Severe Pulmonary Arterial Hypertension Induced by SU5416 and Ovalbumin Immunization

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The combination of chronic hypoxia and treatment of rats with the vascular endothelial growth factor (VEGF) receptor blocker, SU5416, induces pulmonary angio-obliteration, resulting in severe pulmonary arterial hypertension (PAH). Inflammation is thought to contribute to the pathology of PAH. Allergic inflammation caused by ovalbumin (OVA) immunization causes muscularization of pulmonary arteries, but not severe PAH. Whether disturbance of the immune system and allergic inflammation in the setting of lung endothelial cell apoptosis causes PAH is unknown. We investigated the effects of OVA-allergic inflammation on the development of PAH initiated by VEGF blockade-induced lung endothelial cell apoptosis. OVA-immunized rats were treated with SU5416 to induce pulmonary vascular endothelial cell apoptosis. The combination of OVA and SU5416 treatment resulted in severe angio-obliterative PAH, accompanied by increased IL-6 expression in the lungs. c-Kit⁺ and Sca-1⁺ cells were found in and around the lung vascular lesions. Pan-caspase inhibitor, dexamethasone treatment, and depletion of B-lymphocytes using an anti-CD20 antibody suppressed this remodeling. OVA immunization also increased lung tissue hypoxia-induced factor-1 α and VEGF expression. Our results also suggest that the increased expression of hypoxia-induced factor-1 α and IL-6 induced by the allergic lung inflammation may be a component of the pathogenesis of PAH.

Keywords: hypoxia-induced factor-1 α ; IL-6; precursor cell

The presence of inflammatory cells in and around the vascular lesions, which develop in the lungs of patients with various forms of severe pulmonary hypertension (PH), including idiopathic pulmonary arterial hypertension (PAH), have been documented by the early pioneers of pulmonary vascular pathology, C. A. Wagenvoort and D. Heath (1, 2), and a contributing role of the immune system in the pathogenesis of various forms of PAH has been postulated, in part, because cells of the immune system have been demonstrated in and around the complex angioproliferative plexiform lesions (3, 4). At present, the recognized associations between autoimmune disorders and severe PAH (5) have not been mechanistically explored. Pulmonary vascular inflammation remains a probable cause in the pathogenesis of severe PAH as

CLINICAL RELEVANCE

Severe pulmonary arterial hypertension (PAH) induced by SU5416 and chronic hypoxia is characterized by small pulmonary vessel lumen obliteration due to exuberant growth of endothelial cells. T helper (Th) type 2 immune system activation due to ovalbumin allergic inflammation results in lung vessel muscularization, but not endothelial cell growth, angio-obliteration, or pulmonary hypertension (PH). However, animal models and mechanistic studies of immune disorder-associated PH are lacking. Here, we introduce a novel rat model of severe angio-obliterative PH induced by the combination of vascular endothelial growth factor (VEGF) receptor tyrosine kinase inhibition and (ovalbumin) Th2 immune system activation. In this model, severe PAH depends on the initial endothelial cell apoptosis, and is also associated with increased expression of hypoxia-induced factor-1 α , VEGF, and IL-6, and participation of B lymphocytes.

a “second hit” in patients with collagen vascular diseases and interstitial lung diseases (6). The second hit may lead to complex vascular lesions and irreversible obstruction of pulmonary arteries, but whether in the lung inflammatory and immune cells are cause or consequence of the severe PAH remains unknown. Severe angio-obliterative PAH is also a serious complication of multiple autoimmune disorders (5, 7). The pathobiological characteristics shared by idiopathic PH and by autoimmune disease-associated PAH have recently been reviewed (5). It is also known that PH is more severe in immune-insufficient animals. For example, the pulmonary vascular disease is worse in the inflammatory monocrotaline model, if the rats are athymic (i.e., T-lymphocyte deficient) (8), and, whereas lung endothelial cell apoptosis induced by vascular endothelial growth factor (VEGF) receptor blockage causes emphysema and mild PAH in wild-type rats, VEGF receptor inhibition causes severe angioproliferative PAH in immune-insufficient athymic rats (9, 10).

