

Lung Angiogenesis Requires CD4⁺Forkhead Homeobox Protein-3⁺ Regulatory T Cells

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Abstract

Angiogenesis in ischemic organs is modulated by immune cells. Systemic neovascularization of the ischemic lung requires macrophages, with chemokines playing a central role in new vessel growth. Because regulatory T (T_{reg}) cells modulate tumor-induced neovascularization, we questioned whether this CD4⁺ lymphocyte subset impacts blood vessel growth during ischemia. In a model of left lung ischemia, an increase in CD4⁺ CD25⁺ forkhead homeobox protein-3 (Foxp3)⁺ cells was observed 3–5 days after the onset of ischemia in wild-type C57Bl/6 mice. Using transgenic mice where Foxp3⁺ T_{reg} cells can be depleted with diphtheria toxin (DT; Foxp3^{DTR}), we unexpectedly found that Foxp3⁺ T_{reg} depletion led to markedly reduced lung angiogenesis (90% reduction from Foxp3^{gfp} controls). Adoptive transfer studies using CD4⁺ CD25⁺ splenocytes from congenic CD45.1 mice into Foxp3⁺ T_{reg}-depleted mice showed an almost complete recovery of the angiogenic phenotype (80% of Foxp3^{gfp} controls). A survey of lung gene expression of angiogenic (lipopolysaccharide-induced CXC chemokine [LIX], IL-6, IL-17) and angiostatic (IFN- γ , transforming growth factor- β , IL-10) cytokines showed T_{reg}-dependent differences only in LIX (CXCL5) and IL-6. Protein confirmation demonstrated a significant reduction

in LIX in T_{reg}-deficient mice compared with controls 5 days after the onset of ischemia. Phenotyping other inflammatory cells in the lung by multicolor flow cytometry demonstrated a significantly reduced number of macrophages (major histocompatibility complex class II [MHCII]^{int}, CD11C⁺) in T_{reg}-deficient lungs compared with T_{reg}-sufficient lungs. T_{reg} cells are essential for maximal systemic angiogenesis after pulmonary ischemia. One likely mechanism responsible for the decrease in angiogenesis in T_{reg}-depleted mice was the decline in the essential CXC chemokine, LIX.

Keywords: angiogenesis; ischemia; regulatory T cells; CXC chemokines

Clinical Relevance

Angiogenesis in the lung is understudied, yet it is a common feature of a variety of lung pathologies. Control of the growth of new blood vessels by regulatory T cells offers a potential therapy in situations where new vessels are required.

Angiogenesis is pivotal for diverse physiological processes, including embryonic development and growth, wound healing, and endometrial and gestational maintenance (1, 2). Modulation of angiogenesis in the lung is necessary for tissue repair, but is susceptible to dysregulation, and can become prominent and contribute to pathology in chronic

inflammatory conditions, such as cystic fibrosis, asthma, chronic obstructive pulmonary disease, interstitial lung diseases, chronic thromboembolic disease, and lung cancer (3–8). The systemic vasculature in and surrounding the lung is proangiogenic, whereas the pulmonary vasculature does not proliferate under most conditions (9–11). Understanding the

balance of proangiogenic and angiostatic factors is of paramount importance to understanding the regulation of the process of neovascularization. However, the underlying cellular mechanisms involved in lung angiogenesis remain largely unknown. Using a murine model of total left pulmonary artery ischemia, our laboratory has shown that subsequent

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neovascularization arises exclusively from the bronchial artery (12) and intercostal arteries (10), is initiated by reactive oxygen species (ROS) (13), and is dependent on early up-regulation of CXC chemokine growth factors (14). Furthermore, our laboratory has demonstrated the importance of mature lung macrophages in promoting angiogenesis (15), whereas lymphocytes limited the inflammation and ischemia-induced lung angiogenic responses (16). Several reports suggest that lymphocytes are involved in both homeostatic and pathological angiogenesis in peripheral tissue (17, 18). However, the role of lymphocytes or their specific subpopulations in lung angiogenesis has not been fully explored.

Forkhead homeobox protein-3 (Foxp3)⁺ regulatory T (T_{reg}) cells are a distinct subpopulation of CD4⁺ lymphocytes that also express CD25 (IL-2 receptor) and the transcription factor, Foxp3, which is the master regulator of T_{reg} development and suppressive function. Given the important regulatory role of Foxp3⁺ T_{reg} cells in resolving lung inflammation and promoting repair (19), we questioned whether this lymphocyte population similarly served a regulatory role after ischemic injury. Furthermore, we recently demonstrated that Foxp3⁺ T_{reg} cells modulated activated lung macrophages in response to endotoxin-induced lung injury (19). Given the importance of macrophages in promoting angiogenesis after ischemic insult (15), we hypothesized that Foxp3⁺ T_{reg} cells moderate lung angiogenesis through modulation of macrophage function.

In our experimental model of left lung ischemia, we found that wild-type (WT) mice had increased Foxp3⁺ T_{reg} cells over time in their left ischemic lung and draining lymph nodes. Using transgenic mice where Foxp3⁺ T_{reg} cells can be depleted with diphtheria toxin (DT; *Foxp3^{DTR}*), we found that Foxp3⁺ T_{reg} depletion led to markedly reduced lung angiogenesis. Contrary to expectations, our findings demonstrate that Foxp3⁺ T_{reg} cells are required for lung angiogenesis. These observations may have important clinical implications, as Foxp3⁺ T_{reg} cells could be used therapeutically to enhance angiogenesis in ischemic organs or to limit pathological blood vessel formation.

Materials and Methods

Mice

C57BL/6 WT (Jackson Laboratories, Bar Harbor, ME) and *Foxp3^{gfp}* and *Foxp3^{DTR}* mice (gifts of Dr. A. Y. Rudensky, Sloan-Kettering Institute [New York, NY]) were housed in a pathogen-free facility. Procedures were approved by the Johns Hopkins Animal Care and Use Committee (protocol no. MO13M239). The lung ischemic injury model was used as previously described (10, 15). Left pulmonary artery ligation (LPAL) was performed in mice and the left lung was harvested at specified time points.

