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IL-4 Is Proangiogenic in the Lung under Hypoxic Conditions

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Abstract

IL-4-mediated proangiogenic and proinflammatory vascular responses have been implicated in the pathogenesis of chronic lung diseases such as asthma. Although it is well known that hypoxia induces pulmonary angiogenesis and vascular alterations, the underlying mechanism of IL-4 on the pulmonary vasculature under hypoxic conditions remains unknown. In this context, we designed the present study to determine the functional importance of IL-4 for pulmonary angiogenesis under hypoxic conditions using IL-4 knockout (KO) animals. Our results show that hypoxia significantly increased IL-4Rα expression in wild-type (WT) control lungs. Even though hypoxia significantly up-regulated vascular endothelial growth factor (VEGF) receptor expression in the lungs of both genotypes, hypoxia-induced VEGF, VCAM-1, HIF-1α, and ERK phosphorylation were significantly diminished in IL-4 KO lungs as compared with WT control lungs. In addition, hypoxia-induced pulmonary angiogenesis and proliferating activities in the airway and pulmonary artery were significantly suppressed in IL-4 KO lungs as compared with WT control lungs. We also isolated primary lung fibroblasts from these genotypes and stimulated these cells with hypoxia. Hypoxia-induced VEGF production was significantly suppressed in lung fibroblasts from IL-4 KO mice. These in vitro results are in accordance with the in vivo data. Furthermore, we observed a significant increase of hypoxia-induced pulmonary angiogenesis in STAT6 KO mice similar to that in WT controls. In conclusion, IL-4 has proangiogenic properties in the lung under hypoxic conditions via the VEGF pathway, and this is independent of the STAT6 pathway.

The incidence of chronic inflammatory lung diseases is increasing worldwide (1). The pathogenesis of such diseases, including pulmonary hypertension, chronic obstructive pulmonary disease, fibrosis, lung cancer, and asthma are characterized by extensive modifications of the extracellular matrix and by insufficient local oxygen supply. Hypoxia has been identified as an important regulator of cell proliferation, extracellular matrix synthesis, and organ function and is assumed to result from increased tissue mass

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or insufficient vascularization (2). Locally restricted hypoxia increases the secretion of proinflammatory cytokines and growth factors via hypoxia-sensitive transcription factors and feedback in hypoxia-related vascular and tissue remodeling (3). It has also been suggested that hypoxia-sensitive proteins are increased in the mouse model of allergic airway inflammation (4).

IL-4 was initially identified as B cell stimulatory factor 1 (BSF1) based on its important role in the stimulation of B cell IgE production (5). It has subsequently been demonstrated to be a pleiotropic cytokine that plays a key role in Th2 inflammatory responses and, in select circumstances, Th2-induced tissue remodeling (5–13). IL-4 is also known to stimulate fibroblast proliferation, myofibroblast differentiation, and collagen and proteoglycan production in experimental systems (11, 13, 14, 15). Fibroblasts at sites of Th2 inflammation express IL-4R components (16). IL-4 is present at high levels in tissues of patients with chronic inflammatory diseases, including atherosclerotic lesions (17–19). Evidence indicates that IL-4 may play a role in vascular endothelial function through the induction of inflammatory responses, such as up-regulation of VCAM-1 (20–22), MCP-1 (23), and vascular endothelial growth factor (VEGF)[3] (24, 25), which is one of the most potent multifunctional angiogenic factors.

The biological actions of IL-4 are mediated by its binding to its receptor, IL-4R (molecular mass, 139 kDa in the mouse). IL-4R is composed of IL-4Rα and γc subunits or IL-4α and IL-13Rα subunits (26). Binding IL-4 to its receptor results in activation of several intracellular signaling pathways, including the PI3K (27) and the JAK/STAT pathways. Signaling through the JAK/STAT pathway occurs by a tyrosine phosphorylation cascade (28) whereby Jak tyrosine kinases activated by tyrosine phosphorylation in turn phosphorylate the STAT family of transcription factors, leading to STAT dimerization and ultimately, translocation into the nucleus. IL-4 stimulation results in the activation of STAT6 in most cell types. There is evidence that IL-4 stimulates the chemokine MCP-1 via the STAT6 pathway and serves as a potent mitogenic factor in human endothelial cells (ECs) (25, 29, 30).

IL-4 may also be considered as a pro-oxidative cytokine that can increase the oxidative potential of target cells (21). A number of in vitro cellular biology data are consistent with the proatherogenic effect of IL-4. It is reasonable to hypothesize that a similar scenario may apply to the lung. It has been suggested that bronchial biopsies from atopic asthmatics compared with atopic control subjects exhibit increased expression of IL-4α mRNA and protein in the epithelium, subepithelium, and EC layer (31). There is evidence that Th2 cytokines (IL-4 and IL-13) induce VEGF from human airway smooth muscle cells (SMCs) (24, 25).

VEGF appears to play a crucial role in the hypoxia-related mitogenic response in various organs, including the lung. In addition to hypoxia-induced expression of VEGF, various investigators have also documented in vivo increased expression of its receptors in pathologic conditions characterized by hypoxia (32, 33). VEGF is expressed in several parts of the lung and the pleura. It has been shown that changes in VEGF expression play a significant role in the pathophysiology of some of the most common respiratory disorders.

However, the exact role of VEGF in the lung is not yet clear and is controversial, suggesting either a protective or a harmful role.

Moreover, human airway SMCs in culture produce VEGF constitutively and in response to stimulation with a variety of inflammatory mediators, including IL-4 (34). VEGF also enhances expression of the Th2 cytokines IL-4 and IL-13 in the lung (35), suggesting a positive feedback loop with VEGF enhancing Th2 sensitization and inflammation and IL-4 subsequently enhancing VEGF production.

IL-4 has been documented to selectively increase human bronchial tissue expression of VCAM-1 (36). VCAM-1 is an inducible cell surface glycoprotein of several cell types and plays an important role in a number of inflammatory and angiogenic responses. Several cell types, including lung fibroblasts, have been shown to express VCAM-1 in response to IL-4 (37). Then, the subsequent interaction between VCAM-1 on the endothelium and α_4 integrins (which are ligands for VCAM-1) on leukocytes are responsible, at least in part, for the selective recruitment of leukocyte subsets. VCAM-1 is also known to be markedly up-regulated on the human bronchial vascular endothelium of subjects with asthma who have air flow limitation when compared with control subjects (38).

These results are in favor of a proinflammatory and mitogenic effect of IL-4, not only in the systemic cardiac vasculature but also in the pulmonary vasculature. In contrast, in addition to these angiogenic properties, IL-4 paradoxically has immunosuppressive, antitumor, and antiinflammatory activities under certain conditions (39–41). However, whether IL-4 has mitogenic action in the lung resident cells under hypoxic conditions remains unknown. The effect of hypoxia or IL-4 on pulmonary vasculature and tissue remodeling has been studied, but less attention has been paid so far to the mechanism of IL-4 under hypoxia conditions or on the interaction of the cell types involved.

In this context, we designed the present study to determine the functional importance of IL-4 for pulmonary angiogenesis under hypoxic conditions using IL-4 knockout (KO) animals to test the following hypotheses: 1) that hypoxia stimulates IL-4R expression in the lung; 2) that hypoxia-induced pulmonary angiogenesis is dependent on IL-4 signaling; 3) that the proangiogenic effect of IL-4 in the lung under hypoxic conditions is mediated by the STAT6 pathway; and 4) that IL-4 plays a role in proliferative activities in the lung during prolonged exposure to hypoxia. We now show that hypoxia stimulates IL-4Rα expression and that IL-4 is responsible for hypoxia-induced VEGF, VCAM-1, and hypoxiainducible factor (HIF)-1α expression in the lung. These signaling responses are mediated by a p42/p44 MEK/ERK-dependent mechanism, but are not Akt pathway dependent. We further show that IL-4 possesses mitogenic properties in hypoxia-induced pulmonary angiogenesis and proliferative activities in the lung.