A recent study by Daley and colleagues (11) demonstrated impressive pulmonary vascular muscularization in mice in response to *Aspergillus* or ovalbumin (OVA) antigen immunization, indicating that lung vascular remodeling is under the control of the immune system. In this study, the authors showed that, in mice, the triggering of a T helper (Th) type 2-skewed immune response resulted in lung vessel muscularization, but not endothelial cell growth, angio-obliteration, or PH. Witzernath and colleagues (12) demonstrated increased pulmonary vascular reactivity in isolated perfused mouse lungs after OVA treatment, and other investigator groups (13, 14) have also shown muscularized pulmonary arterioles after OVA immunization. However, a model of severe PAH and right heart failure after antigen

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challenge has not been reported. Because, in wild-type rats, the combination of VEGF receptor (VEGF-R) blockade, which causes initial apoptosis of lung endothelial cells (the “first hit” or “initiator”), and chronic hypoxic exposure (the second hit or “promoter”) generates severe angio-obliterative PAH and right heart failure (15–17), we postulated that, in rats, chronic hypoxia, as a second hit, could be replaced by inflammation and activation of the immune system. This postulate is based on the concept of “hypoxic inflammation” as the root cause of angio-obliterative PAH; for short, in the setting of lung endothelial cell damage, either hypoxia or inflammation/immune system imbalance are required for angio-obliterative PAH to occur. Here, we replaced chronic hypoxia with the OVA immunization strategy used previously in the study by Daley and colleagues (11). One rationale for substituting OVA immunization for chronic hypoxia was that hypoxia and inflammation have in common the activation of the critically important transcription factor, hypoxia-induced factor (HIF)-1 α (18). Another rationale was provided by published studies that have linked the inflammatory and angiogenic cytokine, IL-6, with the development of pulmonary vascular diseases (19–22). We thus hypothesized that lung endothelial cell apoptosis—induced by VEGF receptor blockade—combined with the OVA-induced immune system activation would cause severe PAH.

A consensus statement resulting from a recent National Heart, Lung, and Blood Institute–sponsored workshop pointed out the need for the development of animal models “that . . . closely mimic the hemodynamics and the pathophysiology and occlusive neointimal and preform pulmonary arteriopathy of human PAH” (23). Here, we report a model of immune response–associated severe PAH and show that the pathobiology of severe PAH in this model depends on the initial endothelial cell apoptosis and the participation of B lymphocytes.

MATERIALS AND METHODS

Animals

The protocol was approved by the Institutional Animal Care and Use Committee of the Virginia Commonwealth University. As shown in Figure 1, male Sprague-Dawley rats (4 wk old) were sensitized with 1 mg of OVA complexed with Imject Alum (Thermo Fisher Scientific, Pittsburgh, PA) on Days 1 and 7 intraperitoneally. On Day 14 after the initial sensitization, the rats were challenged for 30 minutes with an aerosol of 1% OVA in PBS, two times per week for 4 weeks. SU5416 (Su; 20 mg/kg body weight [BW]) was injected subcutaneously once a week on the day after OVA inhalation. In one series of experiments, dexamethasone (2 mg/kg BW) was injected intraperitoneally before OVA inhalation once a week, and Z-Asp (4 mg/kg BW) was injected intraperitoneally once a day, 5 days/week. Both control and Su-treated groups received OVA sensitization and PBS inhalation. As shown in Figure 1B, other groups of male Sprague-Dawley rats (4 wk old) that were sensitized with OVA/Imject Alum received three injections of normal mouse IgG or mouse monoclonal anti-CD20 antibody (generously provided by Genentech, San Francisco, CA) at 7 mg/kg on Days 14, 17, and 21. After the first injection of control IgG or anti-CD20 antibody, the rats received OVA inhalation and Su treatment as above. Hemodynamic measurements were performed on Day 44.

