Absence of T Cells Confers Increased Pulmonary Arterial Hypertension and Vascular Remodeling

Laimute Taraseviciene-Stewart[1]*, Mark R. Nicolls[1]*, Donatas Kraskauskas[1], Robertas Scerbavicius[1], Nana Burns[1], Carlyne Cool[2], Kathy Wood[2], Jane E. Parr[2], Susan A. Boackle[3], and Norbert F. Voelkel[1]

[1]Division of Pulmonary Sciences, Department of Medicine, [2]Department of Pathology, and [3]Division of Rheumatology, Department of Medicine, University of Colorado Health Sciences Center, Denver, Colorado

***Rationale*: Severe pulmonary arterial hypertension (SPH) is a frequently lethal condition characterized by pulmonary vascular remodeling and right heart strain or failure. SPH is also often associated with autoimmune and collagen vascular disorders.**

***Objectives*: To study the effects of T cells on the development of experimental SPH.**

***Methods*: Athymic nude rats lacking T cells were treated with a single subcutaneous injection of vascular endothelial growth factor (VEGF) receptor blocker SU5416 (20 mg/kg) to induce pulmonary vascular endothelial cell apoptosis. Immunohistochemical analysis and IL-4 levels of the lung tissue were performed. Cell death and proliferation were assessed by Western blot and immunohistochemistry.**

***Measurements and Main Results*: In contrast to SU5416-treated euthymic rats that develop SPH only in combination with chronic hypoxia, athymic nude rats developed SPH and vascular remodeling (similar to clinical SPH) at normoxic conditions as demonstrated by measurements of pulmonary artery pressure and right ventricle hypertrophy. Pulmonary arterioles became occluded with proliferating endothelial cells and were surrounded by mast cells, B cells, and macrophages. IL-4, proliferating cell nuclear antigen, and collagen type I levels were markedly increased in SU5416-treated athymic rat lungs. Antibody deposition was noted along the vascular endothelium in rats with SPH. Finally, protection from SPH was conferred by immune challenge with spleen cells from euthymic nude rats.**

***Conclusions*: These studies demonstrate the importance of a complete, intact immune system in protecting against pulmonary angioproliferation in this new model of SPH as well as the importance of intact VEGF receptor signaling for lung endothelial cell homeostasis.**

Keywords: pulmonary hypertension; T cells; apoptosis; proliferation

AT A GLANCE COMMENTARY

Scientific Knowledge on the Subject

There is no experimental model of immune mechanism–dependent severe pulmonary hypertension.

What This Study Adds to the Field

T-cell deficiency confers increased pulmonary arterial hypertension and vascular remodeling.

Severe pulmonary arterial hypertension (SPH) is one manifestation of a number of collagen vascular and autoimmune diseases and viral infections. Scleroderma, systemic lupus erythematosis, Sjögren's syndrome, polymyositis, Hashimoto's thyroiditis, HIV, and human herpes virus-8 have all been associated with the development of SPH (1–5). These conditions are often associated with autoimmune antibodies as well as defects in the CD4 T-cell compartment (2–4, 6–9). SPH has also been described after splenectomy (10). Thus, it is possible that T-cell deficiencies (in either function or number) may contribute to pulmonary vascular injury or disease.

Pathologically, SPH can present with luminal obliteration (vascular lesions) of small precapillary arteries (11, 12). Histologically, all layers of the pulmonary arterioles—intima, media, and adventitia—are involved, and inflammatory cells are present in SPH (13). Notably, inflammation in the perivascular space includes accumulation of mast cells and lymphocytes (11, 14, 15). Given the association of autoimmune phenomena with SPH, such as detection of anti–nuclear and anti–endothelial cell antibodies (16–21), the finding of antibody-complement deposits in the lungs of patients with SPH (22, 23), and the presence of inflammatory cells around plexiform lesions in SPH, there appears to be a basis for an immune-mediated component in SPH pathogenesis. We sought to specifically address the contribution of T cells to the development of SPH.

Our group has developed a model of angioproliferative SPH that uses the vascular endothelial growth factor receptor (VEGFR) inhibitor SU5416 (24). This drug causes early pulmonary vascular endothelial cell apoptosis and, in combination with chronic hypoxia exposure, SPH associated with the development of abnormal endothelial cell proliferation (20). *In vitro* studies suggest that these lumen-occupying endothelial cells are apoptosis-resistant and share features with malignant cells, such as increased survivin expression (25). These cumulative findings suggest to us that early inflammation after vascular injury results in pulmonary vascular obliteration, which then has significant hemodynamic consequences (26). To address the question of whether T cells contribute to the development of SPH or, alternatively, protect against pulmonary disease, we treated athymic nude rats ($rnu^{-/-}$, T-cell deficient) and their heterozygote counterparts ($rnu^{-/+}$, T-cell replete) with the VEGFR blocker SU5416. The absence of T cells made animals vulnerable to the development of SPH after vascular injury to the extent that SPH uniquely developed in a normoxic environment with pathology similar to clinical SPH. SPH was characterized by perivascular mononuclear, mast, and B-cell infiltration and anti–endothelial cell antibodies. This finding suggests that T cells normally have a modulatory function after vascular injury that may prevent an overly exuberant inflammatory response. In the absence of such modulatory activity, it is possible that inflammatory cells (including

(*Received in original form August 21, 2006; accepted in final form March 30, 2007*)

Supported by National Institutes of Health grants 1PO1 HL66254-01A1 (N.F.V.) and HL 02662-01 (M.R.N.).

*These authors contributed equally to this article.

Correspondence and requests for reprints should be addressed to Laimute Taraseviciene-Stewart, Ph.D., Division of Pulmonary Sciences and Critical Care Medicine, 4200 East Ninth Avenue, C272, Denver, CO 80262. E-mail: laima.taraseviciene@uchsc.edu

Am J Respir Crit Care Med Vol 175. pp 1280–1289, 2007
Originally Published in Press as DOI: 10.1164/rccm.200608-1189OC on April 5, 2007
Internet address: www.atsjournals.org

autoreactive B cells) propagate vascular injury, which, in turn, fosters the development of SPH (27).

METHODS

Animals

The experimental protocol was approved by the Animal Care and Use Committee of the University of Colorado Health Sciences Center. Athymic nude National Institutes of Health (NIH)–rnu (rnu^{-}/rnu^{-}) and NIH–rnu^{+} ($rnu^{-/+}$) male rats (6 wk old) were injected subcutaneously with a single dose of SU5416 (20 mg/kg). SU5416 was suspended in CMC (0.5% [wt/vol] carboxymethylcellulose sodium, 0.9% [wt/vol] sodium chloride, 0.4% [vol/vol] polysorbate 80, 0.9% [vol/vol] benzyl alcohol in deionized water). Control rats received only diluent CMC. The animals (6 animals/group) were kept at normoxic conditions (Denver altitude [DA], 1,600 m) for 3 weeks (24).

Assessment of Pulmonary Hypertension

At the end of the treatment period, the rats were weighed, and then anesthetized with intramuscular ketamine hydrochloride (60 mg/kg) and xylazine (8 mg/kg) administration. The pulmonary artery pressure (PAP) and the right ventricle/left ventricle plus septum weight ratio (RV/LV+S) were determined as previously reported (24).

Assessment of Pulmonary Arteriolar Lumen Potency

A quantitative analysis of luminal obstruction was performed by counting at least 200 small pulmonary arteries (outer diameter < 50 μm) per lung section from the SU5416-treated rat group. Vessels were assessed for occlusive lesions on hematoxylin–eosin slides and scored as follows: (*1*) no evidence of lumen occlusion (open), (*2*) partial ($< 50\%$) luminal occlusion, and (*3*) full luminal occlusion (closed).

Immune Challenge

To assess the effects of immune challenge on the development of SPH, 10×10^6 spleen cells from euthymic ($rnu^{-/+}$) nude rats were injected intraperitoneally into athymic nude rnu^{-}/rnu^{-} rats 7 days before SU5416 administration.

Lung Morphology

Freshly excised lungs from all animals were inflated with 0.5% low-melt agarose and processed as previously described (28).

