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Vascular endothelial growth factor (VEGF) induces remodeling and enhances T_H2-mediated sensitization and inflammation in the lung

Chun Geun Lee[1,4], **Holger Link**[1,4], **Peter Baluk**[2], **Robert J Homer**[3], **Svetlana Chapoval**[1], **Vineet Bhandari**[1], **Min Jong Kang**[1], **Lauren Cohn**[1], **Yoon Keun Kim**[1], **Donald M McDonald**[2], and **Jack A Elias**[1]

[1]Section of Pulmonary and Critical Care Medicine, Department of Internal Medicine, Yale University School of Medicine, 300 Cedar Street, 441c TAC, New Haven, Connecticut 06520-8057, USA

[2]Cardiovascular Research Institute, Department of Anatomy and Comprehensive Cancer Center, University of California, San Francisco, 513 Paramus Avenue, San Francisco, California 94143-0130, USA

[3]Department of Pathology, Yale University School of Medicine, 310 Cedar Street, New Haven, Connecticut 06520, USA

Abstract

Exaggerated levels of VEGF (vascular endothelial growth factor) are present in persons with asthma, but the role(s) of VEGF in normal and asthmatic lungs has not been defined. We generated lung-targeted $VEGF_{165}$ transgenic mice and evaluated the role of VEGF in T-helper type 2 cell (T_H2)-mediated inflammation. In these mice, VEGF induced, through IL-13–dependent and –independent pathways, an asthma-like phenotype with inflammation, parenchymal and vascular remodeling, edema, mucus metaplasia, myocyte hyperplasia and airway hyper-responsiveness. VEGF also enhanced respiratory antigen sensitization and T_H2 inflammation and increased the number of activated DC2 dendritic cells. In antigen-induced inflammation, VEGF was produced by epithelial cells and preferentially by T_H2 versus T_H1 cells. In this setting, it had a critical role in T_H2 inflammation, cytokine production and physiologic dysregulation. Thus, VEGF is a mediator of vascular and extravascular remodeling and inflammation that enhances antigen sensitization and is crucial in adaptive T_H2 inflammation. VEGF regulation may be therapeutic in asthma and other T_H2 disorders.

Exaggerated T_H2 inflammation and airway remodeling are cornerstones in the pathogenesis of asthma[1–3]. In keeping with the importance of neovascularization in inflammation and remodeling, a number of investigators have characterized vascular responses in asthmatic tissues. These studies demonstrated prominent increases in vessel number, vessel size, vascular surface area and vascular leakage, and important correlations between these alterations and disease severity[4–12]. As a result, it has been assumed that asthmatic inflammation stimulates the growth of new blood vessels[9,11,12] and that these vascular

Correspondence should be addressed to J.E. (jack.elias@yale.edu).
[4]These authors contributed equally to this work.

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alterations contribute to the airway obstruction or airway hyper-responsiveness (AHR), or both, in this disorder[12–14]. Yet the mechanisms that generate these vascular alterations have not been defined. In addition, the possibility that the processes inducing these alterations also contribute to the pathogenesis of the inflammatory and immune alterations that are central to asthma has not been investigated.

Vascular endothelial growth factor (VEGF) was originally described as vascular permeability factor (VPF) based on its ability to generate tissue edema[15]. It has subsequently been understood to be a multifunctional angiogenic regulator that stimulates epithelial cell proliferation, blood vessel formation and endothelial cell survival[16,17]. Exaggerated levels of VEGF have been detected in tissues and biologic samples from people with asthma[10,11,18,19], where these levels correlate directly with disease activity[7] and inversely with airway caliber and airway responsiveness[10,11,18,19]. VEGF has been postulated to contribute to asthmatic tissue edema through its effect on vascular permeability[12–14]. However, the role of VEGF in the pathogenesis of other aspects of the asthmatic phenotype and the effector functions of VEGF in the lung have not been defined. Even the contribution of VEGF to asthmatic vascular alterations is not clear because VEGF has been reported to lack angiogenic properties in the respiratory tract[20,21].

The lung is unique amongst mucosal compartments in that it is constantly exposed to airborne particulates. In normal humans, the immune response differentiates between harmless agents that should not induce sensitization and potentially injurious pathogens against which an immune response is warranted. In contrast, the lungs of atopic asthmatics show an enhanced ability to sensitize and mount pathologic T_H2 responses after exposure to largely innocuous allergens[22–24]. Infection-elicited innate immune responses are known to have an essential role in the development of adaptive T_H2 immunity[22,23]. This is nicely illustrated by respiratory syncytial virus (RSV), which enhances aeroallergen sensitization and contributes to the development of asthma[22,25]. It has been proposed that antiviral innate immune responses contribute to the generation or maintenance, or both, of adaptive T_H2 immunity by increasing mucosal permeability and altering local dendritic cells (DCs)[22]. The validity of these assumptions has not been tested and the mediators of these effects have not been defined. In addition, although earlier work from our laboratories demonstrated that RSV stimulates epithelial VEGF elaboration *in vitro* and in humans *in vivo*[26], the ability of VEGF to facilitate immune sensitization has not been evaluated.

We hypothesized that VEGF is a critical mediator in asthma and T_H2 inflammation. To test this hypothesis, we generated and characterized transgenic mice in which $VEGF_{165}$, the 165-amino-acid isoform of VEGF, was overexpressed in the airway and evaluated the role of VEGF in antigen-induced T_H2 inflammation. These studies demonstrate that VEGF is a potent stimulator of angiogenesis, edema, inflammation, vascular remodeling, parenchymal remodeling and physiologic dysregulation. They also link innate and adaptive T_H2 immunity by demonstrating that VEGF enhances antigen sensitization and demonstrate that epithelial and T_H2 cell–derived VEGF have central roles in T_H2 inflammation.

RESULTS

Generation of inducible transgenic mice

VEGF overexpression during lung development causes fetal death[27]. To avoid this, we used a dual-construct transgenic system, developed in our laboratory[28], that can be regulated externally (Fig. 1a). We identified four dual-transgenic CC10-rtTA-VEGF founder mice. At 1 month of age, they were randomized to receive normal water or doxycycline (dox)-containing water. In the wild-type mice given normal or dox water and the transgenic mice given normal water, VEGF levels in bronchoalveolar lavage fluids (BAL) were ≤15 pg/ml.

Increased levels of BAL VEGF were noted within 24 h and steady-state levels between 0.05 and 12 ng/ml were seen after 1 week of dox administration (data not shown). These levels of VEGF are comparable with the levels in biologic fluids from normal humans and those with asthma or RSV infection[18,19,26,29–31]. In all cases, RT-PCR of multiple organs and tissues showed that VEGF was produced in a lung-specific fashion (data not shown). Qualitatively similar, dose-dependent alterations were seen in all mice with levels of BAL VEGF ≥0.1 ng/ml as summarized below.