Materials and Methods

Animals and low oxygen exposure

Wild-type (WT) control, IL-4 KO, and STAT6 KO mice (all with a C57/BL6 background) were purchased from The Jackson Laboratory. Recent study has suggested that genetic

factors regulate the tissue responses induced by IL-4 in different inbred murine strains (42). Thus, we used only C57BL/6 background mice (WT, IL-4 KO, and STAT6 KO), not BALB/c mice, because pulmonary inflammation and remodeling in response to IL-4 are significantly greater in C57BL/6 mice. Housing and procedures involving experimental animals were approved by the Animal Care and Use Committee of Johns Hopkins University (Baltimore, MD). In vivo hypoxia studies were performed as previously described (43). Briefly, animals were placed in a Plexiglas chamber maintained at 10% O_2 (hypoxic group; $n = 6$) or in a chamber open to room air (normoxic group; $n = 6$) for specific times with a 12:12-h light-dark cycle. Hypoxia was maintained using a Pro:ox model 350 unit (Reming Bioinstruments) that controlled the fractional concentration of O_2 in inspired gas by solenoid-controlled infusion of N_2 (Roberts Oxygen) balanced against an inward leak of air through holes in the chamber. Carbon dioxide and ammonia vapors were removed by filtration through soda lime and charcoal. All in vivo experiments were repeated at least three times.

Primary fibroblast cell culture

We used a well-established method to isolate lung fibroblasts from mice (44, 45). After filtration, released cells were centrifuged, washed, and cultured in DMEM supplemented with 10% heat-inactivated plasma-derived FBS, 100 U/ml penicillin, 100 μg/ml streptomycin, 0.25 μg/ml Fungizone (all above reagents were purchased from Invitrogen), 1% ITS (insulin, transferrin, and selenium; Sigma-Aldrich), 5 ng/ml platelet-derived growth factor, and 10 ng/ml epidermal growth factor (R&D Systems). Cells were passaged by trypsinization, used at the third to fifth passage after primary culture, and were mesenchymal vimentin positive, a general marker for fibroblasts (46). After plating as indicated, the cells were allowed to grow until they were almost (~85%) confluent before being used in the indicated experiments.

In vitro hypoxia stimulation and determination of VEGF levels in cultured cells

Levels of VEGF were assessed with an ELISA kit (R&D Systems) according to the manufacturer's recommendations. In vitro hypoxia stimulation was performed as described previously (47). Briefly, 1×10^6 cells in 6-well plates were serum starved overnight and placed in a modular incubator chamber (Billups-Rothenberg) and flushed at 2 pounds per square inch for 15 min with a gas mixture of 1% O_2, 5% CO_2, and balance N_2. The chamber was sealed and placed in an incubator at 37°C for 24 h. For the inhibition study, cells were pretreated with 1 μM U0126 (EMD Chemicals), a specific ERK inhibitor, for 30 min and then incubated for 16 h with or without hypoxia conditions. Cell culture medium from each well was collected for cytokine determination. OD values of the tested samples were compared with the values obtained from a serial dilution of respective recombinant cytokines.

Western blotting

Protein samples were prepared using extraction buffer as described previously (48). Briefly, the samples were separated by SDS-PAGE and analyzed using primary Abs at 1/1000 dilutions. HRP-conjugated secondary Abs against mouse or rabbit IgG were used at 1/3000 dilution, and the signal was visualized using an ECL substrate kit (Amersham Biosciences).

Primary Abs against VEGF, VEGF receptor (VEGFR) 1, VEGFR2, VCAM-1, HIF-1α, and IL-4Rα were purchased from Santa Cruz Biotechnology. We also used anti-phospho-ERK, anti-ERK, anti- phospho-Akt (Ser^{473}), anti-Akt (Cell Signaling Technology), and anti-vascular endothelium cadherin (VE-cadherin) Ab (Cayman Chemical). To ensure equal protein loading and transfer, blots were stripped and reprobed with either anti-β-tubulin, anti-p44/42 ERK, or anti-Akt Abs, followed by the appropriate HRP-conjugated secondary Ab. Each band was quantified by NIH ImageJ software.

Immunohistochemistry

Mice were sacrificed 4 days after the low oxygen exposure and the lungs were harvested for H&E and immunohistochemistry as described previously (48). Paraffin sections were stained with H&E and an anti-Von Willebrand factor (vWF) Ab (DakoCytomation) at 1/200 dilution or an anti-proliferating cell nuclear Ag (PCNA) Ab (Cell Signaling Technology) at 1/1000 dilution. For vWF staining, sections were counterstained with Mayer's hematoxylin. Sections were visualized through a Nikon Eclipse TE2000-E microscope (Nikon Instruments). vWF-positive and PCNA-positive cells were counted in five randomly chosen low power microscopic fields (×200 original magnification) per lung obtained from at least three separate animals for each treatment. Immunohistochemical negative control sections received identical treatments except for exposure to the primary Ab and showed no specific staining.

Statistical procedures

Differences between multiple groups were compared by two-way ANOVA followed by an appropriate multiple comparison test. Two-group analysis was performed by Student's *t* test. A value of $p < 0.05$ was considered significant.

Results

Hypoxia up-regulates the expression of IL-4Rα and VEGFRs in the lung

The biological actions of IL-4 are mediated by its binding to its receptor, IL-4R. Because an Ab against IL-4Rα blocked the formation of tube-like structures in collagen gel by microvascular ECs in response to IL-4, it is believed that IL-4 serves as proangiogenic factor via IL-4Rα (49).

By immunoblot analysis we determined the expression of IL-4Rα in the lungs from WT animals with or without hypoxia stimulation. Untreated lungs (normoxia) constitutively expressed the IL-4Rα, and after hypoxia treatment for 4 days we observed a significant increase of the IL-4Rα expression (Fig. 1A). The constitutively expressed IL-4Rα in the lung of the IL-4 KO mouse was significantly lower than that of WT controls, but neither showed changes in the hypoxia-treated lung (data not shown). We also examined the effect of hypoxia on VEGFR1 and VEGFR2 expression in the lungs from both WT and IL-4 KO mice (Fig. 1B). VEGFR2 is considered as the most crucial receptor for transmitting cellular signals for angiogenesis (50). Hypoxia stimulation caused significant increase of both VEGFR1 and VEGFR2 in the lungs from both WT and IL-4 KO mice at similar levels

as compared with nontreated (normoxia) animals, suggesting that the VEGFR reaction in response to hypoxia is similar in WT and IL-4 KO mice.

Hypoxia-induced VEGF, VCAM-1, and HIF-1α expression is suppressed in the lungs from IL-4 KO mice

VEGF appears to play a crucial role in the hypoxia-related mitogenic response in various organs, including the lung. In addition to hypoxia-induced expression of VEGF, various investigators have documented that in vivo expression of its receptors is increased in pathologic conditions characterized by hypoxia (32).

