

Regulatory T Cells Negatively Regulate Neovasculature of Airway Remodeling via DLL4-Notch Signaling¹

Miao-Tzu Huang,* Yang-Shia Dai,[†] Yu-Bin Chou,* Yi-Hsiu Juan,[‡] Chih-Chiang Wang,* and Bor-Luen Chiang^{2§¶}

Regulatory T cells (Treg) have been shown to prevent the development of allergic asthma; however, the role of Treg in asthma with established airway remodeling is unknown. To address this, we exploited an OVA-induced chronic asthma mouse model wherein Treg were adoptively transferred to the mice at chronic stage of the model. We found that among the structural alterations of airway remodeling, Treg selectively reduced the vessel numbers in both peritracheal and peribronchial regions and the lung parenchyma. Extracellular matrix deposition, mucus metaplasia, muscular hyperplasia, and vasodilation, as were also induced by chronic allergen challenge, were not affected by Treg. TUNEL staining of the lung sections revealed an increased endothelial cell (EC) apoptosis in mice receiving Treg transfers compared with their asthmatic counterparts. By using Matrigel angiogenesis assays, we showed that Treg inhibited EC angiogenesis both in vitro and in vivo. Treg preferentially expressed Notch ligand DLL4, and an anti-DLL4 blocking Ab abrogated the inhibitory effect of Treg on EC tube formation. In vivo, decreased airway and lung vessel numbers as well as ameliorated airway hyperresponsiveness after Treg transfers were reverted when Treg-derived DLL4 signal was blocked by the anti-DLL4 Ab. Our findings demonstrate a novel function of Treg whereby Treg down-regulate remodeling angiogenesis via proapoptotic DLL4-Notch signaling, and suggest a therapeutic potential of Treg in alleviating airway hyperresponsiveness of chronic asthma. *The Journal of Immunology*, 2009, 183: 4745–4754.

The mammalian Notch family molecules include four Notch receptors, Notch1–4, and five Notch ligands, Jagged1, Jagged2, DLL1, DLL3, and DLL4. Notch activation has been identified as an important signaling pathway in the development of many cell populations (1, 2). Differentiation and function of peripheral T lymphocytes are also regulated by Notch signaling (3, 4). In the context of regulatory T cells (Treg),³ they were found to differentially express Notch3 and Notch4, and from varied data of different groups, Jagged1, DLL1, and DLL4 (5, 6). In addition, the differentiation and expansion of Treg were reportedly induced by Jagged engagement and Notch3 activation (7–10). The contact-dependent immune suppressive function of Treg can also be mediated by Notch signaling pathway. To this end, it was found that Treg triggered Notch1 activation of the target cells by membrane-bound TGF- β , and blockage of Notch1 signaling re-

versed the immune suppression function of Treg (6). In accordance with these findings, the induction of tolerance by high-dose intranasal peptide delivery in a murine model was accompanied by the emergence of DLL1-expressing T cells that inhibited neighboring T cell activation through DLL1-Notch1 interaction (11).

Notch signaling pathway also regulates multiple aspects of vasculogenesis and angiogenesis, including endothelial cell (EC) proliferation and migration, smooth muscle differentiation, and vascular patterning (12, 13). It has been shown that Notch1 and DLL4 expression of the EC was enhanced by vascular endothelial growth factor (VEGF) and basic fibroblast factor (14, 15), and VEGF function was, in a negative feedback manner, inhibited by the up-regulated DLL4 through down-regulation of EC VEGF-R2 expression (16). In the context of allergic asthma, VEGF has been shown to induce airway remodeling and enhance Th2-mediated lung inflammation (17). The negative regulatory role of DLL4 in VEGF function therefore suggests that DLL4 signaling pathway may play a protective role in asthma by limiting the vicious cycle of inflammation remodeling maintained at least in part by VEGF.

Airway remodeling in asthma is elicited by repeated acute or chronic allergen exposures that persist beyond resolution of allergen-induced inflammation, occurs early in childhood asthma, and may even predate the onset of symptoms (18–20). A causal association is believed to exist between airway remodeling and sustained functional impairment in asthma (21–23). The structural alterations of airway remodeling include epithelial hyperplasia, goblet cell metaplasia, airway smooth muscle (ASM) hyperplasia, and deposition of extracellular matrix (ECM). In addition, airway remodeling also includes increased subepithelial vascularity and microvascular leakage. In human asthma, both dilated vessels and increased vessel numbers of the airways were reported by studies of resected lungs and endobronchial biopsies (24–29). The vascular changes were positively associated with submucosal deposition of angiogenic factors and were found to correlate with severity of asthma, suggesting an active angiogenic process and a pathogenic

Departments of *Medical Research, [†]Dermatology, [‡]Internal Medicine, and [§]Pediatrics, National Taiwan University Hospital, Taipei, Taiwan; and [¶]Graduate Institute of Clinical Medicine, School of Medicine, National Taiwan University, Taipei, Taiwan
Received for publication December 31, 2008. Accepted for publication August 5, 2009.

The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked *advertisement* in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

¹ This work was supported by grants from the National Taiwan University Hospital (NTUH-94A21, NTUH-95A08, NTUH-96A07, and NTUH-97A02) and the National Science Council, Taiwan (NSC94-2312-B-002-009, NSC95-2320-B-002-059, and NSC96-2314-B-002-037-MY3).

² Address correspondence and reprint requests to Dr. Bor-Luen Chiang, National Taiwan University Hospital and Graduate Institute of Clinical Medicine, School of Medicine, National Taiwan University, No. 7, Chung-Shan S RD, Taipei, Taiwan 100. E-mail address: gicmbor@ntu.edu.tw

³ Abbreviations used in this paper: Treg, regulatory T cell; α -SMA, α -smooth muscle actin; AHR, airway hyperresponsiveness; ASM, airway smooth muscle; BALF, bronchoalveolar lavage fluid; EC, endothelial cell; ECM, extracellular matrix; MPEC, murine pulmonary EC; ProCol, procollagen; Teff, effector T cell; VEGF, vascular endothelial growth factor.

Copyright © 2009 by The American Association of Immunologists, Inc. 0022-1767/09/\$2.00

role of remodeling angiogenesis in chronic asthma. Nonetheless, the pathogenesis and therapeutic implications of the vascular alterations in asthmatic airway remodeling are just beginning to be appreciated, and it was only recently that active angiogenesis with mobilization of endothelial progenitor cells into asthmatic lungs was demonstrated in animals (30–32).

Imbalanced Th1/Th2 immune microenvironment in asthma has been attributed to inadequate function of Treg (33–35), and reintroduction of Treg to asthmatic subjects suppresses Th2 response and reverts the asthma phenotype (36–38). However, despite the knowledge of Treg in alleviation and prevention of acute allergic asthma, their role in chronic asthma with established airway remodeling has not been thoroughly investigated. In the present study, we exploited a murine model of airway remodeling with adoptive Treg transfer. We found that Treg selectively reduced airway and pulmonary neovasculature via mechanisms involving EC apoptosis. We demonstrated that Treg preferentially expressed Notch ligand DLL4 and blockage of DLL4-Notch signaling abrogated the apoptotic antiangiogenic effects of Treg. Collectively, we identify a new role for Treg in chronic airway inflammation whereby Treg inhibited angiogenesis of airway remodeling via DLL4-Notch signaling pathway.

Materials and Methods

Mice

Female BALB/c and OVA_{323–339}-specific DO11.10 mice were purchased from The Jackson Laboratory and bred in the National Taiwan University animal facility. Animal procedures were reviewed and approved by the National Taiwan University Animal Ethics Committee.

OVA-induced chronic airway inflammation

Chronic airway inflammation was induced according to the schematic protocol shown in Fig. 1A. Briefly, BALB/c mice were sensitized by two i.p. injections of 50 and 20 μ g of OVA (grade V; Sigma-Aldrich) emulsified in 1% alum (Pierce) on days 0 and 12, followed by aerosol OVA (5%) challenge for 30 min everyday for 5 days and alternate day for 4 wk. Control mice were given PBS as the same schedule. One week after the last OVA challenge, mice were given three weekly i.v. transfers of $1 \sim 1.5 \times 10^6$ Treg or effector T cells (Teff) from OVA-fed DO11.10 mice. When indicated, Treg were preincubated with the anti-DLL4 blocking Ab, YW152F (30 μ g/ml; a gift provided by M. Yang, Genentech, South San Francisco, CA), or a human IgG1 isotype control Ab (Alexis) for 30 min before transfer.

Measurement of airway hyperresponsiveness (AHR)

AHR manifested as increased lung resistance was measured by the Buxco Pulmonary Mechanics System, according to manufacturer's instructions (Buxco Electronics). Briefly, mice were anesthetized and a tracheal cannula was inserted via tracheotomy. Mechanical ventilation with 150 breaths/min, tidal volume of 0.3 ml, and positive end-expiratory pressure of 4 cm H₂O was provided via the tracheal cannula, and increasing concentrations of methacholine (0–50 mg/ml) were administered via the aerosol delivery system after 5-min equilibration. Pulmonary parameters were recorded for 3 min after 3-min exposure of aerosol methacholine. The measurements were analyzed by using the BioSystem XA software, and lung resistance was expressed as cmH₂O $\times$ s/ml.

Isolation of CD4⁺CD25⁺ Treg

DO11.10 mice were fed with OVA-containing drinking water (20 mg/ml) for 5 days, and splenic CD4⁺CD25⁺ Treg were isolated by using the MACS Treg isolation kit (Miltenyi Biotec). Treg purity was examined by flow cytometry, and a purity of at least >90% was required for each adoptive transfer. For isolation of CD4⁺CD25⁺ T cells from the lung tissues, leukocytes were first released from the tissues by digestion with collagenase-IV (1 mg/ml) and DNase-I (0.5 mg/ml) at 37°C for 40 min, followed by purification of CD4⁺CD25⁺ T_H1, as described above. Isolated CD4⁺CD25⁺ T cells were stained with CD4 and FoxP3 (eBioscience) and examined with flow cytometry to investigate the percentage of FoxP3-expressing Treg in the CD4⁺CD25⁺ T cell pool.