Effect on neovascularization and edema

Lycopersicon esculentum lectin analysis demonstrated that blood vessels in the tracheas and intrapulmonary bronchi of wild-type mice given normal or dox water and transgenic mice given normal water were arranged in cascades with capillaries crossing between arterioles and venules (Fig. 1b,c). This bronchial circulation became less dense as it extended from the extrapulmonary to the intrapulmonary bronchi (Fig. 1b). In the transgenic mice, as little as 3 d of dox generated endothelial sprouts, mostly arising from the venules (Fig. 1b). Vascular density (the percent of the airway covered with vessels) was maximal after 7 d of dox (42.3 ± 5.4 versus 23.5 ± 1.3%; $P < 0.01$) (Fig. 1c) and remained elevated for at least a month thereafter. The new vessels were larger than the capillaries of the control airways (11.86 ± 0.37 versus 8.90 ± 0.14 μm, $P < 0.001$) and, as seen in asthma[4], had migrated into the lamina reticularis and occasionally into the epithelium (Fig. 1c,d). The endothelial cells of these vessels were thin, had occasional fenestrations, and were enveloped by pericyte processes and basement membranes. In dox-treated transgenic mice, there was a significant increase in lung wet/dry ratios, Evans blue dye extravasation and edema compared to the wild-type and transgenic controls (Fig. 1e,f and data not shown) ($P < 0.01$ comparing dox-treated transgenic mice to all other groups). Thus, VEGF is a potent inducer of angiogenesis and edema in the mouse airway and lung.

Histologic and physiologic evaluation

Lungs from wild-type mice given normal or dox water and transgenic mice given normal water could not be distinguished from lungs from control mice given normal water (Fig. 2a and data not shown). In contrast, VEGF overexpression caused conspicuous alterations that persisted throughout the 5-month study interval. Inflammation was seen after 2 d of dox. At early time points, increases in tissue mononuclear cells, B lymphocytes and occasional clusters of eosinophils were noted (Fig. 2a and data not shown). At later time points, $CD4^+$ and $CD8^+$ T cells were also more abundant (1.82% ± 0.3 to 9.92 ± 0.9% for $CD4^+$ cells, 1.14 ± 0.4 to 8.64 ± 0.7% for $CD8^+$ cells, $P < 0.01$ comparing wild-type to transgenic for both). Similarly, total cell recovery and the recovery of macrophages, lymphocytes and eosinophils were increased in BAL from dox-treated transgenic mice (Fig. 2b). Mucus metaplasia, characterized by D-PAS and Alcian blue staining of airway epithelial cells, increased *Muc5ac* and epithelial *gob-5* (*Clca3*) gene expression (Fig. 2c and Supplementary Fig. 1 online) and myocyte hyperplasia with enlarged airway smooth muscle bundles (Fig. 2d) were seen after 7 d of dox. Histologic and biochemical increases in collagen were first seen after 2 months and were most prominent after 4 months of VEGF overexpression (Fig. 2d,e). At these time points, VEGF also stimulated the production and spontaneous activation of TGF-β_1 (Fig. 2f). Thus, VEGF is a potent stimulator of airway inflammation and airway remodeling with mucus metaplasia, subepithelial fibrosis and smooth muscle hyperplasia.

Responsiveness to methacholine was also assessed. When compared to wild-type mice given normal or dox water and transgenic mice given normal water, transgenic mice given dox water for as few as 7 d show impressive AHR (Fig. 2g and data not shown). Thus, VEGF induces AHR in the mouse lung.

Induction and role of IL-13

We also undertook studies to determine whether VEGF mediated its effects by stimulating T_H2 cytokines. The levels of mRNA encoding IL-4, IL-5 and IL-9 in wild-type and transgenic mice given normal water or dox water were near or below the limits of detection of our assays (data not shown). Similarly, mRNA encoding IL-13 could not be detected in RNA from wild-type mice given normal or dox water or transgenic mice given normal water (Fig. 3a and data not shown). In contrast, the lungs of transgenic mice given dox showed increased expression of IL-13 mRNA (Fig. 3a).

To define the role of IL-13 in the VEGF phenotype, we compared the effects of VEGF in *Il13*$^{+/+}$ and *Il13*$^{-/-}$ mice. In the absence of IL-13, the ability of VEGF to induce mucus metaplasia was completely abrogated (Fig. 3b). In contrast, VEGF induction of tissue and BAL inflammation, neovascularization, AHR, myocyte hyperplasia, subepithelial fibrosis, TGF-β_1 elaboration and dendritic cell alterations (see below) were unaltered in *Il13*$^{-/-}$ mice (Fig. 3c,d and data not shown). These studies document the IL-13 dependence of VEGF-induced mucus metaplasia. Thus, the preponderance of the VEGF phenotype is mediated through an IL-13–independent mechanism.

Effect on sensitization, T_H2 inflammation and DCs

To determine whether VEGF altered respiratory antigen sensitization, wild-type and transgenic mice were exposed to ovalbumin (OVA) by aerosol and systemic sensitization was assessed by quantifying OVA-induced splenocyte proliferation and OVA-specific IgG_1. OVA did not cause significant sensitization in wild-type mice given normal or dox water or in transgenic mice given normal water. It did, however, engender marked sensitivity in dox-treated transgenic mice (Fig. 4a,b). In agreement with this observation, transgenic mice showed increased BAL and tissue eosinophilic inflammation (Fig. 4c) and enhanced AHR (Fig. 4d) after aerosol antigen sensitization and challenge. Similar results were obtained when recombinant VEGF was administered with intranasal OVA to wild-type mice (data not shown). In both experimental systems, alterations in OVA-specific IgG_{2a} and keyhole limpet hemocyanin (KLH)–induced splenocyte proliferation could not be observed (data not shown). In addition, enhanced respiratory antigen sensitization was not seen when transgenic CC10-IL-11 mice that overexpress IL-11 were similarly challenged (data not shown). Thus, VEGF selectively increases T_H2 antigen sensitization and inflammation in the lung.

The number and state of activation of parenchymal DCs were similar in wild-type mice given normal or dox water and transgenic mice given normal water. In contrast, in transgenic mice, as little as 7 d of dox increased the number of $CD11c^{high}$ major histocompatibility (MHC) II^{high} DCs in pulmonary tissues (Fig. 4e). These cells had increased CD11b and decreased CD8α expression (Fig. 4f). These DC2 cells had the characteristics of activated DCs because they expressed increased levels of MHC class II, CD80/B7.1, CD86/B7.2 and CD40 and decreased levels of ICOSL/B.7h (Fig. 4f). Thus, VEGF increases the number and state of activation of DC2 cells in pulmonary tissues.

Role(s) of Vegf in T_H2 inflammation

Next we undertook studies to evaluate the role(s) of Vegf in antigen-induced T_H2 inflammation. Eosinophil-, lymphocyte- and macrophage-rich inflammation and AHR were seen in wild-type mice sensitized and challenged with OVA (Fig. 5a–c and data not shown). This response was associated with increased Vegf levels in BAL (5.9 ± 3.5 (range 5–15) pg/ml for controls versus 24.5 ± 2.2 (range 20–35) pg/ml for sensitized and challenged mice) ($P < 0.01$). This Vegf could be detected in $CD3^+$ T cells and in a variety of airway epithelial cells including CC10-positive Clara cells (Fig. 5d and Supplementary Fig. 2 online). Studies

using *in vitro*–polarized mouse T cells also showed that T_H2 cells are potent producers of Vegf when compared to T_H1 cells or naive $CD4^+$ cells when stimulated with antigen in the presence of antigen-presenting cells (APCs) or with PMA and ionomycin in the absence of APC (Fig. 5e and Supplementary Fig. 3 online). Notably, the VEGF inhibitor SU1498 markedly decreased BAL and tissue inflammation and AHR (Fig. 5a–c). Similar results were seen with the $VEGF_{R1R2}$ TRAP (Supplementary Fig. 4 online). SU1498 and the TRAP also decreased antigen-stimulated IL-13 and IL-4 production (Fig. 5f and data not shown). This response was not OVA-specific, because Vegf induction was readily observed and similar decreases in inflammation and AHR were seen in mice with a null mutation of the T-bet transcription factor that spontaneously develop an asthma-like phenotype[32] treated in a similar fashion (Fig. 5g,h and data not shown). Thus, Vegf is produced by epithelial cells and T_H2 cells and plays a critical role in T_H2 inflammation, cytokine elaboration and AHR.