After hemodynamic measurements, the thoracic cavities were opened by midline incision, and blood was obtained by cardiac puncture. The right lung was removed and placed into RNAlater (Ambion, Grand Island, NY) or frozen in liquid nitrogen; the left lung was inflated with 0.5% low-melting agarose at a constant pressure of 25 cm H₂O, fixed in 10% formalin for 48 hours, and used for morphometry and immunohistochemical analysis. Right ventricular (RV) hypertrophy was measured as a ratio of RV weight to left ventricular (LV) plus septal (S) weight (RV/LV + S).

Hemodynamic Measurements

Hemodynamic measurements were made using a 4.5-mm conductance catheter (Millar Instruments, Houston, TX) and the Powerlab data

Figure 1. Schematic representation of the animal protocol of a combination of SU5416 (Su) treatment with ovalbumin (OVA) immunization. (A) Male Sprague-Dawley rats were sensitized with 1 mg of OVA on Days 1 and 7 intraperitoneally (solid arrowheads). On Day 14 after the initial sensitization, the rats were challenged for 30 minutes with an aerosol of 1% OVA in PBS, two times per week for 4 weeks (arrows). Su (20 mg/kg body weight [BW]) was injected subcutaneously once a week on the day after OVA inhalation (open arrowheads). In one series of experiments, dexamethasone (2 mg/kg BW) was injected intraperitoneally before OVA inhalation once a week (open arrows), and Z-Asp (4 mg/kg BW) was injected intraperitoneally once a day, 5 days/week (gray arrows). Both control and Su-treated groups received OVA sensitization and PBS inhalation. (B) Other groups of male Sprague-Dawley rats that were sensitized with OVA/Imject Alum, received three injections of normal mouse IgG or mouse monoclonal anti-CD20 antibody at 7 mg/kg on Days 14, 17, and 21 (open arrows). After the first injection of control IgG or anti-CD20 antibody, the rats received OVA inhalation and Su treatment as above. Hemodynamic measurements were performed on Day 44.

acquisition system (AD Instruments, Colorado Springs, CO). The rats were anesthetized with isoflurane, intubated, and placed in a supine position and ventilated. After a median sternotomy, the RV outflow tract was punctured with a 23-G needle and the catheter was introduced into the RV cavity and pulmonary artery through the incision; placement was verified via the pressure waveform. The RV pressure and pulmonary arterial pressures were continuously monitored and recorded using Chart 5 pro software (AD Instruments), as previously reported (17).

Please see the online supplement for histological evaluation and real-time RT-PCR and Western blot analyses.

Statistical Analysis

Results are expressed as means ($\pm$ SE). Statistical analysis was performed using ANOVA with Bonferroni corrections for multiple comparisons. Correlations were analyzed by the Pearson correlation coefficient. Comparisons were considered statistically significant at *P* less than 0.05.

RESULTS

The Combination of VEGF-R Blockade with OVA Immunization Causes PH, which Is Prevented by Treatment with Dexamethasone, a Pan-Caspase Inhibitor and an Anti-B Lymphocyte Antibody

The OVA immunization strategy employed in animals treated with four (once a week) injections of the VEGF receptor/tyrosine kinase inhibitor, Su, as shown in Figure 1A, resulted in the development of severe PH and RV hypertrophy when compared with animals

treated with OVA immunization alone or with Su alone. In contrast, when Su-treated and OVA-immunized (OVA-Su) rats were also, from the start of the protocol, treated with either dexamethasone or the pan-caspase inhibitor, Z-Asp-CH₂-DCB (Z-Asp), or a B lymphocyte-depleting antibody (mouse anti-CD20 antibody), the increase in the mean pulmonary arterial pressure was significantly reduced. These three prevention strategies also reduced the increase in RV hypertrophy, although the dexamethasone treatment prevented the development of RV hypertrophy more effectively than the Z-Asp treatment. Taken together, our results show that the combination of Su treatment and OVA immunization causes severe, and even fatal, PH, which can be significantly prevented by steroid treatment, prevention of apoptosis, and elimination of B lymphocytes (Figures 2A and 2B).