Cell proliferation

T cells (5×10^4) and irradiated APC (1×10^5) were cocultured with 200 μ g/ml OVA, pulsed with 1 μ Ci of ³H at 72 h, and quantified for [³H] thymidine incorporation 16 h later. Data compared against unstimulated cells were calculated as cpm (stimulated cells – unstimulated cells)/cpm (unstimulated cells – medium only), and were expressed as stimulation index.

Flow cytometry

Surface and intracellular staining were performed as instructed (eBioscience and BD Biosciences). Fluorescence intensity of Ab was compared with that of isotype control and expressed as relative fluorescence intensity.

Histology

Paraffin-embedded lung and trachea sections were stained by H&E for general histology study. Goblet cells were examined by periodic acid-Schiff and were stained in red. Connective tissues were assessed by Masson's trichrome, which labeled muscle and mature fibrins in red, collagen and old fibrins in blue, and RBC in yellow.

Immunohistochemistry

Lung tissues were fixed with zinc fixatives and carefully cut into appropriate blocks for each lobe at the same way and embedded in paraffin. Lung sections were blocked with 10% normal serum, followed by sequential incubation with primary Abs (CD31, MEC13.3, BD Pharmingen; α -smooth muscle actin (α -SMA) and α -calponin, Sigma-Aldrich; procollagen (ProCol)-I, M60, Santa Cruz Biotechnology), biotinylated secondary Abs, HRP, or AP-conjugated avidin-biotin complex (Vector Laboratories), and visualized by AEC or fast red (Sigma-Aldrich). Vessels were identified by CD31 immunoreactivity, and vessel size was measured by the shortest internal diameter of the vessels. Peritracheal vessels were defined as those internal to tracheal cartilages, peribronchial vessels as those in immediate vicinity of bronchioles, and the lung vessels as those associated with respiratory bronchioles, respiratory ducts, and the alveoli. For α -SMA staining, the area of positive labeling was compared with the length of associated airway epithelium and expressed as α -SMA index. ProCol-I expression was quantified by measuring the staining intensity against a selected intensity threshold by using the Image ProPlus software.

TUNEL staining

EC apoptosis was detected by TUNEL staining, as per manufacturer's instructions (Calbiochem), followed by CD31 labeling of the vessels. The apoptotic EC were identified by dark-brown staining of the nuclei located within the vessel walls labeled in red. The numbers of TUNEL⁺ EC of the whole lung section were normalized by perimeter of individual vessel. Calculation of TUNEL⁺ EC and quantification of other immunohistochemistry staining were performed by two persons, with one blinded. The data were consistent between the two observers.

Isolation of murine pulmonary EC (MPEC)

Lung tissues were digested with collagenase A plus trypsin, incubated with anti-CD105 Ab (MY7/18, 10 μ g/ml; eBioscience), and positively selected by MACS magnetic secondary Ab (Miltenyi Biotec). MPEC were cultured with 10% DMEM containing EC growth supplement in tissue culture wares precoated with gelatin (Sigma-Aldrich). MPEC was identified by the characteristic cobble-stone morphology and by positive CD105 expression when examined by flow cytometry. A purity of >95% was constantly obtained by this protocol.

Matrigel tube formation assay

MPEC were seeded onto Matrigel (BD Bioscience) at 0.3×10^5 cells/well, and branches from each EC were quantified at 4 h. In indicated experiments, MPEC were preincubated 16 h before assay with recombinant Jagged1-Fc or DLL4-Fc (10 μ g/ml; R&D Systems), Treg, Teff, or Ab-treated Treg (anti-DLL4 Ab, Genentech; polyclonal anti-Jagged1 Ab, Abcam; 30 μ g/ml).

Matrigel plug assay

Matrigel was gently mixed with 1.5×10^6 Treg or Teff and s.c. injected at flank of the mice. Matrigel plugs were harvested and stained with H&E 7 days later. Vascular lumen in five representative $\times 100$ fields from each of three consecutive sections was measured by using the Image ProPlus software and presented as percentage of vessel-occupying area/ $\times 100$ field.

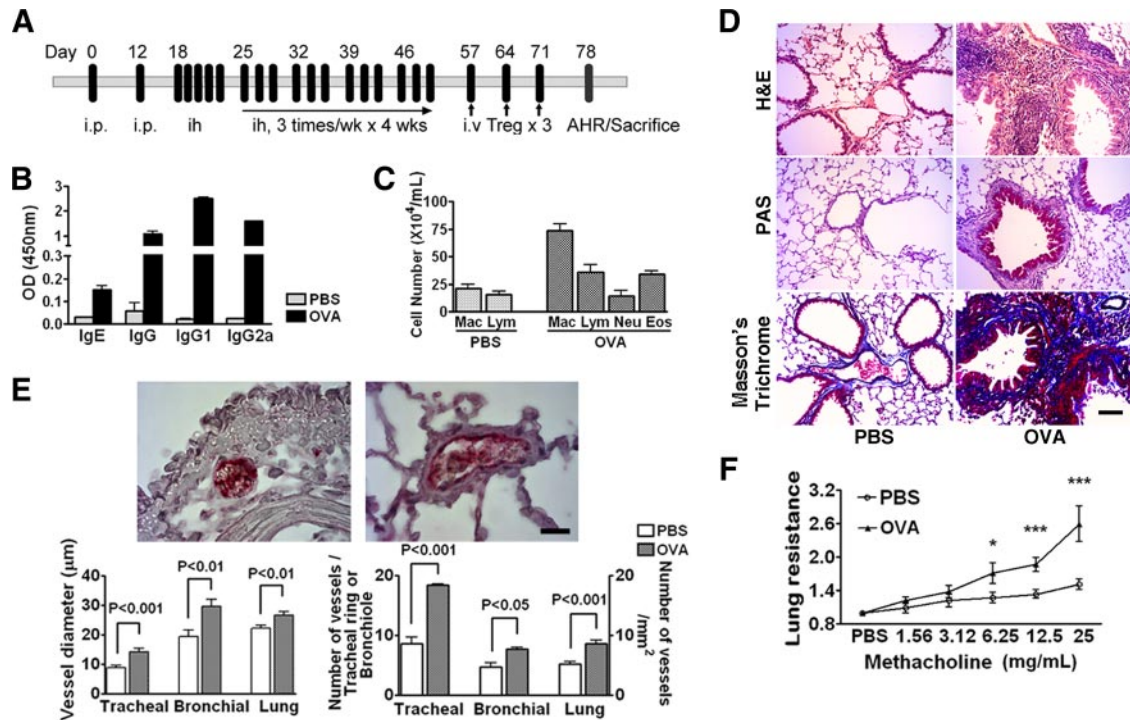


FIGURE 1. Establishment of chronic allergic airway inflammation. Mice were sensitized and challenged, as described in *Materials and Methods*. Unless indicated, immunologic and histopathologic profiles were examined at the end of the fourth week, alternate-day OVA exposure (days 53–55). *A*, Study protocol of chronic airway inflammation. *B*, OVA-specific Abs in sera measured by ELISA during chronic phase of OVA exposure, d37. *C*, BALF leukocytes were determined by cytopspins. A total of 500 cells was counted for each slide. *D*, Photomicrographs showing lung sections stained with H&E, periodic acid-Schiff, and Masson's trichrome, respectively. Scale bar = 100 μ m. *E*, Photomicrographs showing peritracheal (left) and pulmonary blood vessels (right) labeled in red by anti-CD31 immunostaining. Bars showed vessel sizes (left) and total vessel numbers (right). Scale bar = 20 μ m. *F*, AHR was recorded through an intratracheal cannula while the animals were exposed to increasing concentrations of methacholine (0–25 mg/ml). Graphs are representative of four separate experiments; five to six mice/group.

Ab, cytokine, and protein analysis

OVA-specific Abs in sera were measured by ELISA, as previously described (39). Cytokine expression in bronchoalveolar lavage fluid (BALF), lung homogenates, or culture supernatants was measured by ELISA kits, per manufacturer's instruction (R&D Systems). Expression of CD105 (MY7/18; eBioscience), α -SMA, and ProCol-I in lung homogenates was detected by Western blotting, and integrated density was measured with ChemImager 5500 (Alpha Innotech). Alternatively, cDNA was amplified with ProCol gene-specific primers and SYBR supermix (Applied Biosystems) in an ABI Prism 7900 system. Data were normalized with GAPDH expression.

Statistical analysis

Data were expressed as mean $\pm$ SD. Statistical comparisons between groups were made by ANOVA or unpaired Student's *t* test. A *p* value of less than 0.05 was considered significant.

Results

Repeated allergen exposure induces chronic airway inflammation and airway remodeling

Chronic airway inflammation was induced according to protocol depicted in Fig. 1A. Production of OVA-specific Abs, recruitment of leukocytes, including eosinophils, neutrophils, and mononuclear cells, and characteristic histologic changes, including bronchial epithelial hyperplasia, goblet cell metaplasia, ASM hyperplasia, and subepithelial ECM deposition, were elicited by this protocol (Fig. 1, B–D).

In the context of vascular alterations, we found that vascular sizes in peritracheal and peribronchial regions and in lung parenchyma were significantly increased after repeated allergen challenges (Fig. 1E, left bars). In addition, vessel numbers were also

significantly increased in these airway compartments (Fig. 1E, right bars). These findings confirm that vascular changes in airway remodeling involve both vasodilation and new vessel formation, i.e., angiogenesis. It is also clear from our study that vascular alterations in airway remodeling are not confined to bronchial-associated vessels, as have been widely perceived, but also occur in microcirculation of the lung parenchyma. AHR was assessed by methacholine challenge, and enhanced lung resistance was found in OVA-treated mice compared with controls (Fig. 1F).

Collectively, we demonstrate the elicitation of characteristic Ag-specific immune responses and the associated structural and functional changes in airway remodeling by using an allergen-induced chronic airway inflammation model, which enables further exploration on the effect of Treg in airway remodeling of chronic asthma.