Reversibility of the VEGF phenotype

To define the reversibility of VEGF-induced alterations, wild-type and transgenic mice were treated with dox for 2 weeks. They were then switched to normal water and characterized at intervals thereafter. Transgenic VEGF was a potent stimulator of inflammation, vascular remodeling, mucus metaplasia, mucin gene expression, DC alterations, smooth muscle hyperplasia and AHR. Two weeks after cessation of transgenic VEGF elaboration, BAL and tissue inflammation (Fig. 6a and data not shown), DC numbers, mucus metaplasia, mucin gene expression (Fig. 6b and data not shown) and angiogenesis (Fig. 6c) had returned to basal levels. In contrast, 1 month after the cessation of transgene expression, smooth muscle hyperplasia and AHR were still readily apparent in lungs from transgenic mice (Fig. 6d,e). Thus, many VEGF-induced alterations are readily reversible after the cessation of Vegf elaboration; however, VEGF-induced smooth muscle and physiologic abnormalities have a lesser propensity toward normalization.

DISCUSSION

Leonardo da Vinci is generally credited with the discovery that new blood vessels form at sites of pulmonary pathology[12]. It has since become clear that he was viewing bronchial vascular remodeling and angiogenesis at sites of infection, inflammation and neoplasia[12]. An increase in vessel size, number and surface area and the exaggerated expression of VEGF and VEGF receptors is well documented in the asthmatic airway[4–8,10,11,18,19]. Notably, the mechanisms of asthmatic vascular remodeling and the vascular and nonvascular contributions of VEGF to asthma pathogenesis have not been defined. To address this issue, we generated and characterized lung-targeted $VEGF_{165}$ transgenic mice and evaluated the role of VEGF in pulmonary T_H2 inflammation. These studies show that levels of VEGF that are comparable to those in human tissues and biologic fluids[18,19,29–31] induce an asthma-like phenotype characterized by inflammation, edema, angiogenesis, vascular remodeling, mucus metaplasia, subepithelial fibrosis, smooth muscle hyperplasia and AHR. They also demonstrate that VEGF enhances respiratory antigen sensitization, augments antigen-induced T_H2 inflammation, increases the accumulation and activation of pulmonary DCs and has a key role in antigen-induced T_H2 inflammation and cytokine elaboration. Prior studies from our laboratories and others demonstrated that VEGF is produced during innate immune responses induced by RSV and endotoxin[26,33]. Taken together, these studies demonstrate that VEGF has a critical role in pulmonary T_H2 inflammation, and provide important insights into a number of aspects of asthma pathogenesis.

First, these studies demonstrate that the VEGF produced during innate immune responses can generate asthma-like inflammation, airway and vascular remodeling and physiologic dysregulation. They also demonstrate that, in contrast to some earlier reports[20,21], VEGF is a

potent mediator of vascular remodeling and angiogenesis in the lung. It is presently believed that asthmatic airway remodeling is caused by chronic T_H2 inflammation. The present studies demonstrate that remodeling can also be caused by innate immune responses and highlight the importance of VEGF in the genesis of these alterations. This provides a potential explanation for the observation that airway remodeling can be seen in childhood asthma well before the ravages of chronic T_H2 inflammation would be expected[34].

Second, these studies demonstrate that VEGF augments respiratory T_H2 sensitization while simultaneously increasing tissue permeability and the number and activation of pulmonary DCs. These findings suggest that RSV and endotoxin may enhance antigen sensitization and T_H2 inflammation by inducing VEGF and that this induction may explain how RSV infection early in life contributes to the development of asthma[22,25]. The exaggerated levels of VEGF in asthma may also contribute to the proclivity of asthmatics to become sensitized to respiratory antigens. The ability of cockroach antigen to directly stimulate epithelial VEGF elaboration[14] may also account for the impressive levels of sensitization that are caused by even low-level exposure to this antigen[35]. Additional investigation will be required to determine whether VEGF-facilitated sensitization is mediated by the vascular leak, DC alterations and/or vascular alterations that it induces. It is clear, however, that VEGF can link innate and adaptive immunity by predisposing the lung (and possibly other organs) to antigen sensitization and, after antigen exposure, pathologic T_H2 cytokine production and inflammation. Previous studies from our laboratories demonstrated that IL-13 also stimulates lung Vegf production[36]. When viewed together, these studies define a positive feedback loop with VEGF enhancing T_H2 sensitization and inflammation and IL-13 subsequently enhancing VEGF production. This interaction may contribute to the severity and or chronicity of VEGF or IL-13–mediated disorders.

Lastly, in accord with studies in an isocyanate model[37], we demonstrated that VEGF signaling is required for antigen-induced T_H2 inflammation and AHR. In the present studies, we have added to these findings by demonstrating that specific Vegf neutralization abrogates this antigen-induced response and by characterizing the role(s) of Vegf in classic aeroallergen-induced and genetic asthma models. Notably, we have also defined the mechanism of this inhibition by demonstrating that VEGF is induced in both models, that Vegf is produced by epithelial cells and T cells in the allergen-challenged lung, that Vegf is selectively elaborated by T_H2 versus T_H1 cells and that Vegf is required for antigen-induced T_H2 cytokine elaboration. These effects of VEGF may relate to its ability to increase and activate DCs. In fact, our studies demonstrated that VEGF increases the number of DC2 cells and generates change in DC phenotype (B-7^{low} ICOS-L^{high} to B-7^{high} ICOS-L^{low}) similar to that seen in the allergic antigen-challenged lung[38]. Alternatively, VEGF may have previously unappreciated regulatory effects on T cells, which are known to express the VEGF receptors[10]. On superficial analysis, these findings would appear to conflict with studies that suggest that VEGF inhibits T cell development, is induced during T_H1 inflammation and inhibits DC function[39,40]. It is important to point out, however, that these studies focused on the role of VEGF in antitumor and mycobacterial responses and that a T_H1-to-T_H2 or DC1-to-DC2 shift, such as is described here, would appear to be inhibitory in those settings.

Our studies demonstrate that VEGF-induced alterations differ in their degree of VEGF dependence with inflammation, mucus metaplasia, angiogenesis and DC alterations reversing rapidly, whereas smooth muscle hyperplasia and AHR did not reverse over similar intervals. These findings suggest that therapies that inhibit VEGF can ameliorate inflammation, angiogenesis and mucus responses, even in people with established disease. If VEGF-induced DC alterations have an essential role in pulmonary antigen sensitization and T_H2 inflammation, it is reasonable to believe that anti-VEGF–based therapies will also

ameliorate these responses. The demonstration that VEGF-induced smooth muscle and physiologic alterations are relatively less VEGF-dependent also has important implications. First, it suggests that these responses may not reverse or may require longer periods of time to reverse with anti-VEGF–based therapies. It also demonstrates that even short periods of VEGF expression can have long-lasting tissue remodeling and physiologic consequences. Lastly, it provides pathogenic mechanisms that can account for the dissociation of inflammation and AHR and the persistent AHR and remodeling that are seen in asthma and chronic antigen-driven experimental systems[41].

