Targeted Delivery of Pulmonary Arterial Endothelial Cells Overexpressing Interleukin-8 Receptors Attenuates Monocrotaline-Induced Pulmonary Vascular Remodeling

Jinyan Fu, Yiu-Fai Chen, Xiangmin Zhao, Judy R. Creighton, Yuanyuan Guo, Fadi G. Hage, Suzanne Oparil, Daisy D. Xing

Objective—Interleukin-8 (IL-8) receptors IL8RA and IL8RB (IL8RA/B) on neutrophil membranes bind to IL-8 with high affinity and play a critical role in neutrophil recruitment to sites of injury and inflammation. This study tested the hypothesis that administration of rat pulmonary arterial endothelial cells (ECs) overexpressing IL8RA/B can accelerate the adhesion of ECs to the injured lung and inhibit monocrotaline-induced pulmonary inflammation, arterial thickening and hypertension, and right ventricular hypertrophy.

Approach and Results—The treatment groups included 10-week-old ovariectomized Sprague-Dawley rats that received subcutaneous injection of PBS (vehicle), a single injection of monocrotaline (monocrotaline alone, 60 mg/kg, SC), monocrotaline followed by intravenous transduction of ECs transduced with the empty adenoviral vector (null-EC), and monocrotaline followed by intravenous transduction of ECs overexpressing IL8RA/B (1.5×10⁶ cells/rat). Two days or 4 weeks after monocrotaline treatment, endothelial nitric oxide synthase, inducible nitric oxide synthase, cytokine-induced neutrophil chemoattractant-2β (IL-8 equivalent in rat), and monocyte chemoattractant protein-1 expression, neutrophil and macrophage infiltration into pulmonary arterioles, and arteriolar and alveolar morphology were measured by histological and immunohistochemical techniques. Proinflammatory cytokine/chemokine protein levels were measured by Multiplex rat-specific magnetic bead–based sandwich immunoassay in total lung homogenates. Transfusion of ECs overexpressing IL8RA/B significantly reduced monocrotaline-induced neutrophil infiltration and proinflammatory mediator (IL-8, monocyte chemoattractant protein-1, inducible nitric oxide synthase, cytokine-induced neutrophil chemoattractant, and macrophage inflammatory protein-2) expression in lungs and pulmonary arterioles and alveoli, pulmonary arterial pressure, and pulmonary arterial and right ventricular hypertrophy and remodeling.

Conclusions—These provocative findings suggest that targeted delivery of ECs overexpressing IL8RA/B is effective in repairing the injured pulmonary vasculature. (Arterioscler Thromb Vasc Biol. 2014;34:1539-1547.)

Key Words: cell- and tissue-based therapy ■ receptors, interleukin-8

The neutrophil chemoattractant interleukin-8 (IL-8; also named cytokine-induced neutrophil chemoattractant [CINC]-2β in rat) is expressed in the setting of acute vascular injury. Neutrophils migrate to injured tissue, triggering an initial proinflammatory response and facilitating the influx of other types of inflammatory cells (eg, monocytes/macrophages). Selective IL-8 receptors (IL8RA and IL8RB; also named C-X-C chemokine receptor [CXCR]1 and CXCR2) on the cell membrane of neutrophils bind to IL-8 or N-acetylated proline–glycine–proline (a specific collagen-derived tripeptide fragment expressed in injured tissue and 3-dimensionally structurally similar to IL-8)¹,² and serve as homing devices to orient neutrophils to target injured tissue.

See accompanying editorial on page 1336

We have recently developed an innovative strategy to improve the efficiency of cell-based therapy by intravenously transfusing rat aortic endothelial cells (RAECs) overexpressing IL8RA and IL8RB (IL-8 receptors A and B [IL8RA/B]) into rats with balloon injury of the carotid artery³ or myocardial infarction caused by ligation of the left anterior descending coronary artery.⁴ We demonstrated that acute intravenous transfusion of RAECs equipped with IL8RA/B results in targeting and adherence of the transformed RAECs to injured carotid artery³ or left ventricle (LV),⁵ decreasing infiltration of activated neutrophils, inhibiting proinflammatory responses, reducing neointima formation in balloon injured carotid artery,³ or enhancing neovascularization in left anterior descending ligation–injured LV.⁴

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Using the rat model of monocrotaline-induced lung injury, the present study tested the hypothesis that acute administration of pulmonary arterial endothelial cells (ECs) equipped with IL8RA/B alleviates monocrotaline-induced pulmonary vascular hypertrophy/remodeling and right ventricular (RV) hypertrophy by decreasing neutrophil infiltration and proinflammatory mediator production, as well as enhancing endothelial nitric oxide synthase (eNOS) expression in ECs. Rat ECs transduced with adenoviral vectors carrying neutrophil IL8RA/B were transfused into the femoral vein of rats 24 hours after monocrotaline treatment. We demonstrated that, compared with the control groups, administration of ECs overexpressing IL8RA/B reduced monocrotaline-induced neutrophil infiltration and proinflammatory mediator expression in pulmonary arterioles and alveoli, as well as pulmonary arterial and RV hypertrophy and remodeling in rats after experimentally induced pulmonary vascular injury.