Treg exert immune suppression via direct cell-cell contact

Oral feeding of OVA has been shown to activate and expand Treg in DO11.10 mice (40). Using this feeding protocol, over 60% of the isolated CD4⁺CD25⁺ T cells expressed the Treg-specific transcription factor, FoxP3 (Fig. 2A). Consistent with previous reports, we found that Treg secreted IL-10 and TGF- β in response to OVA stimulation *in vitro* (Fig. 2B). We also examined the CD4⁺CD25⁺FoxP3⁺ population and found that ~50% of these cells expressed CD69, ~30% expressed CD103, ~10% expressed surface CTLA4, and less than 20% of them expressed L-selectin. Combined with intracellular cytokine staining, the CD25⁺FoxP3⁺ T cells could very probably represent a mixed population

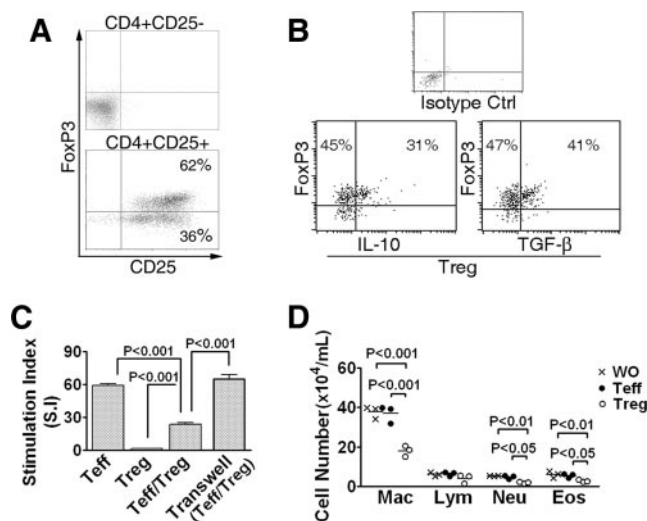


FIGURE 2. Functional characterization of Treg. CD4⁺CD25⁺ Treg and CD4⁺CD25⁻ Teff were isolated from OVA-fed DO11.10 mice by using MACS Treg isolation kit, as described in *Materials and Methods*. Treg and/or Teff were incubated with irradiated APC at 1:2 ratio plus OVA (200 μ g/ml) for 72 h. Alternatively, Treg and Teff were incubated in a Transwell system in which Treg were stimulated in the upper chamber and Teff in the lower chamber. **A**, Dot plots showing FoxP3 expression of CD4⁺CD25⁺ Treg cells. **B**, Cytokine production by Treg in response to OVA stimulation as measured by intracellular cytokine staining and flow cytometry. $n = 4$. **C**, Proliferation response to 72-h OVA stimulation detected by ³H incorporation. Data were representative of three independent experiments performed in triplicates. **D**, BALF leukocyte collected 1 day after OVA challenge. OVA-sensitized mice were given i.v. Treg or Teff 1 day before aerosol OVA challenge. Data shown are representative of two experiments. $n = 3$ mice/group.

containing activated effector T cells as well as induced IL-10/TGF- β -secreting Treg. Furthermore, whereas Teff showed vigorous proliferation to OVA stimulation, Treg hardly proliferated. Proliferation of Teff was significantly inhibited by Treg coculture, and the suppressive effect was abrogated when the coculture was performed in a Transwell system (Fig. 2C). The immune suppressive function of the Treg was further validated in an acute asthma model wherein OVA-sensitized mice adoptively transferred with Treg showed significantly reduced BALF leukocyte infiltration after OVA challenge compared with control mice (Fig. 2D).

Treg reduce neovascularity of airway remodeling and ameliorate AHR

Having characterized the chronic airway inflammation model and the immunologic profile of Treg, we examined the effects of Treg in airway remodeling after three weekly cell transfers. In the context of vascular changes (Fig. 3A), despite the fact that no allergen challenge was given since before commencing cell transfer, vessel numbers in airways of OVA-treated mice receiving i.v. HBSS (the WO group) were persistently increased. Noticeably, Treg transfers reduced vessel numbers in all three airway compartments to the levels comparable with PBS controls. Unlike Treg, however, Teff transfers had no effect on the vessel numbers (Fig. 3A, upper panel). Increased vascular size also persisted after cessation of allergen challenge, and irrespective of the cell type transferred, the vessel sizes were comparable between the OVA groups (Fig. 3A, lower panel).

ASM quantified by α -SMA immunoreactivity (Fig. 3B) and ECM deposition examined by ProCol-I immunostaining (Fig. 3C, photomicrograph) showed no statistical difference among the OVA

groups. Because α -SMA also stains myofibroblasts and fibroblasts, we verified the data by using α -calponin immunostaining and we found a similar staining pattern as α -SMA with no statistical difference among the OVA groups by α -calponin staining (Fig. 3B, bottom photomicrograph). ECM deposition was also analyzed by ProCol-I, -III, and -V gene expression, which showed that only ProCol-V was increased in OVA-treated mice. However, expression of the three ProCol was also comparable between OVA groups (Fig. 3C, bars). Quantification of blood vessels, ASM, and ECM was also performed by using total lung homogenates and Western blotting (Fig. 3D). Using CD105 as an EC marker, we found a significantly decreased CD105 expression in lung homogenates of Treg-transferred mice compared with that of WO or Teff mice. In addition, increased α -SMA and marginally enhanced ProCol-I expression were found in the OVA groups compared with PBS controls; however, expression of the two molecules was of no statistical difference among the OVA groups (densitometry not shown). These data suggest a selective inhibitory effect of Treg on the maintenance of neovascularity in airway remodeling.

In the current experimental settings, leukocyte infiltration in BALF decreased with time after cessation of allergen exposure (data not shown). Nonetheless, total BALF leukocytes of OVA-treated mice were persistently increased. Interestingly, we found a selective reduction of BALF eosinophils, but not other leukocyte subtypes, in Treg-transferred mice (Fig. 3E). Although it is possible that Treg may have downplayed remodeling angiogenesis through messenger cells such as eosinophils, mechanisms involving direct interaction between the EC and Treg could not be excluded.

The extent of AHR also decreased with time in the absence of allergen challenge. However, AHR of OVA-treated mice (WO and Teff) were persistently enhanced compared with PBS controls, although at a lesser amplitude; the enhanced lung resistance was significantly normalized after Treg transfers (Fig. 3F). Because we observed no difference in ASM mass and ECM deposition between OVA groups, it is conceivable that improved AHR in Treg-transferred mice is attributed to decreased airway/pulmonary vessels.

Although nearly 50% of the Teff transferred in this model were IFN- γ positive, adoptive transfer of Teff had no effect on airway inflammation and AHR. The reason for the lack of modulatory effects on experimental allergic airway disease by Teff in our model may be explained by the fact that Teff secreted IFN- γ only upon stimulation with Ag in vitro. Teff cultured without Ag stimulation did not secrete or secreted only trace of IFN- γ . In our model, adoptive T cell transfers were performed without further allergen challenge of the mice. In the absence of adequate allergen stimulation, it might not be a permissive condition for Teff to exert substantial additive effect on leukocyte recruitment or development of AHR.

Treg induce apoptosis of vascular EC and decrease VEGF expression of the lungs

Having found that the airway and lung vessels of Treg-transferred mice were reduced to the levels of PBS controls, we speculated that Treg exerted an antiangiogenic effect by interfering with maintenance of the neovascularity. To test this, we investigated apoptosis of the EC by TUNEL staining of the lung sections (Fig. 4A). We found that TUNEL-positive EC in the lungs of Treg-transferred mice were significantly higher than that of the WO or Teff mice, suggesting that the antiangiogenic effect of Treg was mediated by mechanisms involving the induction of EC apoptosis. We also examined the apoptotic effect of Treg on the EC by in vitro culture system wherein serum-deprived subconfluent MPEC were

