Pirfenidone inhibits inflammatory responses and ameliorates allograft injury in a rat lung transplant model

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Objective: Tumor necrosis factor α is a proinflammatory cytokine that has been proved to play a crucial role in inducing posttransplantation lung injury. The present study was performed to determine whether pirfenidone, a new nonpeptide drug with potent anti–tumor necrosis factor α activity, promotes protection against acute allograft injury through inhibiting pulmonary inflammatory responses in a rat model of orthotopic lung transplantation.

Methods: Three transplant groups were formed: isografts, untreated allografts, and allografts treated with pirfenidone (0.5% chow starting on day 1 after transplantation). The implants were harvested on day 21 after transplantation. Acute cellular rejection grade and degree of allograft injury were evaluated on the basis of hematoxylin-and-eosin staining. The pulmonary inflammatory response and inflammation-induced oxidative stress were assessed on the basis of neutrophil accumulation (myeloperoxidase immunoreactivity and enzymatic activity) and iron deposition (Prussian blue staining). In addition, circulating levels of tissue necrosis factor α in all animals were measured.

Results: The degree of allograft injury was significantly reduced in pirfenidone-treated allografts relative to untreated allografts ($P < .01$). The beneficial effect of pirfenidone was associated with decreased lung myeloperoxidase immunoreactivity ($P < .05$) and enzymatic activity ($P < .01$). Moreover, the untreated allografts contained a high concentration of iron, which was strikingly reduced by pirfenidone. Treatment with pirfenidone resulted in a lower level of plasma tissue necrosis factor α, which correlated positively with lung myeloperoxidase enzymatic activity ($P < .0001$).

Conclusion: These results suggest that pirfenidone, with its anti–tissue necrosis factor α activity, reduced neutrophil recruitment and iron accumulation, hence limiting the acute lung allograft injury.

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Compared with other solid organs, the lung is highly immunogenic.[1] Transplantation of this organ thus results in a relatively high incidence of acute and chronic allograft rejection.[2,3] The acute lung allograft rejection is an inflammatory process characterized by pronounced perivascular and peribronchiolar infiltration of inflammatory cells that could invade and destroy vessels and airways and eventually lead to dysfunction of the lung allograft.[2,3] Indeed, it was reported that excessive inflammatory responses and overproduction of proinflammatory cytokines exacerbate acute lung allograft injury.[4-6] Pirfenidone (5-methyl-1-phenyl-2-[1H]-pyridone) is a new nonpeptide drug with excellent oral absorption and hepatic metabolism.[7] In an open-label phase II study in patients with end-stage idiopathic pulmonary fibrosis, treatment with pirfenidone safely restored pulmonary function and improved survival.[8] In addition to its well-documented antifibrotic property, pirfenidone is also a potent anti-inflammatory agent. It was found that pirfenidone could effectively inhibit bleomycin-induced acute lung inflammation[9]

and selectively decreased the activity and synthesis of a major proinflammatory cytokine, tumor necrosis factor α (TNF-α).[10-14] It has been established that TNF-α plays an important role in the pathogenesis of acute lung allograft rejection.[4-6] Given that pirfenidone is a currently available therapeutic agent for human use,[7,8] it is thus very interesting to determine whether this compound, with its potent anti-TNF-α property, promotes protection against acute allograft injury by limiting the pulmonary inflammatory response. This study evaluated whether pirfenidone reduced lung allograft injury and the inflammatory response in a rat lung transplant model.

Methods

Materials

All chemicals and reagents were purchased from Sigma Chemical Company (St Louis, Mo) unless otherwise specified.

Animal Model

Specific pathogen-free male Lewis and Sprague-Dawley (SD) rats (weight, approximately 300 g) were purchased from Harlan Sprague-Dawley (Indianapolis, Ind) and housed and cared for in the Animal Care Center at the University of Florida in accordance with institutional guidelines. The experimental protocol was approved by the Animal Care Committee of the University of Florida.