In our present study, even though 4 days of hypoxia increased lung VEGFR expression in both WT and IL-4 KO mice (Fig. 1B), hypoxia-induced VEGF expression in the lung was significantly suppressed in the lungs from hypoxia-treated IL-4 KO mice as compared with hypoxia-treated WT mice (Fig. 2A). Similar to this result, hypoxia-induced VCAM-1 expression was also suppressed in the lungs from IL-4 KO mice as compared with WT mice (Fig. 2A). HIF-1α also plays a critical role in angiogenesis by regulating the expression of key angiogenic growth factors, including VEGF, under hypoxic conditions (51). In this context, we analyzed the expression of HIF-1α in the lungs from these animals. Hypoxia-induced HIF-1α expression was decreased in the lungs from IL-4 KO mice as compared with WT mice (Fig. 2B). These results suggest that IL-4 plays an important role in hypoxia induction of VEGF, VCAM-1, and HIF-1α expressions in the lung.

Hypoxia-induced ERK phosphorylation, but not Akt phosphorylation, is suppressed in the lungs from IL-4 KO mice

IL-4 is known to initiate activation of MEK subfamilies, including p38 MEK and ERK, in the lung resident cells (52). However, the mechanism of this signaling under hypoxia conditions remains unknown. It has been well documented that hypoxia up-regulates MEK subfamilies and acts as a proliferative stimulus in the pulmonary artery (53). MEKs are also known as major mediators of down-stream signaling of the VEGF-VEFGR2 pathway (54). Hypoxia stimuli induced significant ERK phosphorylation in the lungs of WT mice, and this effect was diminished in IL-4 KO mice (Fig. 3A). The Akt-PI3K pathway has also been implicated in mitogenic responses of the pulmonary vasculature and VEGF signaling; however, we did not see any significant difference in hypoxia-induced Akt phosphorylation between WT and IL-4 KO mice (Fig. 3B). These results suggest that IL-4 signaling is involved in hypoxia-induced mitogenic action in the lung via ERK pathways, but not Akt pathways.

Hypoxia-induced VEGF production is suppressed in pulmonary fibroblasts from IL-4 KO mice

Our in vivo results suggest that hypoxia-induced VEGF production is significantly suppressed in IL-4 KO lungs (Fig. 2A); nevertheless, the induction of VEGFR expression is prominent (Fig. 1B), indicating that the capability for VEGF production is suppressed in IL-4 KO mice.

It has been suggested that fibroblasts produce proangiogenic factors, induce an angiogenic phenotype in microvascular ECs in vitro, and secrete active matrix metalloproteinase 9, which is implicated in the proteolysis of the basement membrane early during the invasion stage of angiogenesis (55). It has also reported that hypoxic pulmonary artery fibroblasts trigger the proliferation of vascular SMCs by a HIF-dependent mechanism (56). In addition, cultured fibrocytes constitutively secrete several growth factors such as VEGF that induce EC migration and proliferation, and vascular tubule formation (55). In this context, we have isolated pulmonary fibroblasts from both WT and IL-4 KO mice and treated these cells with hypoxia for 24 h. In vitro hypoxia stimuli significantly increased VEGF production in pulmonary fibroblasts from WT mice; however, VEGF production was completely suppressed in the cells from IL-4 KO mice (Fig. 4A). We also analyzed whether hypoxia-induced VEGF production depends on an ERK pathway by using the specific ERK inhibitor U0126. As seen in Fig. 4B, hypoxia-induced VEGF induction in pulmonary fibroblasts from WT mice was significantly suppressed by the coincubation of U0126. This in vitro result is keeping with our prior in vivo observations that hypoxia-induced VEGF production is, at least in part, an ERK-dependent pathway in the lung.

Hypoxia-induced pulmonary angiogenesis is suppressed in IL-4 KO mice, but not in STAT6 KO mice

In the present study, we demonstrated that hypoxia-induced angiogenic and mitogenic signaling is suppressed in the lungs of IL-4 KO mice. Next we examined hypoxia-induced pulmonary angiogenesis of these animals. We quantified pulmonary angiogenesis by anti-VE-cadherin, also known as cadherin-5, as an EC-specific adhesion molecule and a marker for ECs (57). Hypoxia stimuli caused a nearly 2.4 ± 0.04- to 2.9 ± 0.03-fold increase in ECs in the lungs of WT mice (Fig. 5, A and B); however, VE-cadherin expression was not increased in the lungs from hypoxia-treated IL-4 KO mice (Fig. 5A). Because there is evidence that IL-4 stimulates EC adhesion molecules via the STAT6 pathway and serves as a potent mitogenic factor in human ECs (58), we also examined hypoxia-induced pulmonary angiogenesis in the lungs of STAT6 KO mice. Interestingly, lungs from hypoxia-treated STAT6 KO mice showed a significant increase of VE-cadherin expression similar to that in hypoxia-treated WT mice (Fig. 5B). These data suggest that hypoxia-induced pulmonary angiogenesis is dependent on the IL-4 pathway but does not require STAT6 activation.

To investigate the effect of acute hypoxia on the remodeling of pulmonary arterioles directly, histological sections of lungs from WT and IL-4 KO mice exposed to 10% O_2 for 4 days were prepared for morphometric analysis (Fig. 6A). On day 4 of hypoxia stimulation, both alveolar and pulmonary vasculature remodeling were not quite obvious; however, both airway epithelial cells and pulmonary arteries were thickened in WT controls, and this hypoxia-induced cell proliferation in airway epithelial cells and pulmonary arteries was attenuated in IL-4 KO mice (Fig. 6A). To support the result of angiogenesis quantification using immunoblotting for VE-cadherin, we also compared vWF-positive cells, another specific marker for ECs, in the lungs of these genotypes with or without hypoxia stimulation, and these cells were quantified. As seen in Fig. 6, B and C, both WT and STAT6 KO mice showed significant increases of hypoxia-induced vWF-positive cells in the lungs as compared with normoxia controls of each genotype; however, these phenomena did not

occur in the lungs of IL-4 KO mice. These results are in accordance with angiogenesis quantification by comparing VE-cadherin expression in these genotypes (Fig. 5, A and B).

Hypoxia-induced cell proliferation activity in the pulmonary vasculature is suppressed in IL-4 KO mice

To examine the effect of hypoxia on the proliferative activity within the pulmonary vasculature, lung sections were stained for PCNA (a marker for proliferating cells). Exposure to hypoxia for 4 days induced intense expression of PCNA (6.2 ± 0.2-fold) in the area of pulmonary arteries in hypoxia-treated WT controls; however, the fold increase of PCNA-positive cells was significantly decreased in the lungs of hypoxia-treated IL-4 KO mice (3.7 ± 0.2-fold; Fig. 7, A and B). These data suggest that IL-4 plays a role in hypoxia-induced angiogenesis and cell proliferation in the lung.

Discussion

The major finding of the present study is that IL-4 has proangiogenic and proinflammatory properties in the lung during exposure to chronic hypoxia and that this effect is dependent on hypoxia induction of the VEGF signaling pathway.

Hypoxia plays a key role in VEGF gene expression both in vivo and in vitro. The human VEGF gene contains two hypoxia-sensitive elements and several consensus binding sites for growth factor-regulated transcription factors (59). It is reasonable to speculate that the paracrine or autocrine release of cytokines, such as IL-4, can stimulate VEGF production to cooperate with local hypoxia in regulating VEGF release in the microenvironment.