Materials and Methods
Materials and Methods are available in the online-only Supplement.

Results
Transfusion of IL8RA/B-ECs Improved the Growth and Survival of Monocrotaline-Treated Rats
Intravenous transfusion of ECs overexpressing IL8RA/B (IL8RA/B-EC, 1.5×10^6 cells per rat in 500 μL saline, 1 day after monocrotaline injection [60 mg/kg, SC]) resulted in greater weight gain than in monocrotaline-treated and monocrotaline+null-EC–treated (ECs transduced with control empty adenovirus) rats at 3 to 4 weeks after monocrotaline treatment (Figure 1A). Rats that received monocrotaline alone began to die within 3 to 4 weeks of treatment; survival was 25% in monocrotaline-alone rats, 50% in monocrotaline+null-EC rats, and 66.7% in monocrotaline+IL8RA/B rats at 9 weeks after monocrotaline treatment (Figure 1B). All dead rats exhibited significant RV hypertrophy. The ratio of RV/(LV+septum) weight (Fulton index) of monocrotaline-alone rats at death was 0.476±0.031 (n=12), which was significantly greater (P<0.05) than that of vehicle control rats, 0.248±0.010 (n=12).

Transfusion of IL8RA/B-ECs Partially Corrected the Monocrotaline-Induced Pulmonary Artery Midsystolic Notch
Doppler imaging echocardiographic analysis was used to visualize pulmonary artery outflow. We used the change in the shape of the pulmonary artery waveform as an index of pulmonary arterial hypertension at 4 weeks after monocrotaline treatment according to the report of Jones et al. As shown in Figure 2A, the development of a pulmonary artery midsystolic notch in monocrotaline-treated and monocrotaline+IL8RA/B-EC–treated rats was significant at 4 weeks after the monocrotaline treatment. Transfusion of IL8RA/B-ECs partially corrected the monocrotaline-induced midsystolic pulmonary artery notch. RV weight (measured after rats were euthanized) was related to the change in waveform of pulmonary artery flow.
Figure 2. A, Representative pulmonary outflow waveforms show monocrotaline (MCT)-induced midsystolic pulmonary artery notching. Doppler of pulmonary outflow was recorded in the parasternal view at the level of the aortic valve. Five rats were measured in each group 4 weeks after MCT treatment. Vehicle control rats did not receive MCT injection or endothelial cell (EC) transfection; MCT-alone rats received MCT injection (80 mg/kg, SC) without EC transfection; MCT+null-EC rats received MCT injection followed by intravenous transfection of ECs (1.5×10^6 cells per rat, 24 hours after the MCT injection) transduced with control empty adenovirus; and MCT+ECs overexpressing interleukin-8 receptors A and B (IL8RA/B-EC) rats received MCT injection followed by intravenous transfection of IL8RA/B-EC (1.5×10^6 cells per rat, 24 hours after the MCT injection). Note that (1) the midsystolic pulmonary artery notching was associated with right ventricular (RV) hypertension [RV/(left ventricle (LV)+septum (S)) weight ratio], an index of pulmonary arterial hypertension; and (2) transfection of IL8RA/B-EC prevented the MCT-induced change in the shape of the pulmonary arterial waveform. B, Transfusion of IL8RA/B-EC attenuated MCT-induced RV hypertension and (C) RV hypertrophy at 4 weeks after MCT injection. Mean RV pressure was measured directly by inserting a 23-gauge needle (connected to a transducer) into RV after the chest was opened using a Grass Model 7 polygraph. Note that the RV pressure measured using the open chest method was lower than that obtained in the same model using a closed chest method.° RV weights were normalized by the weights of LV and S (LV+S; Fulton index). Results are means±SEM; n=number of rats. *P<0.05 vs respective vehicle control group; #P<0.05 vs respective MCT-alone group. One-way ANOVA was used to analyze the data. When the overall F test result of ANOVA was significant, a multiple-comparison Tukey post hoc test was applied.

Transfusion of IL8RA/B-ECs Inhibited Monocrotaline-Induced RV Hypertrophy and Hypertension at 4 Weeks After Monocrotaline Treatment

Monocrotaline-treated and monocrotaline-null-EC–treated rats developed increased mean RV pressure (an index of chronic pulmonary arterial hypertension, assessed by inserting a 23-gauge needle directly into the RV in anesthetized open-chest rats; Figure 2B). The chronic pulmonary arterial hypertension in monocrotaline-treated and monocrotaline-null-EC–treated rats was confirmed by the presence of significant RV hypertrophy [RV/(LV+S) ratios; Figure 2C]. Transfusion of ECs that overexpress IL8RA/B significantly inhibited monocrotaline-induced RV hypertension and hypertrophy, whereas transfusion of null-ECs had no effect.