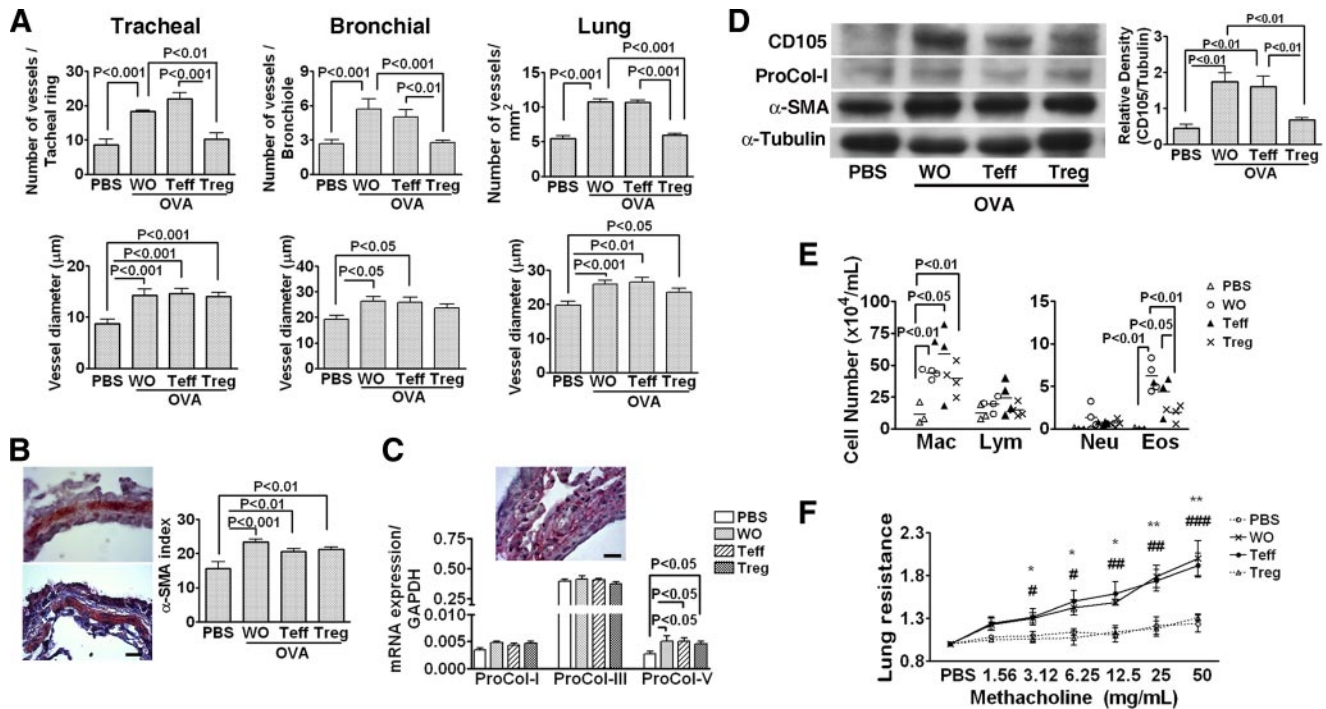


FIGURE 3. Adoptive Treg transfers reduce remodeling angiogenesis and AHR. Mice were sensitized and repeatedly challenged with aerosol OVA, as depicted in the protocol. Six days after the last OVA challenge, mice were given three weekly transfers of Treg or Teff. OVA- and PBS-sensitized mice given PBS without containing cells were used as positive (WO) and negative (PBS) controls, respectively. Unless indicated, mice were killed 1 wk after the third cell transfer (d78). *A*, Modulation of vascular changes by Treg. *Upper panel*, Vessel numbers; *lower panel*, vascular sizes. Blood vessels were identified by CD31 immunoreactivity. *B*, Quantification of ASM by α -SMA immunostaining. Photomicrographs show staining of ASM by α -SMA (*upper*) and α -calponin (*bottom*) labeled in brown. The area of positive α -SMA labeling in the subepithelial region was compared against the length of associated airway segment and expressed as α -SMA index. Scale bar = 20 μ m. *C*, Photomicrograph shows the scattered subepithelial and interstitial staining pattern of ProCol-I labeled in red. Bars show gene expression of ProCol-I, -III, and -V quantified by real-time PCR. $n = 3$; six to eight mice/group. Scale bar = 20 μ m. *D*, Western blots for CD105, ProCol-I, and α -SMA. Bars show densitometry of CD105 using α -tubulin as a loading control. $n = 5$ mice/group. *E*, BALF leukocytes enumerated a total of 500 cells per cytoslide. $n = 4$. *F*, AHR was measured 5–6 days after the last cell transfer. Data are from four experiments, each containing six to eight mice/group. *, Comparison of Treg with WO, and #, Treg with Teff. * and #, $p < 0.05$; ** and ##, $p < 0.01$; *** and ###, $p < 0.001$.

incubated with Teff or Treg for 18–24 h and apoptosis was detected by annexin-V/propidium iodide staining and flow cytometry. The results showed a higher apoptosis (annexin-V positive/propidium iodide negative) in MPEC cocultured with Treg compared with that with Teff (8.5 vs 1.5%; data not shown), supporting a proapoptotic effect of Treg on EC.

Cytokines in BALF and lung homogenates were evaluated by ELISA (Fig. 4, *B* and *C*), and it showed that although Treg secreted IL-10 and TGF- β upon Ag activation in vitro, there was no preferential expression of these two cytokines in Treg-transferred mice. Noticeably, VEGF in both BALF and lung homogenates of Treg-transferred mice was significantly decreased. These data suggest a role for VEGF, and less likely IL-10 or TGF- β , in Treg-mediated regulation of remodeling angiogenesis, and also support a cell contact-dependent mechanism in Treg-mediated modulation.

Airway epithelium has been shown to participate in the pathogenesis of asthma by producing a wide range of inflammatory and angiogenic mediators. It is possible that Treg exerted regulation on VEGF secretion of the airway epithelium in our model. We therefore assessed the role of Treg in VEGF production of an airway epithelial cell line, LA4 cells (ATCC; CCL-196; Fig. 4*D*). Under steady state, LA4 constitutively produced VEGF, and the secretion was enhanced by incubation with BALF taken from the WO mice. In contrast, Treg BALF had no effect on the constitutive VEGF production of LA4 cells, and Treg coincubation did not affect WO BALF-stimulated VEGF secretion (WO/Treg). These data hence exclude a direct regulatory role of Treg on epithelial VEGF pro-

duction in asthmatic milieu. Suppressed, but not completely abrogated, VEGF production in Treg-transferred mice may very likely be consequent to and be a part of a negative feed-forward loop constituted of increased EC apoptosis and decreased angiogenesis after Treg transfers.

We next examined whether direct interaction between transferred Treg and the EC occurred in our model. OVA-treated mice were given the first adoptive Treg transfer as the study protocol, and lung tissues were collected at 30 min and 1, 2, and 3 h after transfer. By immunostaining with FoxP3 and CD31, we found that direct contact of Treg with the EC indeed took place in vivo, and it peaked before 1 h after transfer in the current experimental settings (Fig. 4*E*). These findings support the hypothesis that Treg deliver proapoptotic signaling to the EC via direct cell-cell interaction, and this process, involving at least in part VEGF secretion, modulates angiogenesis during airway remodeling.

Treg inhibit angiogenesis by direct interaction with the EC

Showing that Treg directly interacted with the EC after transfer, we next verified whether Treg regulate angiogenesis via direct contact with the EC by using primary murine EC isolated from the lung tissues and an in vitro Matrigel tube formation assay (Fig. 5*A*). We found that preincubating Treg with EC significantly reduced EC tube formation on Matrigel, and the inhibitory effect was abrogated when EC were cocultured with Treg in the Transwell system (Fig. 5*B*). These findings verify that inhibition of angiogenesis by Treg, like their immune suppressive function, requires

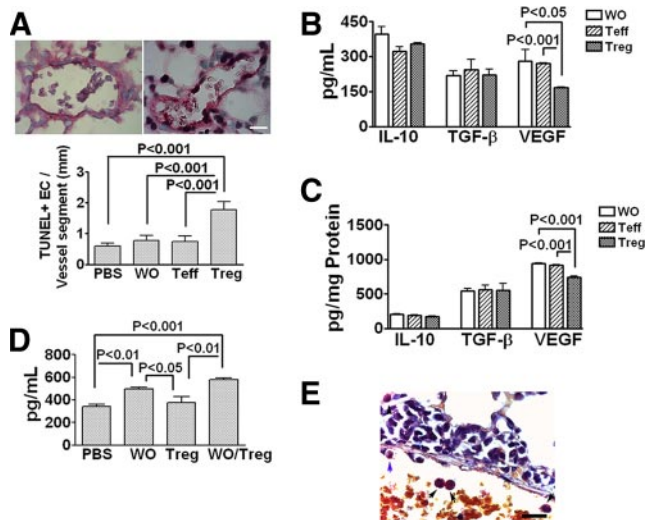


FIGURE 4. Treg transfers induce EC apoptosis and decrease VEGF production of the lungs. **A**, Apoptosis of EC detected by TUNEL staining of the lung sections. Photomicrographs show the TUNEL-negative (left) and TUNEL-positive (dark brown labeling; right) vessels. Data are representative of two separate experiments; five mice/group. Scale bar = 20 μ m. **B**, BALF and **C**, lung homogenate cytokines detected by ELISA. $n = 5$ mice/group, performed in triplicates. **D**, VEGF secretion of airway epithelial cells. LA4 cells were incubated with BALF collected after the last cell transfer. When indicated, Treg were added to LA4 cells 16 h after incubation with BALF of the WO mice (WO/Treg). Supernatants were collected after 72 h and analyzed with ELISA. Data represent three separate experiments performed in triplicates. **E**, Photomicrograph shows direct interaction of FoxP3-positive Treg with the vessel after adoptive transfer. Lung tissue was prepared at 30 min to 3 h after Treg transfer. Black arrow, Treg inside the vessel; blue arrow, Treg interacting with the EC; black arrowhead, Treg in the lung parenchyma. Scale bar = 20 μ m.

direct cell contact between Treg and the EC. The antiangiogenic effect of Treg was further validated by using an in vivo Matrigel plug assay that showed significantly reduced neovascularity formation in plugs containing Treg compared with HBSS- or Teff-containing control plugs (Fig. 5C).

Treg-derived DLL4-Notch signaling suppresses angiogenesis

Notch signaling pathway has been shown to direct differentiation and function of T cells, including delivering instructive signals in the pathogenesis of allergic diseases (41, 42). In supporting this, we found that Treg preferentially expressed Notch2, Notch3, Notch4, and DLL4 compared with Teff (>10-fold increase; Fig. 6A). In addition, we found that expression of Notch receptors was more prominent than the ligands in subconfluent EC, whereas sparse and confluent EC expressed similar amount of Notch molecules (Fig. 6B). The confluence-dependent expression pattern supports a role for Notch signaling during angiogenesis.

We next examined the role of the two differentially Treg-expressed Notch ligands, Jagged1 and DLL4, in angiogenesis in vitro. We found that preincubation with DLL4-Fc, but not Jagged1-Fc, significantly inhibited EC tube formation on Matrigel (Fig. 6C), suggesting DLL4 as a candidate molecule in Treg-mediated antiangiogenic function.

The humanized anti-DLL4 Ab, YW-152F, has been shown to neutralize both human and murine DLL4, and blockage of DLL4-Notch signaling resulted in aberrant EC proliferation in vivo (43). In our study, no detrimental effect on Treg survival was found by treatment with this anti-DLL4 Ab compared with treatment with isotype control Ab (Fig. 6D). Furthermore, by in vitro OVA stim-

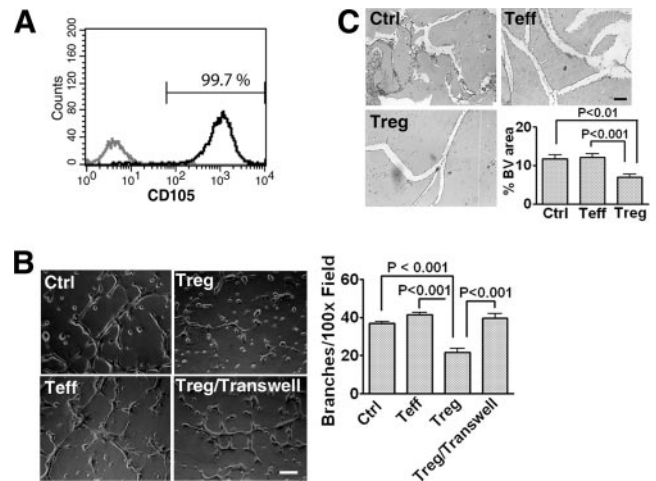


FIGURE 5. Treg inhibit EC angiogenesis via direct cell-cell contacts. **A**, MPEC were isolated from lung tissues by using CD105 MACS-positive selection. Purity of MPEC was examined by flow cytometry. **B**, Regulation of EC tube formation on Matrigel by Treg. MPEC were preincubated with Treg or Teff for 16 h, followed by tube formation assay performed at 0.3×10^5 EC/cm² in Matrigel-coated 24-well plates. When indicated, Treg and MPEC were cocultured in a Transwell to avoid direct cell contacts. Photomicrographs show EC tube formation on Matrigel taken at 4 h of the assay. Total branches of each EC from at least three representative $\times 100$ fields/well were counted. Data represent three separate experiments performed in triplicates. Scale bar = 100 μ m. **C**, Regulation of angiogenesis by Treg in Matrigel plug assays. A total of 1.5×10^6 Treg or Teff was mixed with 0.3 ml of Matrigel and s.c. injected at the ventral side of the mice. Matrigel without containing cells were used as controls. Photomicrographs show neovessels in H&E-stained Matrigel plug sections harvested 7 days later. Percentage of vessel-containing area was calculated from representative $\times 100$ fields in three consecutive sections. Data are representative of three independent experiments with five to six mice/group. Scale bar = 100 μ m.

ulation, we found that the presence of anti-DLL4 blocking Ab did not alter either the anergic state or suppressive property of Treg (Fig. 6E).