In the present study, our data suggest that hypoxia-induced VEGF expression and pulmonary angiogenesis are diminished in IL-4 KO mice as compared with WT controls. IL-4 is also known to initiate activation of MEK subfamilies, including p42/p44 MEK/ERK, in bronchial smooth muscle and epithelial cells (52, 60). It has been well documented that hypoxia up-regulates MEK subfamilies and acts as a proliferative stimulus in the pulmonary artery (53, 61). In the present study, we showed that ERK, which is also a downstream signaling molecule of the VEGF pathway, is involved, at least in part, in this process. It has also been shown that the ERK signal transduction pathway is important in VCAM-1 expression (62). In the present study, our data show that hypoxia-induced VCAM-1 expression is diminished in IL-4 KO lungs, suggesting that IL-4 plays an important role in hypoxia-induced VCAM-1 expression and the subsequent inflammatory responses.

We also have isolated lung fibroblast cells from both IL-4 KOs and WT control animals, and stimulated these cells with hypoxia. Cultured fibrocytes constitutively secrete angiogenic factors such as VEGF and collagen and thus are important contributors to new blood vessel formation (63). Tissue remodeling is the result of modified fibroblast proliferation and a significant modification of the local extracellular matrix (53). These events subsequently increase the proliferation of vascular SMCs and therefore affect neovascularization, and hypoxic pulmonary artery fibroblasts trigger proliferation of SMCs by a HIF-dependent pathway (56). These observations are in accordance with our present study. In this study, HIF-1α expression was significantly decreased in the lungs from hypoxia-treated IL-4

KO mice as compared with WT mice (Fig. 2B), partly explaining the changes of VEGF expression in this genotype.

Activation of fibroblasts with various stimuli induces the expression of α-smooth muscle action and the development of an enriched microfilamentous system in the cytoplasm. The accumulation of myofibroblasts (α-smooth muscle action-expressing fibroblasts) in the pulmonary artery adventitia of hypoxic animals is well documented, and their presence is speculated to contribute to high pulmonary vascular resistance (64). These are identified as key participants in the tissue remodeling that occurs during wound healing and various vascular diseases and fibrotic disorders (63). These myofibroblasts are believed to contribute to many of the functional abnormalities reported in remodeled vessels. In the current study, our in vitro data also revealed that hypoxia-induced VEGF production in fibroblasts from IL-4 KOs was significantly suppressed as compared with WT controls. These results are in accordance with our in vivo experiments.

A prominent feature of airway inflammation in asthma is the infiltration of high numbers of eosinophils into the lung tissue (65). Eosinophils constitute a small population of circulating or tissue-resident leukocytes, and the associated infiltration of these cells suggests the existence of molecular mechanisms responsible for their selective recruitment into tissues. The endothelial cell adhesion molecules P-selectin and VCAM-1 are important in eosinophil tethering, rolling, and firm adhesion on endothelium (66, 67). IL-4 also selectively regulates the expression of VCAM-1 on human ECs (68) and increases the adherence of eosinophils to ECs in a VCAM-1-dependent manner (67). Induction of P-selectin in ECs is also regulated by IL-4 in a STAT-6-dependent manner (58), and IL-4 signal transduction includes activation of STAT6 (69). Although a number of studies have established a key role for IL-4 in VCAM-1 expression, a connection between IL-4-mediated VCAM-1 expression in the lung under hypoxic conditions has not been examined. In this context, we also examined whether STAT6 is a critical factor for hypoxia-induced pulmonary angiogenesis and its signaling using STAT6 KO animals. Interestingly, our results demonstrate that hypoxia-induced VCAM-1, as well as VEGF expression, is significantly reduced in IL-4 KO lungs but not in STAT6 KO lungs (data not shown), suggesting that hypoxia-induced VCAM-1 and VEGF induction in the lung does not require a STAT6 pathway.

In addition, it is believed that VCAM-1 up-regulation occurs by stabilization of its mRNA in response to IL-4, as the VCAM-1 promoter dose not contain any STAT6-binding sites (68). Previous studies have suggested that the distal region of the IL-4Rα-chain is required for induction of STAT6 tyrosine phosphorylation (70). However, this region of the receptor is not required for cell growth, suggesting that induction of STAT6 tyrosine phosphorylation is not required for a mitogenic response (70). This is in accordance with our result showing that STAT6 was not required by hypoxia-induced pulmonary angiogenesis.

In summary, these studies demonstrate that IL-4 has proangiogenic and proinflammatory properties in the lung under hypoxic conditions. Our data show that hypoxia can stimulate IL-4Rα expression as well as VEGFR expression and that IL-4 is essential for hypoxia-induced VEGF and VCAM-1 expression in the lung. Induction of pulmonary angiogenesis and proliferative activity in response to hypoxia also requires IL-4. These signaling

processes are, at least in part, mediated by the ERK-dependent pathway but not the Akt pathway. Even though IL-4 signal transduction includes activation of STAT6 in several cell types, STAT6 does not appear to be essential for hypoxia-induced angiogenic properties in the lung, suggesting that the proangiogenic properties of IL-4 under hypoxic conditions are independent of STAT6 signaling. Additional investigations of the genes that mediate and the mechanisms responsible for the responses induced by IL-4 in animal models, as well as their relevance in humans, are warranted.

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Abbreviations used in this paper used in this paper:

VEGF	vascular endothelial growth factor
EC	endothelial cell
HIF	hypoxia inducible factor
KO	knockout
PCNA	proliferating cell nuclear Ag
SMC	smooth muscle cell
VE-cadherin	vascular endothelium cadherin
VEGFR	VEGF receptor
vWF	Von Willebrand factor
WT	wild type

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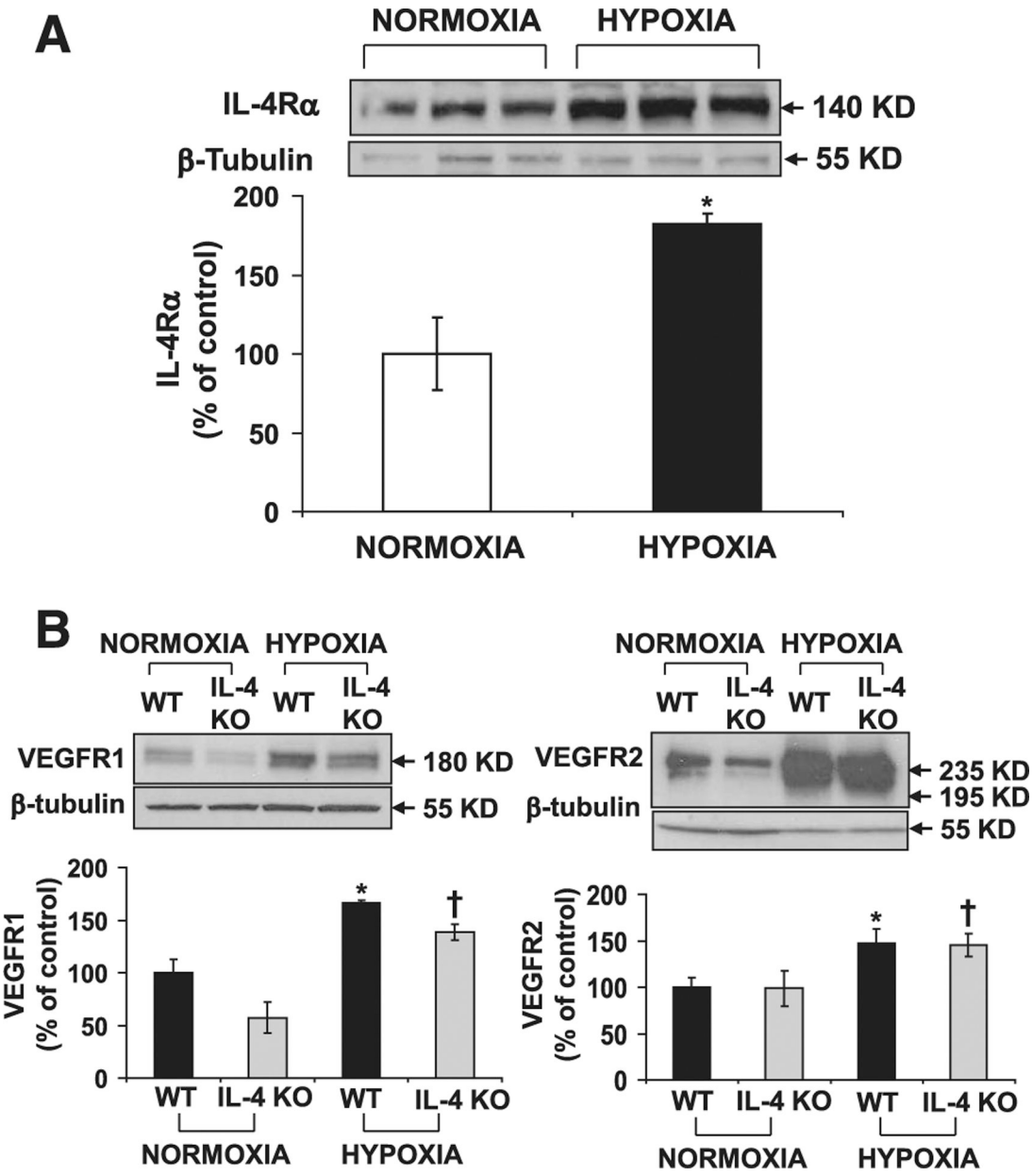

