Effector T Cells and Ischemia-Induced Systemic Angiogenesis in the Lung

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Abstract

Lymphocytes have been shown to modulate angiogenesis. Our previous work showed that T regulatory (Treg) cell depletion prevented angiogenesis. In the present study, we sought to examine T-cell populations during lung angiogenesis and subsequent angiostasis. In a mouse model of ischemia-induced systemic angiogenesis in the lung, we examined the time course (0–35 d) of neovascularization and T-cell phenotypes within the lung after left pulmonary artery ligation (LPAL). T cells increased and reached a maximum by 10 days after LPAL and then progressively decreased, suggestive of a modulatory role during the early phase of new vessel growth. Because others have shown IFN- γ to be angiostatic in tumor models, we focused on this effector T-cell cytokine to control the magnitude of angiogenesis. Results showed that IFN- γ protein is secreted at low levels after LPAL and that mice required Treg depletion to see the full effect of effector T cells. Using Foxp3^{DTR} and diphtheria toxin to deplete T regulatory cells, increased numbers

of effector T cells (CD8⁺) and/or increased capacity to secrete the prominent angiostatic cytokine IFN- γ (CD4⁺) were seen. *In vitro* culture of mouse systemic and pulmonary microvascular endothelial cells with IFN- γ showed increased endothelial cell apoptosis. CD8^{-/-} mice and IFN- γ R^{-/-} mice showed enhanced angiogenesis compared with wild-type mice, confirming that, in this model, IFN- γ limits the extent of systemic neovascularization in the lung.

Keywords: angiogenesis; angiostasis; ischemia; IFN- γ ; regulatory T-cells

Clinical Relevance

Angiogenesis in the lung is poorly understood. Our results demonstrate that after ischemia, neovascularization is regulated by effector T cells through IFN- γ secretion.

The role of inflammatory cells in angiogenesis is well documented. The work of others in peripheral organs and our own studies in the lung show an essential role for lung macrophages immediately after the onset of tissue ischemia (1–4). A reduction in lung macrophages was correlated with a reduction in the magnitude of angiogenesis (5). Others have shown that lymphocytes play a pivotal role in the extent of neovascularization in several models, demonstrating both pro- and antiangiogenic responses (6–8). Specifically,

CD4⁺ and CD8⁺ T lymphocytes have been suggested to play a role in vascular remodeling (6, 9). Previous results from our laboratory show enhanced angiogenesis in Rag1^{-/-} mice lacking both T and B lymphocytes (5). Recently, our laboratory has shown that mice deficient in T-regulatory (Treg) cells lack the normal angiogenic response to pulmonary ischemia (1). The role of specific lymphocyte subpopulations in lung angiogenesis has not been fully explored. However, the fact that lymphocytes and their cytokine

products can alter the growth of new vessels suggests that the balance of inflammatory cells dictates overall the growth phase after an acute ischemic insult. It is unclear whether during chronic ischemia that dynamic balance is responsible for slowing neovascularization and promoting eventual angiostasis.

IFN- γ is the quintessential Th1 cytokine produced by effector T cells (10–12). This cytokine has been shown to be a potent antiangiogenic agent that acts directly on endothelial cells and through

(Received in original form March 17, 2015; accepted in final form July 27, 2015)

This work was supported by National Institutes of Health grants HL10342 and HL113392.

Author Contributions: Conception and design: Q.Z., F.D.A., and E.M.W. Experimental work: Q.Z., J.J., and A.M. Analysis and interpretation: Q.Z., J.J., A.M., and E.M.W. Writing the manuscript: Q.Z. and E.M.W.