Combined OVA-Su Treatment Causes Angio-Obliterative Pulmonary Lesions

Figure 2C shows representative lung tissue samples that illustrate that OVA immunization alone caused some degree of perivascular

accumulation of inflammatory cells (some of these cells are eosinophils; Figure 2C, *arrows*), but, importantly, no lumen obliteration. However, the combined OVA-Su treatment causes highly abnormal lung artery remodeling, which can be characterized by thickening of the media—which occasionally is undergoing fibrinoid necrosis (pink, faded out smooth muscle cells; data not shown)—lumen obliteration, and perivascular cell infiltrates. Dexamethasone treatment prevented all of these manifestations, and both Z-Asp treatment and B-lymphocyte depletion protected against the development of lumen-obliterating lesions (Figure 2D). These results indicate that, in the OVA-Su model, inhibition of inflammation and of apoptosis prevent pulmonary vascular lumen obstructions. In addition, the data strongly indicate that B lymphocytes are contributing to the formation of the vascular lesions.

To demonstrate that the vascular lesions are also composed of proliferating cells, we stained lung tissue sections with an antibody directed against proliferating cell nuclear antigen, and found proliferating cells in and around the vascular lesions in the lungs from OVA-Su-treated animals. The accumulation of these proliferating cells was prevented by dexamethasone

Figure 2. The effect of Su treatment and OVA immunization on (A) mean pulmonary artery pressure (mPAP), (B) RV hypertrophy, and (C) small pulmonary arteries. The combination of OVA immunization and Su treatment resulted in an increase of the mPAP. The mPAP was significantly suppressed by dexamethasone (Dxa), the pan-caspase inhibitor, Z-Asp-CH₂-DCB (Z-Asp), and anti-B-lymphocyte antibody treatment (CD20ab) (A). RV hypertrophy was increased by the combination of OVA immunization and Su treatment, and suppressed by Dxa (OVA + Su + Dxa) and Z-Asp (OVA + Su + Z-Asp). B-lymphocyte depletion by CD20ab treatment (OVA + Su + CD20ab) significantly prevented RV hypertrophy when compared with normal mouse IgG (OVA + Su + IgG) (B). Representative photographs of small pulmonary arteries from control, OVA-immunized (OVA), Su-treated (Su), and OVA and Su-treated (OVA + Su) rats. OVA immunization alone caused some degree of perivascular accumulation of eosinophils (*arrows*) without lumen obliteration. The combined OVA-Su treatment generated severe lumen obliteration and perivascular cell infiltrates (C). Dxa and Z-Asp prevented the vascular obliteration and perivascular infiltrations (OVA + Su + Dxa, OVA + Su + Z-Asp). B-lymphocyte depletion (OVA + Su + CD20ab) also prevented the vascular obliteration compared with normal mouse IgG administration (OVA + Su + IgG) (D).

treatment and B-cell depletion (Figure 3A). Western blot data using nuclear protein extracts from these lungs confirm the immunohistochemistry (IHC) data (Figure 3B).

Precursor Cells, Immune Cells, and Immune Complex Formation Are Present in and around the Pulmonary Vascular Lesions in OVA-Su-Treated Animals

To explore the possibility that precursor cells participate in the formation of the vascular lesions, we employed IHC, and identified that c-Kit⁺ cells and Sca-1⁺ cells were present in and around the lesions. Dual staining for c-Kit and von Willebrand factor (vWF) or α -smooth muscle actin demonstrated that the c-Kit⁺ cells are situated in the perivascular niche. Similarly, dual staining for Sca-1 and vWF or smooth muscle actin showed that Sca-1⁺ cells were found in and around the perivascular lesions (Figure 4A). In addition to cells displaying precursor markers, we also identified CD8⁺ and CD20⁺ lymphocytes in the perivascular space of the OVA-Su animals (see Figure E1 in the online supplement).

Rats subjected to the OVA-Su protocol showed, by immune fluorescence microscopy, deposits in lung arteries; these deposits decorate lumen-obliterating endothelial cells when probed with anti-Rat IgG antibodies. Such deposits were absent in the lungs from rats treated with Su alone and the lungs from OVA-Su rats where B lymphocytes had been depleted (Figure 4B).