Preparation of Cell Suspensions

Left lungs were collected in dissociator tubes (2 mg/ml Dulbecco's modified Eagles medium, collagenase D; 40 U/ml DNase I; and HEPES). Tissues were homogenized, incubated (37°C, 30 min), strained (70 μm), red blood cells removed (ammonium chloride potassium buffer), and cells were washed (cold PBS). Endothelial cell analysis followed the digestion protocol previously described (20).

Antibodies and Flow Cytometry

Fluorescence-conjugated anti-mouse antibodies were used to identify lung T cells, macrophages, and endothelial cells. A complete list of antibodies, concentrations, vendors, and detailed staining methods with gating strategy are provided in the MATERIALS AND METHODS section in the online supplement. Cells were acquired using

BD LSRII (Becton Dickinson Life Science Research II, Franklin Lakes, NJ) and analyzed with FlowJo (Tree Star, Ashland, OR).

Foxp3^{DTR} Mice and DT Administration

To eliminate Foxp3⁺ T_{reg} cells, we used transgenic *Foxp3^{DTR}* mice (21) and eliminated Foxp3⁺ T_{reg} cells *in vivo* through DT (Sigma, St. Louis, MO) administration (20 ng/g or 10 ng/g, intraperitoneal) (21, 22). *Foxp3^{gfp}* reporter mice that express an N-terminal green fluorescent protein–Foxp3 fusion protein served as controls (23).

Angiogenic Index

Functional angiogenic perfusion of the left lung was determined by infusing fluorescent microspheres (10 μm; Invitrogen, Grand Island, NY) into the aorta as described previously (15).

Isolation of CD4⁺ CD25⁺ T Cells and Adoptive Transfer

Splenocytes were collected from naive congenic CD45.1, C57BL/6 mice. CD4⁺CD25⁺ T cells were purified by magnetic cell sorting (T_{reg} isolation kit; Miltenyi, San Diego, CA). Isolated cells (1 × 10⁶) or PBS (100 μl) were given to recipient mice intravenously 24 hours before LPAL.

Real-Time PCR

RNA was isolated from left lung using standard techniques followed by quantitative PCR (SYBR Green gene-specific primers; Table 1). Results were normalized (β₂-microglobulin) and fold change reported (versus 0 h left lung). We selected genes

Table 1. Cytokine Primer Sequences

Gene	Forward/Reverse	Sequence 5' to 3'
Mouse IL-10	Forward	TCG GCC AGA GCC ACA TG
	Reverse	TTA AGG AGT CGG TTA GCA AGT ATG TTG
Mouse IL-17A	Forward	GGA CTC TCC ACC GCA ATG AA
	Reverse	GCA CTG AGC TTC CCA GAT CAC
Mouse IFN-γ	Forward	TTG CCA AGT TTG AGG TCA ACA A
	Reverse	TGG TGG ACC ACT CGG ATG A
Mouse IL-6	Forward	TCG GAG GCT TAA TTA CAC ATG TTC
	Reverse	TGC CAT TGC ACA ACT CTT TTC T
Mouse LIX	Forward	GCCGCTGGCATTCTGTT
	Reverse	GGGCAGCTTCAGCTAGATGCT
Mouse TGF-β	Forward	CGGAGAGCCCTGGATACCA
	Reverse	GCCGCACACAGCAGTTCTT
Mouse β2 microglobulin	Forward	AAA TGC TGA AGA ACG GGA AAA
	Reverse	ATA GAA AGA CCA GTC CTT GCT GAA G

Definition of abbreviations: LIX, lipopolysaccharide-induced CXC chemokine; TGF-β, transforming growth factor-β.

with expected proangiogenic (IL-6, lipopolysaccharide-induced CXC chemokine [LIX], IL-17) and antiangiogenic (IFN- γ , transforming growth factor [TGF]- β , IL-10) effects.

Protein Analysis

Left lung was homogenized, centrifuged, and supernatants collected to determine LIX (CXCL5) and IL-6 by ELISA (R&D Systems, Minneapolis, MN).

Immunohistochemistry

Sections of left lungs of mice were stained with fluorescent anti-F4/80 for lung macrophages, anti-CXCL5 (LIX), and 4',6-diamidino-2-phenylindole to identify cell nuclei (see the MATERIALS AND METHODS section in online supplement). Fluorescent images were obtained with a microscope (Olympus IX-51; Olympus, Center Valley, PA) using a Sencam High Performance camera (Cooke, Auburn Hills, MI).

In Vitro Macrophage Cytokine Secretion

Single-cell suspensions of left lung (1×10^6 cells/ml) were dispensed (six-well

plates, 10% fenureek seed extract in Dulbecco's modified Eagles medium) and incubated (2 h at 37°C). Nonadherent cells were discarded, and fresh media with/without 100 μ M H₂O₂ was added to adherent fraction and incubated. Supernatants at 4 hours were analyzed for LIX and IL-6 (ELISA; R&D Systems).

Statistical Analysis

Data are presented as the mean (\pm SE); *t* tests were used to compare single times between strains. Time courses were analyzed with repeated measures ANOVA and Newman-Keuls multiple comparisons. A *P* value less than 0.05 was accepted as significant.

Results

Foxp3⁺ T_{reg} Cells Increase after Lung Ischemia

We sought to determine whether lung lymphocyte subpopulations were dynamically regulated in the ischemic lung. The percentage of CD4⁺Foxp3⁺

T_{reg} cells increased significantly 3 days after LPAL and peaked 7 days after lung ischemia (Figures 1A and 1B; *n* = 5–7 mice/time point, *P* < 0.0001). There was no change in Foxp3⁺ T_{reg} cell proliferation in the lung by intracellular ki67 staining (Figure 1C).