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This article has an online supplement, which is accessible from this issue's table of contents at www.atsjournals.org

Am J Respir Cell Mol Biol Vol 54, Iss 3, pp 394–401, Mar 2016

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Originally Published in Press as DOI: 10.1165/rcmb.2015-0087OC on August 5, 2015

Internet address: www.atsjournals.org

the secondary release of other angiostatic cytokines (13). The capacity for IFN- γ to inhibit angiogenesis has been demonstrated primarily in tumor models (8, 14, 15). Whether effector T cells and IFN- γ play a similar inhibitory role during recovery from tissue ischemia has not been widely studied. In a skin wound healing model, depletion of CD4⁺ or CD8⁺ T cells separately showed no difference in angiogenesis despite differences in IFN- γ expression and inflammatory cell profile (7). Stable and colleagues showed decreased angiogenesis in response to hindlimb ischemia in CD4-null mice (9). However, little information exists regarding systemic angiogenesis during lung ischemia as it relates to T-cell recruitment or modulation of new vessel growth.

Lung ischemia, induced by obstruction of a pulmonary artery, has been shown to promote systemic angiogenesis involving bronchial and/or thoracic intercostal arteries that invade the ischemic lung. The extent of systemic neovascularization of the lung was shown to increase over time in mice (16), rats (17, 18), dogs (19), sheep (20), and humans (21, 22). However, new systemic perfusion did not increase to the expected level of normal pulmonary blood flow to the subserved lung. We have shown previously in a limited series that a plateau in angiogenesis is reached by 3 to 4 weeks after the onset of left lung ischemia induced by left pulmonary artery ligation (16). The time course of changes in systemic perfusion in this model is summarized in Figure 1. After a short period of complete lung ischemia, new vessels from intercostal arteries invade the lung within 5 days (23) (see Figure E1 in the online supplement). This is followed by the angiogenic phase with systemic vessel proliferation and enlargement (23, 24). Approximately 3 weeks later, growth slows to a period of relative angiostasis (16). In the present study, we questioned whether specific

lymphocyte subpopulations contribute to the expected late angiostasis of the ligated left lung. We hypothesized that macrophage-derived growth factors essential for early neovascularization were replaced by antiangiogenic factors from lymphocytes during the late period of angiostasis. Our results demonstrate that lymphocyte influx into the ischemic left lung reaches a maximum by 10 days after the onset of ischemia and progressively declines. We found that IFN- γ levels were detectable during lung angiogenesis, augmented in the absence of Treg cells, and displayed potent endothelial apoptotic effects. Consistent with our findings, IFN- γ receptor 1-null mice showed enhanced angiogenesis. Our results suggest a modulating influence of IFN- γ to limit angiogenesis in this noninfectious model.

Materials and Methods

Mice

C57BL/6 wild-type (WT), CD4-null, CD8-null, and IFN- γ receptor 1-null (male, 6–8 wk old; Jackson Labs, Bar Harbor, ME) mice were housed in a pathogen-free facility. *Foxp3^{gfp}* and *Foxp3^{DTR}* mice, gifts of Dr. Alexander Y. Rudensky (Sloan-Kettering Institute), were bred on site. The Johns Hopkins Animal Care and Use Committee approved all experimental procedures (Protocol #MO13M239). Left lung ischemia was studied as previously described where anesthetized (2% isoflurane), ventilated (120 breaths/min, 0.2 ml/breath) mice were subjected to left pulmonary artery ligation (LPAL) (16, 25).

Angiogenesis Index

Systemic neovascularization of the lung was determined at designated times (2, 3, 4, and 5 wk) after LPAL by fluorescent bead

(10 μ m; Invitrogen, Grand Island, NY) infusion (2, 24, 25). Microspheres lodged in the left lung were quantified after tissue digestion and fluorescent dye extraction. Validation of this technique as an angiogenic index compared with changes in lung vascular morphometry is shown in Figure E1. Some mice were treated with anti-mouse IFN- γ (1 mg intraperitoneally) (Clone R4-6A2; Bio X Cell, West Lebanon, NH) 2 hours before and 5 days after LPAL (8, 26). Separate WT mice were studied concurrently with knockout mice to control for reagent/operator differences. Data are presented as percentage of microspheres in the left lung relative to the total delivered (angiogenesis index).

Preparation of Cell Suspensions

Single-cell suspensions of left lungs were acquired for T-cell phenotyping according to previously described methods (1). Further details are provided in the online supplement.