Lung Tissue IL-6 Is Increased by the Combined OVA-Su Treatment

We next screened for the expression of the genes encoding IL-4, -5, -6, and -13 in the lung tissue samples (Figure E2), and then focused

on the tissue expression of IL-6 protein. We found that IL-6 protein was dramatically expressed in the lungs from OVA-Su-treated rats and in the cells obliterating the lumen of the arterioles (Figure 5A). Of interest, by IHC, we found little to no IL-6 expression in the vessel walls of the patent lung vessels from the dexamethasone treated and B cell-depleted animals. IHC also showed that the IL-6 antibody was decorating endothelial cells. This finding is also documented by dual immune fluorescence staining of vWF- and IL-6-positive cells (Figure E3B). The Western blot data also support the histological data that IL-6 is highly expressed (Figures 5B and 5C), and, remarkably, the amount of expressed IL-6 protein correlated with the mean pulmonary arterial pressure (Figure 5D).

HIF-1 α and VEGF Are Overexpressed in Lungs from OVA- and OVA-Su-Treated Rats

Western blot analysis using lung tissue protein extracts shows that OVA immunization increases the expression of both HIF-1 α and VEGF proteins; this effect is also observed in the animals treated with the combination of OVA-Su. Although dexamethasone and B-cell depletion prevent the increase in HIF-1 α and VEGF protein expression in the OVA-Su-treated animals, caspase inhibition fails to inhibit the increase of lung tissue HIF-1 α protein expression in the OVA-Su-treated rats (Figure 6), likely because of the triggering of the HIF-1 α response by OVA (24).

DISCUSSION

The clinical spectrum of PH disease forms has been organized and categorized by a World Health Organization expert group (25, 26), and PH and pulmonary vascular remodeling occur in

Figure 3. Cell proliferation of small pulmonary arteries and lungs. (A) Representative photographs of immunohistochemical staining of proliferating cell nuclear antigen (PCNA) of small pulmonary arteries. The combined OVA + Su treatment increased PCNA-positive cells in and around the small arteries. Dxa, Z-Asp, and CD20ab treatment decreased the PCNA-positive cells in and around the small pulmonary vessels. (B) Western blot analysis of PCNA in lung nuclear protein extracts. PCNA expression was significantly increased by the combined OVA + Su treatment. The increased PCNA expression was significantly suppressed by Dxa, Z-Asp, and CD20ab treatment. The bar graph shows the ratio of PCNA protein expression relative to that of Lamin B. Data are expressed as mean ($\pm$ SE) ($n = 6$). * $P < 0.05$ versus control; $^{\dagger}P < 0.05$ versus OVA + Su; $^{\#}P < 0.05$ versus OVA + Su + IgG.

Figure 4. Expression of precursor cells and antibody deposition in OVA-Su-treated rat small pulmonary arteries. (A) Dual staining for c-Kit (green) and von Willebrand factor (vWF) (red), and for c-Kit (green) and α -smooth muscle actin (SMA) (red), show that the c-Kit⁺ cells are identified in and around the small angio-obliterating lesions. Dual staining for Sca-1 (green) and vWF (red), and for Sca-1 (green) and α -SMA (red), shows that the Sca-1⁺ cells are identified in and around the small angio-obliterating lesions. The blue color shows 4,6-diamidino-2-phenylindole (DAPI) staining. (B) Representative photographs of dual staining for rat-IgG (red) and vWF (green) shows that

there is no detectable antibody deposition in pulmonary arteries from rats treated with Su alone, whereas OVA + Su-treated animals show abundant IgG-positive cells in the angio-obliterating vessels. B-lymphocyte depletion (OVA + Su + CD20ab) suppressed the expression of IgG-positive cells in and around the small vessels compared with mouse normal IgG administration (OVA + Su + IgG). The blue color shows DAPI staining.