FIGURE 1.

IL-4R*α* and VEGFR expression in the mouse lung induced by hypoxia. *A*, The expression of IL-4R*α* in the lungs from WT mice. *B*, The expression of VEGFR1 and VEGFR2 in the lungs from WT and IL-4 KO mice. Data are normalized to *β*-tubulin expression and expressed as a percentage of normoxia controls, and the values in *A* and *B* represent the mean ± SEM of evaluations in a minimum of three animals per group. These experiments were repeated three times with similar results. *, $p < 0.05$ vs normoxia controls ($n = 3 \pm$ SD; Student's *t* test). †, p = NS, indicating the effect of genotype on the hypoxia-stimulated response ($n = 3 \pm$ SD; two-way ANOVA). KD, Kilodalton.

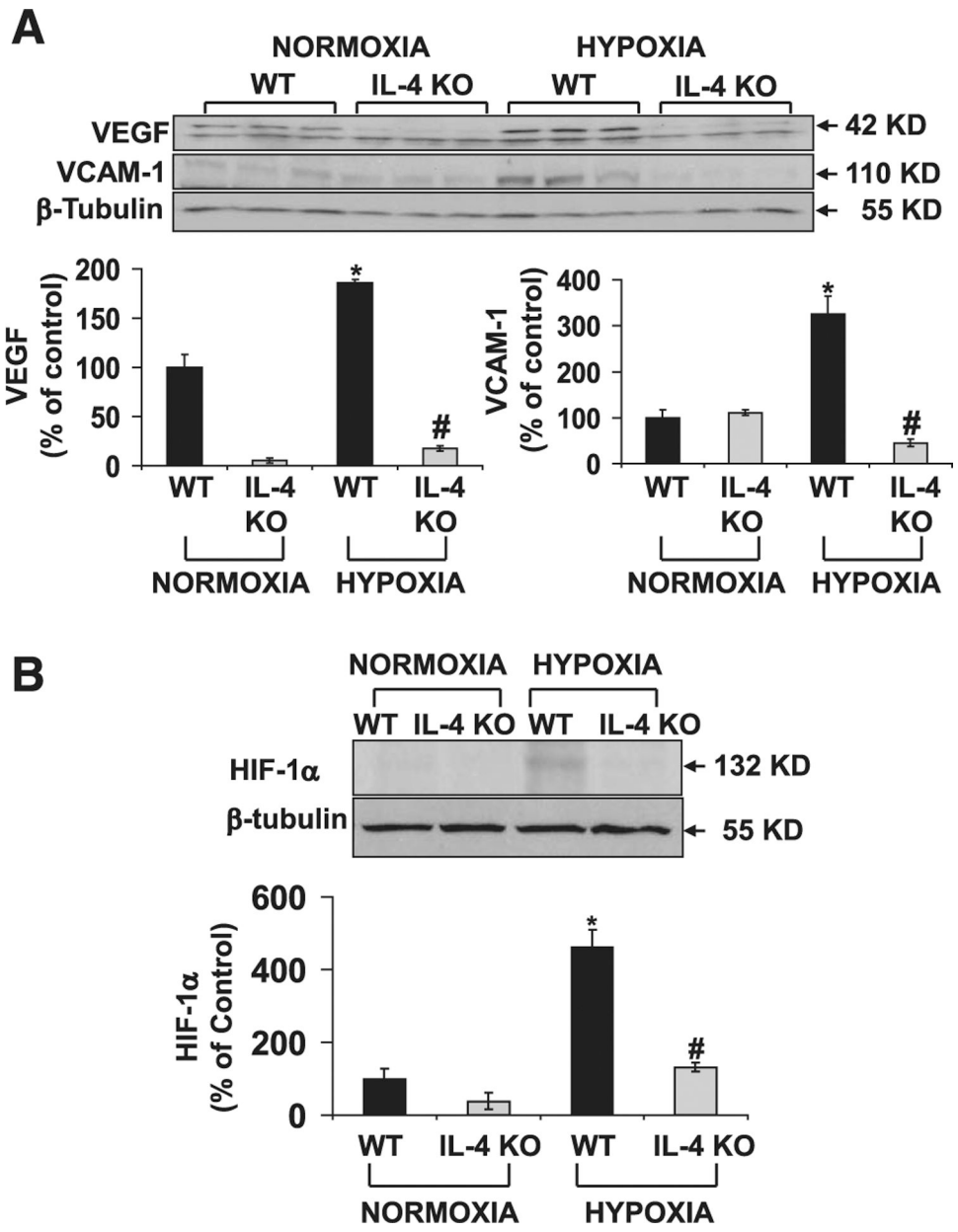

FIGURE 2.

VEGF, VCAM-1, and HIF-1α expression levels in WT and IL-4 KO lungs subjected to hypoxia. *A* and *B*, Immunoblot analysis for VEGF and VCAM-1 (*A*) and HIF-1 α (*B*) in lung tissue extracts. Hypoxia increased VEGF (*A*, *top panel*), VCAM-1 (*A*, *middle panel*), and HIF-1α (*B*) expression in WT lungs, but far less in IL-4 KO lungs. Data are normalized to β-tubulin expression and expressed as a percentage of normoxia controls. These experiments were repeated three times with similar results. *, $p < 0.05$ vs normoxia (WT); #, $p < 0.001$ vs hypoxia (WT) ($n = 3 \pm$ SD; two-way ANOVA). KD, Kilodalton.