Antibodies and Flow Cytometry

Fluorescence-conjugated anti-mouse antibodies were used to label inflammatory cells (details are provided in the online supplement). Cell counts were acquired on a BD LSRII. Data were analyzed with FlowJo software (Tree Star, Ashland, OR).

Immunohistochemistry

Mice were anesthetized, and left lungs were infused with embedding material to ensure optimal cutting temperature (OCT), frozen, and cut into coronal sections. Immunofluorescence staining was used to assess the distribution of CD3⁺ cells colocalized with CD31⁺ endothelium and apoptotic cells (annexin V⁺). Further details are provided in the online supplement.

T-Cell Stimulation *In Vitro*

Single-cell suspensions of the left lung were restimulated with phorbol 12-myristate 13-acetate and ionomycin, and IFN- γ was measured using FACS analysis. Further details are provided in the online supplement.

Foxp3^{DTR} Mice and Diphtheria Toxin Administration

To skew mice toward predominantly effector T cells, Treg cells were eliminated using transgenic *Foxp3^{DTR}* mice and diphtheria toxin as previously reported (1). Further details are provided in the online supplement.

Figure 1. Summary of the time course of systemic blood vessel growth to the lung after left lung ischemia. After a short period of complete lung ischemia, new vessels from intercostal arteries invade the lung within 5 days (23). This is followed by the angiogenic phase with vessel proliferation and enlargement (23, 24). By approximately 3 weeks after the onset of ischemia, vessel growth slows to a period of relative angiostasis (16).

Figure 2. Functional perfusion of the left lung (angiogenesis index) 21 through 35 days after the onset of ischemia in wild-type (WT) and *Rag-1*^{-/-} mice. A significant difference in angiogenesis exists between WT mice and mice without lymphocytes only at the 21-day time point. The average angiogenic index 14 days after left pulmonary artery ligation (LPAL) (D14) is provided to put changes in context with current work (5). Figure E1 in the online supplement shows morphologic changes as new systemic vessels invade the ischemic lung. **P* = 0.02 (*n* = 2–6 mice/time point, including 12 CD57BL/6 mice and 15 *Rag-1*^{-/-} mice). D, days.

ELISA

IFN- γ in homogenized left lung tissue and plasma was measured using a mouse IFN- γ kit (R&D, Minneapolis, MN). Further details are provided in the online supplement.

Endothelial Cells *In Vitro*

Lung microvascular and aortic endothelial cells were isolated from C57BL/6 mice as previously described (27) (details are provided in the online supplement). For cell apoptosis staining, endothelial cells were stained with an Annexin V kit (eBioscience, San Diego, CA) according to the manufacturer's protocol. Samples were evaluated on a BD Aria (San Jose, CA) and analyzed with FlowJo.

Statistical Analysis

Data are presented as the mean $\pm$ SE. ANOVA was used to compare multiple groups, followed by Fisher's LSD. *P* < 0.05 was accepted as significant.

Results

Lymphocytes Modulate Early, but Not Late, Angiogenesis Index

To confirm the late time course of ischemia-induced angiogenesis after LPAL, systemic

blood flow to the left lung was determined. Figure 2 shows that in WT mice, a progressive increase in functional angiogenesis was observed that reached a plateau by D28 (*n* = 12 mice). This observation is consistent with that previously suggested (16) and is shown in context with historical data of an earlier time point (D14) (5). Functional angiogenesis in *Rag-1*^{-/-} mice, devoid of T and B lymphocytes (*n* = 15 mice), was significantly different from WT only at D14 (5) and D21 (*P* = 0.02). Because a similar plateau in perfusion is reached by both strains, lymphocytes appeared not to play a major role in angiostasis at late time points by this assessment.