The orthotopic transplantation of the left lung was performed as previously reported,[15] with slight modifications. In brief, the donor rat was anesthetized in a glass chamber constantly perfused with 5% isoflurane in oxygen for a period of 10 minutes. The animal was then intubated orotracheally with a 14-gauge Teflon angiocatheter and mechanically ventilated with a volume-controlled ventilator (Harvard Rodent Ventilator, model 683; Harvard Apparatus, South Natick, Mass) with 2% isoflurane in oxygen at a rate of 80 breaths/min, a tidal volume of 10 mL/kg, and an inspired fraction of oxygen of 1.0. The animal was heparinized (1000 U/kg body weight administered intravenously), and a median laparosternotomy was performed. The main pulmonary artery was cannulated through an anterior incision in the right ventricular outflow tract, and the lungs were flushed with 20 mL of ice-cold low-potassium-dextran preservation solution (Perfadex; Vitrolife, Gotenberg, Sweden) at a pressure of 20 cm H_2O. The entire heart-lung block was removed, with the lungs inflated at tidal volume. Sixteen-gauge and 14-gauge angiocatheter cuffs were placed in the pulmonary artery and pulmonary vein, respectively. The lung with cuffs was stored in the low-potassium-dextran preservation solution at 4°C in a sterile beaker until implantation.

The recipient rat was intubated orotracheally and ventilated with 2% isoflurane in oxygen at a rate of 80 breaths/min, a tidal volume of 10 mL/kg, an inspired fraction of oxygen of 1.0, and a positive end-expiratory pressure of 2 cm H_2O. A left thoracotomy was made through the fourth intercostal space, and the left pulmonary artery, vein, and bronchus were isolated and clamped proximally with microvascular clips, respectively. A small ventral incision was made in each of the blood vessels to enable introduction of the respective donor's blood vessels and their cuffs. The donor and recipient bronchi were anastomosed with a running 8-0 Prolene suture (Ethicon, Inc, Somerville, NJ). Perfusion and ventilation of the graft were restored by removing clamps from the pulmonary vein, artery, and bronchus, respectively. All transplantation procedures were performed by one author (H.L.) under sterile conditions. Isoflurane ($\leq$5%) was the only anesthetic agent given to the donor and recipient animals throughout the experimental period to minimize the possible negative effects of anesthetics on lung graft function.

Experimental Designs

Three transplantation groups with 6 transplantations in each group were studied: SD donor and SD recipient (isografts), Lewis to SD rats (untreated allografts), and Lewis to pirfenidone-fed SD rats (pirfenidone-treated allografts). Pirfenidone (Department of Chemistry, University of Florida), 0.5% in chow, was started on day 1 after transplantation. The pirfenidone dose was selected on the basis of prevention of allograft injury in other animal models in previous studies.[16] In addition, 6 left lungs taken from body weight–matched untreated male SD rats were used as a normal control group. No immunosuppressive therapy was administered to animals at any time during the experimental period. All animals were killed 21 days after transplantation.

At the end of each study, animals were intubated orotracheally and ventilated with 2% isoflurane in oxygen. One milliliter of blood was collected from the abdominal vein in each animal. Then the lungs were flushed through the main pulmonary artery with ice-cold saline until they turned white. A straight, fine hemostatic forceps was applied across the transplanted (left) lung just below the hilum so that the lung was divided into 2 parts. The lower part was snap-frozen in liquid nitrogen for biochemical studies, and the remaining lung was inflated with 2 mL of 10% neutral-buffered formalin, fixed overnight, and embedded in paraffin wax.

Morphologic Evaluation

The paraffin-embedded lung block was cut into 4-μm sections and stained with hematoxylin and eosin. The severity of acute cellular rejection (ACR) was graded as A0 to 4 on the basis of the revised "Working Formation for the Classification of Pulmonary Allograft Rejection" according to the presence and extent of perivascular and interstitial mononuclear cell infiltrates,[17] as described previously.[15] Preservation of alveolar architecture was determined by converting the light microscopy fields into digital format with an Optronics digital camera with Magna Fire software (Optronics, Goleta, Calif) to assess the degree of lung graft injury in general. The images were analyzed with Spot Advanced software (Diagnostic Instruments, Sterling Heights, Mich). The damaged alveolar architecture includes edema, congestion, hemorrhage, hyaline membranes, and necrosis. The degree of lung injury was expressed as the percentage of area with damaged alveolar architecture to total lung area. The morphologic study was performed by a lung pathologist (P.D.) in a blinded fashion.

