein expression of IL-10 were analyzed using reverse transcription polymerase chain reaction as well as western blot and quantitative analysis, respectively. * $P < 0.05$ vs. the control group. Tregs, CD4⁺CD25⁺ regulatory T cells; IL-10, interleukin 10.

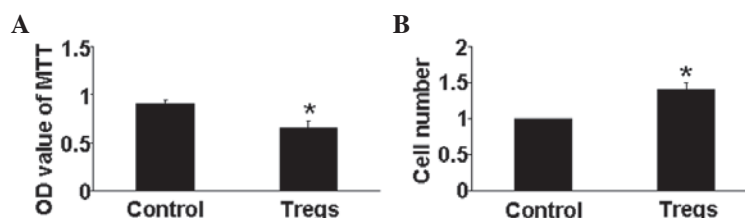


Figure 4. Tregs reduce hypoxia-induced human pulmonary artery smooth muscle cell proliferation. Cell proliferation was assessed using (A) MTT assay and (B) cell counting assay. * $P < 0.05$ vs. the control group. Tregs, CD4⁺CD25⁺ regulatory T cells; OD, optical density.

Tregs treatment, RVSP was significantly reduced compared with that of the hypoxia control group ($P < 0.05$) (Fig. 1A). Chronic severe PAH is a cardiopulmonary disease which may affect RV hypertrophy, which is promoted through exposure to chronic hypoxia (4). In the present study, the Fulton's index was measured for the assessment of RV hypertrophy. The results showed that exposure to hypoxia was associated with an increase in the Fulton's index; however, the increased index observed in the hypoxic control group was partially reversed in the Tregs group ($P < 0.05$) (Fig. 1B). This therefore indicated that Tregs may improve hypoxia-induced pulmonary hypertension and vascular remodeling.

Tregs downregulate proinflammatory cytokine expression and upregulate anti-inflammatory cytokine expression in vivo. RT-qPCR and western blot analysis were used to determine the

mRNA and protein expression, respectively, of pro-inflammatory cytokines, including MCP-1, IL-1 β and IL-6 (Fig. 2), as well as the anti-inflammatory cytokine IL-10 in the lungs of mice in each group. The results showed that mRNA and protein expression levels of each of the pro-inflammatory cytokines were significantly downregulated in the Tregs group compared with those of the hypoxia control group ($P < 0.05$) (Fig. 2A-G). In addition, the mRNA and protein expression levels of IL-10 were found to be significantly upregulated in the Tregs groups compared with that of the hypoxia control group ($P < 0.05$) (Fig. 3). These results indicated that Tregs regulated the inflammatory response through reducing the expression of proinflammatory cytokines and increasing that of anti-inflammatory cytokines.

Tregs reduce hypoxia-induced HPASMCs proliferation in vitro. The effect of Tregs on HPASMCs proliferation