Lung T-Lymphocyte Time Course

To determine T-lymphocyte recruitment to the lung, the complete time course from the onset of ischemia (D0) through D35 was determined in dissociated left lung. Figure 3 shows these changes (*n* = 3 mice/time point; **P* < 0.05 from D0). A significant increase in each T-cell group occurred after LPAL (*P* < 0.001). Peak recruitment for each group occurred at early time points (D7–D14) after LPAL and coincided with the time when a functional vasculature had been established (23). By D21, the overall percentage of CD3⁺ T cells was not different from baseline (D0) and remained at a low level. These results are consistent with the findings shown in Figure 2 suggesting that T cells do not play a role in angiostasis late (>21 d) after ischemic injury because they are not increased at these late time points and that WT and *Rag-1*^{-/-} mice showed similar plateau levels of perfusion. Because the peak increase in CD3⁺ T cells occurred by D10 after LPAL, subsequent experiments focused on changes in the lung up to D14. Immunohistochemistry was used to confirm that T cells localized to the pleural region of the left lung where blood vessels have been shown to predominate (23). Figure 4 shows images of frozen lung sections 10 days after LPAL. CD31⁺ blood vessels (top left) colocalize with CD3⁺ cells (top right and merged composite). Because we have shown previously and in Figure E1D that angiogenic vessels appear initially most prominently within the visceral pleura (23), the accumulation of T cells confirms their presence at the site of active angiogenesis.

Angiogenesis in CD8^{-/-} and CD4^{-/-} Mice

Based on the observed increases in CD8⁺ and CD4⁺ T cells (Figure 3), we determined whether phenotypic changes in angiogenesis occurred in mice without CD8⁺ and CD4⁺ T cells. Figure 5 shows systemic perfusion of the left lung 14 days after LPAL in WT, CD8^{-/-}, and CD4^{-/-} mice studied concurrently. Each point represents one mouse. CD8^{-/-} mice showed enhanced angiogenesis compared with WT (95% increase compared with WT; *P* < 0.05), yet CD4^{-/-} showed a variable phenotype. We showed recently that one subpopulation of CD4⁺ cells—Treg cells (CD4⁺, CD25⁺ Foxp3⁺)—was required for normal angiogenesis (1). Hence, we suggest that subpopulations of CD4⁺ cells contribute differently to angiogenic outcome, and consequently a variable phenotype is seen in the CD4^{-/-} mice.

In Vitro T-Cell Production of IFN- γ

We next sought to characterize the capacity for CD8⁺ and CD4⁺ T cells in the ischemic lung to release the angiostatic cytokine IFN- γ . We studied WT mice and lungs from mice without Treg cells to skew toward increased populations of CD4⁺ and CD8⁺ effector T cells. We showed previously that Treg cell depletion completely prevented ischemia-induced angiogenesis (1). We recovered CD8⁺ and CD4⁺ T cells in the lung 5 days after LPAL. The rationale for this time point was based on our previous study (1) and that the recovered T cells represented the initial

Figure 3. Time course of changes in lung T cells. A significant change in CD3⁺, CD4⁺, and CD8⁺ cells (as percentage of live cells) was observed after LPAL (*P* < 0.001). Recruitment of each group at D7 and D10 was significantly greater than at D0. **P* < 0.05 from D0 (*n* = 3 mice/time point).

Figure 4. Lung immunohistochemistry D10 after LPAL. Anti-CD31⁺ staining (green, upper left panel) shows vascular network, with arrows indicating pleural surface. CD3⁺ cells (red, upper right panel) are prominent in the visceral pleura where angiogenic vessels first appear (23). Composite image (lower right panel) shows colocalization of CD3⁺ cells with CD31⁺ endothelium. DAPI (blue stain) represents all cell nuclei. DAPI, 4',6-diamidino-2-phenylindole.

recruited population. Average results are shown in Figure 6. Compared with WT, DT-treated *Foxp3^{DTR}* mice showed a large increase CD8⁺ cells as a percentage of live cells (upper right panel; $P < 0.01$ from WT and *Foxp3^{gfp}* controls) and a trend toward an increased percentage of CD4⁺ cells (upper left panel; $P = 0.08$; $n = 3-4$ mice/group). We measured changes in IFN- γ upon CD8⁺ and CD4⁺ T-cell stimulation. CD4⁺ cells from left lungs of DT-treated *Foxp3^{DTR}* mice showed a large increase in IFN- γ production (lower left panel;

$P = 0.002$ versus WT; $n = 3-6$ mice/group). These results confirm that mice without Treg cells have increased percentages of effector T cells (CD8⁺) and/or an increased capacity to secrete the prominent angiostatic cytokine IFN- γ (CD4⁺).