In summary, these studies indicate that VEGF is a potent stimulator of inflammation, airway and vascular remodeling and physiologic dysregulation that augments antigen sensitization and T_H2 inflammation and increases the number and activation of DCs. They also demonstrate that these effects are mediated by IL-13–dependent and –independent pathways and highlight the impressive reversibility of some, but not all, of these VEGF-induced responses. Lastly, they show that VEGF production is a critical event in T_H2 inflammation and T_H2 cytokine elaboration and that epithelial cells and T_H2 cells are potent producers of VEGF in the antigen-challenged lung. These findings demonstrate how asthma-relevant responses can be induced by innate as well as adaptive inflammation and highlight mechanisms by which innate immune responses can predispose to antigen sensitization and T_H2 inflammation. Thus, these findings provide a rationale for the use of VEGF regulators to prevent and or treat asthma and other T_H2-dominated disorders.

METHODS

Transgenic mice

The CC10-rtTA-VEGF mice in these studies used the Clara cell 10 kDa protein (CC10) promoter and two transgenic constructs to target VEGF to the lung in a way that could be regulated externally. These mice were generated using approaches previously described by our laboratories[28]. Construct 1, CC10-rtTA-hGH, contains the CC10 promoter, the reverse tetracycline transactivator (rtTA) and human growth hormone (hGH) intronic and polyadenylation sequences (Fig. 1a). Construct 2, tet-O-CMV-VEGF-hGH, contains a polymeric tetracycline operator (tet-O), minimal cytomegalovirus (CMV) promoter, human $VEGF_{165}$ cDNA and hGH (Fig. 1a).

All mice were evaluated for the presence of both rtTA and $VEGF_{165}$ using PCR analysis. PCR for $VEGF_{165}$ used the following primers: sense 5′-CCTCCGCGGCCATGAACTTT-3′ and antisense 5′-TCTTTCCGGATCCGAGATCTGG-3′. All founders were bred for at least eight generations onto a C57BL/6 background.

Dox water administration and phenotypic analysis

One-month-old transgenic and wild-type littermate controls were randomized to receive normal water or water containing doxycycline (dox) (0.5 mg/ml) as described[28] and evaluated at intervals thereafter. BAL VEGF quantification, histologic analysis, mRNA analysis, *in situ* hybridization, immunohistochemistry (IHC), collagen quantification, and the quantification of total and bioactive TGF-β_1 were done as previously described[28,36,42,43]. Airway methacholine responsiveness was assessed using noninvasive whole body phlethysmography as previously described[44].

Breeding to *Il13*$^{-/-}$ mice

CC10-rtTA-VEGF mice were bred with wild-type and *Il13*$^{-/-}$ mice (from A.N.J. McKenzie) as previously described[42]

Staining of airway microvasculature

The vasculature was labeled by perfusion with *L. esculentum*, a lectin that binds uniformly to the luminal surface of endothelial cells, as described previously[45,46].

Electron microscopy

After vascular perfusion with paraformaldehyde and glutaraldehyde, tissues were fixed, sectioned, treated with osmium tetroxide followed by aqueous uranyl acetate, dehydrated in acetone, and embedded in epoxy. We stained 0.5 μm sections with toluidine blue for light microscopy, and 50–100 nm sections with 0.8% lead citrate for electron microscopy.

Calculation of wet/dry ratios

Lungs were excised *en bloc* and extrapulmonary tissues were removed. They were then weighed, placed in a desiccating oven at 65 °C for 48 h and reweighed.

Evans blue dye extravasation

Evans blue dye (EBD) was used to assess plasma leakage as previously described[20]. The results are expressed as the ratio of the EBD absorbance at 620 nm of paired lung homogenates and serum.

Evaluation of antigen sensitization

The effects of transgenic VEGF, transgenic IL-11 (ref. 47) or recombinant $VEGF_{165}$ were evaluated. In the case of transgenic VEGF, wild-type and transgenic mice were randomized to normal or dox water. Two weeks later, mice were exposed to aerosolized OVA (2%, grade V, Sigma) twice a day for 10 d. In the case of recombinant VEGF, wild-type mice received ten daily doses of intranasal recombinant VEGF (10 μg/mouse) or vehicle followed 20 min later by the same dose of OVA. After an additional week, representative mice were killed and serum IgG_1 and IgG_{2a} were quantified. The remaining mice received intranasal OVA (25 μg/mouse) and inflammation was assessed 48 h later.

Splenocyte proliferation assay

Splenocytes were harvested 2 d after the last OVA challenge and were incubated (5×10^5 cells/well) in 96-well flat-bottom plates at 37 °C for 48 h in medium alone or with 1, 5, 10, 50 or 100 μg OVA/well. KLH (Pierce) was the antigen control. After 18 h of culture with 1 μCi [^{3}H]thymidine, cellular thymidine incorporation was assessed.

DC analysis

Single cell suspensions from lungs from wild-type and transgenic mice on normal or dox water were prepared as described previously[38]. FACS analysis was performed with monoclonal antibodies from BD Biosciences Pharmingen: anti-I-Abb-biotin, anti-CD3-FITC, anti-CD4-PE (GK1.5), anti-CD8α-PE (53-6.7), anti-CD11b-APC-Cy7 (M1/70), anti-CD11c-FITC, anti-CD40-PE, anti-CD80-PE, anti-CD86-PE and anti-B220-PE. PE-labeled anti-B7h/ICOS-L monoclonal antibodies were obtained from eBioscience. PE-conjugated rat IgG_{2a} and rat IgG_{2b} were isotype controls. SAV-PerCP was used as a second-step reagent. When necessary, cells were preincubated with anti-CD16/CD32 monoclonal antibodies to block cell surface Fc receptors. Cells gated by forward- and side-scatter parameters were analyzed on a FACScalibur flow cytometer (Becton Dickinson) using CELLQuest software.

Role of VEGF in OVA- and T-bet null–stimulated inflammation

OVA sensitization and challenge were undertaken in the presence and absence of the VEGF receptor inhibitor SU1498 (ref. 48) (10 mg/kg/d) as previously described[49]. In selected

experiments the VEGF $Trap_{R1R2}$ (Regeneron) (25 mg/kg), a fusion protein containing the extracellular domains of $VEGF_{R1}$ and $VEGF_{R2}$ coupled to the Fc-portion of IgG[50], was used. T-bet null mutant mice[32] (Jackson Laboratories) were randomized to SU1498 or control vehicle, and 2 weeks later phenotypic characterization was undertaken as described above.

T_H1 and T_H2 cell Vegf production

As previously described by our laboratories[51], CD4 T cells from OT-II mice that are transgenic for OVA-specific TCR were generated and polarized *in vitro* into T_H1 or T_H2 cells. They were then washed and incubated either in the presence and absence of APCs (syngeneic T-depleted splenocytes) and antigen ($pOVA_{323–339}$, 15 μg/ml) for 24 h, or with phorbol-12 myristate 13-acetate (10 ng/ml, Sigma) and ionomycin (500 ng/ml, Sigma). Cell supernatant VEGF was quantified by ELISA as described above.