Lung Angiogenesis Is Markedly Reduced in the Absence of Foxp3⁺ T_{reg} Cells

To determine whether Foxp3⁺ T_{reg} cells participate in lung angiogenesis after ischemia, we used DT-treated transgenic Foxp3^{DTR} mice and Foxp3^{gfp} mice (controls). Foxp3^{gfp} and WT mice had similar increases in the percent of Foxp3⁺ T_{reg} cells (9% versus 6% of CD4⁺ T cells at 5 d [D5] after LPAL, respectively). We optimized the DT treatment scheme to deplete Foxp3⁺ T_{reg} cells over a 2-week period and achieved over 85% depletion of Foxp3⁺ T_{reg} cells (Figures 2A and 2B). Systemic angiogenesis of the left lung was assessed 14 days after LPAL (Figure 2C). Foxp3⁺ T_{reg}-depleted animals had a 90% reduction in angiogenic response in the left

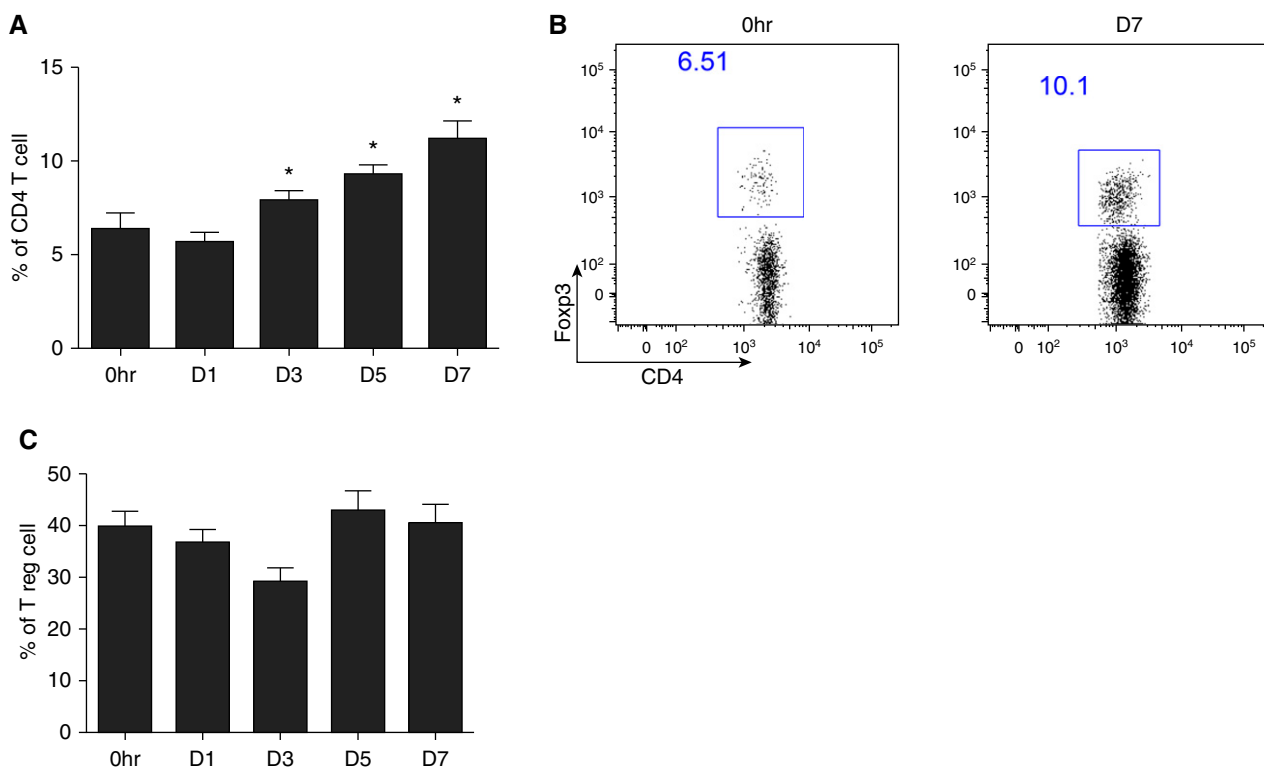


Figure 1. Regulatory T (T_{reg}) profiles after the induction of pulmonary ischemia in wild-type mice (*n* = 5–7 mice/time point). **P* < 0.05, different from 0 h control. (A) Average changes in lung T_{reg} cells (% of CD4⁺ T cells) at the onset of left lung ischemia (0 h) through the first 7 days (D7) after ligating the left pulmonary artery. A progressive increase in the percent of lung T_{reg} cells is seen over the course of the first 7 days of ischemia. (B) Representative dot plot showing changes in CD4⁺ forkhead homeobox protein-3 (Foxp3)⁺ cells immediately (0 h) and 7 days (D7) after the onset of ischemia. (C) T_{reg} cells (CD4⁺, Foxp3⁺ cells) showed no changes in the intracellular proliferation marker, ki67, over the time course of 7 days.