Transfusion of IL8RA/B-ECs Inhibited Monocrotaline-Induced Pulmonary Arterial Hypertrophy and Remodeling at 4 Weeks After Monocrotaline Treatment

Monocrotaline treatment markedly increased the medial area and decreased adventitial tissue in 50- to 200-μm pulmonary arterioles 4 weeks after the monocrotaline treatment (Figure 3). Transfusion with IL8RA/B-ECs prevented the development of medial hypertrophy and preserved the adventitial architecture of these vessels. In contrast, transfusion with null-ECs had no effect on monocrotaline-induced pulmonary vascular hypertrophy and remodeling.

Transfusion of IL8RA/B-ECs prevented monocrotaline-induced decreases in eNOS and increases in inducible NOS (iNOS) in pulmonary arterioles and alveoli at 2 days after monocrotaline treatment (and 1 day after IL8RA/B-EC treatment)

Immunohistochemical staining demonstrated large decreases in the number of eNOS-positive cells in pulmonary arterioles and alveoli of monocrotaline-treated and monocrotaline-null-EC–treated rats (Figure 4, top and Figures II and III in the online-only Data Supplement). Transfusion with IL8RA/B-ECs attenuated this effect on eNOS expression in pulmonary arterioles and alveoli of rats treated with monocrotaline. In contrast, monocrotaline treatment resulted in large increases in the number of iNOS-positive cells in pulmonary arterioles and alveoli (Figure 4, bottom and Figures IV and V in the online-only Data Supplement). Transfusion with IL8RA/B-ECs, but not null-ECs, significantly inhibited the monocrotaline-induced increase in iNOS expression in both arterioles and alveoli in injured lungs. The number of iNOS-positive cells in the injured lung was negatively correlated with the number of eNOS-positive...
cells ($R=-0.887$, $P<0.01$ for arterioles and $R=-0.552$, $P<0.05$ for alveoli). Transfusion of IL8RA/B-ECs inhibited monocrotaline-induced increases in IL-8 (CINC-2β in rat) and monocyte chemoattractant protein-1 (MCP-1)–positive cells in injured lungs at 2 days after monocrotaline treatment.

Immunohistochemical staining demonstrated large increases in the number of IL-8– and MCP-1–positive cells in arterioles and alveoli in lungs of monocrotaline-treated and monocrotaline+null-EC–treated rats (Figure 5 and Figures VI–IX in the online-only Data Supplement). Transfusion with IL8RA/B-ECs resulted in major reductions in the number of IL-8 and MCP-1 cells in these vessels compared with monocrotaline-treated and monocrotaline+null-EC–treated rats. The number of IL-8–positive cells in the injured lung was positively correlated with the number of MCP-1–positive cells ($R=0.823$, $P<0.01$ for arterioles and $R=0.614$, $P<0.05$ for alveoli).

Transfusion of IL8RA/B-ECs Inhibited Neutrophil and Monocyte/Macrophage Infiltration in Injured Lungs at 2 Days After Monocrotaline Treatment

Immunohistochemical staining demonstrated large numbers of neutrophils (myeloperoxidase-positive) and monocytes/macrophages (ED1-positive) in arterioles and alveoli in lungs of monocrotaline-treated and monocrotaline+null-EC–treated rats. Numbers of neutrophils and monocytes/macrophages were greatly reduced after IL8RA/B-EC treatment (Figure 6 and Figures X–XIII in the online-only Data Supplement). The numbers of neutrophil and monocyte/macrophage were positively correlated with numbers of IL-8– and MCP-1–positive (Figure 5) cells in pulmonary arterioles ($R=0.655$, $P<0.05$ for neutrophil/IL-8 and $R=0.916$, $P<0.01$ for monocyte/macrophage) and alveoli ($R=0.741$, $P<0.05$ for neutrophil/IL-8 and $R=0.260$, $P<0.05$ for monocyte/macrophage), respectively.