In Vivo IFN- γ Protein

To further confirm IFN- γ secretion, protein was measured in left lung homogenate 5 days after the onset of ischemia in each of the three experimental groups (Figure 7A). Mice without Treg cells showed a large and significant increase in left lung IFN- γ protein compared with WT and treated controls (*Foxp3^{gfp}*; $n = 5-6$ mice/group; $P < 0.0001$). These results demonstrate that low levels of IFN- γ are present in the WT mouse lung 5 days after LPAL. However, when mice are skewed toward increased T-effector cells by removing Treg control, a significant increase in IFN- γ is revealed. In another series of mice, serum IFN- γ protein was measured over the 14-day time course after LPAL in WT mice and in Treg cell-depleted mice and their controls. Using standard detection methods, IFN- γ protein was detectable only in the serum

Figure 5. Depletion of CD8⁺ T cells enhances angiogenesis 14 days after LPAL. Each point represents one mouse. * $P < 0.05$.

of mice without Treg cells ($n = 2-6$ samples/time point) (Figure 7B). Statistical analysis demonstrated a significant strain effect but no time effect ($P < 0.0001$). Hence, combining all time points within each group showed a highly significant increase in serum IFN- γ protein in Treg cell-deficient mice (*Foxp3^{DTR}*) compared with both WT and treated controls ($n = 14, 21$, and 20, respectively; $P < 0.0002$) (Figure 7C).

IFN- γ Causes Endothelial Cell Apoptosis

Given that increased IFN- γ levels correlated with attenuated lung angiogenesis after ischemia, we next determined *in vivo* the level of endothelial cell apoptosis. We studied the left lung 14 days after LPAL in WT mice and mice skewed toward predominant T-effector cells. Treg cell-deficient mice (*Foxp3^{DTR}*), which showed increased levels of IFN- γ in lung and serum, also show enhanced endothelial cell apoptosis (Annexin V⁺) by immunostaining and by FACS (Figures 8A and 8B). These mice were shown previously to have no systemic angiogenesis (1).

We next evaluated the capacity of IFN- γ to directly affect lung endothelial cells and to induce apoptosis *in vitro* (Figure 8C). We studied aortic endothelial cells and lung microvascular endothelial cells, both of which contribute to ischemia-induced vasculature formation. Stimulation of endothelial cells with IFN- γ (100 ng/ml) had a strong apoptotic effect in resting cells (1% serum). For comparison, the very low rate of apoptosis is shown for both cell types when stimulated only with high serum levels (20%). These experiments demonstrate that IFN- γ can directly alter endothelial cell viability.

IFN- γ Effects on Angiogenesis

To confirm that IFN- γ is angiostatic and limits angiogenesis, we delivered anti-IFN- γ at a dose previously confirmed to neutralize IFN- γ activity in an *in vivo* tumor model (8). No difference in angiogenesis was observed 14 days after LPAL compared with WT/PBS controls (Figure 9). However, *IFN- γ R^{-/-}* mice showed a significant increase in the level of systemic angiogenesis compared with WT/PBS control mice (125% increase compared with WT; $n = 5-6$ /group; $P < 0.01$). This observation demonstrates that the angiostatic effect of circulating or secreted