We therefore took advantage of this Ab to investigate the role of DLL4-Notch signaling in Treg antiangiogenic effect. We found that blocking Treg-derived DLL4 signaling, but not Jagged1 signaling, rescued EC tube formation on Matrigel (Fig. 6F). The abrogation of Treg antiangiogenic effect by DLL4 signaling blockage validates a specific role of DLL4 in Treg-mediated antiangiogenic function.

Treg inhibit angiogenesis of airway remodeling via DLL4-Notch signaling in vivo

The role of DLL4 signaling in Treg-mediated antiangiogenesis was further verified in the chronic asthma model wherein Treg were pretreated with the anti-DLL4 Ab (Treg/ α -DLL4) or human IgG1 isotype control Ab (Treg), thoroughly washed to remove unbound Ab, and adoptively transferred to OVA-treated mice. Consistently, reduced airway and pulmonary vessel numbers were found in Treg-transferred mice. However, when Treg were pretreated with the anti-DLL4 blocking Ab, the decreased vasculature in Treg-transferred mice was reversed (Fig. 7A). To examine the possible effects of retained anti-DLL4 Ab, OVA-treated mice were given three weekly i.v. injections of 4.5 μ g of anti-DLL4 Ab, the same amount of Ab incubated with one aliquot of Treg ($1 \sim 1.5 \times 10^6$) transferred to each mice, and lung vessel numbers examined 1 wk after the last Ab injection showed no difference in mice receiving this amount of Ab compared with mice receiving

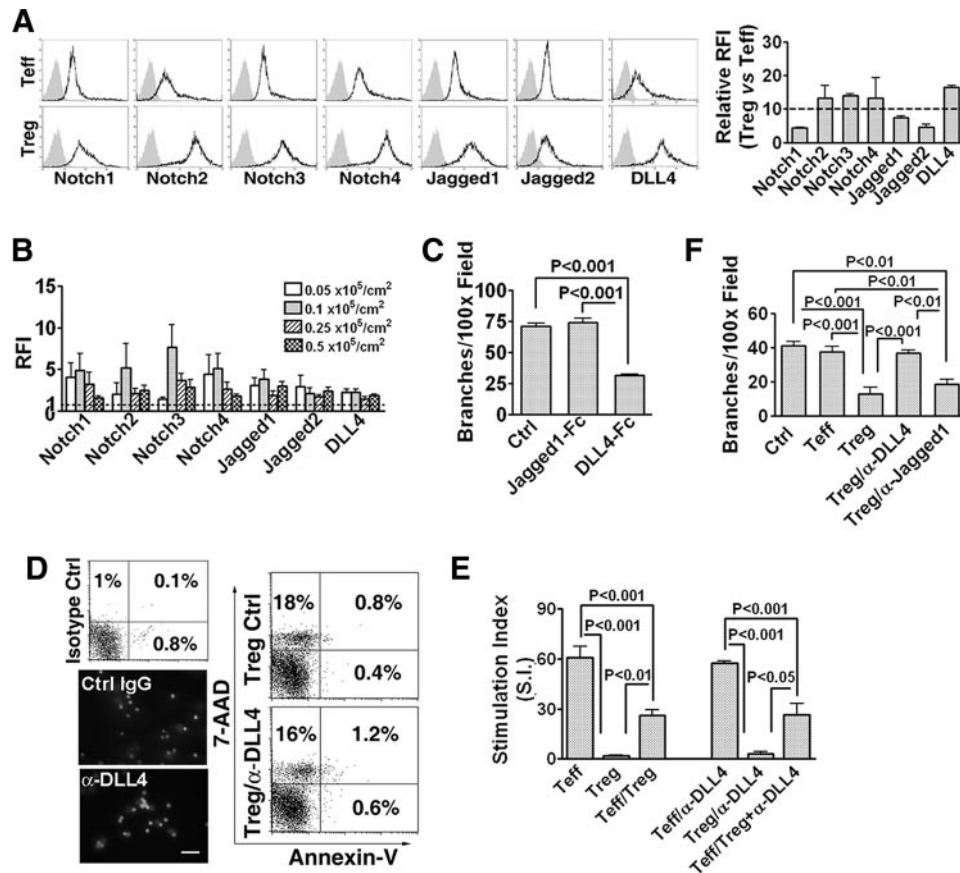


FIGURE 6. Treg preferentially express Notch ligand DLL4, and blockage of DLL4-Notch signaling abrogates Treg-mediated antiangiogenic effect in vitro. **A**, Expression of Notch on Treg and Teff analyzed by flow cytometry. Histograms show the representative staining profile of individual Notch molecule. Gray-shaded area indicates isotype control Ab (goat IgG) staining. Expression was calculated by comparing relative fluorescence intensity of individual molecule on Treg with that of Teff. Data are pooled from three experiments. **B**, Expression of Notch by MPEC at different stage of angiogenesis. MPEC were seeded at density 0.05, 0.1, 0.25, and $0.5 \times 10^5/\text{cm}^2$. At 72 h after seeding, when cells seeded at $0.5 \times 10^5/\text{cm}^2$ reached confluent, those at 0.1 and $0.25 \times 10^5/\text{cm}^2$ subconfluent, and those at $0.05 \times 10^5/\text{cm}^2$ remained sparse, cells were examined for Notch expression by flow cytometry. Data shown are pooled from three experiments. **C**, Modulation of EC tube formation on Matrigel by DLL4- and Jagged1-Fc proteins. MPEC were preincubated with recombinant DLL4-Fc or Jagged1-Fc (10 $\mu\text{g}/\text{ml}$) for 16 h before performing Matrigel tube formation assay. $n = 3$, performed in triplicates. **D**, Apoptosis of Treg after anti-DLL4 Ab treatment was measured by acridine orange staining of Treg cytopins (photomicrographs) and annexin-V/7-aminoactinomycin D plus flow cytometry (dot plots). Treg were isolated from OVA-fed DO11.10 mice, as described, and incubated with the anti-DLL4 Ab (30 $\mu\text{g}/\text{ml}$) for 40 min before being analyzed for apoptosis. $n = 2$, performed in duplicates. Scale bar = 50 μm . **E**, Proliferation response to 72-h OVA stimulation at the presence of anti-DLL4 Ab (5 $\mu\text{g}/\text{ml}$) detected by ^3H incorporation. $\text{CD4}^+\text{CD25}^+$ Treg and $\text{CD4}^+\text{CD25}^-$ Teff from OVA-fed DO11.10 mice were isolated and cultured, as described in *Materials and Methods*. $n = 3$, performed in triplicates. **F**, Modulation of EC tube formation by anti-DLL4 Ab-treated Treg. Treg were treated with anti-DLL4 Ab or anti-Jagged1 Ab for 40 min before incubation with MPEC. Matrigel tube formation assay was performed 16 h later. $n = 3$ and performed in triplicates.

PBS or control Ab (data not shown). Likewise, EC apoptosis and VEGF expression in lung homogenates were both rescued by inhibiting Treg-derived DLL4 signaling (Fig. 7, *B* and *C*). IL-10 and IFN- γ expression in lung homogenates were comparable between each group (Fig. 7*C*).

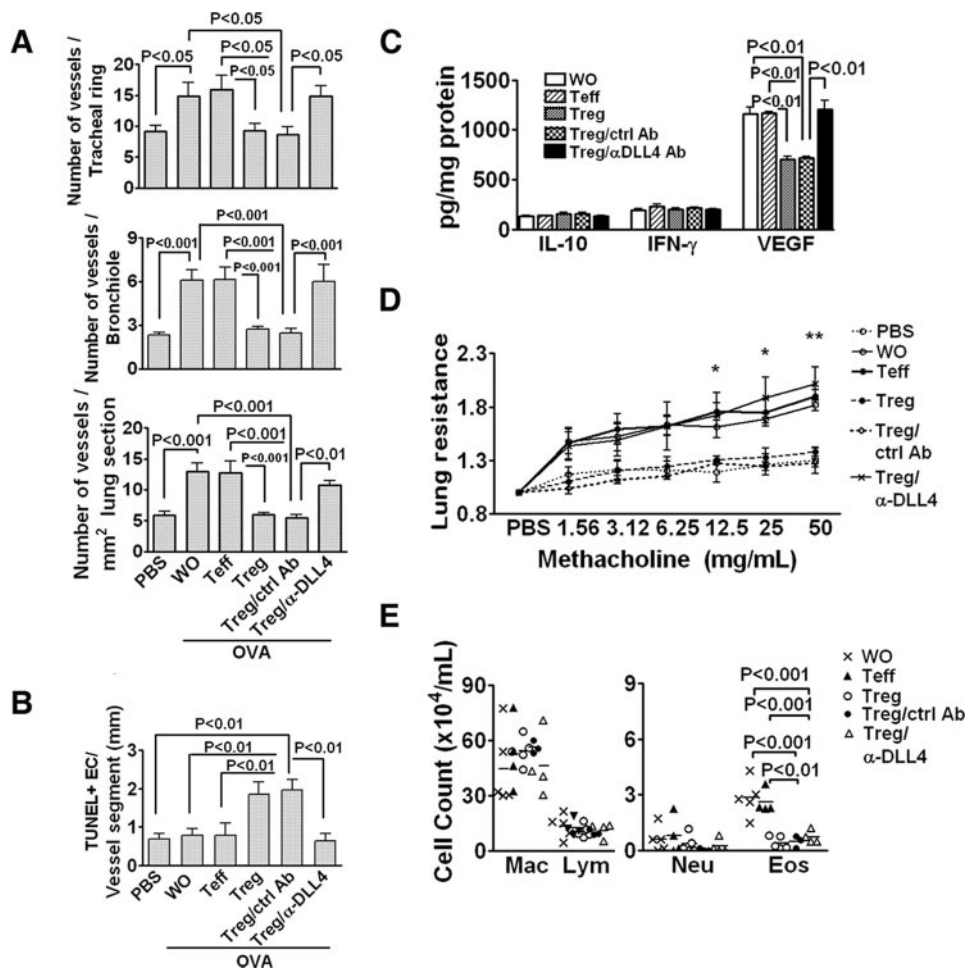
AHR, which was reduced after Treg transfers, was converted to the levels of WO and Teff in mice receiving Treg/ α -DLL4 (Fig. 7*D*). However, reduced BALF eosinophils after Treg transfers were not affected by blockage of DLL4 signaling, indicating that regulation of eosinophil infiltration by Treg was independent of Treg-derived DLL4 signaling (Fig. 7*E*). Treatment of Treg with isotype control Ab (Treg/ctrl Ab) did not affect the regulation exerted by Treg. Collectively, these *in vivo* data verified the role of DLL4 signaling in the proapoptotic antiangiogenic effect of Treg during airway remodeling.