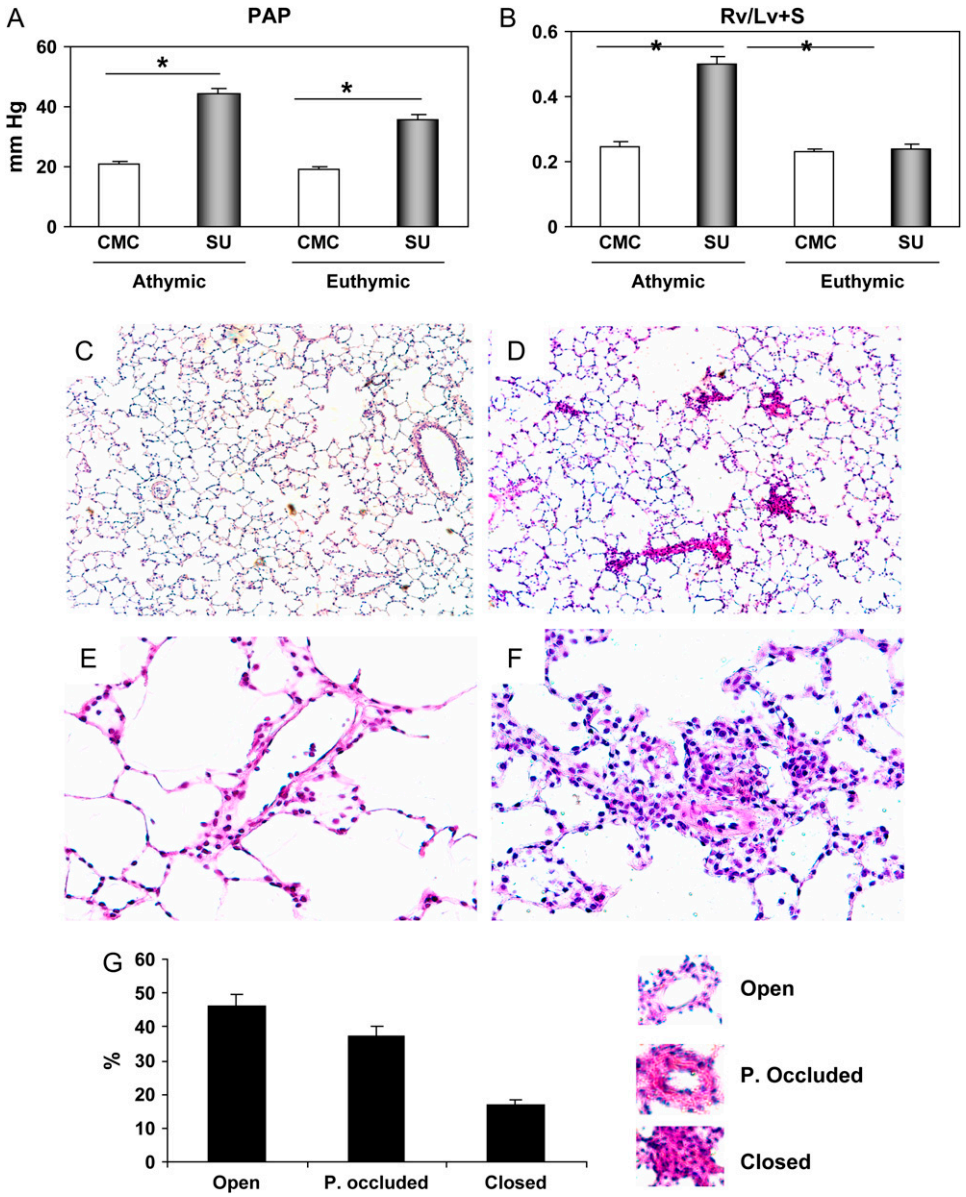

Figure 1. Mean pulmonary artery pressure (PAP) and measurement of right ventricular (RV) hypertrophy (RV weight as a fraction of the weight of the left ventricle plus septum; RV/LV+S) of athymic and euthymic rats treated with vehicle (CMC) or with a single subcutaneous injection of SU5416 (20 mg/kg). PAP (*A*) and RV/LV+S ratio (*B*) at normoxic conditions (Denver altitude, 1,600 m; Pb = 630 mm Hg, inspired P_{O_2} = 122 mm Hg). (*C–F*) Histology of untreated (*C*, *E*) and SU5416-treated (*D*, *F*) athymic rat lungs at Denver altitude. (*G*) Quantitative analysis of open, partially occluded (P. Occluded) and closed precapillary vessels in SU5416-treated athymic rat lungs. Hematoxylin-and-eosin staining, original magnification ×100 in *C* and *D* and ×400 in *E* and *F*. *$p < 0.01$.

Antibodies

Factor VIII–related antigen (polyclonal antibody used at 1/250 dilution for immunohistochemistry; DakoCytomation, Carpinteria, CA); smooth muscle α-actin (1/150 dilution for immunohistochemistry); sheep horseradish peroxidase (HRP)–conjugated antibodies, anti-rat IgG (Sigma, St. Louis, MO); anti-mouse HRP–conjugated antibody (Vector Laboratories, Burlingame, CA); mouse monoclonal anti-Flk-1, (VEGFR-2) clone A-3; rabbit polyclonal anti-CD19 (Santa Cruz Biotechnology, Santa Cruz, CA); anti-CD68, anti-CD20 (DakoCytomation); goat anti-rabbit HRP– and swine anti-goat HRP–conjugated antibodies (BioSource International, Camarillo, CA). Rabbit antibody to cleaved caspase-3 (Asp175), mouse anti–proliferating cell nuclear antigen (anti-PCNA) (PC10) (Cell Signaling Technology, Beverly, MA, distributed by New England Biolabs), and anti-rat collagen type I (Chemicon International, Temecula, CA). Commercially available kits for immunohistochemistry, Vectastain rabbit or mouse Elite (PK-6101, PK-6102; Vector Laboratories), were used.

Immunohistochemistry

After deparaffinization of the sections, rehydration, pretreatment with microwave (2 × 10 min in citrate buffer, pH 6.0), and washing in phosphate-buffered saline (PBS), pH 7.4, endogenous peroxidase was blocked with 0.3% H_2O_2 for 10 minutes and slides were then incubated with normal goat serum for 15 minutes at room temperature (RT). The primary antibodies were incubated for 1 hour at 37°C, followed by washings in PBS and incubation with the secondary biotinylated antibody for 1 hour at RT. Bound antibodies were detected by the use of the avidin–biotin complex for 30 minutes at RT. Peroxidase activity was visualized with 3,3′-diaminobenzidine, followed by counterstaining with hematoxylin and embedding in DePeX (Serva, Heidelberg, Germany). Negative controls included omission of the primary antibody and its replacement by rabbit nonimmune serum. For the mouse kit, a biotinylated anti-mouse IgG, rat adsorbed (BA-2001; Vector Laboratories) at 10 mg/ml, was used to avoid any background staining. Mast cells in the paraffin-embedded lung tissue sections were detected by Giemsa stain. Antibody deposition was detected using an anti-rat IgG antibody (Sigma).

Western Blot Analysis

Frozen rat lung tissue was homogenized in homogenization buffer (HB) (20 mM *N*-2-hydroxyethylpiperazine-*N*′-ethane sulfonic acid [4-(2-hydroxyethyl)-1-piperazine-ethane sulfonic acid], pH 7.4, 1 mM dithiothreitol, 10% glycerol, 0.1% Triton X-100) and centrifuged at 10,000 rpm for 10 minutes. Protein concentration in the supernatant was determined by Bradford assay using Bradford reagent (Sigma). Proteins (25 μg) were subjected to electrophoresis on 4 to 12% gradient NuPAGE Bis-Tris gels (Invitrogen, Carlsbad, CA) and transferred to PolyScreen PVDF Transfer membrane (Life Science Products, Frederick, CO) in NuPAGE transfer buffer containing 10% methanol. Prestained molecular mass marker proteins (Bio-Rad, Hercules, CA) were used as standards for the sodium dodecyl sulfate–polyacrylamide gel electrophoresis. Western blots were visualized using Renaissance Western Blot Chemiluminescence Reagent (Perkin Elmer, Boston MA).

Statistical Analyses

Statistical significance was determined using Student's unpaired *t* test ($p < 0.05$). Values are expressed as mean ± SE.