Transfusion of IL8RA/B-ECs prevented monocrotaline-induced increases in CINC-1 (rat homolog to human IL-8) and macrophage inflammatory protein (MIP)-2 expression in lungs at 2 days after monocrotaline treatment (and 1 day after IL8RA/B-EC treatment)

In separate groups of rats, Multiplex rat-specific magnetic bead–based sandwich immunoassay demonstrated significant increases in protein levels of proinflammatory mediators CINC1 and MIP-2 in whole lung homogenates of rats treated with monocrotaline (Figure 7B). In contrast, transfusion with IL8RA/B-ECs significantly decreased CINC1 and MIP-2 and increased MIP-1α protein expression in lungs of monocrotaline+IL8RA/B-EC rats compared with those of vehicle control or monocrotaline-treated rats (Figure 7D). Monocrotaline+null-EC treatment was also associated with decreased CINC1 and increased MIP-1α levels (Figure 7C and 7D). Neither monocrotaline treatment nor EC transfusion altered levels of the other cytokines/chemokines measured (eg, interleukin-1β, interleukin-10, MCP-1), vascular...
Targeted Delivery of ECs to Repair Pulmonary Vascular Injury

Alveoli

These anti-inflammatory effects of targeting ECs to the monocytes/macrophages in lungs of rats treated with monocrotaline, as well as infiltration of neutrophils and IL8RA/B homing devices significantly enhances eNOS transfusion of pulmonary arterial ECs overexpressing IL-8 receptors A and B (IL8RA/B-EC) or null-EC transfusion on monocrotaline (MCT)-induced decrease in endothelial nitric oxide synthase (eNOS) expression and increase in inducible nitric oxide synthase (iNOS) expression in pulmonary arterioles and alveoli at 4 weeks after MCT treatment. Positive immunostained eNOS or iNOS cells on arterioles or alveoli in each x400 microscopic field were counted, and 10 microscopic fields on each lung section were randomly selected for counting. n= number of rats. Results are means±SEM. *P<0.05 vs respective MCT-alone and MCT+null-EC groups. One-way ANOVA was used to analyze the data, followed by a multiple-comparison Tukey post hoc test. Representative micrographs are shown in Figures II through V in the online-only Data Supplement.

Figure 4. Effects of endothelial cells (ECs) overexpressing interleukin-8 receptors A and B (IL8RA/B-EC) or null-EC transfection on monocrotaline (MCT)-induced decrease in endothelial nitric oxide synthase (eNOS) expression and increase in inducible nitric oxide synthase (iNOS) expression in pulmonary arterioles and alveoli at 4 weeks after MCT treatment. Positive immunostained eNOS or iNOS cells on arterioles or alveoli in each x400 microscopic field were counted, and 10 microscopic fields on each lung section were randomly selected for counting. n= number of rats. Results are means±SEM. *P<0.05 vs respective MCT-alone and MCT+null-EC groups. One-way ANOVA was used to analyze the data, followed by a multiple-comparison Tukey post hoc test. Representative micrographs are shown in Figures II through V in the online-only Data Supplement.

endothelial growth factor, and regulated on activation, normal T-cell expressed and secreted (chemokine ligand 5). The concentrations of cytokine/chemokine in lungs of vehicle control rats (in pg/μg, means±SEM, n=7) were as follows: MIP-1α (0.143±0.011); interleukin-1β (1.059±0.087); interleukin-10 (0.143±0.011); MCP-1 (0.189±0.091); CINC1 (0.211±0.044); vascular endothelial growth factor (4.951±0.515); MIP-2 (0.123±0.025); and regulated on activation, normal T-cell expressed and secreted (chemokine ligand 5). The concentrations of cytokine/chemokine in lungs of vehicle control rats (in pg/μg, means±SEM, n=7) were as follows: MIP-1α (0.143±0.011); interleukin-1β (1.059±0.087); interleukin-10 (0.143±0.011); MCP-1 (0.189±0.091); CINC1 (0.211±0.044); vascular endothelial growth factor (4.951±0.515); MIP-2 (0.123±0.025); and regulated on activation, normal T-cell expressed and secreted (chemokine ligand 5). Levels of tumor necrosis factor-α and interleukin-6 were too low to be detected by sensitized ELISA.

Discussion

The present study has shown for the first time that intravenous transfusion of pulmonary arterial ECs overexpressing IL8RA/B homing devices significantly enhances eNOS expression and inhibits expression of iNOS and other proinflammatory mediators, as well as infiltration of neutrophils and monocytes/macrophages in lungs of rats treated with monocrotaline. These anti-inflammatory effects of targeting ECs to the injured lung effectively prevented pulmonary vascular hypertrophy and remodeling and loss of RV function after monocrotaline treatment. These results support our hypothesis that ECs overexpressing IL8RA/B mimic the behavior of neutrophils that target IL-8 and N-acetylated proline–glycine–proline expressed in acutely injured lungs, thus inhibiting proinflammatory responses, accelerating endothelial repair, and decreasing vascular hypertrophy and remodeling in injured lungs, thus preserving RV function after acute injury by monocrotaline. Targeting delivery of ECs equipped with the homing device (IL-8 receptors) to the site of pulmonary injury, as demonstrated in this study, provides a potential novel strategy for the treatment of pulmonary vascular diseases.