Assessment of reversibility

VEGF transgenic mice were randomized to dox or normal water for 2–4 weeks and then placed on normal water. VEGF-induced alterations were assessed at the end of the period of dox administration and after 2–4 weeks of normal water.

Statistics

Data were assessed using the Student's *t*-test, ANOVA or Wilcoxon rank sum test as appropriate. Data are expressed as mean ± s.e.m. and are representative of evaluations in a minimum of six mice.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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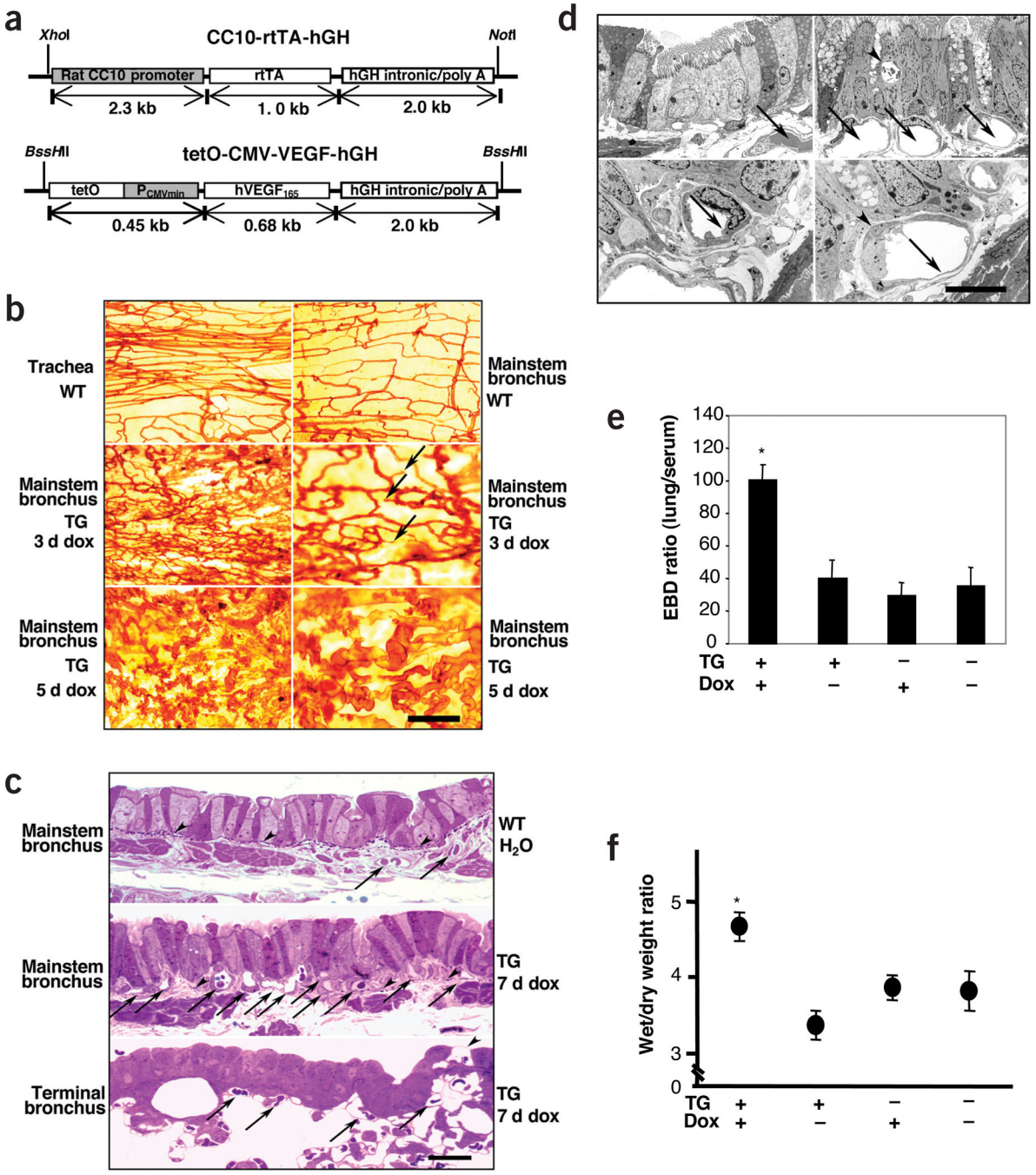

Figure 1.

Consequences of transgenic VEGF expression. (**a**) Constructs in CC10-rtTA-VEGF mice. (**b**) *Lycopersicon esculentum* staining (arrows, endothelial sprouts; scale bar, 100 μm in upper, middle and lower left subpanels, and 50 μm in middle and lower right subpanels). (**c**) Toluidine blue–stained bronchi from wild-type (WT) and transgenic (TG) mice. (Arrows, blood vessels; arrowheads, subepithelial elastic lamina; scale bar, 20 μm.) (**d**) Electron micrographs of tissue from WT mouse on normal water (upper and lower left) and TG mouse given dox for 7 d (upper and lower right) (In upper panels, arrows, blood vessels; arrowhead, epithelial vacuole). Endothelial cell in lower left has a thick wall, whereas that in

lower right has a thin wall and pericyte processes (arrowheads). Scale bar, 25 μm in upper and 10 μm in lower subpanels. (**e**,**f**) Evans Blue dye (EBD) and wet/dry ratios compare the permeability in WT and TG mice given normal or dox water for 7 d (*$P < 0.01$ versus the other three groups.)