idiopathic forms or associated with a large number of conditions or diseases. Chronic inflammation is a component of many lung diseases, such as chronic obstructive pulmonary disease, interstitial pulmonary fibrosis, sarcoidosis, scleroderma, and schistosomiasis—all of which can be associated with lung vessel remodeling and PH. Human schistosomiasis is known as a common cause of PAH, and in schistosomiasis the pulmonary vascular disease is likely driven by the host response to parasite antigens. The initial reaction to schistosoma infection is a Th1 response due to the antigens released by schistosomules during acute infection. After the initial response, the immune reaction becomes chronic and the immune response is dominated by Th2 activities (27). A likely too simplistic, but perhaps useful distinction, of the morphology of the vessels in the lungs from patients with severe PAH and vessels in the lungs from animals with severe PAH is the distinction between patent muscularized arteries and arterioles that are partially or completely obliterated by cells (16). Furthermore, it appears that, experimentally in animal models, the development of angio-obliterative PAH requires more than “one hit”; for example, chronic hypoxia or monocrotaline alone do not cause vessel obliteration, whereas vessel occlusion develops when monocrotaline is combined with pneumonectomy (high blood flow through the remaining lung) (28), or when chronic hypoxia is combined with the lung endothelial cell apoptosis-inducing VEGF receptor blocker (Su) treatment (15–17). Here, we demonstrate that OVA immunization, which is associated with increased tissue concentrations of HIF-1 α and VEGF proteins, can replace chronic hypoxia as the second hit: the combination of VEGF-R blockade and OVA treatment results in the development of severe, B lymphocyte-dependent, angio-obliterative PAH. These findings are in clear contrast to the nonobliterating muscularization in OVA-immunized mice (11), which do not develop severe PAH.

Whereas Su treatment *alone* generates chronic inhibition of the VEGF-R1 and VEGF-R2 receptor tyrosine kinases and induces pulmonary vascular endothelial cell apoptosis (29, 30), emphysema, and mild PH (15), OVA immunization *alone* causes, in mice, bronchial epithelial hyperplasia and eosinophilic airway hyperreactivity, but not severe PH (11). In the new OVA-Su model

of severe angioproliferative PAH described here, inflammation clearly plays a mechanistically important role, as do B lymphocytes. As previously shown in the Su/chronic hypoxia model (15, 17), lung cell apoptosis prevention in this OVA-Su model (Figure 2) also inhibits the development of angio-obliteration and severe PAH. This supports the concept that endothelial cell apoptosis is one of the essential mechanistic components required for the development of angioproliferative PAH (15, 31). We hypothesize that the OVA-triggered Th2 immune response induces the production of mediators of pulmonary vascular cell proliferation, such as HIF-1 α , VEGF, and IL-13 (11, 32, 33).

Whereas, so far, we have emphasized the two components of the model that we postulate to be of pathobiological importance (VEGF-R blockade-induced apoptosis and OVA-induced inflammation), we also must consider that, in this rat model, the VEGF receptor blockade may have modified the OVA allergen response—and that this modification could be important for the development of the angio-obliterative PAH. There are several reports in the literature that point out that VEGF plays a role in allergic inflammation (34). Both B and T lymphocytes express VEGF receptors, both receptors are capable of inhibiting dendritic cell function, and VEGF-R blockade also compromises lymphocyte functions (35). Indeed, our data (*see the online supplement*) confirm the findings of Lee and colleagues (34), who showed, in mice sensitized and challenged with OVA, that VEGF-R blockade decreased the OVA-stimulated production of IL-4 and -13. FOXP3⁺ regulatory T cells express VEGF-R2, and VEGF-R2 blockade can suppress proliferation of regulatory T cells. Li and colleagues (36) showed that VEGF receptor blockade reduced the number of regulatory T cells in tumor-bearing mice, and Watanabe and colleagues (37) demonstrated in mice that VEGF-R blockade suppressed contact hypersensitivity in a mouse model. Thus, it is possible that Su in this new model of PAH has modified the OVA-induced immune response, perhaps by affecting regulatory T cells.