Previous studies using chronic allergen challenge models showed an expansion of Treg (44, 45). The expansion includes actual proliferation of Treg in response to chronic Ag stimulation

and also peripheral conversion of Teff to Treg under appropriate environmental cues, such as the presence of TGF- β and dendritic cell-derived retinoic acid, inhibited costimulation or IL-2 production, and the presence of subimmunogenic Ag conditions (46–50). To address this issue, we isolated endogenous Treg from lung tissues of OVA-treated BALB/c mice after repeated OVA challenges (Fig. S1).⁴ In accordance with previous reports, we found an expansion of $\text{CD4}^+\text{CD25}^+$ T cells in the OVA group compared with the PBS controls. However, an average of only 30–40% of the $\text{CD4}^+\text{CD25}^+$ T cells were FoxP3 positive, a percentage lower than that isolated from PBS controls (~60%) or the spleen (~70%) of DO11.10 mice, suggesting expansion of activated Teff cells, in addition to Treg, in the $\text{CD4}^+\text{CD25}^+$ T cell pool after repeated allergen challenges. The number of the Treg population remained similar during the course of chronic allergen challenges,

⁴ The online version of this article contains supplemental material.

FIGURE 7. DLL4 signaling blockage reverts the proapoptotic antiangiogenic effects of Treg in vivo. Mice sensitized and repeatedly challenged with OVA were given Treg, as described. In an indicated group, Treg were preincubated with the anti-DLL4 Ab before adoptive transfers. Mice were killed 1 wk after the last cell transfer. **A**, Vessel numbers were enumerated by using CD31-stained lung sections. **B**, Apoptosis of EC was detected by TUNEL staining of the lung sections. **C**, Cytokine expression in lung homogenates. $n = 6$ mice, performed in triplicates. **D**, BALF leukocytes were determined by cytopins. A total of 500 cells was counted in each slide. **E**, AHR performed within 1 wk after the last cell transfer. Data are representative of two separate experiments. $n = 6-8$ mice/group.



and there were approximately $1-2 \times 10^5$ CD4⁺CD25⁺ T cells per lung. Therefore, each lung contains no more than 1×10^5 FoxP3⁺ Treg. The inability of the in situ Treg to exert regulation on airway remodeling may be due to the low cell number and the spatial location of these induced Treg in the lung. Adoptive transfer using in situ CD4⁺CD25⁺ T cells was not performed due to the low cell number and the relatively lower frequency of FoxP3-positive Treg in the CD4⁺CD25⁺ T cell pool. We also examined the expression profile of Notch receptors and ligands on these in situ Treg, and it showed a comparable profile with what was observed in splenic Treg generated from the Ag-feeding model (data not shown).

In addition, previous studies demonstrated the emergence of an anergic population with stable CD25 expression and suppressive function between 3 and 7 days after Teff transfer or later (47, 49). In this study, we found a selective antiangiogenic effect of Treg via a cell contact-dependent mechanism involving Treg-derived DLL4 ligation. Hence, physical contact between Treg and the EC is crucial for the regulation to proceed. Our findings that no beneficial effect in vascular load or AHR accompanying improved vascularity was observed after Teff transfer could be explained by the reasons that, although in vivo conversion of CD4⁺CD25⁺ cells to CD4⁺CD25⁺Foxp3⁺ Treg may indeed occur, the conversion would not occur until several days after transfer. Therefore, the differentiated Treg may not gain optimal access to the EC as the freshly transferred Treg do, and the extent of regulation is also limited by the lesser number of cells differentiated into Treg compared with the number of Treg transferred.

Discussion

In this study, we demonstrate a unique role of Treg in selectively inhibiting neovascularity formation of airway remodeling by using a chronic asthma model and adoptive Treg transfers. Although we and others (51) have found a protracted course in reverting the ECM and ASM alterations after prolonged allergen challenge, adoptive Treg transfer was nonetheless shown to alleviate established airway inflammation and ECM/mucus remodeling (52). The factors causing variable effects of Treg transfer in airway remodeling may be attributed to ongoing allergen exposures, numbers, and frequency of Treg transferred, as well as differences in the study readouts.

Cross-talk between T cells and EC has been studied in the field of transplant immunology in which Ag presentation by EC resulted in generation of anergic T cells and graft survival. In contrast, it has been shown that suppressor T and Treg induced a tolerogenic EC that subsequently inhibited helper and cytotoxic T cell activation (53). Regulation of EC angiogenesis by Treg has not been reported before, nor has the role of Treg in established airway remodeling. In our study, we found that Treg transfers induced EC apoptosis and reduced VEGF expression of the lungs. Furthermore, we found that endothelial progenitor cells in Treg-transferred mice were significantly decreased compared with asthmatic mice receiving Teff or PBS controls (M.-T. Huang and B.-L. Chiang, unpublished data). VEGF has been reportedly secreted during asthma by many inflammatory cells, such as mast cells, eosinophils, neutrophils, macrophages, and lymphocytes, and also

by structural cells, including fibroblasts, smooth muscle cells, and epithelial and EC (54). All of these are possible sources of VEGF in our model. In our current study, we found a selective reduction of eosinophils in mice receiving Treg transfers. It is possible that reduced VEGF expression in these mice is due to decreased eosinophils. However, whereas blockage of Treg-derived DLL4 signaling reverted VEGF expression, eosinophil infiltration in the lung was not affected, suggesting that eosinophils are less likely the regulated source of VEGF in this model. Furthermore, we demonstrated that the addition of Treg to LA4 cells had no effect on stimulated VEGF secretion, which eliminated a direct regulatory role of Treg on VEGF secretion by the airway epithelium. Combined with the immunohistochemistry data in this study, we hypothesize that direct interaction of Treg with the EC via DLL4 delivers an apoptotic antiangiogenic signal, which, at least in part, involves regulation of endothelial VEGF secretion. In supporting our hypothesis, previous study showed that DLL4 selectively inhibited VEGF functions by acting as a negative regulator of VEGF-R2 expression on the EC. In the case of our model, inhibited VEGF function by DLL4 signaling may, in a feed-forward manner, decrease VEGF secretion by the EC, leading to apoptosis of the EC. The vessel numbers after Treg transfers were reduced to the levels comparable to PBS controls, suggesting that Treg most likely interfered with maintenance of the vasculature. From our study, deprivation of VEGF and the proapoptotic signals derived from Treg offered the most plausible causes for regression of the vasculature. Our findings hence suggest that in addition to a preventive role in allergic airway diseases, Treg could also be a therapeutic strategy in chronic airway remodeling.

By genetic approaches, recombinant proteins, or blocking Abs, DLL4-Notch signaling has been shown to restrict endothelial tip cell formation, and hence, plays a negative regulatory role in vascular sprouting and vessel branching that are required for normal vascular network formation during angiogenesis (16, 55, 56). Therefore, DLL4-Notch signaling promotes the timely formation of a well-differentiated vascular network and maintains the vascular network in a mature and functional state by rendering EC unresponsive to angiogenic cues such as VEGF. In several tumor models, blockage of DLL4 signaling was found to cause aberrant EC proliferation and defective vessel maturation and perfusion. Nonproductive angiogenesis after DLL4 signaling blockage led to the paradoxical findings of increased tumor vessels while inhibiting tumor growth at the same time (43, 57, 58). These findings are intriguing because neovasculature generated in this manner, i.e., increased, but poorly perfused vasculature, would have detrimental effects in asthma. In this regard, our findings that DLL4 signaling delivered by Treg inhibited angiogenesis of airway remodeling are in line with previous studies on the role of DLL4 in angiogenesis. Treg-derived DLL4 signaling is therefore a beneficial target in preventing nonfunctional angiogenesis in chronic lung diseases.

Our study is unique in that we thoroughly evaluated the vascular component of airway remodeling in asthma. We found that vasodilation and angiogenesis occurred not only in peritracheal and peribronchial vessels, but also in microcirculation of the lung parenchyma, and both of which persisted after cessation of allergen challenges. Previous studies on the vascular changes of airway remodeling have mainly focused on bronchial-associated vessels, i.e., vessels of the bronchial circulation, which is probably a consequence of the perception that asthma is an airway disease and the accessibility of appropriate lung tissues. Nonetheless, a peripheral or parenchymal component of asthma has been recognized by studies showing that chronic allergic airway inflammation involves both central and peripheral airways and induces not only bronchial, but also parenchymal remodeling (59–61). In supporting this fur-

ther, recent studies on allergen- or infection-induced chronic airway inflammation have also demonstrated vascular remodeling and angiogenesis in nonbronchial-associated pulmonary vessels, such as those associated with the gas exchange region of the lungs (31, 62). Therefore, our findings add to the list supporting a pulmonary microvascular component in the airway remodeling complex.