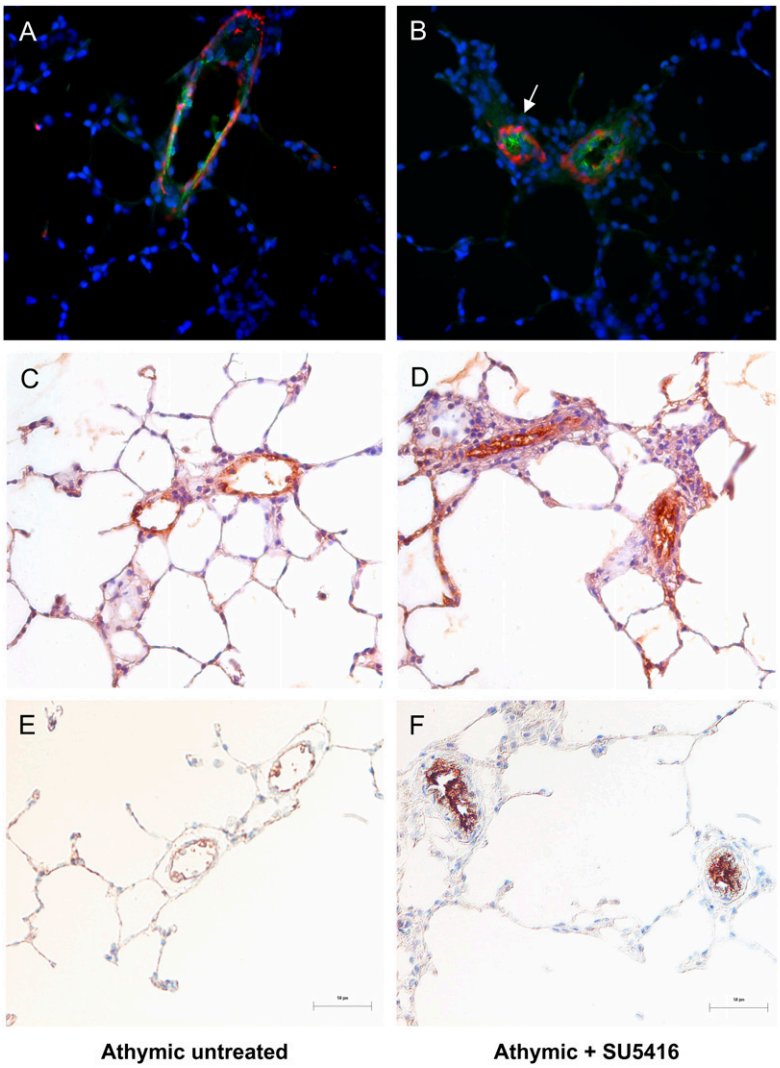

Figure 2. Immunohistochemistry of pulmonary vascular lesions. Triple fluorescence staining of untreated (*A*) and SU5416-treated (*B*) athymic rat lung at Denver altitude. *Red*, smooth muscle actin; *green*, factor VIII; *blue*, DAPI (4′,6-Diamidino-2-phenylindole). Pulmonary vascular lesions in SU5416-treated athymic rat at Denver altitude show intense staining for the endothelial cell marker factor VIII (*B*, *D*) and VEGFR2 (vascular endothelial growth factor receptor 2) (*F*) as compared with normal lung vessels (*A*, *C*, *E*). Original magnification, ×400.

RESULTS

PAP and Right Ventricular Hypertrophy in SU5416-treated Athymic and Euthymic Rats

There is no significant difference in PAP of athymic and euthymic rats during normoxia at DA (1,600 m), whereas treatment with the VEGFR inhibitor SU5416 at DA for 3 weeks leads to the development of SPH in athymic nude ($rnu^{-/-}$) rats (that lack T lymphocytes). The mean PAP in athymic SU5416-treated animals (n = 12) was significantly higher (44 ± 3.3 mm Hg) when compared with SU5416-treated syngeneic euthymic ($rnu^{-/+}$) rats (n = 9) (31 ± 2.8 mm Hg), whereas the mean PAP of untreated athymic and euthymic rats was the same (20 ± 2.5 mm Hg) (Figure 1A). The high PAP in athymic SU-5416–treated rats was accompanied by right ventricular hypertrophy (Figure 1B). The RV/LV+S in athymic SU5416-treated animals kept at DA was 0.55 ± 0.03, when compared with SU5416-treated euthymic rats (0.24 ± 0.02, $p < 0.05$). There was no significant difference in the RV/LV+S ratio between untreated athymic, euthymic, and SU5416-treated euthymic rats (Figure 1B). Previously, we demonstrated that immunocompetent Sprague-Dawley rats treated with SU5416 develop SPH only when exposed to chronic hypoxia for 3 weeks (24). When exposed to 3 weeks of chronic hypoxia alone, euthymic ($rnu^{-/+}$) rats developed SPH that was comparable to that observed in Sprague-Dawley rats (data not shown). Athymic ($rnu^{-/-}$), chronically hypoxic SU5416-treated rats developed high PAPs, and 50% of them died within 2 to 3 weeks, whereas none of the chronically hypoxic SU5416-treated Sprague-Dawley rats died (data not shown). As shown in Figure 1, under normoxic conditions, SU5416-treated athymic ($rnu^{-/-}$) animals (Figures 1D and 1F) but not euthymic ($rnu^{-/+}$) animals (Figures 1C and 1E) developed significant vascular remodeling.

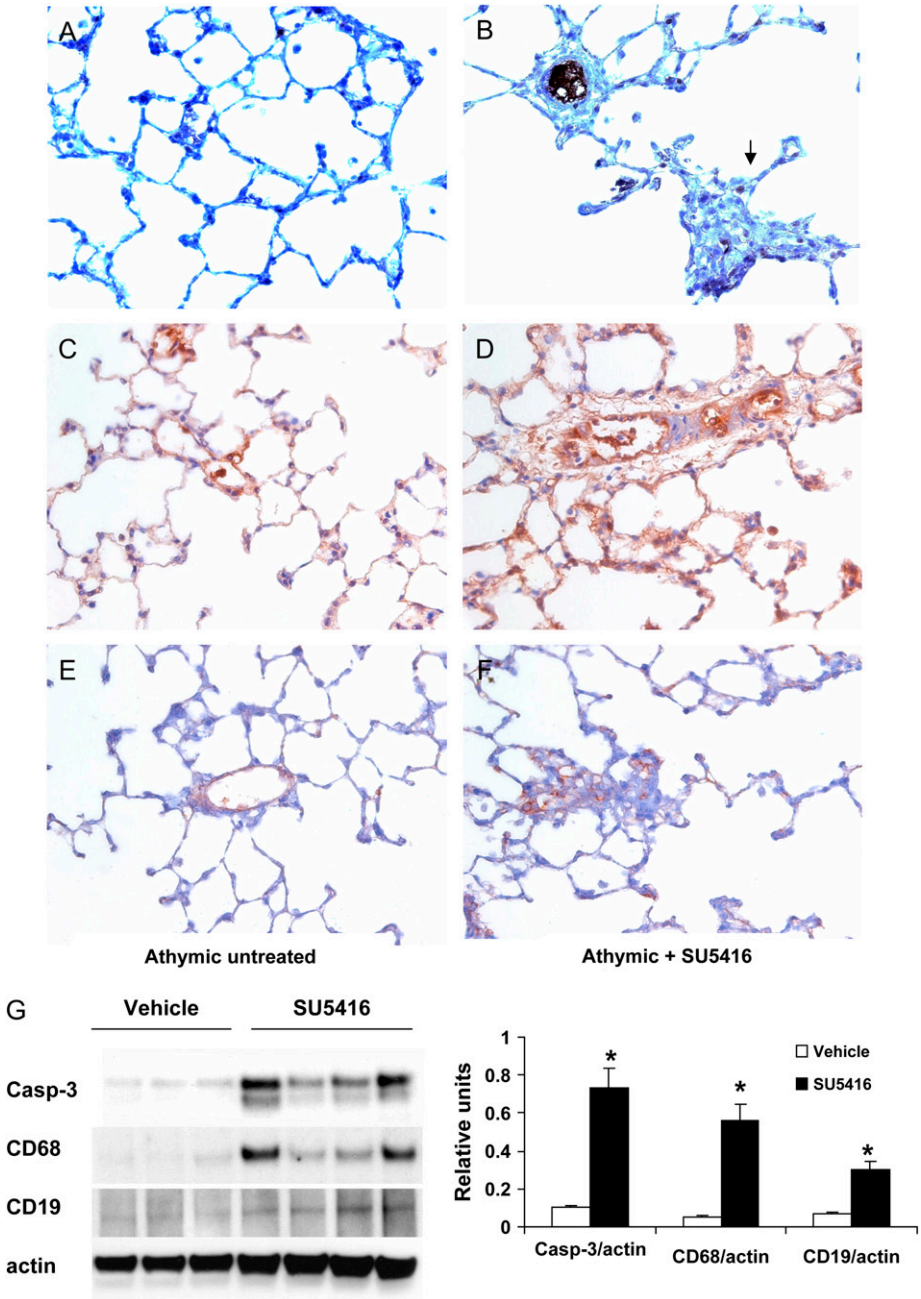

Figure 3. Immunohistochemical staining of activated caspase-3 (*A*, *B*); CD68 (*C*, *D*), and CD20 (*E*, *F*) of untreated (*A*, *C*, *E*) and SU5416-treated (*B*, *D*, *F*) athymic rat lungs. Original magnification, ×400. (*G*) Western blot and quantitative analysis of activated caspase-3 (2 wk after SU5416 injection), CD68, and CD19 (3 wk after SU5416 injection) in the whole lung extracts of untreated and SU5416-treated athymic rats. *$p < 0.01$.