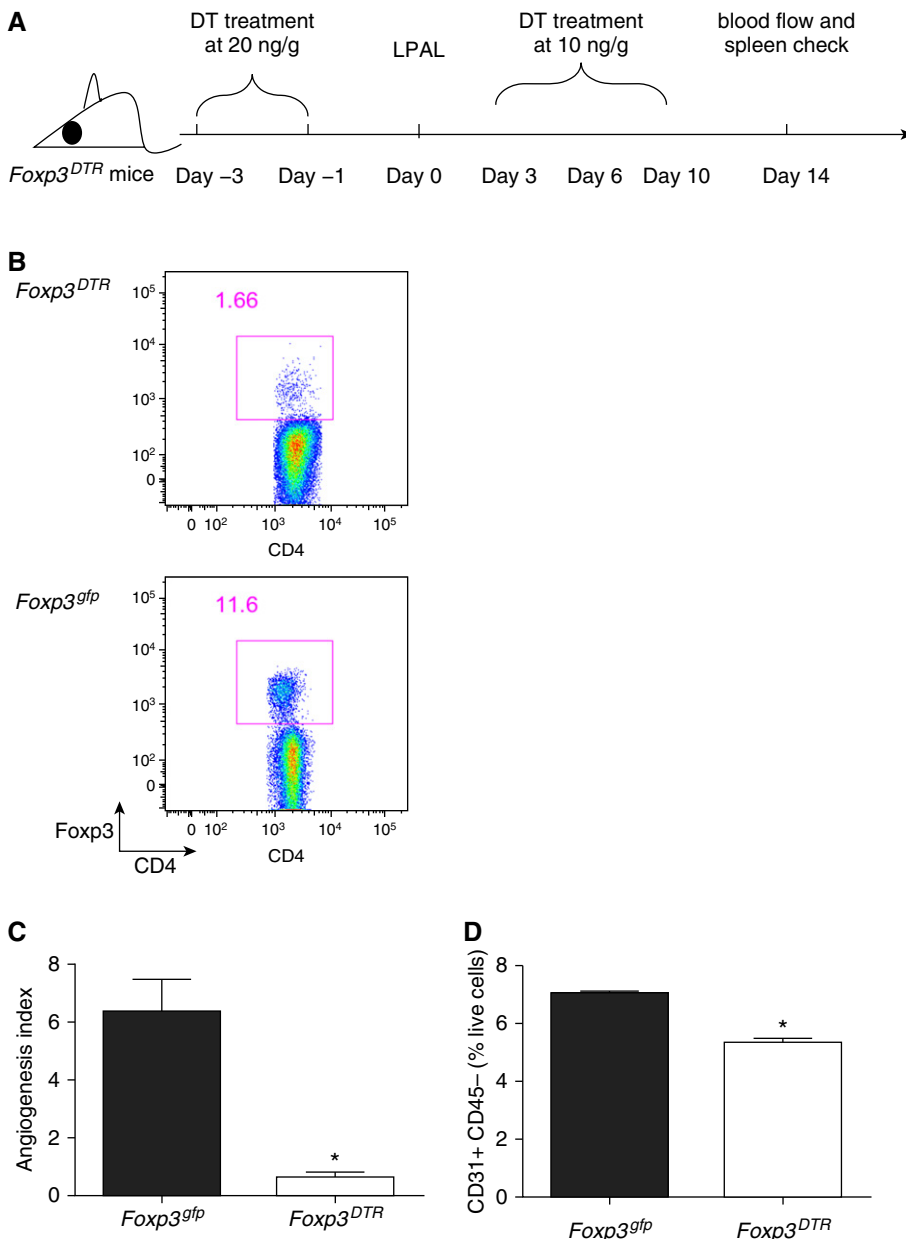


Figure 2. T_{reg} cell depletion prevents angiogenesis. (A) Treatment regimen to deplete T_{reg} cells in the $Foxp3^{DTR}$ mouse strain before and after left pulmonary artery ligation (LPAL) with diphtheria toxin (DT), and time of determination of angiogenesis (blood flow at Day 14). (B) Representative dot plot showing effectiveness of depletion treatment. $CD4^{+} Foxp3^{+}$ cells in T_{reg} -sufficient ($Foxp3^{gfp}$ mouse strain) spleens are much greater than in T_{reg} -deficient ($Foxp3^{DTR}$) spleens 14 days after LPAL. (C) Functional angiogenesis index assessed by perfusion to the left lung 14 days after LPAL. A significant reduction in angiogenesis was observed in mice without T_{reg} cells ($n = 6-8$ mice/group). $*P < 0.0001$. (D) $CD31^{+} CD45^{-}$ endothelial cells (% live cells) in the left lung 5 days after LPAL. A significant reduction in endothelial cells was seen in left lungs from $Foxp3^{DTR}$ mice compared with control $Foxp3^{gfp}$ lungs by 5 days after the induction of ischemia ($n = 2-3$ /group). $*P = 0.003$.

ischemic lung compared with $Foxp3^{gfp}$ controls (Figure 2B; $n = 6-8$ mice/group; $P < 0.0001$). Consistent with the functional angiogenic index, flow cytometry to detect $CD31^{+} CD45^{-}$ endothelial cells as early as

5 days after LPAL showed an average 25% reduction in the $Foxp3^{+} T_{reg}$ -depleted mice (Figure 2D; $n = 2-3$ /group; $P = 0.003$). Gating strategy for endothelial cells is shown in Figure E2 in the online

supplement. These results confirmed that, in the absence of T_{reg} cells, systemic angiogenesis and lung endothelial cell numbers are significantly reduced during ischemia.

Transfer of $Foxp3^{+} T_{reg}$ Cells Restores Lung Angiogenesis

To further confirm that $Foxp3^{+} T_{reg}$ cells are required to promote lung angiogenesis after ischemia, we used the T_{reg} depletion scheme shown in Figure 2A. In addition, 1 day before LPAL, mice were given a retro-orbital injection of PBS or T_{reg} cells isolated from congenic CD45.1 spleens and sorted using sterile technique. In contrast to endogenous T_{reg} cells, the adoptively transferred cells were not susceptible to exogenous DT depletion. After 14 days of lung ischemia, the $Foxp3^{+} T_{reg}$ -depleted group, that received PBS, showed a marked reduction in angiogenesis (Figure 3A; $n = 5-6$ mice/treatment) similar to that seen in Figure 2B. In contrast, the $Foxp3^{+} T_{reg}$ -depleted group that was adoptively transferred with congenic WT T_{reg} cells 1 day before LPAL showed a significant restoration of the normal angiogenic response observed after LPAL ($P = 0.001$). Adoptively transferred CD45.1 T_{reg} cells were confirmed in spleens 14 days after lung ischemia (Figure 3B), and were approximately the same as seen in $Foxp3^{gfp}$ controls (Figure 2B). These experiments reinforced the observation demonstrating a role for $Foxp3^{+} T_{reg}$ cells in promoting lung neovascularization after ischemia.