AHR is characteristic of asthmatic airways, and the phenomenon of AHR is at best partially restored by corticosteroids in both clinical and experimental settings. Both airway inflammation and airway remodeling contribute to the development of AHR. Histological studies and computer-assisted modeling revealed that increased ASM and excessive contractility are the major causes for the development of AHR. Nonetheless, increased airway vascular indices have also been related to airflow limitation and lung function impairments. Increased vascularity and the leaky property of the vessels cumulatively lead to mucosal and adventitial swelling, and contribute to disproportionate reduction of airway calibre for a given degree of ASM contraction. One major finding in our study is that Treg transfers at the stage of established airway remodeling selectively reduced pulmonary vasculature and alleviated AHR of the recipient mice, despite no observable changes of ASM hyperplasia and ECM deposition. Our findings hence support a long-overlooked importance of remodeling angiogenesis during airway remodeling.

Although a prominent role of Treg in preventing the development of allergic diseases has been defined, the role of Treg in established airway remodeling has not been investigated thoroughly, nor has regulation of EC angiogenesis by Treg. From immunological perspective, our findings pose important implication in that Treg not only control aberrant inflammation, which causes the disease, but also downplay detrimental pathological angiogenesis accompanying chronic inflammation. Future studies on Treg-mediated antiangiogenic effect in other disease models will contribute further to bench-to-bedside application of Treg.

Acknowledgments

We are grateful for the scientific discussions with Dr. Pei-Jer Chen, Graduate Institute of Clinical Medicine, National Taiwan University.

Disclosures

The authors have no financial conflict of interest.

References

- Bray, S. J. 2006. Notch signalling: a simple pathway becomes complex. *Nat. Rev. Mol. Cell Biol.* 7: 678–689.
- Ehebauer, M., P. Hayward, and A. M. Arias. 2006. Notch, a universal arbiter of cell fate decisions. *Science* 314: 1414–1415.
- Dallman, M. J., E. Smith, R. A. Benson, and J. R. Lamb. 2005. Notch: control of lymphocyte differentiation in the periphery. *Curr. Opin. Immunol.* 17: 259–266.
- Tanigaki, K., and T. Honjo. 2007. Regulation of lymphocyte development by Notch signaling. *Nat. Immunol.* 8: 451–456.
- Ng, W. F., P. J. Duggan, F. Ponchel, G. Matarese, G. Lombardi, A. D. Edwards, J. D. Isaacs, and R. I. Lechler. 2001. Human CD4⁺CD25⁺ cells: a naturally occurring population of regulatory T cells. *Blood* 98: 2736–2744.
- Ostroukhova, M., Z. Qi, T. B. Oriss, B. xon-McCarthy, P. Ray, and A. Ray. 2006. Treg-mediated immunosuppression involves activation of the Notch-HES1 axis by membrane-bound TGF- β . *J. Clin. Invest.* 116: 996–1004.
- Anastasi, E., A. F. Campese, D. Bellavia, A. Bulotta, A. Balestri, M. Pascucci, S. Checquolo, R. Gradini, U. Lendahl, L. Frati, et al. 2003. Expression of activated Notch3 in transgenic mice enhances generation of T regulatory cells and protects against experimental autoimmune diabetes. *J. Immunol.* 171: 4504–4511.
- Hoyne, G. F., I. Le Roux, M. Corsin-Jimenez, K. Tan, J. Dunne, L. M. G. Forsyth, M. J. Dallman, M. J. Owen, D. Ish-Horowicz, and J. R. Lamb. 2000. Serrate1-induced Notch signalling regulates the decision between immunity and tolerance made by peripheral CD4⁺ T cells. *Int. Immunol.* 12: 177–185.
- Kared, H., H. dle-Biasette, E. Fois, A. Masson, J. F. Bach, L. Chatenoud, E. Schneider, and F. Zavala. 2006. Jagged2-expressing hematopoietic progenitors promote regulatory T cell expansion in the periphery through Notch signaling. *Immunity* 25: 823–834.