To begin to dissect the mechanisms that, in this new OVA-Su model, lead to angio-obliteration and PAH, we selected three different prevention strategies. We found that a pan-caspase inhibitor prevented the development of PAH, thus strongly

Figure 5. IL-6 expression in OVA-Su-treated rat lungs. (A) Representative photographs of immunohistochemical staining of IL-6 of small pulmonary arteries. The combination OVA + Su treatment increased the number of IL-6-positive cells in and around the small vessels. Dxa, Z-Asp, and CD20ab prevented the increase of the IL-6-positive cells in and around the small pulmonary arteries. (B) Western blot analysis of IL-6 in whole-lung protein extracts. IL-6 expression was significantly increased by the combined OVA + Su treatment. The increased IL-6 expression was suppressed by Dxa, Z-asp, and CD20ab treatment. (C) The bar graph shows the ratio of IL-6 protein expression relative to that of β-actin. Data are expressed as mean ($\pm$ SE) ($n = 6$). * $P < 0.05$ versus control; † $P < 0.05$ versus OVA + Su; # $P < 0.05$ versus OVA + Su + IgG. (D) Correlation analysis between mPAP and IL-6 expression, as measured by Western blot. A strong correlation ($R^2=0.6769$) was observed between mPAP and IL-6 protein expression.

supporting the important role of apoptosis in this model (15). We chose as our second prevention strategy high-dose (cytotoxic) corticosteroid administration, as a proof of principle strategy,

and show here that dexamethasone prevents inflammation, vascular obliteration, IL-6 production, and increased HIF-1 α and VEGF lung tissue expression (Figures 2, 5, and 6). It is possible

Figure 6. Hypoxia-induced factor (HIF)-1 α and vascular endothelial growth factor (VEGF) protein expression in OVA-Su-treated rat lungs; Western blot analysis of HIF-1 α and VEGF of whole-lung protein extracts. HIF-1 α and VEGF expression were significantly increased by OVA immunization. The increased HIF-1 α and VEGF expression was significantly suppressed by Dxa, Z-Asp, and CD20ab treatment. The bar graph shows the ratio of HIF-1 α and VEGF protein expression relative to that of Lamin A/C or β-actin. Data are expressed as mean ($\pm$ SE) ($n = 6$). * $P < 0.05$ versus control; † $P < 0.05$ versus OVA + Su; # $P < 0.05$ versus OVA + Su + IgG.

that dexamethasone in this model acts as a lymphocyte toxic agent, and, if so, the suppression of the OVA-induced immune response and the suppression of the pulmonary vascular disease development by dexamethasone may not be surprising. Dexamethasone affects the action of IL-6, and a detailed analysis of the repression of IL-6 gene expression by dexamethasone has been provided by Ray and colleagues (38). Of interest, in patients with the POEMS (polyneuropathy, organomegaly, endocrinopathy, monoclonal gammopathy, and skin changes) syndrome, it has been reported that PAH was observed in 25–48% of the patients (39, 40). POEMS syndrome is known as a rare disease, characterized by a monoclonal plasma cell disorder associated with high serum inflammatory cytokines (40). Although the precise mechanisms of PAH in patients with POEMS syndrome remains uncertain, the pathological changes of pulmonary arteries are, to some degree, similar to the changes seen in the patients with idiopathic PAH, including medial hypertrophy and plexiform lesions (39). Interestingly, high-dose steroid therapy is sometimes effective in treating POEMS syndrome-associated PAH (41). It is possible that dexamethasone and B-cell antibody treatment in our model disarm the second hit of the OVA-induced immune response. Our results showing suppressive effects of pulmonary vascular remodeling by high-dose steroid and B-cell depletion may indicate that modification of B cell-associated inflammation and/or inhibitors of Th2 signaling could be a future treatment for PAH in patients having immune disorders, such as POEMS syndrome.