Lung Inflammatory Milieu Modulated by $Foxp3^{+} T_{reg}$ Cells

To begin to understand mechanisms underlying $Foxp3^{+} T_{reg}$ -mediated lung angiogenesis after ischemia, we measured at intervals, several prominent proangiogenic and antiangiogenic cytokines known to be involved in ischemia-induced vascular responses. Ischemic lung homogenate mRNA levels for IL-6 and LIX (CXCL5) were significantly higher in mice with intact $Foxp3^{+} T_{reg}$ cells compared with T_{reg} -depleted animals (Figure 4A; $n = 3-4$ mice/time point/group; $P < 0.05$). Other mediators studied (IFN- γ , TGF- β , IL-17 and IL-10) were not different between $Foxp3^{+} T_{reg}$ -sufficient or -depleted mice. To validate the mRNA results, we performed ELISA on ischemic lung homogenates to confirm significantly higher levels of LIX, but not IL-6 in $Foxp3^{+}$

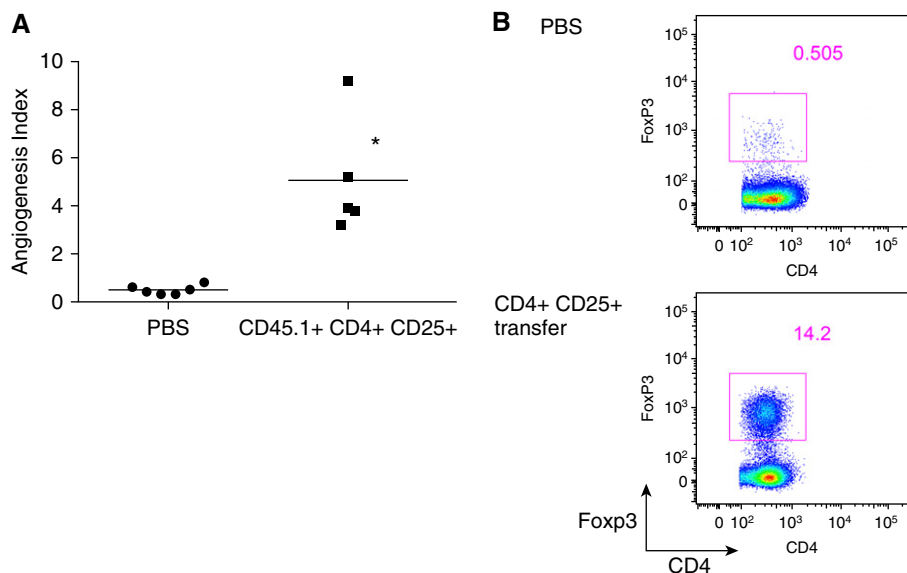


Figure 3. Transfer of normal Foxp3⁺ T_{reg} cells restores lung angiogenesis. (A) Foxp3^{DTR} mice treated with DT and vehicle showed reduced angiogenesis (each point represents one mouse and the horizontal line is group mean). However, a significant increase in angiogenesis occurred in T_{reg}-depleted mice after adoptive transfer of CD45.1⁺CD4⁺CD25⁺ splenocytes before LPAL ($n = 5-6$ mice/group). * $P = 0.001$. (B) Representative dot plot of cells from spleens showing effectiveness of depletion treatment (PBS) and adoptive transfer (CD4⁺CD25⁺). CD4⁺Foxp3⁺ cells in the T_{reg}-sufficient (Foxp3^{gfp}) spleen are much greater than in T_{reg}-deficient (Foxp3^{DTR}) spleens 14 days after LPAL.

T_{reg}-sufficient mice (Figure 4B; $n = 5-6$ mice/time point/group; $P < 0.001$). Of note, the differences in LIX were measured at D5, whereas differences in mRNA levels were detected earlier at D3 after lung ischemia. Our studies support the notion that T_{reg} cells allow a more favorable proangiogenic milieu in the ischemic lung characterized by higher lung LIX levels.

Foxp3⁺ T_{reg} Cells Promote Increased Macrophages during Lung Ischemia

To explore lung macrophage modulation by Foxp3⁺ T_{reg} cells, we further immunophenotyped macrophages in dissociated left lung by multicolor flow cytometry at the onset of ischemia (0 h) and at intervals after LPAL. We found similar percent live macrophages in the lungs (major histocompatibility complex class II [MHCII]^{int}, CD11C⁺) initially and during the first 3 days after LPAL in T_{reg}-depleted Foxp3^{DTR} and T_{reg}-sufficient Foxp3^{gfp} mice, with ever-increasing numbers. However, by D5 after LPAL, Foxp3^{gfp} ischemic left lung had nearly threefold more macrophages than their Foxp3^{DTR} counterparts (Figure 5A; $n = 6-9$ mice/time point/group; $P < 0.01$).

Evaluation of activation markers CD86 (M1 activation), and mannose receptor (M2 activation), and mannose receptor (M2 activation) showed no difference between Foxp3^{gfp} and Foxp3^{DTR} mice as assessed by mean fluorescence intensity. However, overall mannose receptor staining was much greater than CD86 in both groups (data not shown). To begin to determine the source of LIX in left lungs, immunohistochemistry was performed using frozen sections from both groups of mice ($n = 1$ mouse/group at 0 h, 2 mice/group at 5D). Figure 5B shows a representative example of a 5D Foxp3^{gfp} left lung showing F4/80⁺ cells colocalized with LIX protein. In all sections evaluated, macrophage-LIX colocalization was confirmed.