Lungs from SU5416-treated athymic rats showed arteriolar lesions characterized by endothelial cell proliferation; many of the vessels demonstrated near-complete lumen occlusion of precapillary intraalveolar arteries (Figures 1D and 1F), whereas the untreated athymic rnu$^-$/rnu$^-$ rat lungs were histologically normal (Figures 1C and 1E). Quantitative analysis (Figure 1G) shows that, in SU5426-treated athymic rat lungs, almost 40% of the precapillary pulmonary vessels were partially occluded and 20% were totally occluded, whereas no vessel occlusion was observed in vehicle-treated rat lungs (Figures 1C and 1E). As stated, none of the SU5416-treated rnu$^-$/rnu$^+$ animals showed lung vessel lumen occlusion.

Histology of Vehicle (CMC)- and SU5416-treated Athymic Rat Lungs

Double fluorescent staining for the endothelial cell marker factor VIII (green) and smooth muscle actin (red) showed that the lumen-obliterating vascular lesions (Figure 2, *arrow*) in SU5416-treated normoxic athymic rat lungs were indeed caused by proliferation of endothelial cells. Cells in the lesions expressed the endothelial cell marker factor VIII (Figures 2B and 2D) and VEGFR-2/KDR (Figure 2F). As in idiopathic pulmonary hypertension (PH) (29), these cells did not form a monolayer as seen in the untreated rat lungs (Figures 2A, 2C, and 2E) but piled up to fill the vascular lumen (Figures 2B, 2D, and 2F). There was no lumen obliteration in vehicle-treated rnu$^-$/rnu$^+$ or rnu$^-$/rnu$^-$ nude rat lungs (Figures 2A, 2C, and 2E).

SU5416 Treatment Causes Vascular Endothelial Cell Death and Accumulation of B Cells and CD68$^+$ Cells in Athymic Rat Lungs

Earlier, we demonstrated that, in SU5416-treated Sprague-Dawley rats exposed to chronic hypoxia, at 1 to 2 weeks of exposure there was significant lung endothelial cell death, whereas at a later time point of 3 weeks, proliferation of endothelial cells occurred (24). Immunohistochemistry of activated caspase-3 revealed the presence of a large number of apoptotic cells in SU5416-treated athymic rat lungs at 2 weeks after the treatment (Figure 3B), whereas there was no cell death in vehicle-treated rat lungs (Figure 3A). At 3 weeks after SU5416 injection, there was also an elevated number of CD68$^+$ macrophages, monocytes, and neutrophils (Figure 3D) and CD20$^+$ B cells in the vascular lesions of SU5416-treated athymic rat lungs when compared with vehicle-treated athymic controls (Figures 3C and 3D). Immunohistochemical findings were confirmed by Western blot (Figure 3G). Quantitative analysis of Western blot protein bands showed significant up-regulation of active caspase-3, CD68, and CD19 protein expression in whole lung extracts of SU5416-treated athymic rats when compared with vehicle-treated control rats, supporting the immunohistochemical findings.

SU5416 Treatment Causes Proliferation of Endothelial Cells in Athymic Rat Pulmonary Vascular Lesions

Immunohistochemistry for PCNA showed proliferating cells in vascular lesions of SU5416-treated athymic rat lungs (Figure 4B), whereas there were no proliferating cells in vehicle-only–treated athymic rat lungs (Figure 4A). Western blot analysis showed an almost threefold up-regulation of PCNA protein expression in the whole lung extracts of SU5416-treated athymic rats as compared with vehicle-treated control rats (Figure 4C).

Mast Cells and IL-4 Levels in SU5416-treated Athymic Rat Lungs

The SU5416-treated athymic rat lungs (Figure 5B) were characterized by infiltration with mast cells, whereas in vehicle-treated

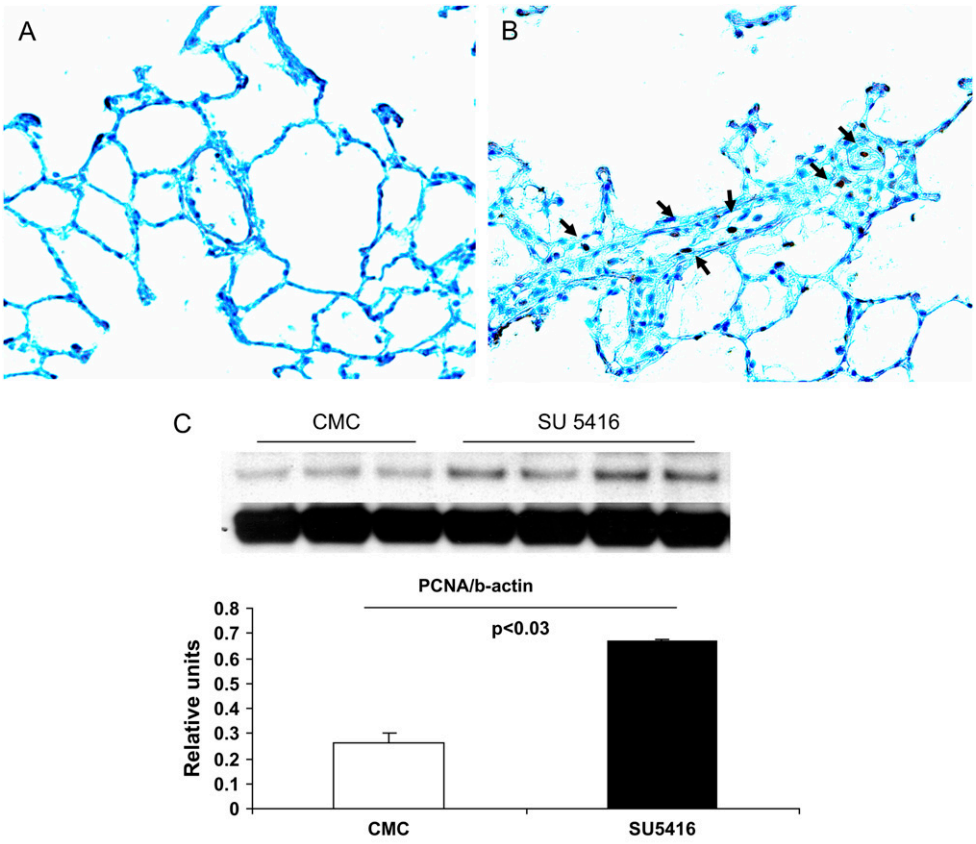

Figure 4. Immunohistochemistry for proliferating cell nuclear antigen (PCNA) in untreated (*A*) and SU5416-treated (*B*) athymic rat lung tissue. (*C*) Western blot and quantitative analysis of PCNA in the whole lung extracts from vehicle (CMC) and SU5416-treated athymic rats (3 wk after SU5416 injection).