Myeloperoxidase Expression and Enzymatic Activity

Separate paraffin-embedded sections were immunohistochemically stained with myeloperoxidase (MPO) by the avidin-biotin-peroxidase complex method.[18] In brief, after antigen retrieval with Trilogy solution (Cell Marque Corp, Hot Springs, Ark), the lung sections were incubated overnight at 4°C with primary polyclonal

anti-MPO antibody (NeoMarkers, Fremont, Calif) at 1:200 dilutions. After being washed 3 times with phosphate-buffered saline, the sections were further incubated for 1 hour at room temperature with biotinylated goat anti-rabbit secondary antibody (Santa Cruz Biotechnology, Santa Cruz, Calif) at a dilution of 1:400. The specific MPO binding was detected with streptavidin-biotinylated horseradish peroxidase complex (Vectstain ABC kit; Vector Laboratories Inc, Burlingame, Calif) and visualized with diaminobenzidine. Nuclei were counterstained with hematoxylin. Negative control sections were included in each staining run, with the primary antibody being replaced by normal rabbit immunoglobulin G (Santa Cruz Biotechnology). None of the negative control sections showed positive staining. The number of MPO-positive cells was counted in 10 randomly chosen microscopic high-power fields (400×) per graft in a blinded fashion.[15]

Quantitative MPO enzymatic activity in lung tissue was determined with a commercial MPO assay kit (CytoStore, Alberta, Canada). In brief, frozen lung tissue was homogenized on ice in 50 mmol/L Tris-HCl buffer (pH 7.4) containing 2% protease inhibitor cocktail and 5 mg/mL hexadecyltrimethylammonium bromide. The homogenized samples were centrifuged for 15 minutes at 3000*g* (4°C), and 20 μL of the supernatants was collected and mixed with 200 μL of 20 mmol/L potassium phosphate buffer (pH 7.0) containing 0.167 mg/mL *o*-dianisidine dihydrochloride and 0.0005% hydrogen peroxide. Change in absorbance over 1 minute was measured spectrophotometrically at 450 nm. Protein concentration for all samples was determined by using a BCA protein assay kit (Pierce, Rockford, IL), with bovine serum albumin as the standard. The final results of MPO activity were expressed as the change in absorbance per minute per milligram of protein.

Assessment of Iron Deposition

Prussian blue staining is widely used for assessment of iron deposition in tissues.[19,20] In brief, after deparaffinization and rehydration, the lung sections were treated with 20% hydrochloric acid solution to liberate ferric iron and further treated with 10% aqueous potassium ferrocyanide solution to produce insoluble ferric ferrocyanide. The sections were then counterstained with eosin. This staining identifies ferric iron as bright blue and nuclei as red. The standard procedure for this staining was provided by the clinical histology laboratory at the Veterans Affairs Hospital, University of Florida.

Determination of Plasma TNF-α Level

The plasma concentration of TNF-α was determined by a commercial rat TNF-α ELISA kit (R&D systems, Minneapolis, Minn), according to the manufacturer's instruction. The rat TNF-α assay has no cross-reactivity with other cytokines, such as interleukin 1 (IL-1), IL-2, or IL-4. The final results were expressed as picograms per milliliter.

Statistical Analysis

Data are expressed as means ± standard error of the mean (SEM), and statistical analyses were performed with the Prism statistical program (GraphPad, San Diego, Calif). A nonparametric analysis of variance, the Kruskal-Wallis test with the Dunn multiple comparisons test, was used to determine the difference between ACR grades. One-way analysis of variance with the Newman-Keuls test was used to evaluate the degree of allograft injury, the number of MPO-positive cells, MPO enzymatic activity, and plasma TNF-α levels between the different groups. Linear correlation was evaluated with the Pearson *r* coefficient.