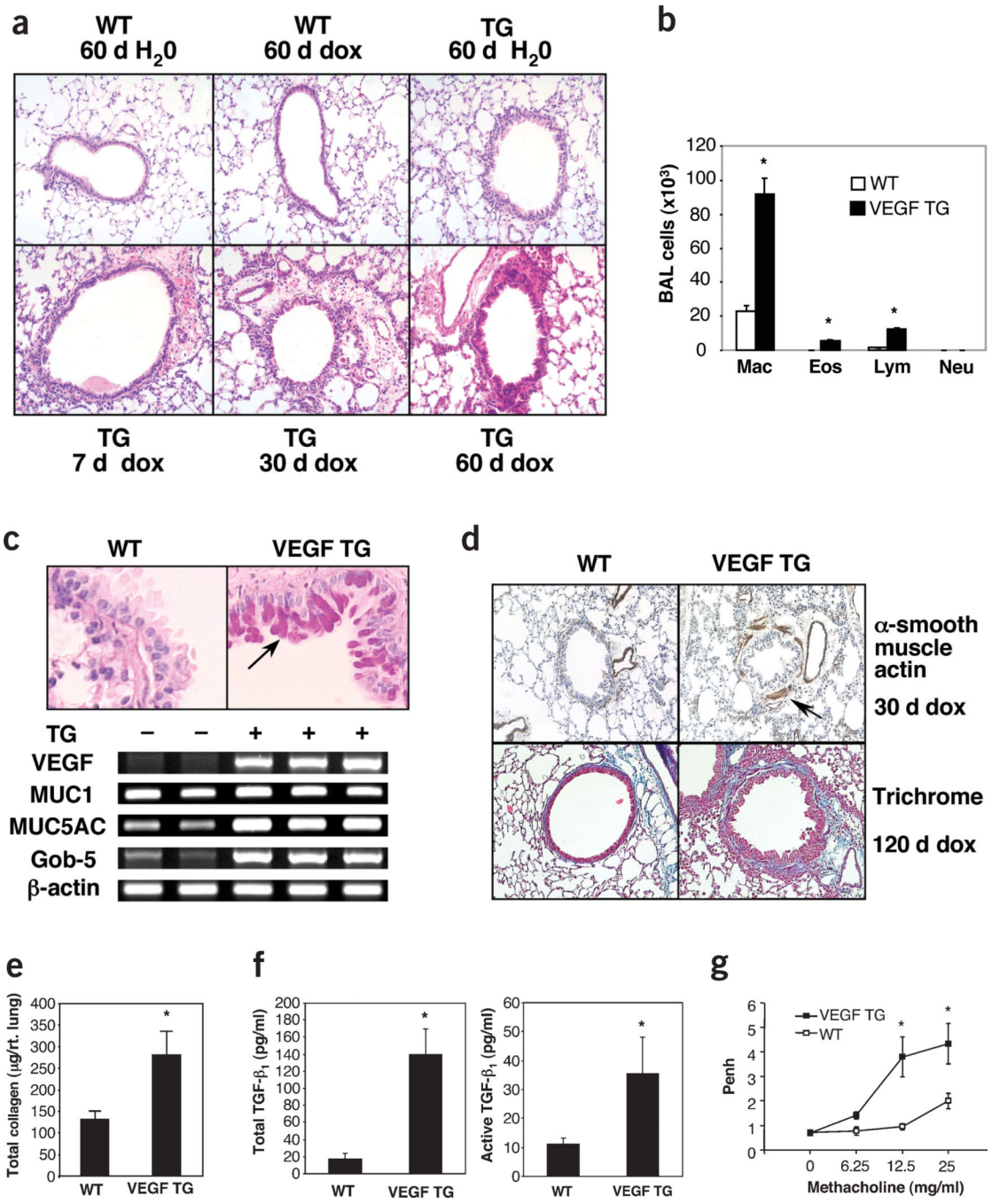

Figure 2.

Structural alterations in transgenic (TG) mice. (**a**) H&E staining (×10 original magnification) for wild-type (WT) and TG mice given dox for the noted intervals. (**b**) BAL from WT and TG mice given dox for 1 month (*$P < 0.05$ versus same cells in WT). (**c**) Levels of mucus (top) and mucin and *gob-5* mRNA (bottom) were evaluated with D-PAS staining and RT-PCR evaluations (arrow, epithelial staining) (×20). (**d**) α-smooth muscle actin immunohistochemistry and tissue fibrosis (Mallory's trichrome staining) are compared in lungs from WT and TG mice (×10). (**e**) Sircol evaluations were used to compare the collagen content of lungs from WT and TG mice given dox for 4 months (*$P < 0.01$). (**f**)

ELISA evaluations of acid-activated (left) and untreated (right) BAL fluids from WT and TG mice given dox for 4 months (*$P < 0.01$). (**g**) Methacholine responsiveness of WT and TG mice given dox for 7 d is compared (*$P < 0.01$).

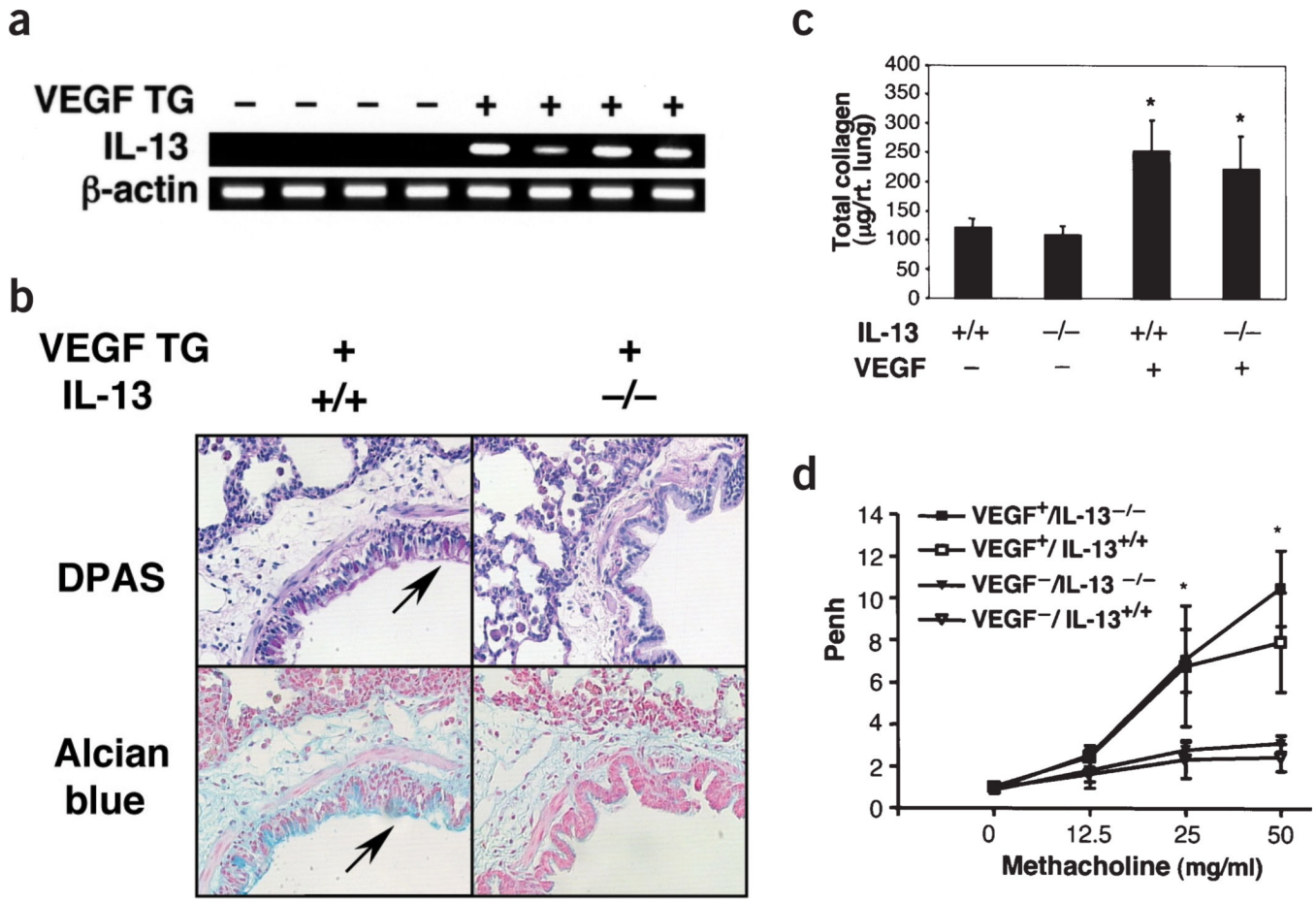

Figure 3.

Role of IL-13 in the VEGF phenotype. (**a**) RT-PCR was used to evaluate the levels of IL-13 mRNA in lung RNA from wild-type (WT) and transgenic (TG) mice given dox for 1 month. (**b**–**d**) Ability of VEGF to induce mucus metaplasia (×20), pulmonary fibrosis and AHR, respectively, are compared in mice with wild-type (*Il13*$^{+/+}$) and null mutant (*Il13*$^{-/-}$) loci. The arrows in **b** highlight positive-staining cells. In **c**, **P* < 0.01 versus VEGF^{-} mice. In **d**, **P* < 0.05 versus VEGF^{-} controls.