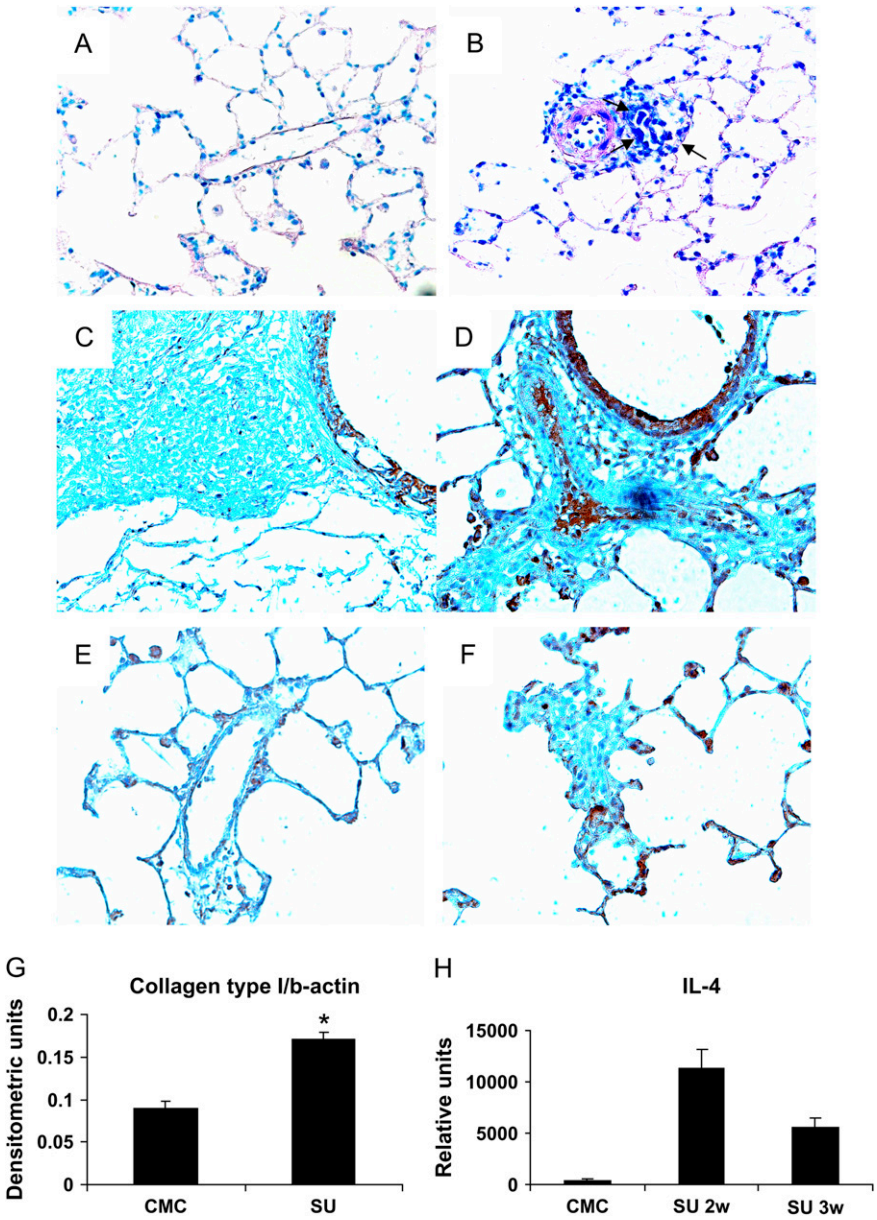

Figure 5. Accumulation of mast cells and collagen in athymic rat lungs. Giemsa staining of mast cells (*A*, *B*) and collagen type I deposition (*C–F*) in untreated (*A*, *C*, *E*), and SU5416-treated (*B*, *D*, *F*) rat lungs. Original magnification, ×400. Western blot quantitative analyses for collagen type I in the whole lung extracts (*G*). Elevated levels of IL-4 production (*H*) were found in SU5416-treated athymic rat lungs as compared with the vehicle-treated controls. *$p < 0.01$.

rat lungs there were only occasional mast cells (Figure 5A). Mast cell granules are the storage site for the neutral serine protease tryptase. One consequence of tryptase release from activated mast cells is the induction of collagen I production (30). For this reason, we measured the collagen I production in rat lungs. Immunohistochemical detection of collagen type I shows that, in SU5416-treated athymic rats, there is a marked increase of collagen type I (Figures 5D and 5F) as compared with vehicle-treated athymic control rats (Figures 5C and 5E). The quantitative analysis of collagen type I performed by Western blot (Figure 5G) shows a threefold increase in collagen type I expression in the whole lung extracts of SU5416-treated athymic rats. It is known that mast cells can be a significant source of cytokine IL-4 production. For this reason, lung homogenates from control and athymic SPH animals were assayed with a cytokine array kit that included IL-4, IL-1α, IFN-γ, tumor necrosis factor (TNF)-α, monocyte chemotactic protein-1 (MCP-1), macrophage inflammatory protein-3α (MIP-3α), tissue inhibitor of metalloproteinases-1 (TIMP-1), and IL-10. Of these cytokines and chemokines, IL-4 was the only differentially expressed protein, with significant up-regulation of IL-4 protein by 2 weeks in whole tissue extracts of SU5416-treated athymic rat lungs as compared with vehicle-treated athymic rat lungs (Figures 5C–5F, and 5H).

Histology of Vehicle- and SU5416-treated Athymic Rat Hearts

As shown in Figure 1B, SU5416 treatment of athymic rats lacking T cells causes severe right ventricular hypertrophy at DA compared with vehicle-treated rats. Hematoxylin–eosin staining of the heart tissue (Figures 6A and 6B) shows inflammatory cell infiltrates in SU5416-treated athymic rats (Figure 6B). The immunohistological staining of SU5416-treated athymic rat heart tissue revealed the following: cell death (2 wk after SU5416 injection), as shown by active caspase-3 staining (Figure 6D); abundant cell proliferation, as detected by PCNA staining (Figure 6F); accumulation of mast cells (Figure 6H); and high levels of collagen type I (Figure 6J) compared with vehicle-treated athymic rat hearts (Figures 6C, 6E, 6G, and 6I).

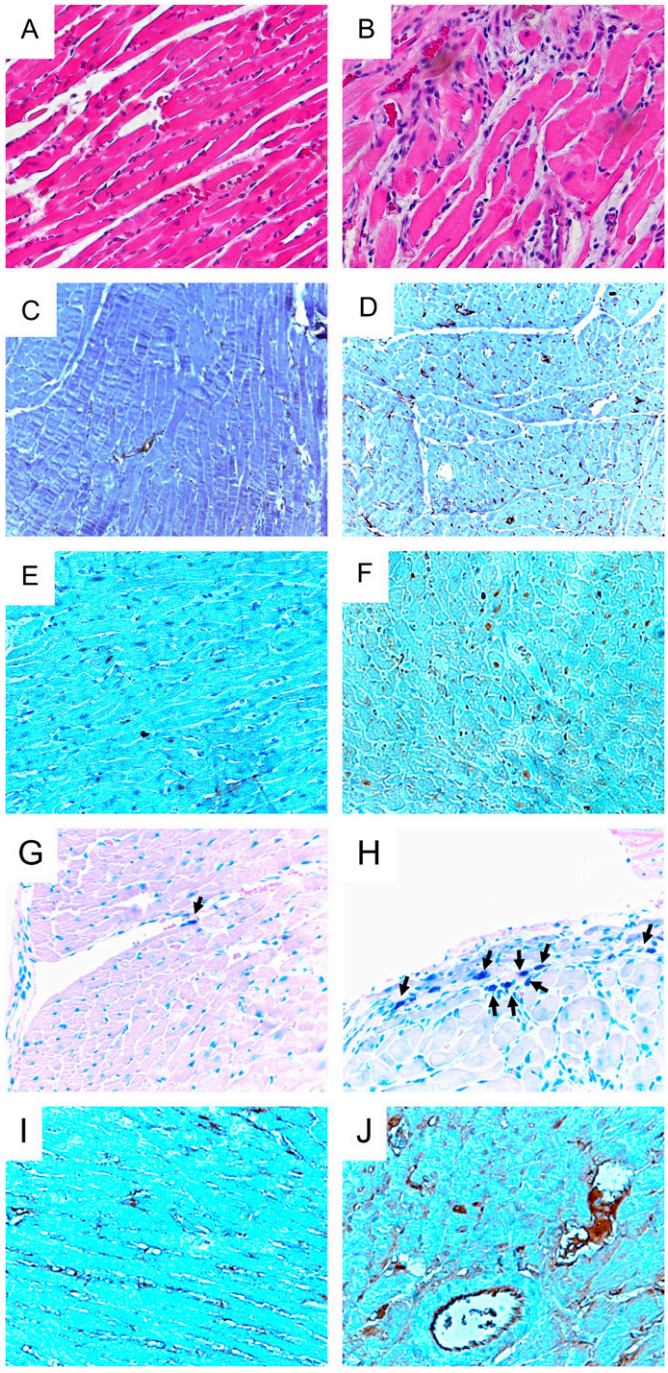

Figure 6. Immunohistochemistry of untreated (*A*, *C*, *E*, *G*) and SU5416-treated (*B*, *D*, *F*, *H*) athymic rat heart tissue. (*A*, *B*) Hematoxylin–eosin; (*C*, *D*) caspase-3; (*E*, *F*) proliferating cell nuclear antigen (PCNA); (*G*, *H*) Giemsa staining of mast cells; (*I* , *J*) collagen type I staining. Original magnification, ×400.