To determine whether there were inherent differences in lung macrophages between the two groups of mice, we studied *in vitro* secretion of proangiogenic cytokines, LIX and IL-6. Macrophages isolated on D5 from T_{reg}-depleted Foxp3^{DTR} and T_{reg}-sufficient Foxp3^{gfp} lungs showed similar patterns of cytokine secretion. Figure 6 demonstrates that, whether under basal conditions or after stimulation with an ROS mimic (H₂O₂), macrophages from the two strains

responded in a similar manner. ROS stimulation caused a significant increase in cytokine release in macrophages from both strains ($P = 0.01$).

Discussion

Organ ischemia is among the leading pathophysiological causes of morbidity and mortality. New blood vessel formation in response to ischemia is critical for the restoration of organ homeostasis. The goal of the present study was to determine the modulating influence of T_{reg} cells (CD4⁺CD25⁺Foxp3⁺) on the process of lung neovascularization during ischemia. Our previous work demonstrated that angiogenesis in the lung is dependent on macrophage-derived chemokine release early after the induction of pulmonary vascular ischemia. Consequently, we predicted that limiting T_{reg} control of chemokine-secreting macrophages would enhance angiogenesis. Contrary to these expectations, results demonstrated that T_{reg} depletion eliminated angiogenesis completely. The prominent CXC chemokine, LIX, was significantly reduced in lung homogenate in T_{reg}-depleted mice, as were the number of macrophages. We conclude that T_{reg} cells are essential for maximal neovascularization after pulmonary ischemia. One mechanism by which T_{reg} cells control the angiogenic process is by limiting the number of chemokine-producing macrophages in the lung.

Our work describes a novel role for T_{reg} cells in modulating angiogenesis after lung ischemia. In general, the role of lymphocytes in angiogenesis has become more apparent. Recently, Du and colleagues (24) demonstrated that tumor-infiltrating lymphocytes produce IL-17 and promote tumor growth by enhancing angiogenesis. Moreover, Tayade (18) reported that lymphocytes promoted endometrial angiogenesis, and thereby contributed to fetal health. An earlier study of graft versus host disease showed that injection of lymphocytes into histoincompatible hosts resulted in formation of networks of new blood vessels (25). These reports suggest that lymphocytes are involved in both homeostatic and pathological angiogenesis in peripheral tissues. However, to our knowledge, the role of lymphocytes or their specific subtypes in lung angiogenesis has not been described.