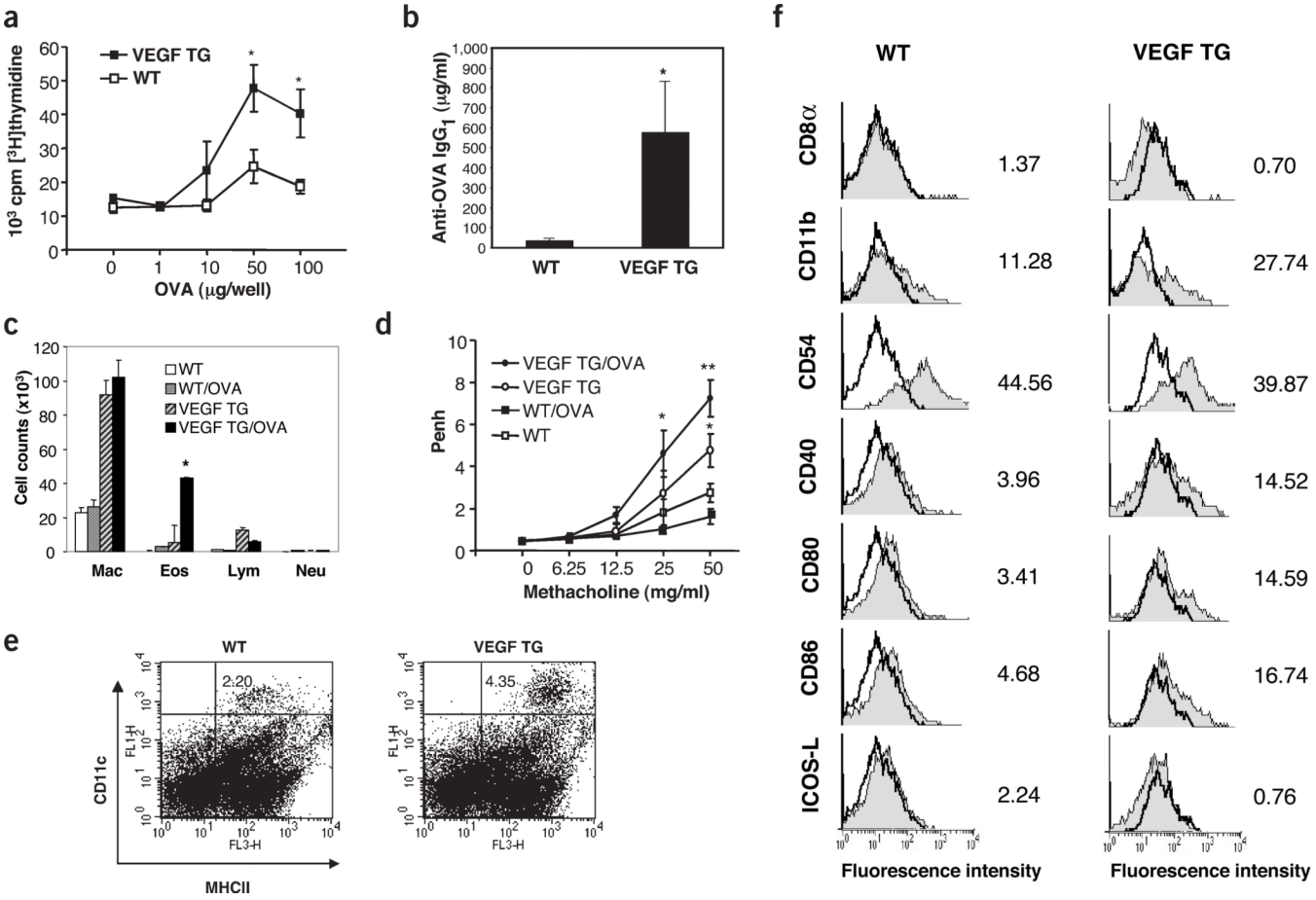

Figure 4.

Immune effects of VEGF. Effect of intranasal OVA in wild-type (WT) and transgenic (TG) mice treated with dox for 2 weeks. (**a**,**b**) OVA-induced spleen cell proliferation (**a**) and OVA-specific IgG_1 (**b**) were assessed (*$P < 0.01$). (**c**,**d**) Dox-treated WT and TG mice were challenged with intranasal OVA, re-challenged after 7 d and, 48 h later, BAL cellularity (**c**) and AHR (**d**) were evaluated. (In **c**, *$P < 0.001$ versus eosinophils in other groups; in **d**, *$P < 0.01$ versus WT and **$P < 0.05$ versus TG mouse challenged with vehicle.) (**e**,**f**) FACS analysis of lung cells isolated from WT mice and TG mice given dox for 7 d and stained with antibodies directed against CD11c, MHC II and with other antibodies as indicated. In **f**: Cells were sorted for CD11c. $CD11c^+$ cells were incubated with test antibodies (gray) and control antibodies (transparent).