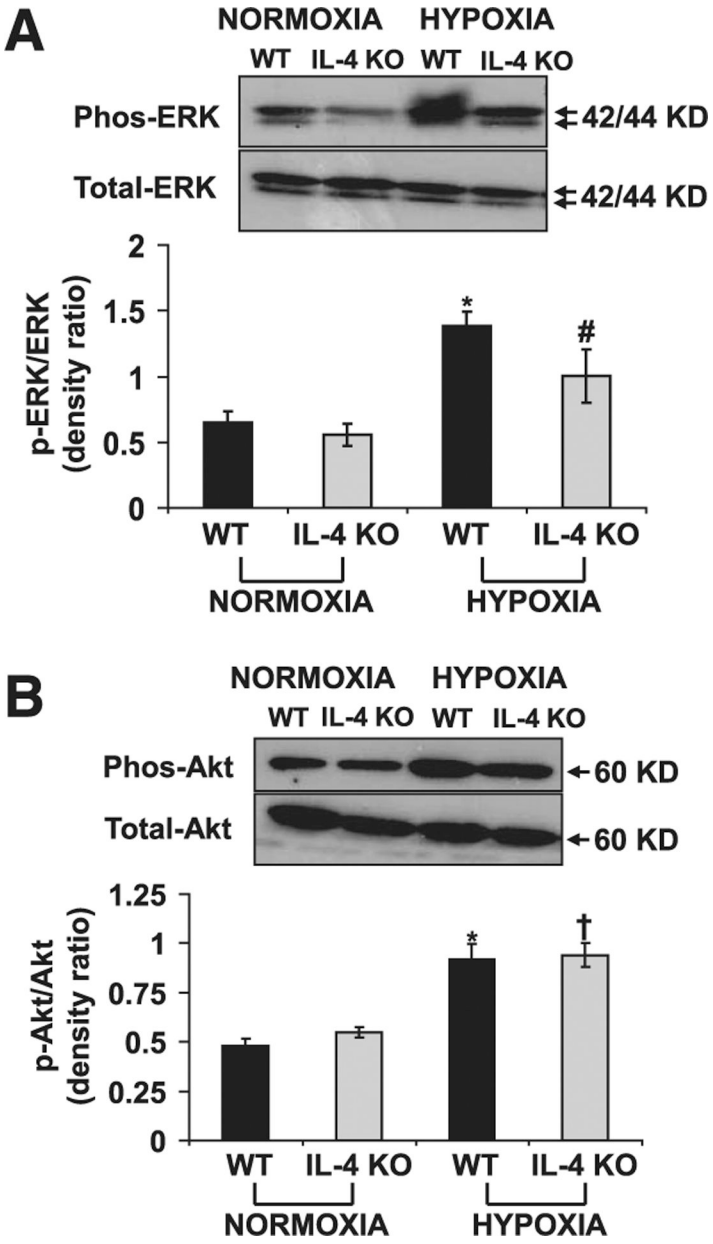

FIGURE 3.

ERK and Akt phosphorylation levels in WT and IL-4 KO lungs subjected to hypoxia. *A*, Response to total-ERK and phospho-ERK (Phos-ERK) to hypoxia in both genotypes and quantification results as a ratio of phospho-ERK to total ERK. Hypoxia induced a marked increase in levels of phospho-ERK in WT lungs. In contrast, hypoxia-induced ERK phosphorylation was diminished in IL-4 KO lungs as compared with WT lungs. *B*, Response to total Akt and phospho-Akt to hypoxia in both genotypes and quantification results as a ratio of phospho-Akt to total Akt (p-Akt/Akt). These experiments were repeated three times with similar results. *, $p < 0.05$ vs normoxia (WT) ($n = 3 \pm$ SD, two-way ANOVA). p values indicate the effect of genotype on the hypoxia-stimulated response (two-way ANOVA, $n = 3 \pm$ SD). #, $p < 0.01$; † $p = 0.24$ (NS). KD, Kilodalton.

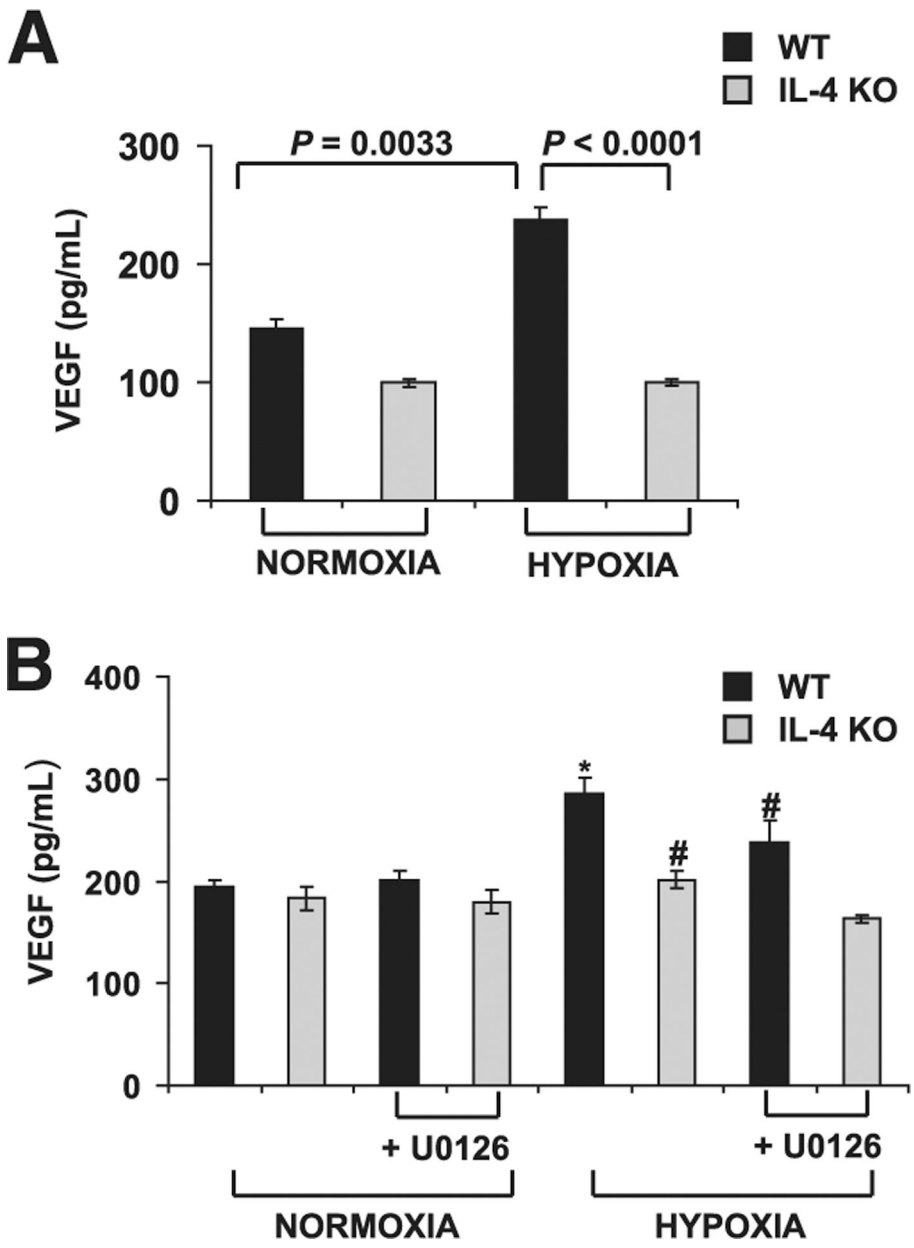

FIGURE 4.

Levels of VEGF from lung primary fibroblasts induced by hypoxia. Primary lung fibroblasts isolated from both genotypes were exposed to hypoxia for 24 h (*A*) and 16 h followed by incubation with 1 μM U0126 (*B*), and VEGF levels in the cell culture medium were quantified. Hypoxia stimulated a significant increase of VEGF production in WT lung fibroblasts (*A*, $p = 0.0033$). *, $p < 0.05$ vs hypoxia-treated WT mice. #, $p < 0.01$, indicating the effect of genotype on the hypoxia-stimulated response (two-way ANOVA; $n = 5 \pm$ SD).