Pulmonary arterial hypertension is a complex disorder that is characterized by remodeling of pulmonary arteries and increased pulmonary vascular resistance, resulting in progressive elevation in pulmonary arterial pressure and subsequent RV failure and death.7,8 Progressive structural remodeling of small pulmonary arteries and arterioles is associated with excessive proliferation of pulmonary arterial smooth muscle cells.

A single subcutaneous monocrotaline injection has been shown to induce pulmonary hypertension during a period of 3 to 4 weeks in rat. The active hepatic metabolite of monocrotaline (monocrotaline pyrrole) selectively damages the pulmonary vascular endothelium and stimulates the proliferation of pulmonary arterial smooth muscle cells, resulting in pulmonary arterial remodeling and hypertrophy, pulmonary arterial hypertension, and RV dysfunction.9 Enhancing regeneration of endothelium (re-endothelialization) in injured vessels is thus a potential therapeutic target to rescue tissue from critical vascular injury. After severe endothelial injury occurs in monocrotaline-treated animals, protection of pulmonary arterial structure and function can decrease pulmonary arterial hypertension, thus preventing RV dysfunction and reducing mortality. Therapeutic endothelial repair has thus become a promising new method of treatment for patients with pulmonary arterial diseases.

Cell- and gene-based therapies for pulmonary arterial diseases have proliferated during the past years, but with limited success.10–12 Cell-based therapies have been shown to alleviate pulmonary hypertension and RV dysfunction, but the majority of the studies are currently at the preclinical level in animal models.11,13–16 An important limitation of cell therapy is that, because the introduced cells lack selective homing devices to target injured tissues, they must be injected locally or directly into the affected tissue to increase therapeutic efficiency. Such invasive procedures are costly and difficult to adapt for widespread clinical use. Other major hurdles for successful cell therapies include (1) cell type selection (eg, stem cells or differentiated cells), (2) cell delivery mode (eg, peripherally or directly into tissue), (3) timing of cell delivery (eg, acute or delayed treatment), (4) rejection of transplanted cells (eg, autologous or heterologous transplantation), (5) age of cells, and most importantly, (6) targeting delivery of cells to damaged organs to maximize therapeutic effects. Several strategies including growth factor delivery,15,17 gene therapy,14,18,19 and stem cell therapy16,17 have been developed to promote vascular repair in pulmonary vascular diseases. Among them, cell-based therapy has emerged as the most promising therapeutic tool for treatment of pulmonary hypertension.

Both nonselective bone marrow–derived stem cells and selective endothelial progenitor cells have been used in rodent models of hypoxia- or monocrotaline-induced pulmonary
arterial remodeling and hypertension.11,14–17 Intratracheal administration of bone marrow–derived stem cells has been shown to attenuate neonatal hyperoxia-induced lung injury.16 Transplantation of bone marrow–derived stem cells overexpressing hepatocyte growth factor, alone or in combination with granulocyte colony–stimulating factor, attenuates the development of monocrotaline-induced pulmonary arterial hypertension in rats.15 Localized jugular vein delivery of prostacyclin-producing endothelial progenitor cells attenuates monocrotaline-induced RV systolic pressure increase, RV hypertrophy, and pulmonary vessel wall thickening. These effects have led to functional recovery of pulmonary arteries.17

A critical limitation for the therapeutic application of exogenous bone marrow–derived stem cells or endothelial progenitor cells is the lack of guidance for the cells to reach target tissue to elicit maximal therapeutic efficiency. Because the introduced cells lack a selective homing device to target the injured tissues, they must be introduced directly into the affected tissue (eg, injured lung tissue or vessels), procedures that are invasive and may be hazardous. In the present study, the injured pulmonary arterioles are the therapeutic target for the treatment of monocrotaline-induced injury. We have generated RAECs overexpressing IL8RA/B that can mimic the behavior of neutrophils in binding to IL-8 that is produced by injured tissues.3,4 We have previously demonstrated that adenoviral transduction did not change the expression of EC makers (von Willebrand factor and CD31) in ECs transduced with Ad-ILRA/RB-green fluorescence protein vector. The percentage of ECs expressing the green fluorescence protein marker peaked (≈90%) at 3 days after transduction and gradually decreased during cell proliferation (50% at passage 1 and <10% at passage 2). Similarly, the intensity of green fluorescence protein staining diminished over time with cell proliferation.6 The in vitro proliferative activity of adenoviral transduced IL8RA/RB-ECs was the same as untransduced control ECs, and the migratory activity of IL8RA/RB-EC to IL-8 (in a dose-dependent manner, assessed by the Boyden chamber technique) was significantly greater compared with untransduced cells.3 We also showed that IL8RA/RB-ECs can compete with neutrophils to attach to activated endothelial monolayers in vitro and that intravenous transfusion of these transduced ECs into rats with balloon-injured carotid arteries or left anterior descending ligation–injured LV resulted in targeting and adhesion of the transduced RAECs to injured arterial or cardiac tissues.3,4 Transfusion of RAECs overexpressing IL8RA/B resulted in decreased neutrophil infiltration and inflammatory mediator expression, accelerated re-endothelialization, neovascularization, and reduced neointima formation in injured arteries3 or left anterior descending