Antibody Deposition

To test whether autoreactive antibodies were present in athymic SU5416-treated rat lungs, we stained lungs with anti-rat IgG antibody. As shown in Figure 7A, there was no positive staining for IgG in vehicle-treated athymic rat lungs, whereas in SU5416-treated athymic rat lung, vascular lesions (the site of B-cell accumulation), there was a positive staining for rat IgG consistent with autoantibody deposition in the pulmonary vasculature (Figure 7B). Screening for circulating antinuclear antibodies was negative in both vehicle- and SU5416-treated athymic rats (data not shown).

Splenocytes Blocked the Development of SU5416-induced PH in Athymic Rats

To determine whether immune challenge protected against the development of exuberant pulmonary remodeling in T-cell–deficient nude rats, splenocytes (which contain T cells, B cells, and other mononuclear cells) from euthymic syngeneic (rnu/$^{-/+}$) animals were administered intraperitoneally into athymic (rnu^{-}/rnu^{-}) animals 1 week before SU5416 injection. We found that the lungs from athymic (rnu^{-}/rnu^{-}) rats reconstituted with immunocompetent spleen cells from rnu^{-}/rnu^{+} rats were protected from the development of exuberant pulmonary vascular lesions (Figure 8B) because the appearance of the lung vessels of the reconstituted animals did not differ from that observed in immunocompetent euthymic rats (Figure 8A). Athymic rnu^{-}/rnu^{-} rats injected with splenocytes from euthymic rnu/$^{+}$ rats did not develop high PAPs (Figure 8C) and there was no evidence of right ventricle hypertrophy (Figure 8D).

DISCUSSION

In the present study, we demonstrated that the absence of T cells enhances the vaso-obliterative remodeling that occurs as a consequence of pulmonary endothelial cell apoptosis and that reconstituting the immune system prevented these effects. Although the induction of SPH in euthymic immunocompetent rats treated with the VEGFR blocker SU5416 requires additional stressors, such as chronic hypoxia (24), or in monocrotaline-treated rats, pneumonectomy-induced high shear stress (31), athymic rats that lack T cells develop obliterative vascular lesions and SPH when treated with a VEGFR blocker under normoxic conditions (without additional stress). Vascular injury with SU5416 is sufficient to cause PH in athymic animals and a mild elevation in PAP in euthymic animals at DA. Of relevance, monocrotaline-treated athymic rats also develop severe vascular remodeling (32) consistent with SPH compared with euthymic rats, illustrating that disparate vascular injuries coupled with a T-cell deficiency appear to provide a two-hit phenomenon favoring the development of SPH. A previous study of athymic rats (32) did not attribute the more severe PH to the absence of T cells but rather postulated that the absence of a thymus resulted in dysregulated mast cells. We conclude, in contrast, that the absence of T cells conferred an increased risk of pulmonary arterial hypertension and vascular remodeling in the current experimental model. Injection of T-cell–containing spleen cells was protective in this study. However, a limitation of this experiment is that Charles River rnu rats are outbred and conferred protection may have been secondary to an immune response to alloantigen or replacement of T-cell function. Therefore, the more conservative term "immune challenge" is more appropriate than "immune reconstitution" given our lack of certainty about genetic homogeneity of these animals. Although this study limitation prevents establishing a conclusive role for T-cell activity, it clearly strongly supports the role of immune responses in the development of SPH in this experimental model.

In these athymic animals with SPH, the small precapillary arteries were largely obliterated by abnormal proliferating endothelial cells both in the normoxic SU5416-challenged as well as the chronic hypoxia-exposed rats. Some vessels were filled with CD20^{+} B cells (Figure 3F) and mast cell accumulation (Figure 5B) occurred frequently in perivascular cellular infiltrates reminiscent of the inflammatory cells in plexiform lesions from patients with idiopathic PH (33). We have hypothesized that

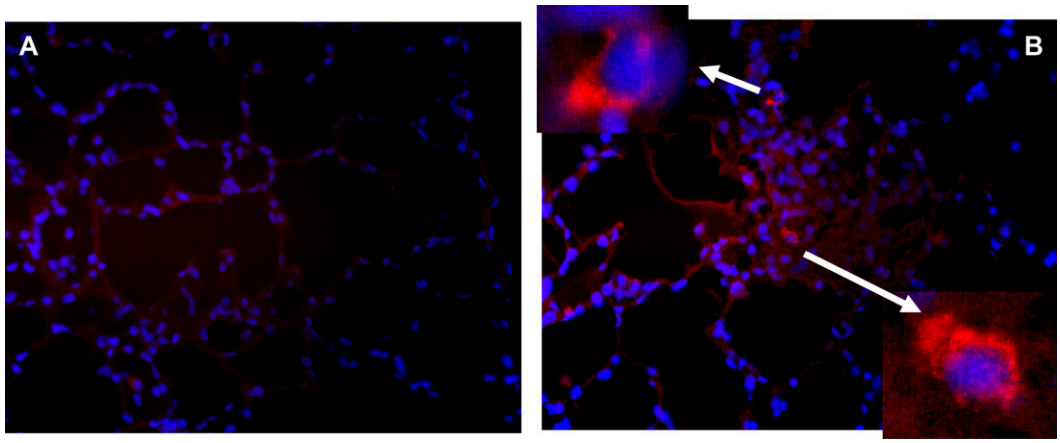

Figure 7. Antibody deposition in SU5416-treated athymic rat lung vascular lesions. Combined double staining images: *red*, anti-rat IgG; *blue*, DAPI staining for nuclei. There is no detectable antibody deposition in untreated athymic rat lungs (*A*), whereas in SU5416-treated animal lungs (*B*), there are IgG-positive cells in the vasculature (*see inserts*). Original magnification, ×400. *Arrows* point from cells with antibody deposition shown in detail in the *inserts*.

angioproliferative pulmonary vascular remodeling begins with endothelial cell apoptosis, which is followed by exuberant intraluminal endothelial cell growth (24, 25). In support of this concept, activated caspase-3^+ cells were observed in some vessels and absence of caspase-3^+ cells (absence of apoptosis was noted) in fully obliterated arterioles within the SU5416-treated (early time point, 2 wk after SU5416 injection) athymic rat lungs (Figure 3B) and PCNA-positive proliferating endothelial cells (late time point, 3 wk after SU5416 treatment) were found in vascular lesions (Figure 4B). B-cell and mast cell infiltrates and caspase-3^+ endothelial cells were observed not only in lung vessels but also in and around myocardial microvessels (data not shown). Apoptosis of myocardial microvascular endothelial cells in the SU5416-treated athymic rats indicates that there is not only lung vessel involvement but also cardiac involvement in this model of immune insufficiency. The dual involvement of pulmonary and cardiac vascular endothelium may explain the lethality of SU5416-treated chronically hypoxic athymic animals, which apparently experience both PH and myocardiopathy. Cardiac muscle involvement in this model thus shares features with scleroderma-associated PH, a particularly malignant form of SPH characterized by myocardial microvascular disease (34, 35).

How inflammatory cells contribute to the pathogenesis of SPH is currently not understood. In addition to $CD68^+$ and $CD20^+$ cells, the current model demonstrated mast cells and vascular antibody deposition in the lungs of athymic rats with SPH. However, no circulating antinuclear antibodies were found in SU5416- or vehicle-treated rats. The presence of mast cells surrounding pulmonary vessels has long been observed, but the relevance of these cells to the disease has not been well understood (32, 36–40). We postulate that, in the current model, in the absence of any possible T-cell regulation, mast cells may be potentiating autoreactive B cells to produce autoantibodies through the local production of IL-4 (41). Beyond being an important link between the innate and adaptive immune system in autoimmune responses (42), mast cells may also be of key