Results

Representative microphotographs of hematoxylin and eosin–stained lung tissue from each of the 4 groups are shown in Figure 1, *A* through *D*. An occasional lung isograft showed very mild lymphocytic infiltrates in perivascular locations, whereas the majority of lung isografts (Figure 1, *B*) displayed no detectable pathologic lesions and were similar to normal left lungs (Figure 1, *A*), indicating that surgical trauma and the ischemia-reperfusion process had very little effect on graft morphologic property. In contrast, untreated lung allografts displayed end-stage ACR, with dense infiltration of mononuclear cells around the vessels and airways and in the interstitium. In addition, there is evidence of necrosis with interstitial edema, thickening of the wall of small airways and vessels, and destruction of alveolar-interstitial structure (Figure 1, *C*). Notably, a markedly reduced interstitial infiltration of mononuclear cells with a widespread preservation of alveolar architecture was found in pirfenidone-treated allografts (Figure 1, *D*). ACR grade was significantly greater in untreated allografts compared with that in the normal lung or isograft groups ($P < .05$). Pirfenidone might have reduced ACR grade, but the ACR in the pirfenidone-treated allograft group was not significantly different ($P = .056$) from that in the untreated allograft group (Figure 1, *E*). Importantly, much less lung injury, as determined on the basis of preservation of alveolar architecture, was found in the pirfenidone-treated allografts (24.2% ± 12.6% of damaged area/total area) in comparison with the untreated allografts (67.4% ± 14.3%, $P < .01$; Figure 1, *F*).

The histologic study revealed that pirfenidone is protective against acute allograft injury. We next explored whether the beneficial effect of pirfenidone was linked to inhibited pulmonary inflammatory responses. For this, we first measured the effect of pirfenidone on lung MPO immunoreactivity and enzymatic activity, which reflects neutrophil recruitment.[18,21,22] Representative microphotographs of MPO-immunostained lung sections of a normal left lung and an untreated lung allograft are shown in Figure 2, *A*. Scarce MPO staining was apparent in the normal left lungs and isografts. By contrast, a large number of MPO-positive cells were seen in untreated allografts (40.8 ± 8.4 MPO-stained cells/400× field), which was significantly reduced ($P < .05$) by pirfenidone (24.8 ± 3.9 MPO-stained cells/400× field; Figure 2, *B*). MPO immunochemical staining was mirrored by MPO enzymatic activity, which showed that untreated allografts contained a higher level of MPO activity (2.0 ± 0.16 Δ absorbance per minute per milligram of protein) in comparison with pirfenidone-