Our third prevention strategy is based on B-lymphocyte depletion, and our data show that OVA-Su-treated and B cell-depleted animals are remarkably protected against the development of angio-obliteration and PAH, although some perivascular infiltrates remained and the pulmonary artery pressure was somewhat higher than in control animals. Because of this result, we wondered whether, in the non-B cell-depleted animals, OVA-Su treatment had produced anti-endothelial antibodies and formation of immune complexes on the endothelium of the small pulmonary arteries. This was indeed observed, whereas immune complexes were not found in the lungs from B cell-depleted OVA-Su animals (Figure 4B). We speculate that the formation of anti-endothelial cell antibodies was a consequence of the Su-induced damage to the lung endothelial cells (29). Whether these antibodies are of pathobiological importance in the OVA-Su model is unknown.

Whereas Su-induced VEGF-R blockade alone induces lung endothelial cell apoptosis, some elevation of lung tissue IL-6 (Figure 5), and increased proliferating cell nuclear antigen (Figure 3) expression, importantly VEGF-R blockade alone does not increase lung tissue HIF-1 α and VEGF protein expression (Figure 6), and is not associated with the appearance of precursor cells in the perivascular niche (Figure 4A). In contrast, OVA immunization alone does cause increased expression of lung tissue HIF-1 α and VEGF, as previously reported (24, 32), but not lung endothelial cell apoptosis (Figure E4), suggesting that increased expression of HIF-1 α , and of its transcriptional target, VEGF, is insufficient to cause angio-obliterative PAH. However, the combination of VEGF-R blockade and OVA immunization adds lung endothelial cell apoptosis, and perhaps VEGF-R blockade-related changes of the immune response to the strong inflammatory response generated by the repeated OVA challenges. Whether IL-6 in the setting of VEGF-R blockade is the only, or one of several, important angiogenesis factors generated by OVA-Su is unclear. The endothelial cell IL-6 expression of the lumen-filling cells leads us to speculate that endothelial cell IL-6 plays a role in the pathogenesis of this model. Such a concept is strongly supported by several publications, which emphasize the pathogenetically important role of

IL-6 in PAH (19, 22, 42). The large increase in the expression of IL-6 in the lungs from OVA-Su rats, which relates to the severity of PAH in this model (Figure 5), could be explained by activation of a IL-6/STAT3/HIF-1 α signaling axis, as recently described by Nilsson and colleagues (43), or involvement of NF- κ B and HIF-1 α (44), which may act synergistically during “hypoxic inflammation” (45) and OVA inflammation. Increasingly, the role of endothelial cell progenitor cells in PAH is being recognized (46–48), and it is possible that IL-6 in this OVA-Su model also play a role in the attraction of precursor cells to the lung perivascular niche (49).

Whether this new model of immune system activation and inflammation-related PAH is a model of one distinct or several human forms of angioproliferative and autoimmune diseases-associated PAH, or whether this model represents various aspects of inflammatory mechanisms participating in the pathobiology of all forms of PAH, or in particular in PAH in patients with Th2 type-skewed schistosoma infection, is presently unclear. This clarification will require a number of different approaches and sets of experiments. We propose that pathobiologically important elements that are shared between human angio-obliterative PAH and this OVA-Su model are endothelial apoptosis (50), vascular and perivascular accumulation of inflammatory cells and cells of the immune system, and a dysregulated immune response. One manifestation of dysregulated immunity is the elevation of plasma levels of IL-1 β and -6 in patients with severe PAH; high circulating levels of IL-6 have recently been associated with a poor prognosis (19).

In conclusion, this new OVA-Su model of severe PAH depends on endothelial cell apoptosis, inflammation, and actions of B lymphocytes. Importantly, PAH in this model—that also may depend on a VEGF receptor blockade-related modification of the typical immune response to the OVA antigen—is preventable. Two unexplored questions are whether the established PAH in this model is IL-6 dependent and can be treated, when established. Future studies will investigate whether, in this OVA-Su model, VEGF-R blockade suppresses regulatory T cell function and whether B-lymphocyte depletion protects against angio-obliteration because of the elimination of anti-endothelial antibody production or by other B cell-dependent mechanisms.

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