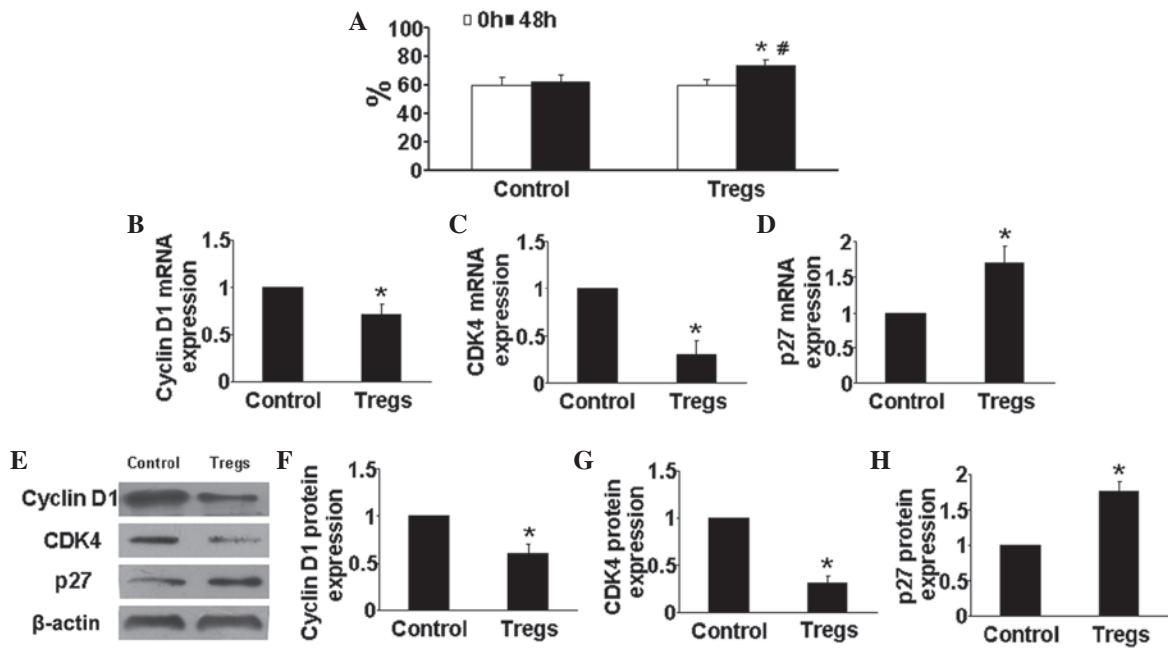


Figure 5. Tregs influence the cell mitosis cycle of human pulmonary artery smooth muscle cells. (A) Tregs arrested HPASMCs in G1/G0-phase under hypoxic conditions for 48 h compared with that of 0 h. mRNA expression of (B) cyclin D1, (C) CDK4 and (D) p27 was assessed using reverse transcription quantitative polymerase chain reaction. (E) Protein expression of (F) cyclin D1, (G) CDK4 and (H) p27 was assessed using western blot and quantitative analysis. * $P < 0.05$ vs. the control group. Tregs, CD4⁺CD25⁺ regulatory T cells; CDK4, cyclin dependent kinase 4.

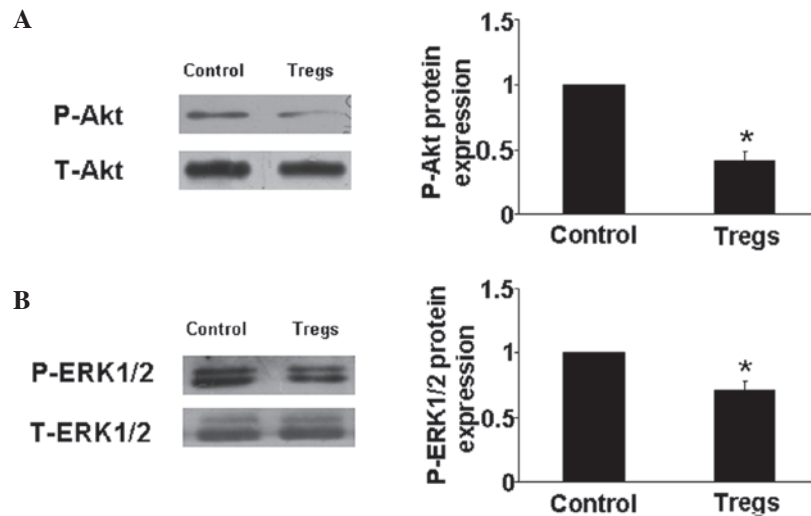


Figure 6. Tregs reduce the phosphorylation of Akt and ERK in human pulmonary artery smooth muscle cells *in vitro*. Phosphorylation of (A) Akt and (B) ERK were assayed using western blot and quantitative analysis. * $P < 0.05$ vs. the control group. Tregs, CD4⁺CD25⁺ regulatory T cells; ERK, extracellular signal-regulated kinase; P-, phosphorylated; T-, total.

under hypoxic conditions was determined using an MTT assay. The results showed that Tregs significantly inhibited HPASMCs proliferation compared with that of the hypoxia control group ($P < 0.05$) (Fig. 4A). In addition, a cell counting assay demonstrated a significant increase in cell number in the Treg groups ($P < 0.05$) (Fig. 4B). Therefore, the regulation of HPASMC proliferation by Tregs may be an important anti-PAH mechanism.