**Figure 5.** Effects of endothelial cells (ECs) overexpressing interleukin-8 receptors A and B (IL8RA/B-EC) or null-EC transduction on monocrotaline (MCT)-induced increase in IL-8 and monocyte chemoattractant protein-1 (MCP-1) expression on pulmonary arterioles and alveoli at 4 weeks after MCT treatment. Positive immunostained neutrophils and monocytes/macrophages in arterioles or alveoli in each ×400 microscopic field were counted, and 10 microscopic fields in each lung section were randomly selected for counting. n= number of rats. Results are means±SEM. *P<0.05 vs respective vehicle control groups. #P<0.05 vs respective MCT-alone and MCT-null-EC groups. One-way ANOVA was used to analyze the data, followed by a multiple-comparison Tukey post hoc test. Representative micrographs are shown in Figures VI through IX in the online-only Data Supplement.

**Figure 6.** Effects of endothelial cells (ECs) overexpressing interleukin-8 receptors A and B (IL8RA/B-EC) or null-EC transduction on monocrotaline (MCT)-induced neutrophil and monocyte/macrophage infiltration at 4 weeks after MCT treatment. Positive immunostained neutrophils and monocytes/macrophages in arterioles or alveoli in each ×400 microscopic field were counted, and 10 microscopic fields on each lung section were randomly selected for counting. n= number of rats. Results are means±SEM. *P<0.05 vs respective vehicle control groups. #P<0.05 vs respective MCT-alone and MCT-null-EC groups. One-way ANOVA was used to analyze the data, followed by a multiple-comparison Tukey post hoc test. Representative microscopic micrographs are shown in Figures X through XIII in the online-only Data Supplement. MPO indicates myeloperoxidase.
ligation–induced myocardial infarction. The goal of the present study was to develop novel approaches and strategies to safely attenuate the contribution of neutrophils to the pulmonary vascular injury response without impairing resistance to infection. This approach targets delivery of ECs equipped with homing device (IL8RA/B) to injured pulmonary vessels, thus efficiently inhibiting neutrophil and monocyte/macrophage infiltration and proinflammatory responses. Targeting delivery of ECs to sites of pulmonary vascular injury provides a novel therapeutic strategy for pulmonary disease.

Findings from the present (using a pulmonary vascular injury model) and our previous studies (using a vascular injury model and a cardiac injury model) not only provide a novel strategy for therapeutic interventions for cardiopulmonary vascular diseases but also fundamentally advance the field of cell-based therapy.

Acute inflammatory responses to monocrotaline injection promote vascular EC apoptosis and pulmonary arterial hypertrophy/fibrosis/remodeling and impair pulmonary arterial and RV function. Neutrophils migrate to injured tissue in response to IL-8 and N-acetylated proline–glycine–proline (a tripeptide fragment of collagen that is 3-dimensionally structurally similar to IL-8) that are expressed and released in large amounts by injured tissues. Interactions between IL-8 and its cognate receptors on neutrophil membranes play a key role in host defense and disease responses after recruitment of activated neutrophils to sites of inflammation. IL-8 binds to selective IL8RA/B on the neutrophil surface and activated IL8RA/B induce expression of chemotactic mediators that trigger local inflammation. Neutrophils are the main leukocyte subset that interacts with the damaged endothelium, triggers the initial proinflammatory response, and facilitates the influx of other classes of inflammatory cells (eg, monocytes/macrophages and T cells in the setting of acute vascular injury). Using this background knowledge, we have developed an innovative strategy to equip ECs with a neutrophil homing device to target their delivery to injured tissues.

We chose eNOS and iNOS as target genes to assess endothelial function in monocrotaline-treated lungs. eNOS has been used as an index of vascular EC function.
endothelium, NO formed via eNOS plays a crucial role in the regulation of vascular blood flow through vasodilatation and decreased vascular resistance. Furthermore, in gene therapy studies, eNOS has been shown to promote re-endothelialization and inhibit intimal hyperplasia in injured blood vessels. In contrast, an increase in iNOS expression in the injured vasculature promotes inflammation and vascular smooth muscle cell migration and proliferation in the setting of atherosclerosis and angioplasty-induced restenosis. A common feature of many forms of vascular dysfunction is the contribution of reactive oxygen species and reactive nitrogen species to vascular injury. Primary sources of reactive oxygen species and reactive nitrogen species in vascular smooth muscle cell are the cytokine-regulated iNOS and NADPH (nicotinamide adenine dinucleotide phosphate) oxidase. Nox-derived reactive oxygen species modulates NO bioavailability by altering the expression of iNOS. Reaction of NO produced by iNOS with superoxide yields peroxynitrite, which contributes to the pathogenesis of vascular dysfunction and hypertension.