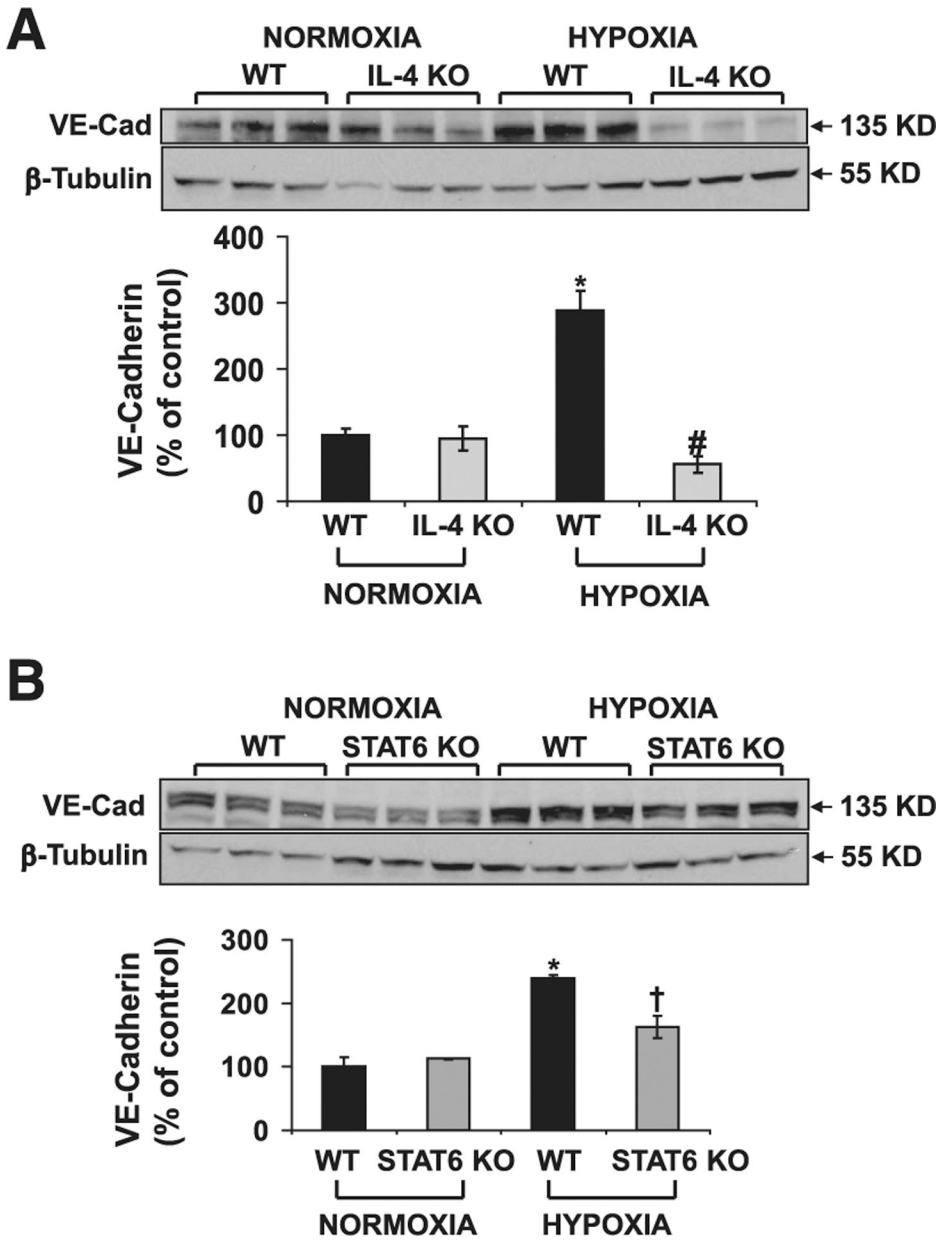

FIGURE 5.

Hypoxia-induced pulmonary angiogenesis in WT, IL-4 KO, and STAT6 KO mice. Pulmonary angiogenesis induced by hypoxia was quantified by immunoblotting for VE-cadherin using lung lysates from WT, IL-4 KO (*A*), and STAT6 KO (*B*) animals. Hypoxia induced a marked increase of pulmonary ECs (VE-cadherin-positive cells) in WT and STAT6 KO lungs. In contrast, there was no increase of pulmonary ECs in IL-4 KO lungs. Data are normalized to β-tubulin expression and expressed as a percentage of normoxia controls. These experiments were repeated three times with similar results. *, $p < 0.01$ vs hypoxia-treated WT animals. p values indicate the effect of genotype on the hypoxia-stimulated response (2-way ANOVA, $n = 3 \pm$ SD). #, $p < 0.01$; †, $p = 0.15$ (NS). KD, Kilodalton.