Tregs arrest HPASMCs in G1/G0-phase under hypoxic conditions. Cell proliferation is dependent on cell cycle transition from G1/G0 to G2/S-phase (17); therefore, in the present study,

the effects of Tregs on cell cycle of HPASMCs was evaluated. As shown in Fig. 5A, compared with that of the hypoxia control group, the Tregs group demonstrated a significantly increase percentage of HPASMCs in the G₁/G₀ phase ($P < 0.05$).

Tregs reduce cyclin D1 and CDK4 expression as well as increase p27 expression. Previous studies shown that cyclin D1, CDK4 and p27 have key roles in proliferation and the cell cycle. In the present study, the expression levels of cyclin D1, CDK4 and p27 in HPASMCs were determined *in vitro*. Compared with those of the control group, the mRNA and protein expression levels of cyclin D1 and CDK4 were markedly

reduced in Tregs-treated HPASMCs ($P < 0.05$) (Fig. 5B, C, E and G), whereas Tregs treatment significantly enhanced p27 mRNA and protein expression compared with that of the hypoxia control group ($P < 0.05$) (Fig. 5D and H). These results suggested that the effect of Tregs on cell cycle regulation may be another mechanism by which it exerts anti-PAH effects.

Tregs decrease Akt and ERK1/2 phosphorylation. It has been previously reported that the Akt and ERK pathways were involved in HPASMCs proliferation and the progression of PAH (4). Therefore, in the present study, western blot analysis was used to determine Akt and ERK protein expression *in vitro*. The results showed that Tregs significantly downregulated the phosphorylation of Akt and ERK compared with those of the hypoxia control group ($P < 0.05$) (Fig. 6). This may therefore be a further protective mechanism of Tregs against PAH.

Discussion

PAH is a fatal disease with unknown etiology. Numerous studies have focused on the development and treatment of PAH; however, there remains to be few therapies which are effective in treating the disease. Tregs suppress the activation and proliferation of effector T cells, prevent autoimmunity and control autoimmune diseases (18). In recent years studies have increasingly focus on the role of immune mechanisms in modulating the disease process. However, whether Tregs have a beneficial and protective effect on PAH remained to be elucidated. The present study provided direct evidence that Tregs treatment prevented the progression of PAH in an animal model. To the best of our knowledge, the present study was the first to show that Tregs significantly suppressed the inflammatory response through enhancing anti-inflammatory cytokine levels, inhibiting HPASMCs proliferation and regulating their cell cycle.

It was hypothesized that exposure to chronic hypoxia may increase vasomotor tone and structural remodeling of the pulmonary vascular bed in PAH patients, leading to pulmonary hypertension. Previous *in vivo* experiments have shown that hypoxia was able to induce pulmonary hyperation (6); in addition, increased right ventricular pressure confirmed the successfully established PAH animal models. In the present study, mice in control group developed a higher RVSP compared with that of those in the normoxia group, which was in accordance with a prior study (6). However, in the present study, RVSP was significantly reduced following Tregs treatment, which suggested that Tregs exerted a beneficial on pulmonary hypertension. Increased pulmonary artery remodeling is characteristic of PAH. An increased afterload resulted in a degree hypertrophy of RV (14). The results of the present study showed that Tregs partially reversed the increased Fulton's index induced by hypoxia, therefore improving the hemodynamic parameters and vascular remodeling in PAH. Furthermore, Tregs therapy did not affect lipid parameters in the animal model, suggesting that the effects of Tregs on PAH were lipid-independent.