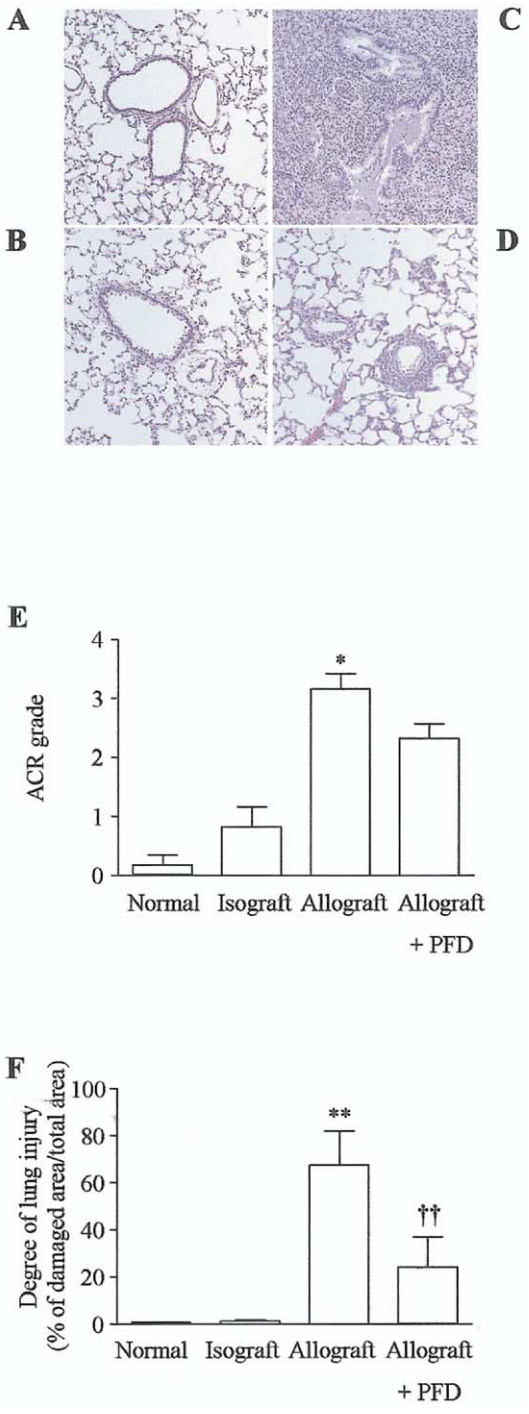

Figure 1. Histologic evaluation of lung grafts. Examples of hematoxylin and eosin–stained sections taken from normal left lung (A), lung isograft (B), untreated (C), and pirfenidone *(PFD)*–treated lung allograft (D). Photomicrographs are representative of 6 rats in each group. (Original magnification 100×.) E shows the grade of ACR in all groups. In F, quantification of lung injury on the basis of the amount of preserved alveolar architecture is shown. Results are presented as means ± SEM (n = 6 each group). *P < .05 and **P < .01 versus normal left lungs or isografts; ††P < .01 versus untreated lung allografts.

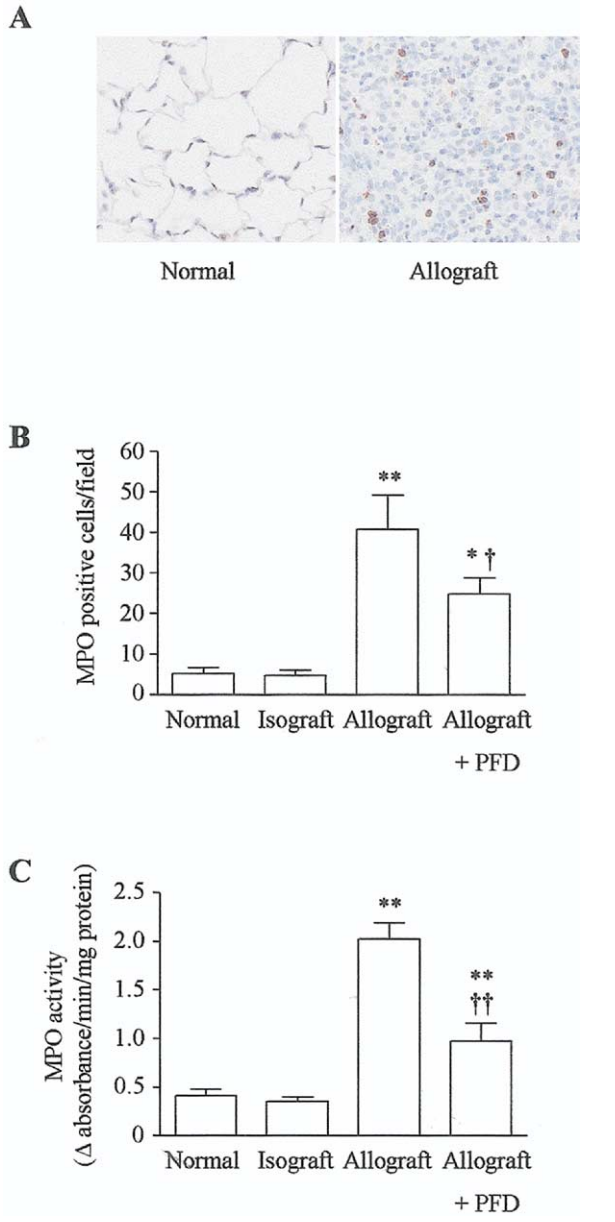

Figure 2. Lung MPO expression and enzymatic activity. MPO immunohistochemically stained sections of a normal left lung and an untreated lung allograft. MPO-positive cells are brown in color (A). The number of MPO-positive cells per high-power field (400×) and lung MPO enzymatic activity in all groups are shown in B and C, respectively. Results are presented as means ± SEM (n = 6 in each group). *P <.05 and **P < .01 versus normal left lungs or isografts; †P < .05 and ††P < .01 versus untreated allografts.

treated allografts (0.97 ± 0.18 Δ absorbance per minute per milligram of protein, $P < .01$ vs untreated allografts; Figure 2, *C*). With the knowledge that the pulmonary inflammatory process is associated with excessive oxidative stress, we