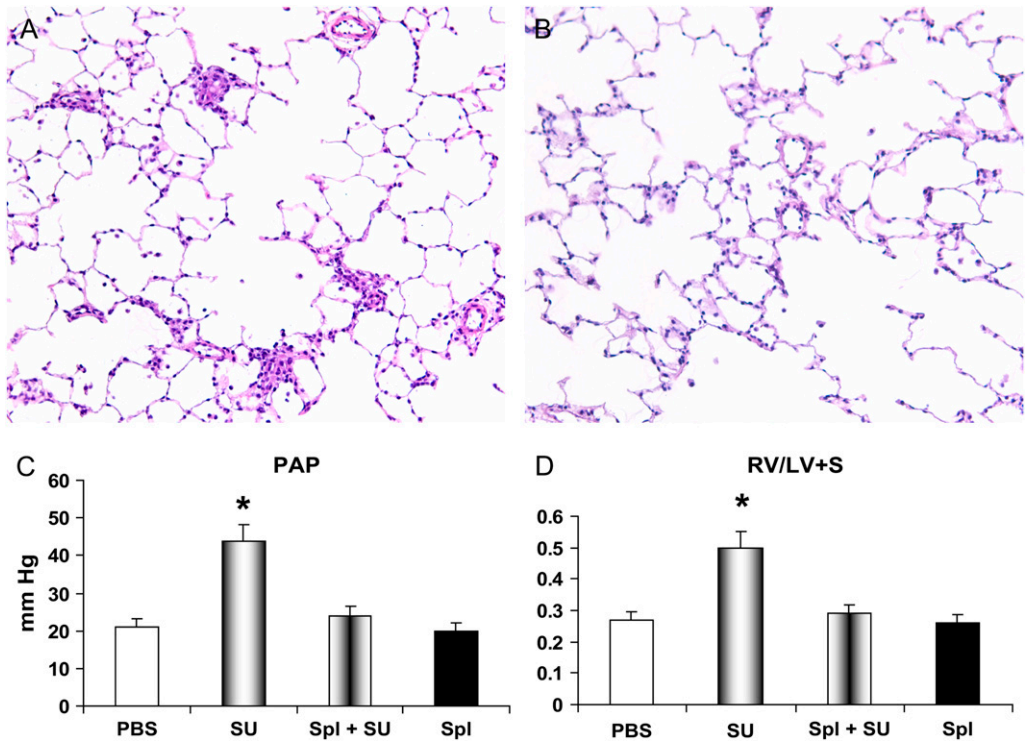

Figure 8. Adoptive transfer of immunocompetent splenocytes protects athymic rats from developing SU5416-induced pulmonary hypertension. (*A*) Hematoxylin–eosin staining of SU5416-treated athymic rat lung shows massive vascular lesions. (*B*) No vascular lesions were found in athymic rats after adoptive transfer of splenocytes from euthymic rats. Original magnification, ×200. (*C*) Pulmonary artery pressure (PAP) and (*D*) RV/LV+S (right ventricular weight as a fraction of the weight of the left ventricle plus septum) measurements in animals treated with SU5416-alone (SU), vehicle (CMC), splenocytes + SU5416 (Spl+SU), and splenocytes alone (Spl). *$p < 0.01$.

importance in autoantibody production (43, 44). Mast cells are also a source of the neutral serine protease tryptase. Although rat mast cell tryptase, unlike tryptases in other species, is a soluble enzyme and is absent in some mast cell subpopulations in mucosa, skin, and lung (45), consistent with increased tryptase activity, we observed the increased deposition of collagen type I in SU5416-treated athymic rat lungs (Figures 5D and 5F) and heart (Figure 6H). Mast cells are also a rich source of non–T-cell-derived IL-4 (46). We found that SU5416-treated rat lungs expressed high levels of IL-4, and we postulate that mast cell infiltration accounts for this phenomenon. Overexpression of IL-4 is associated with autoantibody expression (47), and IL-4 from a mast cell source is sufficient to trigger antibody (41) and autoantibody (43) production. Because pulmonary vascular obliteration in this model is associated with perivascular inflammation and mast cell infiltration, we speculate that mast cell–derived IL-4 could activate B lymphocytes to produce antibodies against endothelial cells via noncognate activation (41). Indeed, we demonstrated the presence of immune complex formation in the lungs from SU5416-treated athymic rats with SPH (Figure 6). The linkage between immune insufficiency and autoantibodies in the current model of SPH pathogenesis is consistent with the notion that a loss of regulatory T-cell activity can result in the development of autoimmune injury (48–50), although we present no data in reference to specific regulatory T-cell function.

The study of dendritic cell (DC) involvement in this model would help to further understanding of the complex links between immune cells and pulmonary vascular remodeling. Indeed, Perros and colleagues (51) have recently demonstrated increased numbers of immature DCs in plexiform lesions of human patients with idiopathic PH and in remodeled pulmonary vessels from rats with monocrotaline-induced PH. If DCs indeed contribute to PH pathogenesis, the next question that arises is what (auto)antigen they might be presenting. Alternatively, DCs might be present inside the lesions to suppress disease progression. This is certainly a possibility in view of the immature state of the DCs.

How a postulated impairment of T-cell function facilitates the development of severe angioproliferative PH remains to be investigated in detail. Our work is, to our knowledge, the first attempt to investigate the role of cells of the immune system in the development of severe angioproliferative PH, a condition which frequently determines the lethal outcome of a variety of autoimmune collagen–vascular disorders (52, 53). Although young athymic (rnu^-/rnu^-) animals clearly lack T lymphocytes, this model is limited by a lack of a complete understanding of the integrated immune functions in this particular rat strain. In conclusion, the data presented here illustrate both the importance of a complete, intact immune system in protecting against pulmonary angioproliferation in this new model of SPH as well as the importance of intact VEGFR signaling for lung endothelial cell homeostasis.

Conflict of Interest Statement: None of the authors has a financial relationship with a commercial entity that has an interest in the subject of this manuscript.

Acknowledgment: The authors thank Kenneth Morris for his valuable technical assistance with the measurements of pulmonary artery pressures. The authors also acknowledge the technical assistance of Vita Kraskauskiene.

References

1. Fagan KA, Badesch DB. Pulmonary hypertension associated with connective tissue disease. *Prog Cardiovasc Dis* 2002;45:225–234.
2. Zandman-Goddard G, Shoenfeld Y. HIV and autoimmunity. *Autoimmun Rev* 2002;1:329–337.
3. Parravinci C, Corbellino M, Paulli M, Magrini U, Lazzarino M, Moore PS, Chang Y. Expression of a virus-derived cytokine, KSHV vIL-6, in HIV-seronegative Castleman's disease. *Am J Pathol* 1997;151:1517–1522.
4. Kemeny L, Kiss M, Gyulai R, Kenderessy AS, Adam E, Nagy F, Dobozy A. Human herpesvirus 8 in classic Kaposi sarcoma. *Acta Microbiol Immunol Hung* 1996;43:391–395.
5. Onji M, Doi Y, Miyaoka H, Masumoto T, Horiike N, Ohta Y. Serum levels of soluble CD4 and CD8 in patients with chronic viral hepatitis. *Hepatogastroenterology* 1994;41:377–379.
6. Degiannis D, Seibold JR, Czarnecki M, Raskova J, Raska K Jr. Soluble and cellular markers of immune activation in patients with systemic sclerosis. *Clin Immunol Immunopathol* 1990;56:259–270.
7. Ingegnoli F, Trabattoni D, Saresella M, Fantini F, Clerici M. Distinct immune profiles characterize patients with diffuse or limited systemic sclerosis. *Clin Immunol* 2003;108:21–28.
8. Crispin JC, Martinez A, Alcocer-Varela J. Quantification of regulatory T cells in patients with systemic lupus erythematosus. *J Autoimmun* 2003;21:273–276.
9. Liu MF, Wang CR, Fung LL, Wu CR. Decreased CD4+CD25+ T cells in peripheral blood of patients with systemic lupus erythematosus. *Scand J Immunol* 2004;59:198–202.
10. Hoeper MM, Niedermeyer J, Hoffmeyer F, Flemming P, Fabel H. Pulmonary hypertension after splenectomy? *Ann Intern Med* 1999;130:506–509.
11. Tuder RM, Groves B, Badesch DB, Voelkel NF. Exuberant endothelial cell growth and elements of inflammation are present in plexiform lesions of pulmonary hypertension. *Am J Pathol* 1994;144:275–285.
12. Cool CD, Stewart JS, Werahera P, Miller GJ, Williams RL, Voelkel NF, Tuder RM. Three-dimensional reconstruction of pulmonary arteries in plexiform pulmonary hypertension using cell-specific markers: evidence for a dynamic and heterogeneous process of pulmonary endothelial cell growth. *Am J Pathol* 1999;155:411–419.
13. Voelkel NF, Taraseviciene-Stewart L, Cool C. Severe angioproliferative pulmonary hypertension: the lung circulation between vasoconstriction and cancer. *Advances in Pulmonary Hypertension*. 2003;4:4–8.
14. Heath D. Pulmonary hypertension in pulmonary parenchymal disease. *Cardiovasc Clin* 1972;4:79–96.
15. Humbert M, Morrell NW, Archer SL, Stenmark KR, MacLean MR, Lang IM, Christman BW, Weir EK, Eickelberg O, Voelkel NF, *et al.* Cellular and molecular pathobiology of pulmonary arterial hypertension. *J Am Coll Cardiol* 2004;43(Suppl):13S–24S.
16. Bodolay E, Csipo I, Gal I, Sipka S, Gyimesi E, Szekanecz Z, Szegedi G. Anti-endothelial cell antibodies in mixed connective tissue disease: frequency and association with clinical symptoms. *Clin Exp Rheumatol* 2004;22:409–415.
17. Negi VS, Tripathy NK, Misra R, Nityanand S. Antiendothelial cell antibodies in scleroderma correlate with severe digital ischemia and pulmonary arterial hypertension. *J Rheumatol* 1998;25:462–466.
18. Renaudineau Y, Dugue C, Dueymes M, Youinou P. Antiendothelial cell antibodies in systemic lupus erythematosus. *Autoimmun Rev* 2002;1:365–372.
19. Song J, Park YB, Lee WK, Lee KH, Lee SK. Clinical associations of anti-endothelial cell antibodies in patients with systemic lupus erythematosus. *Rheumatol Int* 2000;20:1–7.
20. Williams JM, Colman R, Brookes CJ, Savage CO, Harper L. Anti-endothelial cell antibodies from lupus patients bind to apoptotic endothelial cells promoting macrophage phagocytosis but do not induce apoptosis. *Rheumatology (Oxford)* 2005;44:879–884.
21. Worda M, Sgonc R, Dietrich H, Niederegger H, Sundick RS, Gershwin ME, Wick G. In vivo analysis of the apoptosis-inducing effect of anti-endothelial cell antibodies in systemic sclerosis by the chorionallantoic membrane assay. *Arthritis Rheum* 2003;48:2605–2614.
22. Quismorio FP Jr, Sharma O, Koss M, Boylen T, Edmiston AW, Thornton PJ, Tatter D. Immunopathologic and clinical studies in pulmonary hypertension associated with systemic lupus erythematosus. *Semin Arthritis Rheum* 1984;13:349–359.
23. Nakagawa N, Osanai S, Ide H, Nishigaki Y, Takahashi S, Nakano H, Ohsaki Y, Kikuchi K, Tokusashi Y, Obata H. Severe pulmonary hypertension associated with primary Sjogren's syndrome. *Intern Med* 2003;42:1248–1252.
24. Taraseviciene-Stewart L, Kasahara Y, Alger L, Hirth P, Mc Mahon G, Waltenberger J, Voelkel NF, Tuder RM. Inhibition of the VEGF receptor 2 combined with chronic hypoxia causes cell death-dependent pulmonary endothelial cell proliferation and severe pulmonary hypertension. *FASEB J* 2001;15:427–438.
25. Sakao S, Taraseviciene-Stewart L, Lee JD, Wood K, Cool CD, Voelkel NF. Initial apoptosis is followed by increased proliferation of apoptosis-resistant endothelial cells. *FASEB J* 2005;19:1178–1180.