Figure 6. CD4⁺ (left panels) and CD8⁺ (right panels) T cells extracted from the left lung 5 days after LPAL in WT, control (*Foxp3^{gfp}*), and T regulatory (Treg) cell-depleted mice (*Foxp3^{DTR}*) and cell capacity to secrete IFN-γ. Compared with WT, Treg cell-depleted lungs showed a large increase in CD8⁺ cells (upper right panel) ($P < 0.01$ from WT and *Foxp3^{gfp}* controls) and a trend toward increased CD4⁺ cells (upper left panel) ($P = 0.08$; $n = 3-4$ mice/group) as a percentage of live cells. Lower graphs show IFN-γ expression for the two effector T cell types. CD4⁺ cells from Treg cell-depleted mice showed a large increase in secreted IFN-γ (lower left panel). * $P = 0.002$ versus WT ($n = 3-6$ mice/group). Overall, mice without Treg cells showed increased percentages of effector T cells (CD8⁺) and/or an increased capacity to secrete the prominent angiostatic cytokine IFN-γ (CD4⁺).

IFN-γ is attenuated in mice lacking the primary receptor for IFN-γ.

Discussion

The goal of the current study was to determine whether lymphocytes exert an angiostatic effect on systemic

neovascularization of the lung late after ischemic injury. Our results showed that lymphocyte influx into the ischemic left lung reached a maximum by 10 days after the onset of ischemia and progressively declined. This time course did not coincide with the later slowing of angiogenesis, and mice without any lymphocytes reached a similar late plateau level of

neovascularization as WT mice. Thus, we concluded that lymphocytes did not contribute directly to late angiostasis in this model. However, when focusing on the early angiogenic phase (D14), depletion of CD8⁺ T cells promoted greater angiogenesis. Furthermore, when lymphocytes in mice were skewed toward an enhanced CD4⁺/CD8⁺ effector T-cell phenotype by depleting Treg cells, IFN-γ protein levels were significantly higher in serum and in the ischemic lung than in WT mice. These Treg cell-depleted mice were previously shown to have no angiogenesis after ischemia (1). IFN-γ receptor 1 null mice also showed enhanced angiogenesis compared with WT mice. IFN-γ caused a direct increase in lung endothelial cell apoptosis *in vitro*, which was consistent with increased apoptosis of CD31⁺ endothelial cells in lungs of mice showing increased levels of IFN-γ. Our results suggest a modulating influence of IFN-γ to limit neovascularization in this noninfectious model.

The first series of experiments was performed to confirm that new vessel growth after ischemia reached a plateau level and did not continue indefinitely. By D28, angiogenesis reached a maximum of close to 4% of the cardiac output. Given that normal pulmonary blood flow of the left lung is approximately 33% of total cardiac output, this result demonstrates that the neovasculature provides far less than normal pulmonary perfusion yet is adequate to maintain lung metabolism. Furthermore, the plateau in angiogenesis was consistent with previous limited data at late time points (16). Pertinent to the goals of the study,

Figure 7. (A) IFN-γ protein in ischemic left lung of WT, Treg cell-sufficient and Treg cell-deficient mice 5 days after LPAL ($n = 5-6$ mice/group). * $P < 0.0001$ from WT. (B) Time course of IFN-γ in serum (pg/ml) (2-6 samples/time point). Two-way ANOVA shows significant group effect ($P < 0.0001$) but no time effect. (C) Average serum level of IFN-γ after LPAL in WT, control (*Foxp3^{gfp}*), and Treg cell-depleted (*Foxp3^{DTR}*) mice. A significant increase in IFN-γ was detected in Treg cell-depleted mice ($n = 14, 21, 20$, respectively). * $P < 0.0002$.