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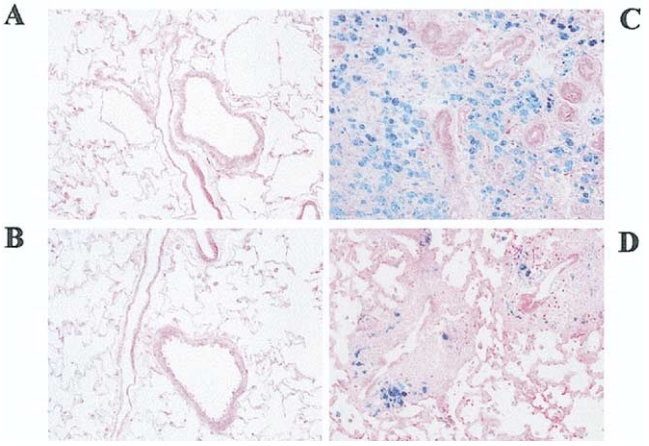

Figure 3. Iron loading in lung tissue. Sample of Prussian blue–stained sections taken from normal left lung (A), lung isograft (B), untreated (C), and pirfenidone-treated lung allograft (D). Photomicrographs are representative of 6 rats in each group. (Original magnification 200×.) Note that the intensive blue positive staining observed in the untreated lung allograft was strikingly reduced in pirfenidone-treated lung allografts.

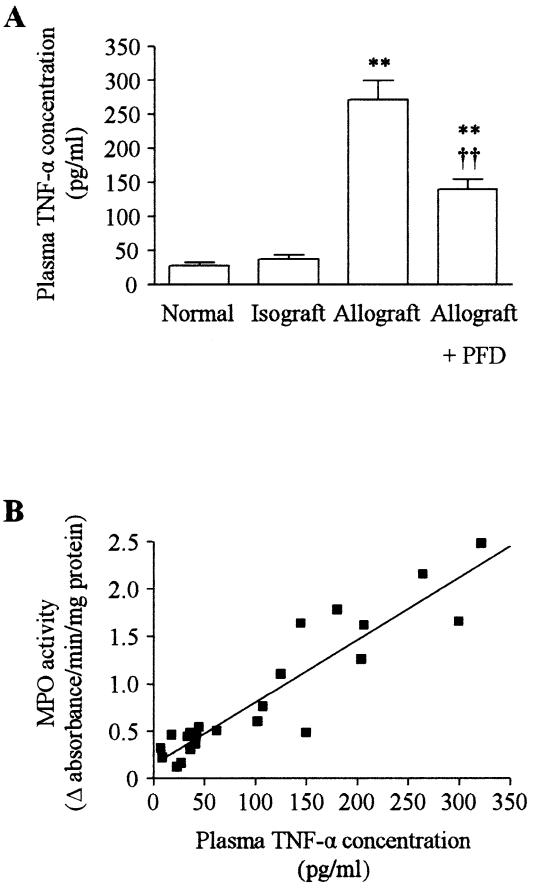

Figure 4. Circulating TNF-α level. A, Plasma TNF-α levels in all groups. Results are presented as means ± SEM (n = 6 in each group). **$P < .01$ versus normal animals or animals receiving isografts; ††$P < .01$ versus untreated animals receiving allografts. B, Plasma TNF-α levels positively correlated with lung MPO activity ($r^2 = 0.88$, $P < .001$).

further characterized the effect of pirfenidone on iron deposition in lung tissue.[23] Indeed, we found that lung sections from normal animals (Figure 3, *A*) or isografts (Figure 3, *B*) stained with Prussian blue displayed no detectable iron. By contrast, intense blue positive staining was consistently observed in sections from untreated allografts (Figure 3, *C*), whereas the amount of stainable iron was strikingly reduced in pirfenidone-treated allografts (Figure 3, *D*).

Finally, we verified the anti-TNF-α activity of pirfenidone and explored the relationship between circulating TNF-α levels and lung MPO activity. We found that plasma TNF-α concentration was similar between normal animals (25.6 ± 5.3 pg/mL) and animals that received isografts (36.2 ± 7.4 pg/mL). In contrast, a marked increase in plasma TNF-α levels was found in animals receiving allografts (271.4 ± 27.6 pg/mL, $P < .01$ vs normal animals or animals receiving isografts). The higher plasma TNF-α concentration was significantly reduced by pirfenidone treatment (138.9 ± 15.1 pg/mL, $P < .01$ vs untreated animals receiving allografts; Figure 4, *A*). In addition, we found that the plasma TNF-α level significantly ($P < .0001$) positively correlated with the lung MPO activity (Figure 4, *B*).