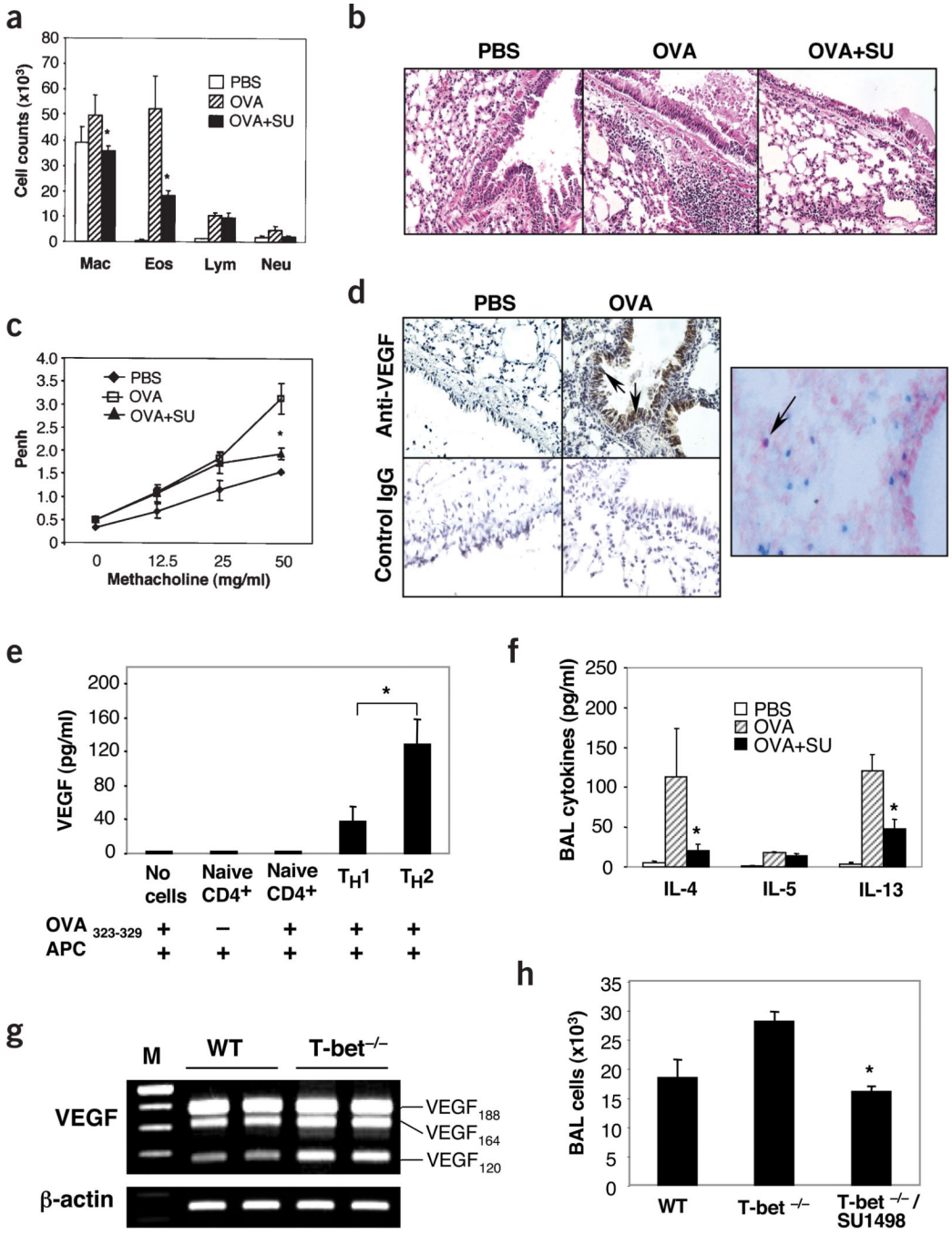

Figure 5.

VEGF in T_H2 inflammation. (**a**–**c**) Comparison of BAL cellularity (**a**), histology (×20; **b**) and AHR (**c**) of mice that were sensitized with OVA and then challenged in the presence or absence of SU1498 (SU) (*$P < 0.01$ versus the absence of inhibitor). (**d**) Staining with anti-VEGF and control antibodies in sensitized mice challenged with OVA or PBS (×20) (left). Right, staining with anti-VEGF (red) and anti-CD3 (blue) (×40). The arrow highlights a VEGF-positive T cell. (**e**) VEGF was quantified by ELISA in supernatants from naive $CD4^+$ cells or polarized T_H1 or T_H2 cells cultured in the presence of APC and in the presence or absence of antigen ($OVA_{323–329}$ peptide). (**f**) Levels of T_H2 cytokines in BAL from OVA-

sensitized mice challenged with OVA in the presence or absence of SU1498. (*$P < 0.01$ for presence versus absence of SU1498.) (**g**) Level of the mRNA encoding VEGF in lungs from wild-type (WT) and T-bet null (T-bet$^{-/-}$) mice. (**h**) BAL cellularity of WT and T-bet$^{-/-}$ mice treated with SU1498 or vehicle control (*$P < 0.01$).

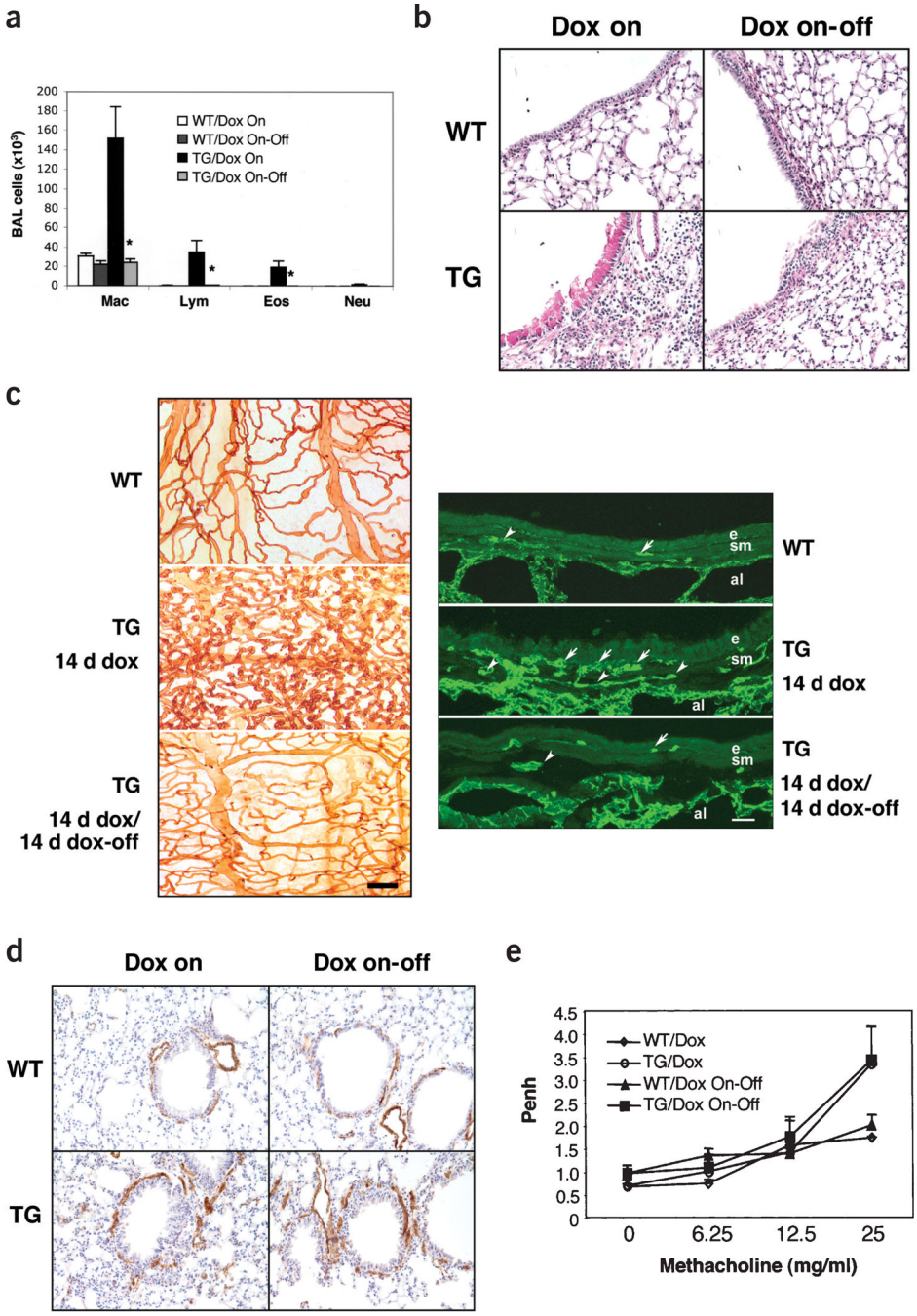

Figure 6.

Reversibility of VEGF effects. (**a**–**e**) Wild-type (WT) and transgenic (TG) mice received dox water for 2 weeks. They were then killed or placed on normal water and evaluated 2 (**a**–**c**) or 4 weeks (**d**,**e**) later. (**a**) Effects of transgene deactivation on BAL cell recovery (*$P <$ 0.01 versus TG mice given dox for 2 weeks); (**b**) mucus metaplasia (×20); (**c**) vascular remodeling, comparing *L. esculentum* evaluations (left) and CD 31 IHC (right); (**d**) smooth muscle hyperplasia (×10); and (**e**) AHR are illustrated. Scale bar in **c**, 100 μm on the left and 20 μm on the right. Arrows, CD31-positive endothelial cells. e, epithelium; sm, smooth muscle cells; a, alveolar space.