Figure 8. (A) Frozen sections of WT, control (*Foxp3^{gfp}*), and Treg-cell-depleted (*Foxp3^{DTR}*) mouse lungs after LPAL, demonstrating increased endothelial cell apoptosis predominantly in Treg cell-depleted mice. Sections show composite images of anti-CD31⁺ endothelium (green), annexin V⁺ apoptotic cells (red), and all cell nuclei (blue). Original magnification: $\times 400$. (B) Using FACS analysis, changes in apoptosis were evaluated 14 days after LPAL in the lungs from the study's three groups. The percentage of apoptosis (annexin V⁺, 7-AAD⁻) in lung endothelial cells (CD31⁺, CD45⁻) after LPAL from Treg cell-depleted mice was significantly increased compared with WT and treatment control mice (*Foxp3^{gfp}*). * $P < 0.05$ ($n = 3-4$ /group). (C) Effects of IFN- γ on endothelial cell apoptosis *in vitro*. Aortic endothelial cells and lung microvascular endothelial cells were challenged with IFN- γ (100 ng/ml), and percentage of apoptosis (annexin V⁺ 7-AAD⁻/CD141⁺) was assessed by FACS. A significant and similar increase in apoptosis over basal levels (1% serum) was observed in both endothelial cell types when challenged with IFN- γ . For comparison, a large decrease in the level of apoptosis was observed when cells were challenged only with high-serum (20%) conditions. * $P < 0.05$ from 1% conditions ($n = 3-4$ /group).

Rag^{-/-} mice that lack all lymphocytes reached a similar plateau level of perfusion, albeit at a faster rate (D21). These findings demonstrated that T cells did not play a role in late angiostasis.

In addition to the functional angiogenesis data, the T-cell profiles within the lung suggested their activation and

recruitment to the lung at an earlier time point after the onset of ischemia. An increase in T lymphocytes was measured within the lung by 7 to 10 days after the onset of ischemia, which coincides with the angiogenic phase and demonstrates that these cells were recruited via the new angiogenic blood vessels (Figure 3) to the

left lung and the visceral pleura, the site specifically where neovessels have been observed (23). These results confirm overall a systemic response to pulmonary ischemia. However, this increase was not sustained, and effector T cells decreased to control levels. The changes in CD4⁺ and CD8⁺ subpopulations lagged Treg changes slightly, as we showed previously that Treg cells specifically increased significantly from D3 to D7 (1). We speculate that Treg cells limited the increase in effector T cells. We subsequently focused on this observation and studied mice where Treg cells were depleted and the full effects of effector T cells were unmasked.

When Treg cells were depleted, we saw increased percentages of CD4⁺ and CD8⁺ T cells and/or the capacity to produce IFN- γ , increased IFN- γ in serum and in the left lung 5 days after LPAL. This suggests that Treg cells control effector T-cell responses during lung angiogenesis by modulating their numbers and IFN- γ production. Only by Treg cell depletion was the angiostatic property of effector T cells by IFN- γ secretion unmasked. This interpretation was supported by the results showing increased angiogenesis in CD8^{-/-}-deficient mice and in IFN- γ R^{-/-}-deficient mice. Overall, our results are consistent with those of others who examined the effects of effector T cells on blood vessel growth in tumor models. Qin and colleagues showed, in several different tumors, that tumor rejection by CD8⁺ T cells is preceded by inhibition of angiogenesis (8). Furthermore, angiostasis in tumors has been shown to be due to release of IFN- γ secreted by effector T cells (8, 28). However, the role of effector T cells in ischemic injury has been less consistent. Studies by Stabile and colleagues in an ischemic hindlimb model showed a necessity for CD8⁺/CD4⁺ effector T cells to promote angiogenesis (6, 9). Yet, in a wound-healing model, Chen and colleagues showed that CD4⁺/CD8⁺ T cells were not required for either wound healing or angiogenesis (7).

To confirm that IFN- γ directly influenced lung endothelial cells, we studied cell apoptosis *in vitro* of systemic endothelial cells and pulmonary microvascular cells. Because both subtypes play a role in the functional vasculature established in this model, we isolated and studied both. We showed a significant increase in endothelial cell apoptosis of

Figure 9. IFN- γ receptor 1-null mice (IFN γ R $^{-/-}$) show enhanced angiogenesis. * $P < 0.01$ compared with WT/PBS ($n = 5$ –6/group).

both subtypes when exposed to a dose of IFN- γ used by others *in vitro* (29). Our results were consistent with others who showed that IFN- γ had a direct inhibitory effect on endothelial cells by significantly down-regulating the Dll4/Notch signaling pathway required for endothelial cell sprouting (15, 30). When we sought to confirm this observation in mice with decreased angiogenesis, we saw a significant increase in the percentage of apoptotic endothelial cells (CD31 $^{+}$ CD45 $^{-}$) only in the Treg cell-depleted mice shown to have increased levels of IFN- γ protein and previously shown to have no angiogenesis (1).