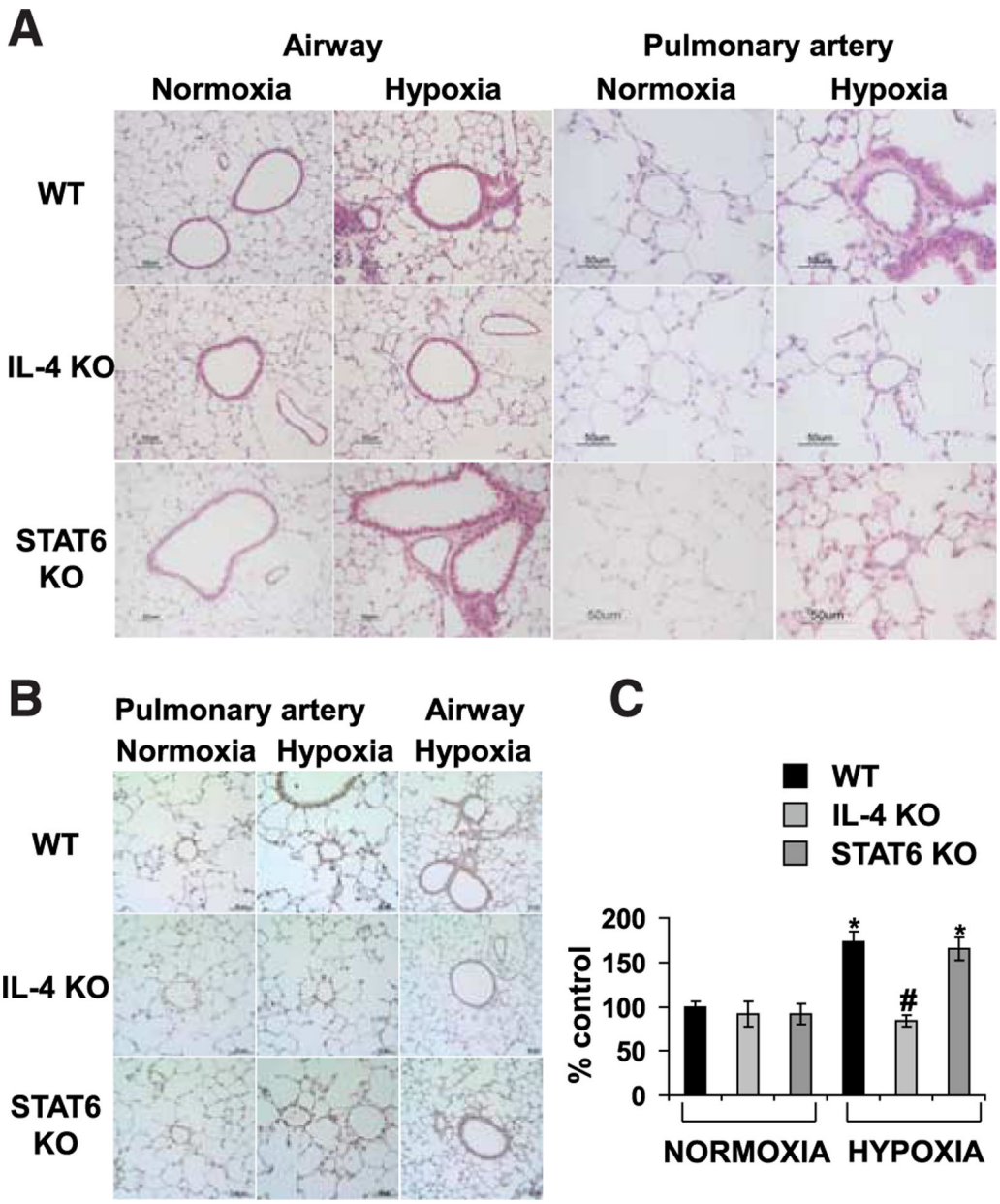

FIGURE 6.

Pulmonary histology and quantification of hypoxia-induced angiogenesis in the lung. Lungs from WT, IL-4 KO, and STAT6 KO mice exposed to normoxia (control) and hypoxia (10% oxygen) for 4 days were formalin-fixed, paraffin-embedded, sectioned, and stained with H&E (*A*) and anti-vWF Ab (*B*). Both airway and pulmonary arteries are shown (scale bars, 50 μm). *A*, The adventitial layer is very thin in normoxic (control) animals. Hypoxia-treated WT and STAT6 KO lungs showed thickened adventitial layer in both airway and pulmonary arteries. In contrast, there was no change in IL-4 KO lungs. *B*, Immunohistochemical analysis of vWF in WT, IL-4 KO, and STAT6 KO lungs. Sections were counterstained with Mayer's hematoxylin (blue staining), and vWF-positive cells are shown as black staining. Increased numbers of vWF-positive cells are observed in airway epithelium, endothelial

cells, and pulmonary arteries in hypoxia-treated WT lungs as compared with normoxia controls, but these are significantly suppressed in hypoxia-treated IL-4 KO lungs. Hypoxia-treated STAT6 KO mice also showed a significant increase of vWF-positive cells, especially in the airway area. *C*, Hypoxia-induced angiogenesis in each genotype and quantification results as a percentage of control of vWF-positive cells as compared with normoxia controls of each genotype. *, $p < 0.05$ vs hypoxia-treated animals of each genotype; #, $p < 0.05$ indicates the effect of genotype on the hypoxia-stimulated response (two-way ANOVA; $n = 5 \pm$ SD).

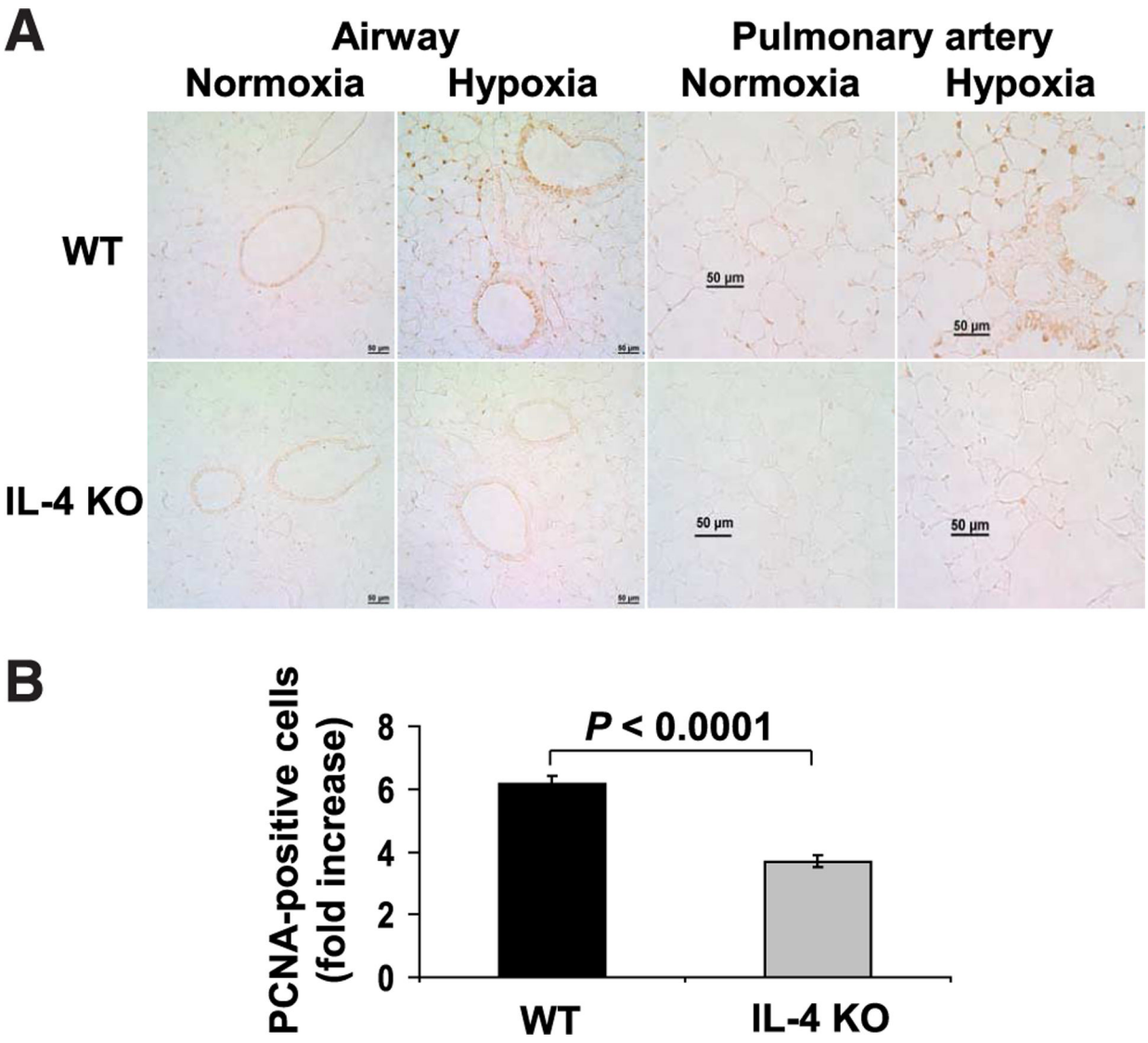

FIGURE 7.

Hypoxia- induced proliferation activity in the lung. *A*, Immunohistochemical analysis of PCNA-positive cells in WT and IL-4 KO lungs. Lungs from WT and IL-4 KO mice exposed to normoxia (control) and hypoxia (10% oxygen) for 4 days were stained with anti-PCNA Ab. Increased numbers of PCNA-positive cells are observed in airway epithelium, endothelial cells, and pulmonary arteries in hypoxia-treated WT lungs as compared with normoxia controls, but these are significantly suppressed in hypoxia-treated IL-4 KO lungs. Scale bars, 50 μm. *B*, Hypoxia-induced proliferation activities in both genotypes and quantification results as a fold increase of PCNA-positive cells as compared with normoxia controls of each genotype. The p value indicates the effect of genotype on the hypoxia-stimulated response (two-way ANOVA; $n = 5 \pm$ SD).