26. Oka M, Homma N, Taraseviciene-Stewart L, Morris K, Kraskauskas D, Voelkel NF, McMurtry I. Rho kinase-mediated vasoconstriction in severe obliterative arterial hypertension in rats [abstract]. *Proc Am Thorac Soc* 2006;3:A758.
27. Taraseviciene-Stewart L, Scerbavicius DK, Burns N, Cool CD, Nicolls MR, Voelkel NF. The protective role of T-lymphocytes in pulmonary vascular remodeling. *Chest* 2005;128:571S–572S.
28. Kasahara Y, Tuder RM, Taraseviciene-Stewart L, Le Cras TD, Abman S, Hirth PK, Waltenberger J, Voelkel NF. Inhibition of VEGF receptors causes lung cell apoptosis and emphysema. *J Clin Invest* 2000;106: 1311–1319.
29. Lee SD, Shroyer KR, Markham NE, Cool CD, Voelkel NF, Tuder RM. Monoclonal endothelial cell proliferation is present in primary but not secondary pulmonary hypertension. *J Clin Invest* 1998;101:927–934.
30. Cairns JA, Walls AF. Mast cell tryptase stimulates the synthesis of type I collagen in human lung fibroblasts. *J Clin Invest* 1997;99:1313–1321.
31. Faul JL, Nishimura T, Berry GJ, Benson GV, Pearl RG, Kao PN. Triptolide attenuates pulmonary arterial hypertension and neointimal formation in rats. *Am J Respir Crit Care Med* 2000;162:2252–2258.
32. Miyata M, Sakuma F, Ito M, Ohira H, Sato Y, Kasukawa R. Athymic nude rats develop severe pulmonary hypertension following monocrotaline administration. *Int Arch Allergy Immunol* 2000;121:246–252.
33. Dorfmuller P, Perros F, Balabanian K, Humbert M. Inflammation in pulmonary arterial hypertension. *Eur Respir J* 2003;22:358–363.
34. Botstein GR, LeRoy EC. Primary heart disease in systemic sclerosis (scleroderma): advances in clinical and pathologic features, pathogenesis, and new therapeutic approaches. *Am Heart J* 1981;102:913–919.
35. Follansbee WP, Curtiss EI, Medsger TA Jr, Steen VD, Uretsky BF, Owens GR, Rodnan GP. Physiologic abnormalities of cardiac function in progressive systemic sclerosis with diffuse scleroderma. *N Engl J Med* 1984;310:142–148.
36. Tucker A, McMurtry IF, Alexander AF, Reeves JT, Grover RF. Lung mast cell density and distribution in chronically hypoxic animals *J Appl Physiol* 1977;42:174–178.
37. Kay JM, Waymire JC, Grover RF. Lung mast cell hyperplasia and pulmonary histamine-forming capacity in hypoxic rats. *Am J Physiol* 1974; 226:178–184.
38. Mitani Y, Ueda M, Maruyama K, Shimpo H, Kojima A, Matsumura M, Aoki K, Sakurai M. Mast cell chymase in pulmonary hypertension. *Thorax* 1999;54:88–90.
39. Heath D, Yacoub M. Lung mast cells in plexogenic pulmonary arteriopathy. *J Clin Pathol* 1991;44:1003–1006.
40. Heath D. Mast cells in the human lung at high altitude. *Int J Biometeorol* 1992;36:210–213.
41. Karulin AY, Hesse MD, Yip HC, Lehmann PV. Indirect IL-4 pathway in type 1 immunity. *J Immunol* 2002;168:545–553.
42. Galli SJ, Nakae S, Tsai M. Mast cells in the development of adaptive immune responses. *Nat Immunol* 2005;6:135–142.
43. Benoist C, Mathis D. Mast cells in autoimmune disease. *Nature* 2002;420: 875–878.
44. Lee DM, Friend DS, Gurish MF, Benoist C, Mathis D, Brenner MB. Mast cells: a cellular link between autoantibodies and inflammatory arthritis. *Science* 2002;297:1689–1692.
45. Valchanov KP, Proctor GB, Hartley RH, Paterson KL, Shori DK. Enzyme histochemistry of rat mast cell tryptase. *Histochem J* 1998; 30:97–103.
46. Sherman MA. The role of STAT6 in mast cell IL-4 production. *Immunol Rev* 2001;179:48–56.
47. Erb KJ, Ruger B, von Brevern M, Ryffel B, Schimpl A, Rivett K. Constitutive expression of interleukin (IL)-4 in vivo causes autoimmune-type disorders in mice. *J Exp Med* 1997;185:329–339.
48. Chatila TA. Role of regulatory T cells in human diseases. *J Allergy Clin Immunol* 2005;116:949–959. [Quiz, 960.]
49. Nicolls MR, Taraseviciene-Stewart L, Rai PR, Badesch DB, Voelkel NF. Autoimmunity and pulmonary hypertension: a perspective. *Eur Respir J* 2005;26:1110–1118.
50. Lan RY, Ansari AA, Lian ZX, Gershwin ME. Regulatory T cells: development, function and role in autoimmunity. *Autoimmun Rev* 2005;4: 351–363.
51. Perros F, Dorfmuller P, Souza R, Durand-Gasselin I, Mussot S, Mazmanian M, Herve P, Emilie D, Simonneau G, Humbert M. Dendritic cell recruitment in lesions of human and experimental pulmonary hypertension. *Eur Respir J* 2007;29:462–468.
52. Hoeper MM. Pulmonary hypertension in collagen vascular disease. *Eur Respir J* 2002;19:571–576.
53. Jara LJ, Medina G, Vera-Lastra O, Amigo MC. Accelerated atherosclerosis, immune response and autoimmune rheumatic diseases. *Autoimmun Rev* 2006;5:195–201.