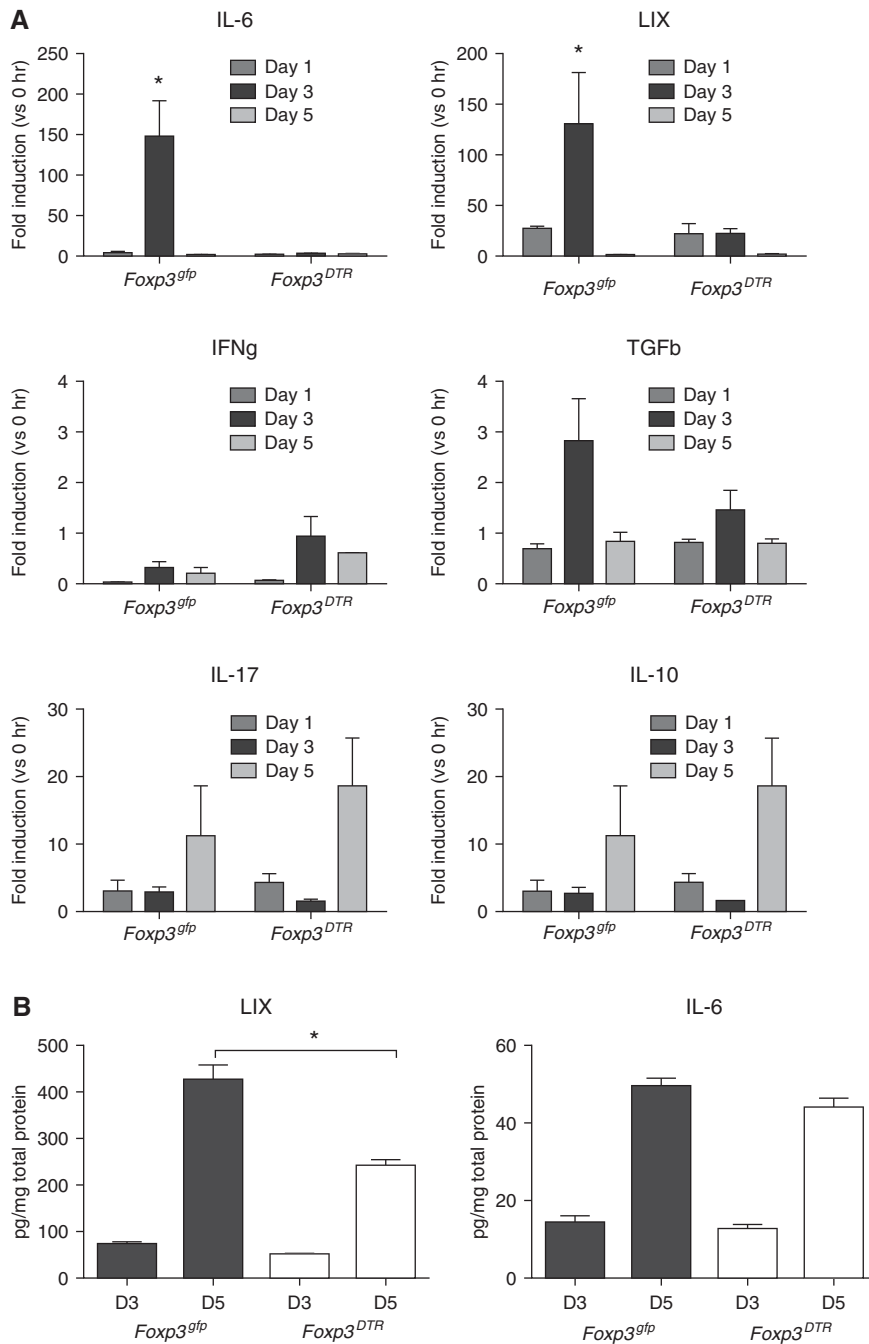


Figure 4. Lung inflammatory milieu is modulated by $Foxp3^+$ T_{reg} cells. (A) Time course of mRNA expression (fold induction from 0 h) of proangiogenic (IL-6, lipopolysaccharide-induced CXC chemokine [LIX], IL-17) and angiostatic (IFN- γ , transforming growth factor [TGF]- β , IL-10) cytokines in the left lungs of T_{reg} -sufficient ($Foxp3^{gfp}$) and T_{reg} -deficient ($Foxp3^{DTR}$) mice. A significant difference in IL-6 and LIX expression was seen between the two strains 3 days after the onset of ischemia ($n = 3-4$ mice/time point/group). * $P < 0.05$. (B) LIX and IL-6 protein of the upper left lung was confirmed at Day 3 (D3) and Day 5 (D5) after LPAL in $Foxp3^{gfp}$ and $Foxp3^{DTR}$ mice ($n = 5-6$ mice/time point/group). * $P < 0.001$. Although significant increases in both LIX and IL-6 were seen in both groups between D3 and D5, the change in LIX protein was significantly attenuated in $Foxp3^{DTR}$ mice. No difference in IL-6 between $Foxp3^{gfp}$ and $Foxp3^{DTR}$ mice was apparent at either time point.

T_{reg} cells have been regarded as master regulators of exuberant immune responses (23). Work from the Rudensky laboratory described profound autoimmunity when using transgenic mice where specific depletion of T_{reg} cells can be achieved (e.g., $Foxp3^{DTR}$). We expected that depletion of T_{reg} cells would have led to an enhanced lung proinflammatory milieu and, subsequently, a greater angiogenic response in the ischemic lung. The robust and consistent differences in phenotype between T_{reg} -sufficient and T_{reg} -deficient mice with regard to measurements of functional angiogenesis were somewhat surprising, given the complex process of angiogenesis after ischemia. Furthermore, the gain of function when T_{reg} cells from CD45.1 splenocytes were adoptively transferred into mice without T_{reg} cells was highly reproducible with little variability (Figure 3). These results highlight the capacity of T_{reg} cells to ostensibly control the process of neovascularization, and reinforce the critical and novel nature of T_{reg} cells as potent proangiogenic cells.

To begin to determine the mechanism for the differences in angiogenesis, we surveyed cytokines known to be involved in ischemia-induced neovascularization. The survey of proangiogenic and suppressive cytokines in the ischemic left lung revealed differences between the T_{reg} -sufficient and T_{reg} -deficient mice in two cytokines that we have shown to be critical growth factors for neovascularization (Figure 4A). Previously, we showed that the CXC chemokines (LIX, MIP-2 α , and KC) and IL-6 were up-regulated early after the onset of ischemia and when blocked with neutralizing antibody for the CXCR1/2 receptor, which binds these chemokines, and, in IL-6 null mice, typical neovascularization was prevented (26, 27). With regard to the CXC chemokines, our work was supportive of the work of Strieter and colleagues (28), who showed, in a number of proangiogenic pathologies, that these proteins bind CXCR1/2 on endothelial cells and promote proliferation and chemotaxis. In the current study, we focused on LIX as representative of the chemokines and IL-6. LIX mRNA and protein were significantly decreased in the T_{reg} -deficient mice, suggesting a possible mechanism for decreased angiogenesis in this experimental group (Figure 4B). Although LIX is produced by several lung

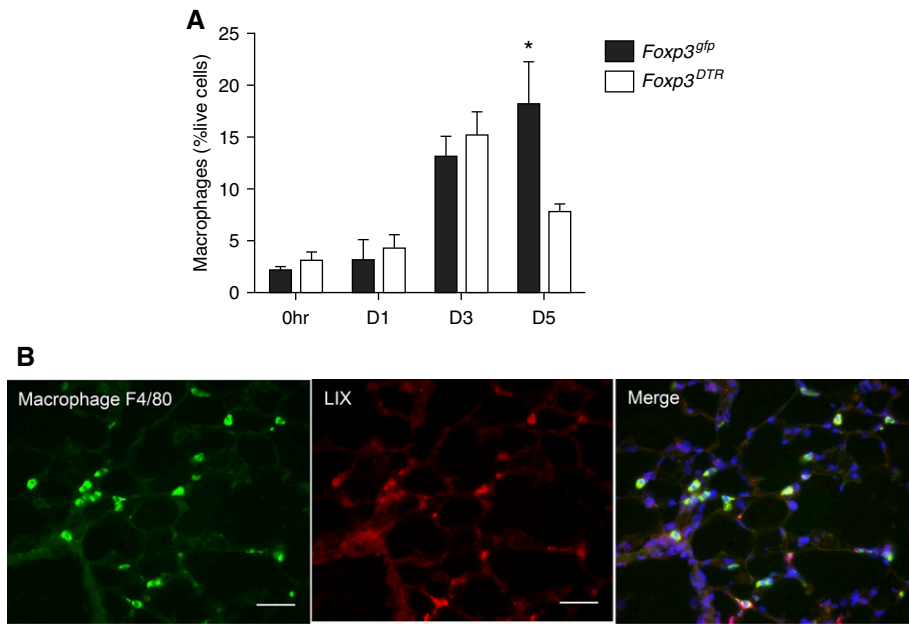


Figure 5. *Foxp3*⁺ T_{reg} cells promote increased macrophage numbers during lung ischemia. (A) The time course of macrophages (major histocompatibility complex class II^{int}, CD11C⁺) in left lungs showed a progressive increase in T_{reg}-sufficient *Foxp3^{gfp}* mice. However, by D5 after LPAL, *Foxp3^{gfp}* ischemic left lungs had nearly threefold more macrophages than their *Foxp3^{DTR}* counterparts ($n = 6-9$ mice/time point/group). * $P < 0.01$, *Foxp3^{gfp}* versus *Foxp3^{DTR}*. (B) Representative immunostaining of macrophage F4/80 (green), LIX protein (red), and the merge showing colocalization (yellow-green) of a left lung section of *Foxp3^{gfp}* lung 5 days after LPAL. 4',6-diamidino-2-phenylindole (blue) labels all cell nuclei. Scale bar, 10 μ m; original magnification, $\times 400$.