Discussion

Pirfenidone has been proved to be effective for patients with idiopathic pulmonary fibrosis[8] and multiple sclerosis.[7] The potential therapeutic adaptation of pirfenidone could be further expanded by the present discovery, in which we found that continuous monotherapy with pirfenidone dramatically reduced the severity of allograft injury in a rat lung transplant model (Figure 1). Given that pirfenidone was tolerated with relatively minor adverse effects and no toxic effect was found in patients,[7,8] the present observation suggests that pirfenidone is a candidate drug to be evaluated for attenuation of posttransplantation lung injury in human subjects.

In the present study a rat lung transplant model with the combination of Lewis donors and SD recipients was used. In this model increased longevity of posttransplantation survival was associated with more severe ACR, whereas ischemia-reperfusion caused very little lung damage.[15] Accordingly, we assessed the lung allografts at a relatively late time point, 21 days after transplantation. Indeed, we found that the untreated lung allografts displayed end-stage ACR with widespread destruction of alveolar-interstitial structure (Figure 1). Moreover, the severe pathologic lesion was accompanied by massive infiltration of neutrophils, as evi-

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dent by markedly increased numbers of MPO-positive cells and MPO enzymatic activity (Figure 2). These findings concur with previous results demonstrating that increased neutrophil recruitment occurred in human lung allografts with higher ACR grades.[20,24] Neutrophils contain MPO, and this enzyme catalyzes the reaction between hydrogen peroxide and chloride to form a highly reactive species, hypochlorous acid.[25] Thus the infiltration of neutrophils in lung allografts would lead to an increase in oxidative stress. The excess oxidative stress was further confirmed by the finding of iron accumulation in untreated lung allografts (Figure 3).[23] It is established that excessive oxidative stress causes tissue damage by means of protein inactivation, lipid peroxidation, and DNA damage.[24,26,27] Indeed, a clinical study revealed that the neutrophil population in bronchoalveolar lavage fluid positively correlated with mortality in human lung recipients.[28] Thus, our finding that pirfenidone dramatically decreased neutrophil recruitment (Figure 2) and iron accumulation (Figure 3) suggests a mechanism by which pirfenidone conferred protection against acute lung allograft injury (Figure 1). Similarly, a recent study showed that pirfenidone prevented endotoxin-induced liver injury through decreased neutrophil infiltration.[29]

There is convincing evidence that pirfenidone is a potent TNF-α antagonist. For example, numerous studies have demonstrated that pirfenidone decreased the synthesis and release of TNF-α in vivo[10,12-14] and in vitro.[12,14] Moreover, it is believed that the drug inhibits the reduction of TNF-α selectively at the translational level.[11] The anti-TNF-α activity of pirfenidone has also been identified in the present study, in which we found that pirfenidone significantly reduced circulating TNF-α levels seen in animals receiving lung allografts (Figure 4). Recent studies have revealed that TNF-α potentiates the recruitment and activation of neutrophils, and inhibition of TNF-α results in a marked reduction of neutrophil infiltration in lung tissue.[4,21,22] Consistent with these, we found that the circulating TNF-α level was positively correlated with lung MPO activity (Figure 4). It thus appears that the anti-TNF-α property of pirfenidone contributed, at least in part, to the reduced neutrophil recruitment seen in the pirfenidone-treated lung allografts. However, it is presently unclear whether the decreased iron accumulation seen in pirfenidone-treated allografts resulted secondarily from the reduced neutrophil recruitment or a direct drug effect. Obviously, additional studies are required to clarify this issue.

Here we report that pirfenidone, with its anti-TNF-α activity, is effective in ameliorating acute lung allograft injury. The compound might mediate the efficacy by inhibiting tissue neutrophil recruitment and iron deposition. These findings lend further support to the notion[4-6] that TNF-α plays a pivotal role in inducing acute posttransplantation lung injury. Notably, an increased TNF-α production has been reported to be associated with more severe ACR in human heart transplantations.[30] Therefore, it would be interesting to test whether treatment with pirfenidone, alone or in combination with classic immunosuppressants, could provide greater benefits to transplantation of thoracic organs (eg, heart-lung) in human subjects.

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