In light of the current results, our past study examining Treg cell control of angiogenesis requires further consideration (1). In that work, Treg cell depletion resulted in changes in macrophage-derived growth factors known to be required for angiogenesis in this model. Thus, we concluded that the decrease in growth factors contributed to the lack of ischemia-induced angiogenesis (1). However, the present results demonstrate that other T-cell effects are playing a role in the ultimate angiogenic phenotype measured. Specifically, T-effector cells contribute a

strong inhibitory, proapoptotic effect when there are no Treg cells. In the past study, although not measured, Treg cell depletion likely increased IFN- γ , which contributed to endothelial cell apoptosis and prevented angiogenesis. In general, it appears that the presence of effector T cells is inversely correlated with the level of angiogenesis by 14 days after LPAL, with Rag-1 $^{-/-}$ mice showing the greatest level of blood vessel growth (no effector T cells) to the other extreme where Treg depletion causes an increase in effector T cells and no angiogenesis (1).

Two observations that are seemingly inconsistent with the overall findings that effector T cells limit angiogenesis through IFN- γ secretion are the angiogenesis results in CD4 $^{-/-}$ mice and in WT mice treated with a neutralizing antibody to IFN- γ . In the CD4 $^{-/-}$ mice, both proangiogenic (Treg [1]) and antiangiogenic (effector) cells were eliminated contributing to a mixed phenotype as observed. In the other series, the dose of neutralizing antibody was based on a transplant model, and the pretreatment coupled with a single postischemia treatment was likely inadequate to neutralize a sustained release of IFN- γ .

The results of this study could be viewed in the broader context of lung vascular remodeling. In pulmonary hypertension, for instance, immune mechanisms are pivotal and contribute to the chronic remodeling and ultimate occlusion of pulmonary arterioles. Ample evidence demonstrates that patients with pulmonary hypertension show increased numbers of perivascular monocytes/macrophages and T lymphocytes at sites of vascular lesions (31). Studies have shown that activated T cells prevented macrophage migration *in vitro* and prevented macrophage recruitment *in vivo* (32).

Yet, other studies have shown that CD4 $^{+}$ T cells contribute to vascular lesions after monocrotaline-induced pulmonary hypertension (33). CD4 $^{+}$ T cells and IFN- γ were required for *Pneumocystis*-induced pulmonary hypertension (34). Pulmonary hypertension is a recognized side effect of patients treated with IFNs for infection (35). Thus, the overall impact of effector T cells and IFN release on the lung vasculatures is still unfolding.

One technical issue requires further comment. Systemic perfusion of the lung as assessed by microspheres provides a functional measurement of angiogenic vessels. The reported blood flow measurements presented as the angiogenic index were made over the course of several years. Slight differences in microsphere extraction reagents, improvement in recovery techniques, and different operators contributed to a slight increase in the D14 measurement of blood flow in WT mice. However, concurrent WT controls have always been studied, are presented, and are used to make statistical comparisons with experimental groups, using the same reagents and performed by the same investigator.

In summary, our results demonstrate that effector T cells limit the extent of angiogenesis after acute pulmonary ischemia through the release of IFN- γ . Unlike an infection model, the levels of IFN- γ protein secretion are relatively low yet directly alter endothelial cell viability. ■

Author disclosures are available with the text of this article at www.atsjournals.org.

Acknowledgments: The authors thank Dr. Alexander Rudensky, Sloan-Kettering Institute, for use of the *Foxp3^{gfp}* and *Foxp3^{DTR}* mice strains and Dr. Jay Bream, Johns Hopkins University, who was instrumental in preliminary studies.

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