10. Yvon, E. S., S. Vigouroux, R. F. Rousseau, E. Biagi, P. Amrolia, G. Dotti, H. J. Wagner, and M. K. Brenner. 2003. Overexpression of the Notch ligand, Jagged-1, induces alloantigen-specific human regulatory T cells. *Blood* 102: 3815–3821.
11. Hoyne, G. F., M. J. Dallman, and J. R. Lamb. 1999. Linked suppression in peripheral T cell tolerance to the house dust mite derived allergen Der p 1. *Int. Arch. Allergy Immunol.* 118: 122–124.
12. Iso, T., Y. Hamamori, and L. Kedes. 2003. Notch signaling in vascular development. *Arterioscler. Thromb. Vasc. Biol.* 23: 543–553.
13. Li, J. L., and A. L. Harris. 2005. Notch signaling from tumor cells: a new mechanism of angiogenesis. *Cancer Cell* 8: 1–3.
14. Liu, Z. J., T. Shirakawa, Y. Li, A. Soma, M. Oka, G. P. Dotto, R. M. Fairman, O. C. Velazquez, and M. Herlyn. 2003. Regulation of Notch1 and Dll4 by vascular endothelial growth factor in arterial endothelial cells: implications for modulating arteriogenesis and angiogenesis. *Mol. Cell. Biol.* 23: 14–25.
15. Patel, N. S., J. L. Li, D. Generali, R. Poulsom, D. W. Cranston, and A. L. Harris. 2005. Up-regulation of δ -like 4 ligand in human tumor vasculature and the role of basal expression in endothelial cell function. *Cancer Res.* 65: 8690–8697.
16. Williams, C. K., J. L. Li, M. Murga, A. L. Harris, and G. Tosato. 2006. Up-regulation of the Notch ligand δ -like 4 inhibits VEGF-induced endothelial cell function. *Blood* 107: 931–939.
17. Lee, C. G., H. Link, P. Baluk, R. J. Homer, S. Chapoval, V. Bhandari, M. J. Kang, L. Cohn, Y. K. Kim, D. M. McDonald, and J. A. Elias. 2004. Vascular endothelial growth factor (VEGF) induces remodeling and enhances TH2-mediated sensitization and inflammation in the lung. *Nat. Med.* 10: 1095–1103.
18. Baldwin, L., and W. R. Roche. 2002. Does remodelling of the airway wall precede asthma? *Paediatr. Respir. Rev.* 3: 315–320.
19. Holgate, S. T., J. Holloway, S. Wilson, F. Bucchieri, S. Puddicombe, and D. E. Davies. 2004. Epithelial-mesenchymal communication in the pathogenesis of chronic asthma. *Proc. Am. Thorac. Soc.* 1: 93–98.
20. Kariyawasam, H. H., M. Aizen, J. Barkans, D. S. Robinson, and A. B. Kay. 2007. Remodeling and airway hyperresponsiveness but not cellular inflammation persist after allergen challenge in asthma. *Am. J. Respir. Crit. Care Med.* 175: 896–904.
21. Bai, T. R., and D. A. Knight. 2005. Structural changes in the airways in asthma: observations and consequences. *Clin. Sci.* 108: 463–477.
22. Elias, J. A., Z. Zhu, G. Chupp, and R. J. Homer. 1999. Airway remodeling in asthma. *J. Clin. Invest.* 104: 1001–1006.
23. Lange, P., J. Parner, J. Vestbo, P. Schnohr, and G. Jensen. 1998. A 15-year follow-up study of ventilatory function in adults with asthma. *N. Engl. J. Med.* 339: 1194–1200.
24. Kuwano, K., C. H. Bosken, P. D. Pare, T. R. Bai, B. R. Wiggs, and J. C. Hogg. 1993. Small airways dimensions in asthma and in chronic obstructive pulmonary disease. *Am. Rev. Respir. Dis.* 148: 1220–1225.
25. Li, X., and J. W. Wilson. 1997. Increased vascularity of the bronchial mucosa in mild asthma. *Am. J. Respir. Crit. Care Med.* 156: 229–233.
26. Vrugt, B., S. Wilson, A. Bron, S. T. Holgate, R. Djukanovic, and R. Aalbers. 2000. Bronchial angiogenesis in severe glucocorticoid-dependent asthma. *Eur. Respir. J.* 15: 1014–1021.
27. Hoshino, M., M. Takahashi, and N. Aoike. 2001. Expression of vascular endothelial growth factor, basic fibroblast growth factor, and angiogenin immunoreactivity in asthmatic airways and its relationship to angiogenesis. *J. Allergy Clin. Immunol.* 107: 295–301.
28. Felts, B. N., D. Wignarajah, L. Zheng, C. Ward, D. Reid, R. Harding, and E. H. Walters. 2006. Increased vascular endothelial growth factor and receptors: relationship to angiogenesis in asthma. *Am. J. Respir. Crit. Care Med.* 173: 1201–1207.
29. Simcock, D. E., V. Kanabar, G. W. Clarke, B. J. O'Connor, T. H. Lee, and S. J. Hirst. 2007. Proangiogenic activity in bronchoalveolar lavage fluid from patients with asthma. *Am. J. Respir. Crit. Care Med.* 176: 146–153.
30. Asosingh, K., S. Swaidani, M. Aronica, and S. C. Erzurum. 2007. Th1- and Th2-dependent endothelial progenitor cell recruitment and angiogenic switch in asthma. *J. Immunol.* 178: 6482–6494.
31. Rydell-Tormanen, K., J. R. Johnson, R. Fattouh, M. Jordana, and J. S. Erjefalt. 2008. Induction of vascular remodeling in the lung by chronic house dust mite exposure. *Am. J. Respir. Cell Mol. Biol.* 39: 61–67.
32. Tigan, B., C. Cannet, H. Karmouty-Quintana, F. X. Ble, S. Zurbrugg, E. Schaeublin, J. R. Fozard, and N. Beckmann. 2007. Lung inflammation and vascular remodeling after repeated allergen challenge detected noninvasively by MRI. *Am. J. Physiol.* 292: L644–L653.
33. Akbari, O., P. Stock, R. H. DeKruyff, and D. T. Umetsu. 2003. Role of regulatory T cells in allergy and asthma. *Curr. Opin. Immunol.* 15: 627–633.
34. Umetsu, D. T., J. J. McIntire, O. Akbari, C. Macaubas, and R. H. DeKruyff. 2002. Asthma: an epidemic of dysregulated immunity. *Nat. Immunol.* 3: 715–720.
35. Robinson, D. S., M. Larche, and S. R. Durham. 2004. Tregs and allergic disease. *J. Clin. Invest.* 114: 1389–1397.
36. Kearley, J., J. E. Barker, D. S. Robinson, and C. M. Lloyd. 2005. Resolution of airway inflammation and hyperreactivity after in vivo transfer of CD4⁺CD25⁺ regulatory T cells is interleukin 10 dependent. *J. Exp. Med.* 202: 1539–1547.
37. Lewkowich, I. P., N. S. Herman, K. W. Schleifer, M. P. Dance, B. L. Chen, K. M. Dienger, A. A. Spores, J. S. Shah, J. Kohl, Y. Belkaid, and M. Wills-Karp. 2005. CD4⁺CD25⁺ T cells protect against experimentally induced asthma and alter pulmonary dendritic cell phenotype and function. *J. Exp. Med.* 202: 1549–1561.
38. Strickland, D. H., P. A. Stumbles, G. R. Zosky, L. S. Subrata, J. A. Thomas, D. J. Turner, P. D. Sly, and P. G. Holt. 2006. Reversal of airway hyperresponsiveness by induction of airway mucosal CD4⁺CD25⁺ regulatory T cells. *J. Exp. Med.* 203: 2649–2660.
39. Huang, H. Y., C. C. Lee, and B. L. Chiang. 2008. Small interfering RNA against interleukin-5 decreases airway eosinophilia and hyper-responsiveness. *Gene Ther.* 15: 660–667.
40. Zhang, X., L. Izikson, L. Liu, and H. L. Weiner. 2001. Activation of CD25⁺CD4⁺ regulatory T cells by oral antigen administration. *J. Immunol.* 167: 4245–4253.
41. Okamoto, M., K. Takeda, A. Joetham, H. Ohnishi, H. Matsuda, C. H. Swasey, B. J. Swanson, K. Yasutomo, A. Dakhama, and E. W. Gelfand. 2008. Essential role of Notch signaling in effector memory CD8⁺ T cell-mediated airway hyperresponsiveness and inflammation. *J. Exp. Med.* 205: 1087–1097.
42. Schaller, M. A., R. Neupane, B. D. Rudd, S. L. Kunkel, L. E. Kallal, P. Lincoln, J. B. Lowe, Y. Man, and N. W. Lukacs. 2007. Notch ligand δ -like 4 regulates disease pathogenesis during respiratory viral infections by modulating Th2 cytokines. *J. Exp. Med.* 204: 2925–2934.
43. Ridgway, J., G. Zhang, Y. Wu, S. Stawicki, W. C. Liang, Y. Chanthery, J. Kowalski, R. J. Watts, C. Callahan, I. Kasman, et al. 2006. Inhibition of Dll4 signalling inhibits tumor growth by deregulating angiogenesis. *Nature* 444: 1083–1087.
44. Strickland, D. H., P. A. Stumbles, G. R. Zosky, L. S. Subrata, J. A. Thomas, D. J. Turner, P. D. Sly, and P. G. Holt. 2006. Reversal of airway hyperresponsiveness by induction of airway mucosal CD4⁺CD25⁺ regulatory T cells. *J. Exp. Med.* 203: 2649–2660.
45. Singh, A., W. F. Carson IV, E. R. Secor, Jr., L. A. Guernsey, R. A. Flavell, R. B. Clark, R. S. Thrall, and C. M. Schramm. 2008. Regulatory role of B cells in a murine model of allergic airway disease. *J. Immunol.* 180: 7318–7326.
46. Zheng, S. G., J. Wang, P. Wang, J. D. Gray, and D. A. Horwitz. 2007. IL-2 is essential for TGF- β to convert naive CD4⁺CD25⁺ cells to CD25⁺Foxp3⁺ regulatory T cells and for expansion of these cells. *J. Immunol.* 178: 2018–2027.
47. Thorstenson, K. M., and A. Khoruts. 2001. Generation of anergic and potentially immunoregulatory CD25⁺CD4⁺ T cells in vivo after induction of peripheral tolerance with intravenous or oral antigen. *J. Immunol.* 167: 188–195.
48. Apostolou, I., and H. von Boehmer. 2004. In vivo instruction of suppressor commitment in naive T cells. *J. Exp. Med.* 199: 1401–1408.
49. Kretschmer, K., I. Apostolou, D. Hawiger, K. Khazaie, M. C. Nussenzweig, and H. von Boehmer. 2005. Inducing and expanding regulatory T cell populations by foreign antigen. *Nat. Immunol.* 6: 1219–1227.
50. Xiao, X., A. Kroemer, W. Gao, N. Ishii, G. Demirci, and X. C. Li. 2008. OX40/OX40L costimulation affects induction of Foxp3⁺ regulatory T cells in part by expanding memory T cells in vivo. *J. Immunol.* 181: 3193–3201.
51. McMillan, S. J., and C. M. Lloyd. 2004. Prolonged allergen challenge in mice leads to persistent airway remodelling. *Clin. Exp. Allergy* 34: 497–507.
52. Kearley, J., D. S. Robinson, and C. M. Lloyd. 2008. CD4⁺CD25⁺ regulatory T cells reverse established allergic airway inflammation and prevent airway remodeling. *J. Allergy Clin. Immunol.* 122: 617–624.
53. Cortesini, N. S.-F., A. I. Colovai, J. S. Manavalan, S. Galluzzo, A. J. Naiyer, J. Liu, G. Vlad, S. Kim-Schulze, L. Scotto, J. Fan, and R. Cortesini. 2004. Role of regulatory and suppressor T-cells in the induction of ILT3⁺ ILT4⁺ tolerogenic endothelial cells in organ allografts. *Transplant Immunol.* 13: 73–82.
54. Puxeddu, I., D. Ribatti, E. Crivellato, and F. Levi-Schaffer. 2005. Mast cells and eosinophils: a novel link between inflammation and angiogenesis in allergic diseases. *J. Allergy Clin. Immunol.* 116: 531–536.
55. Hellstrom, M., L. K. Phng, J. J. Hofmann, E. Wallgard, L. Coultas, P. Lindblom, J. Alva, A. K. Nilsson, L. Karlsson, N. Gaiano, et al. 2007. Dll4 signalling through Notch1 regulates formation of tip cells during angiogenesis. *Nature* 445: 776–780.
56. Lobov, I. B., R. A. Renard, N. Papadopoulos, N. W. Gale, G. Thurston, G. D. Yancopoulos, and S. J. Wiegand. 2007. δ -like ligand 4 (Dll4) is induced by VEGF as a negative regulator of angiogenic sprouting. *Proc. Natl. Acad. Sci. USA* 104: 3219–3224.
57. Scheinet, J. S., W. Jiang, S. R. Kumar, V. Krasnopetrov, C. Borges, E. J. Ley, A. Duarte, and P. S. Gill. 2007. Inhibition of Dll4 mediated signaling induces proliferation of immature vessels and results in poor tissue perfusion. *Blood* 109: 4753–4760.
58. Noguera-Troise, I., C. Daly, N. J. Papadopoulos, S. Coetzee, P. Boland, N. W. Gale, H. Chieh Lin, G. D. Yancopoulos, and G. Thurston. 2006. Blockade of Dll4 inhibits tumor growth by promoting non-productive angiogenesis. *Nature* 444: 1032–1037.
59. Kraft, M., R. Djukanovic, S. Wilson, S. T. Holgate, and R. J. Martin. 1996. Alveolar tissue inflammation in asthma. *Am. J. Respir. Crit. Care Med.* 154: 1505–1510.
60. Busse, W. W., and R. F. Lemanske. 2001. Asthma. *N. Engl. J. Med.* 344: 350–362.
61. Xisto, D. G., L. L. Farias, H. C. Ferreira, M. R. Picanco, D. Amitrano, J. Lapa e Silva, E. M. Negri, T. Mauad, C. Carnielli, L. F. Silva, et al. 2005. Lung parenchyma remodeling in a murine model of chronic allergic inflammation. *Am. J. Respir. Crit. Care Med.* 171: 829–837.
62. Tormanen, K. R., L. Uller, C. G. A. Persson, and J. S. Erjefalt. 2005. Allergen exposure of mouse airways evokes remodeling of both bronchi and large pulmonary vessels. *Am. J. Respir. Crit. Care Med.* 171: 19–25.

Supplemental Figure and Legends

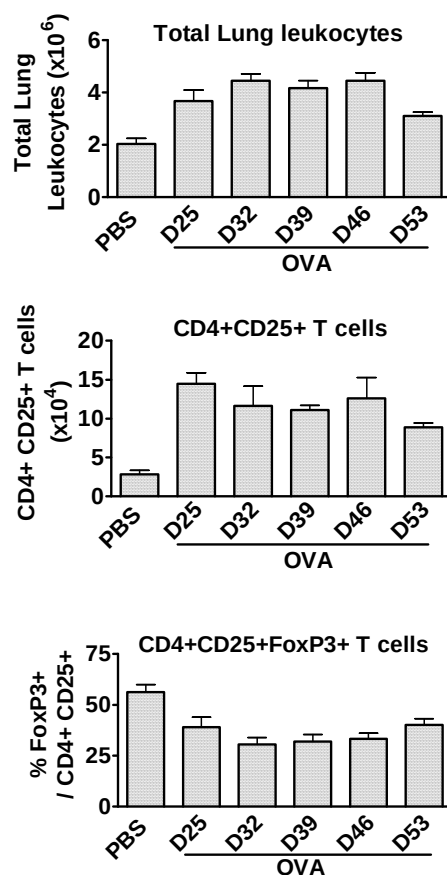


Figure S1. Chronic allergen challenges induce *in situ* expansion of Treg. Leukocytes were released from lung tissues by digestion with collagenase-IV and DNase-I. CD4+CD25+ T cells were purified by using the MACS Magnetic-bead system as described in the Methods and the percentage of FoxP3-expressing Treg in the CD4+CD25+ T cell population was examined by flow cytometry. **A**, Total lung leukocytes; **B**, CD4+CD25+ T cells and **C**, percentage of FoxP3+ T cells among the isolated CD4+CD25+ T cells at indicated time points of the OVA-induced chronic asthma model. Data shown represent 6-9 mice at each time point pooled from 3 independent experiments.