Materials and Methods

Animals and procedures

Ten-wk-old female Sprague-Dawley was used for this study. Rats were maintained at constant humidity (60±5%), temperature (24±1°C), and light cycle (6 A.M. to 6 P.M.) and fed a standard rat pellet diet ad libitum. All protocols were approved by the Institutional Animal Care and Use Committee (IACUC) at the University of Alabama at Birmingham and were consistent with the Guide for the Care and Use of Laboratory Animals published by the National Institutes of Health "Public Health Service Policy on Humane Care and Use of Animals, DHEW Publication No. 96-01, PHS Policy revised in 2002".

Rats were ovariectomized (OVX) in order to eliminate the effects of ovarian hormones on vascular inflammation/remodeling as described previously (1). One week after OVX, rats were randomly divided into four groups: Group-1: Vehicle control rats received s.c. injection of phosphate buffered saline (PBS); Group-2: Monocrotaline (MCT) alone rats received s.c. injection of MCT (60 mg/kg, 60 mg/ml PBS); Group-3: MCT+Null-EC rats received MCT treatment (60 mg/kg, s.c.) and 24 hrs later were transfused i.v. with rat pulmonary arterial endothelial cells (ECs) transduced with an empty adenoviral vector (total $0.5x10^6$ Null-ECs in 500 µl saline at 1 day post MCT treatment); and Group-4: MCT+IL8RA/RB-EC rats received MCT treatment (60 mg/kg, s.c.) and 24 hrs later were transfused i.v. with rat ECs overexpressing IL8RA and RB (total $0.5x10^6$ IL8RA/B-ECs in 500 µl saline at 1 day post MCT treatment). MCT was prepared at 60 mg/ml by mixing 500 mg of MCT (Sigma-Aldrich) in 7.5 ml of PBS and

then adding 0.5 ml of 1 N HCl (to dissolve MCT) and 0.3 ml of 1 N NaOH (to neutralize the pH).

Transfusion of rat pulmonary arterial endothelial cells (ECs) overexpressing IL8RA and IL8RB

Rat pulmonary arterial endothelial cells (ECs) were prepared and characterized as described previously (2,3) and transduced with adenoviral vectors carrying IL8RA and IL8RB cDNAs and the green fluorescent protein (GFP) marker gene or with control empty adenoviral vectors (Null) with a GFP gene as described previously (4,5). Transduced ECs or saline vehicle were transfused into conscious rats through a femoral venous catheter that had been implanted two days before (1 day prior to MCT treatment). The femoral venous catheter was removed one day after the EC transfusion under ketamine-xylazine (80-15 mg/kg, i.p.) anesthesia. An analgesic agent (buprenorphine, 0.05 mg/kg, s.c.) was given BID for 3 days after each survival surgery.

Doppler imaging of pulmonary artery outflow and assessment of right ventricular (RV) pressure and hypertrophy

At 4 wks after MCT treatment ± ECs transfusion, pulse wave Doppler analysis of pulmonary artery outflow was performed in isofluorane (1.5%) anesthetized rats using a Phillips Sonos 5500 ultrasound system equipped with a 15-MHz transducer as described previously (6). Pulmonary flow was recorded using pulse wave Doppler in the parasternal view at the level of the aortic valve. The sample level was placed proximal to the pulmonary valve leaflets and aligned to maximize laminar flow.

Rats were then anesthetized with ketamine (80 mg/kg, i.p.) and xylazine (15 mg/kg, i.p.) and the chest was opened to expose the RV. A 23 gauge needle connected

to a pressure transducer was inserted into RV to measure RV pressure using a Grass Model 7D polygraph as described previously (7). Hearts were quickly removed, dissected in to RV and left ventricle and septum (LV+S) and weighed to calculate the RV/(LV+S) ratios as indices of RV hypertrophy.

Morphometric analysis of pulmonary arteriole as remodeling after MCT treatment ± IL8RA/B-EC transfusion

At 4 wks after MCT treatment ± ECs transfusion, rats were sacrificed and lungs were perfused with PBS followed by 10% formalin. Five µm paraffin-embedded lung sections were stained with hematoxylin and eosin. Quantitative morphometric analysis of pulmonary arteriolar hypertrophy/remodeling was carried out by light microscopy with a Qimaging QiCam digital camera (Qimaging) interfaced with a computer system running Metamorph 6.2v4 software (Universal Imaging) as described previously (8). Pulmonary arterioles (defined as vessels that accompanied bronchi, ranging in external diameter from 50 to 200 µm) were evaluated. Pulmonary arteriole wall area was measured along the shortest curvature of the lumen diameter. Medial area (area between external and internal elastic lamellae) was calculated from total vessel area (area within external elastic lamella) – lumen area (area within internal elastic lamella).

Immunohistochemical analysis of chemokines and NOS expression, inflammatory cell infiltration and localization of transfused ECs in MCT injured lungs

In separate groups of rats, at 2 days after MCT treatment and 1 day after ECs transfusion, lungs were harvested, fixed in 10% formalin and embedded in paraffin. The avidin-biotin-peroxidase immunohistochemical technique was used to detect endothelial NO synthase (eNOS), inducible NOS (iNOS), neutrophils, monocytes/macrophages, IL8

(CINC-2β-cytokine induced neutrophil chemoattractant-2-beta), and monocyte chemotactic protein (MCP)-1 in paraffin-embedded sections (5 μm) of lungs using specific primary antibodies and a Vector Laboratories kit (Biotechnology) as described previously (4,5). To detect the GFP-labeled IL8RA/B-ECs in lung sections of MCT treated rats, fluorescence microscopy was used to examine frozen sections of fresh lung of MCT+IL8-RA/RB-EC rats one day after transfusion of IL8RA/B-ECs.

Cytokines/Chemokines Measurement

Pro-inflammatory cytokine/chemokine protein levels in lung were measured in lung homogenates (200 mg lung tissue in 2 ml T-PER Tissue Protein Extraction Reagent with proteinase inhibitors [Termo Scientific], and centrifuged at 10,000g for 5 min at 4^{o}C) using commercially available Multiplexed rat-specific magnetic beads-based sandwich immunoassay kits with the Luminex xMAP analyzer (Milliplex Rat Cytokine/Chemokine Panel, Millipore) according to manufacturer's instruction.

Statistical analysis

In each *in vivo* experiment, rats were age matched to minimize individual differences. Results were expressed as means±SEM. Statistical analysis was carried out using the SigmaStat statistical package (Version 3.5). The equivalence of variance and normality of the data were tested by SigmaStat statistical program. The primary statistical test was two-way and one-way analysis of variance (ANOVA). When the overall *F* test result of ANOVA was significant, a multiple-comparison post-hoc Tukey test was applied. Student's t-test was used in two-mean comparisons. Differences were reported as significant when $P<0.05$.

References

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