Therefore, the goal to repair the vascular injury is to restore eNOS expression and decrease iNOS expression. In the present study, our IL8RA/B-ECs treatment in monocrotaline-treated rats has successfully reached this goal.

We also demonstrated that, compared with monocrotaline alone and monocrotaline +null-EC rats, transfusion with ECs overexpressing IL8RA/B 1 day after monocrotaline treatment decreased the expression of the proinflammatory chemokatants IL-8 and MCP-1, as well as neutrophil and monocyte/macrophage infiltration into pulmonary arterioles and alveoli (as shown by immunohistochemical analysis in Figures 5 and 6), and reduced pulmonary arterial hypertrophy in association with preserved RV function in rats treated with monocrotaline. IL8RA/B-EC transfusion reversed the monocrotaline treatment–induced increases in CINC1 and MIP-2 protein levels in lungs, confirming the results of the immunohistochemical analysis. Similar to CINC-2β, CINC1 is a rat homolog of human IL-8, and MIP-2 is a member of the family of cysteine-X-cysteine motif chemokines and a neutrophil chemotactic that plays a role in inflammatory, immune, and wound healing responses in lungs. MIP2 and CINCs constitute a chemokine gradient that orchestrates influx of neutrophils into lung, leading to accumulation of neutrophils during acute lung injury.

Our data indicate that transfusion of ECs overexpressing IL8RA/B has protective effects after acute pulmonary vascular injury.

In summary, the present study clearly provides proof of principle that targeted delivery of ECs is effective in repairing injured tissue. Results in Figure 7 demonstrated significant increases in protein levels of several cytokines and chemokines in lung homogenates at 2 days after monocrotaline treatment, clear evidence of lung injury. Expression of these proinflammatory molecules is attenuated by treatment with IL8RA/B-EC (and to a lesser extent with null-EC), indicating reversal of the injury response. We suggest that the targeted delivery of ECs to sites of pulmonary injury provides a novel therapeutic strategy for pulmonary vascular disease. This novel strategy offers advantages over existing methods that include the following: (1) elimination of need for local injection of cells into injured organs because of the homing device. Many existing cell therapies require direct injection of cells into injured organs, which are costly and carry substantial risk for the recipient; (2) selective inhibition of inflammatory responses at the site of injury with healthy ECs to compete with proinflammatory neutrophils and monocytes/macrophages; and (3) preservation of resident cell survival by a combination of inhibiting inflammation and promoting re-endothelialization and revascularization by transplanted ECs. We hypothesize that targeted EC therapy will also have beneficial effects in forms of chronic vascular injury–involved neutrophil and monocyte/macrophage infiltration and inflammation. Future studies are needed to test this hypothesis.

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Disclosures

None.

References


Significance

Cell therapies for pulmonary arterial diseases have proliferated during the past years, but with limited success. A critical limitation is the lack of guidance for the cells to reach target tissue to elicit maximal therapeutic efficiency, thus they must be introduced directly into the affected tissue. Such invasive procedures are costly and difficult to adapt for widespread clinical use. We have developed an innovative strategy to overcome this hurdle by intravenously transfusing rat endothelial cells overexpressing the neutrophil interleukin-8 receptors into rat with pulmonary vascular injury and hypertension induced by monocrotaline. We demonstrated that, equipped with the interleukin-8 receptor homing device, transfused endothelial cells mimic the behavior of neutrophils to bind to interleukin-8 produced in injured lungs, inhibit pro-inflammatory responses, and decrease vascular hypertrophy and remodeling in injured lungs, thus preserving right ventricular function after pulmonary vascular injury induced by monocrotaline.
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Supplemental Figure Legends

**Supplemental Figure I.** Representative fluorescence micrographs (x400) showing GFP-labeled IL8RA/B-ECs in frozen lung section and blood smear of MCT treated rats 24 hrs after transfusion of IL8RA/B-ECs. It is not clear whether the GFP cells were located in blood vessels or lung parenchyma due to the low resolution of the unfixed tissue. Yellow stained cells are GFP-positive cells. Blue stained nuclei are DAPI-labeled nuclei. GFP cells could be seen in blood smears, but not other tissues, including heart, kidney, liver and brain.

**Supplemental Figure II.** Effects of IL8RA/B-EC, Null-EC, or vehicle transfusion on MCT-induced decrease in eNOS expression (brown stained cells) in pulmonary arterioles in rat lungs 4 wks after transfusion of ECs.

**Supplemental Figure III.** Effects of IL8RA/B-EC, Null-EC, or vehicle transfusion on MCT-induced decrease in eNOS expression (brown stained cells) in alveoli in rat lungs 4 wks after transfusion of ECs.