Inflammatory processes involved the pathogenesis of PAH are increasingly considered as major pathogenic components of pulmonary vascular remodeling (19). Infiltration of inflammatory cells and increased levels of inflammatory cytokines

have been observed in the vascular lesions of PAH (20). Previous studies have shown that in comparison with healthy populations, the circulating and pulmonary expression of inflammatory cytokines was significantly increased in patients with PAH (21,22). Another study demonstrated that IL-6 knockout mice did not develop pulmonary hypertension, while the overexpression of IL-6 accelerated spontaneous pulmonary vascular remodeling and progression *in vivo* (23). IL-10, known as a pleiotropic anti-inflammatory cytokine, has been reported to inhibit inflammatory cell infiltration and pro-inflammatory cytokine secretion (24). A previous study reported that overexpression of IL-10 prevented the development of monocrotaline-induced PAH in animal models (25). Furthermore, IL-10, as a potent immunomodulator, was shown to mediate the effects of Tregs (26). The results of the present study showed that Tregs suppressed the mRNA and protein expression of pro-inflammatory cytokines, including MCP-1, IL-1 β and IL-6, as well as upregulated the expression of anti-inflammatory cytokine IL-10. This therefore indicated that Tregs modulated the balance of the inflammatory response, which may a mechanism by which Tregs exert a protective effect against PAH.

The principal phenotype of VSMCs is contraction, which preserves vasodilation and blood flow regulation under physiological conditions. However, VSMCs have been shown to have a 'synthetic' phenotype under pathological conditions, in which they have an increased capacity to proliferate and generate the matrix components of the blood vessel wall, which contributes to vascular remodeling (27). In addition, aberrant HPASMC proliferation has been reported to lead to pulmonary arterial remodeling and contribute to the progression of PAH. Effective inhibition of the aberrant HPASMCs may delay and even cease the deteriorative progress of PAH (28). In the present study, the role of Tregs in hypoxia-induced HPASMCs proliferation was evaluated and the results showed that Tregs effectively inhibited HPASMCs proliferation. Therefore, the anti-PAH properties of Tregs in treated-mice may be attributed to their role in HPASMCs proliferation.

Under hypoxic conditions, an increase number of HPASMCs enter the mitosis phase of the cycle; in addition, the acceleration of the cell cycle is an initial factor in cell proliferation. Hypoxia retains small cell numbers in G0/G1 phase and promotes HPASMCs to enter G2/S phase. In the present study, the effects of Tregs on the cell cycle of HPASMCs was assessed and the results demonstrated that Tregs reversed the effects of hypoxia on the cell cycle. A previous study reported that the balance between cell quiescence and proliferation was regulated by CDKs and CDK inhibitors (29). Cyclin D1 and CDKs, primarily CDK4, are key cell cycle control genes, which were associated with cell proliferation and facilitated the transition of cells from the G1 phase into the S phase (30). Overexpression of CDK4 promotes cell proliferation and inhibition of CDK4 expression may lead to the arrest of the G1 phase and suppression of cell proliferation (31). The present study showed that Tregs significantly reduced Cyclin D1 and CDK4 expression *in vitro*. P27, as one of the key CDK inhibitors, effectively inhibits Cyclin D1-CDK4 protein kinase activity and negatively regulates G1 progression in cells; in addition, the overexpression of p27 induces G1 arrest and decreases HPASMCs proliferation (32). The results of the

present study revealed that Tregs significantly increased p27 mRNA and protein expression, therefore indicating that Tregs promoted G1 phase arrest, which may be the direct mechanism of Tregs against HPASMCs proliferation and PAH.

Akt and ERK are activated via diverse extracellular signals, which trigger cell cascade responses, including cell growth, proliferation, survival and motility. Therefore, in the present study, the expression of Akt and ERK were investigated their in HPASMCs and the results showed that Tregs blocked the activation of Akt and ERK pathway induced through hypoxia.

In conclusion, the results of the present study demonstrated that Tregs protected against hypoxia-induced PAH in mice. These findings provided evidence for a possible targeted therapy for the treatment of pulmonary hypertensive disorders.

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