cell types, immunohistochemistry showed clear colocalization of LIX protein and F4/80⁺ lung macrophages (Figure 5B). The observation that the number of macrophages was significantly reduced coincident with the decline in LIX protein was suggestive of a role for macrophages in the observed difference in LIX protein. However, when studied *in vitro*, macrophages isolated from the lungs of the two mouse strains did not show differences in their *in vitro* capability to secrete cytokines, either under basal conditions or upon oxidant stimulation. Thus, we suggest that one explanation for the decrease in LIX protein in the lung of T_{reg}-deficient mice is the decrease in chemokine-producing macrophages, and not in the inherent ability of these cells to secrete chemokine. At this time, we cannot explain the observation that IL-6 protein appeared similar in both strains. We speculate that the kinetics of this protein may differ from LIX, thereby contributing to the lack of significant changes at the time points of measurement.

It should be noted that, because we saw no differences in gene expression of

known suppressive cytokines, such as IL-10, TGF- β , or IFN- γ , between T_{reg}-sufficient and T_{reg}-deficient lungs, we pursued the proangiogenic cytokines that showed differences. We acknowledge that, at later time points, the suppressive cytokines may play an additional role in modulating the extent of angiogenesis. Because of past work implicating macrophages as critical to the process of ischemia-induced angiogenesis in the lung and the strain-dependent changes in lung macrophages at the time when there were measurable differences in an important proangiogenic chemokine, we focused on the macrophage as a source of growth factors. However, there are likely to be other cells interacting with T_{reg} cells that may contribute as much, if not more, to the profound differences in angiogenesis observed between strains. Our laboratory has recently described that T_{reg} cells can modulate alveolar epithelial proliferation and repair (20); thus, a T_{reg} cell-epithelial cell cross-talk might also exist that could further promote the angiogenic response. Similarly, a T_{reg} cell-endothelial cell interaction might directly promote angiogenesis. ROS-induced

T_{reg} activation has been described (29), and, thus, the ROS release during pulmonary ischemia confirmed by our laboratory and others might promote a compensatory angiogenic response requiring T_{reg} cells (13, 30). For example, T_{reg} cells can express high levels of neuropillin that can bind to vascular endothelial growth factor receptor on endothelial cells to promote their sprouting and, ultimately, new vessel formation (31, 32). Although, in this nonhypoxic model, neither vascular endothelial growth factor nor other hypoxia-inducible factor-1-alpha (HIF-1 α)-regulated growth factors were shown to be up-regulated (14), the direct effect of T_{reg} cell activation of pulmonary endothelium remains to be elucidated and is ongoing in our laboratory.

Also of interest is the observation that differences between T_{reg}-sufficient and T_{reg}-deficient mice become most apparent at 5 days, the point in time at which the new systemic vasculature

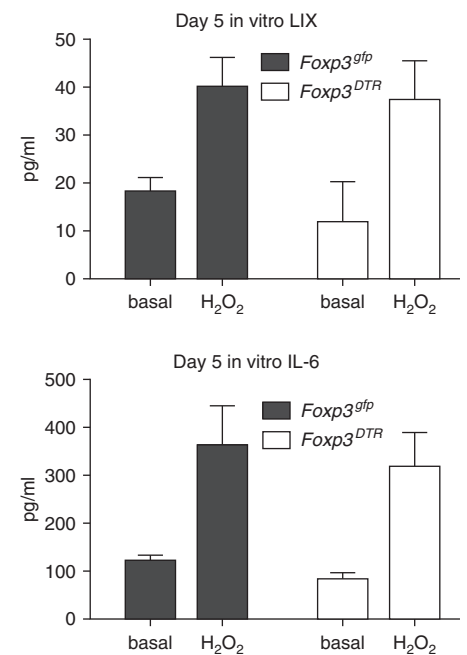


Figure 6. Macrophages from T_{reg}-sufficient *Foxp3^{gfp}* mice and T_{reg}-deficient *Foxp3^{DTR}* show similar levels of cytokine expression *in vitro*. Macrophages isolated from the lungs of T_{reg}-sufficient *Foxp3^{gfp}* mice and T_{reg}-deficient *Foxp3^{DTR}* mice 5 days after the onset of ischemia showed similar basal secretion of LIX and IL-6. Stimulation with a reactive oxidant (H₂O₂) showed a significant increase in cytokine secretion ($n = 3-4$ experiments/group; $P = 0.01$), but no difference between strains.

becomes functional in WT mice (10). Whether the lack of T_{reg} cells prevents macrophage proliferation within the ischemic left lung beyond 3 days, or whether the lack of a fully functional vasculature prevents the influx of macrophages (and chemokine availability) in the recovering ischemic lung, cannot be determined from these studies. Future studies evaluating lung morphometry, including microvessel density, with macrophage and T_{reg} localization, may

provide additional insight into the process of lung neovascularization.

In summary, it is clear that T_{reg} cells are vitally important to the process of lung neovascularization. Although our efforts to understand the mechanism by which T_{reg} cells control ischemia-induced angiogenesis focused on changes in macrophage-derived growth factor release, there are likely other processes, such as direct T_{reg}-endothelial cell proliferative interactions, as well as

suppression of effector T cells. Enhancing and understanding T_{reg}-mediated proangiogenic effects could become a novel strategy for therapeutic angiogenesis required for ischemic organs. ■

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