**Supplemental Figure IV.** Effects of IL8RA/B-EC, Null-EC, or vehicle transfusion on MCT-induced increase in iNOS expression (brown stained cells) in pulmonary arterioles in rat lungs 4 wks after transfusion of ECs.

**Supplemental Figure V.** Effects of IL8RA/B-EC, Null-EC, or vehicle transfusion on MCT-induced increase in iNOS expression (brown stained cells) in alveoli in rat lungs 4 wks after transfusion of ECs.

**Supplemental Figure VI.** Effects of IL8RA/B-EC, Null-EC, or vehicle transfusion on MCT-induced increase in IL-8 (CINC-2•) expression (brown stained cells) in pulmonary arterioles in rat lungs 4 wks after transfusion of ECs. CINC-2• -
cytokine induced neutrophil chemoattractant-2-beta (equivalent to human IL-8)

**Supplemental Figure VII.** Effects of IL8RA/B-EC, Null-EC, or vehicle transfusion on MCT-induced increase in IL8 (CINC-2β) expression (brown stained cells) in alveoli in rat lungs 4 wks after transfusion of ECs.

**Supplemental Figure VIII.** Effects of IL8RA/B-EC, Null-EC, or vehicle transfusion on MCT-induced increase in MCP-1 expression (brown stained cells) in pulmonary arterioles in rat lungs 4 wks after transfusion of ECs. MCP-1 - monocyte chemotactic protein-1.

**Supplemental Figure IX.** Effects of IL8RA/B-EC, Null-EC, or vehicle transfusion on MCT-induced increase in MCP-1 expression (brown stained cells) in alveoli in rat lungs 4 wks after transfusion of ECs.

**Supplemental Figure X.** Effects of IL8RA/B-EC, Null-EC, or vehicle transfusion on MCT-induced infiltration of neutrophils (brown stained cells) in pulmonary arterioles in rat lungs 4 wks after transfusion of ECs.

**Supplemental Figure XI.** Effects of IL8RA/B-EC, Null-EC, or vehicle transfusion on MCT-induced infiltration of neutrophils (brown stained cells) in alveoli in rat lungs 4 wks after transfusion of ECs.

**Supplemental Figure XII.** Effects of IL8RA/B-EC, Null-EC, or vehicle transfusion on MCT-induced infiltration of monocytes/macrophages (brown stained cells) in pulmonary arterioles in rat lungs 4 wks after transfusion of ECs.

**Supplemental Figure XIII.** Effects of IL8RA/B-EC, Null-EC, or vehicle transfusion on MCT-induced infiltration of monocytes/macrophages (brown stained cells) in alveoli in rat lungs 4 wks after transfusion of ECs.
GFP labeled IL8RA/RB-ECs in Blood at 1 day after transfusion. A) Dark field, and B) Bright field
eNOS Positive Cells in Pulmonary Arterioles

Vehicle

MCT Alone

MCT+Null-ECs

MCT+IL8RA/B-ECs

Supplemental Figure II
eNOS Positive Cells in Alveoli

Vehicle

MCT Alone

MCT+Null-ECs

MCT+IL8RA/B-ECs

Supplemental Figure III
iNOS Positive Cells on Pulmonary Arterioles

Vehicle

MCT Alone

MCT+Null-ECs

MCT+IL8RA/B-ECs

Supplemental Figure IV
iNOS Positive Cells in Alveoli

Vehicle

MCT Alone

MCT+Null-ECs

MCT+IL8RA/B-ECs

Supplemental Figure V
IL8 Positive Cells in Pulmonary Arterioles

Vehicle  MCT Alone

MCT+Null-ECs  MCT+IL8RA/B-ECs

Supplemental Figure VI
IL8 Positive Cells in Alveoli

Vehicle

MCT Alone

MCT+Null-ECs

MCT+IL8RA/B-ECs

Supplemental Figure VII
MCP-1 Positive Cells in Pulmonary Arterioles

- Vehicle
- MCT Alone
- MCT+Null-ECs
- MCT+IL8RA/B-ECs
MCP-1 Positive Cells in Alveoli

Vehicle

MCT Alone

MCT+Null-ECs

MCT+IL8RA/B-ECs

Supplemental Figure IX
Neutrophils in Pulmonary Arterioles

Vehicle

MCT Alone

MCT+Null-ECs

MCT+IL8RA/B-ECs

Supplemental Figure X
Supplemental Figure XI

**Neutrophils in Alveoli**

- Vehicle
- MCT Alone
- MCT+Null-ECs
- MCT+IL8RA/B-ECs
Monocytes/Macrophages in Pulmonary Arterioles

Supplemental Figure XII
Monocytes/Macrophages in Alveoli

Vehicle

MCT Alone

MCT+Null-ECs

MCT+IL8RA/B-ECs

Supplemental Figure XIII
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°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


