

*Vascular Biology, Atherosclerosis and Endothelium Biology*

# Angiopoietin-2-Driven Vascular Remodeling in Airway Inflammation

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**physiology.** (*Am J Pathol* 2010, 177:000–000; DOI: 10.2353/ajpath.2010.100059)

Vascular remodeling is a well-documented feature of chronic inflammatory airway diseases such as asthma and chronic bronchitis.<sup>1–4</sup> Such persistent inflammatory conditions are accompanied by changes in the molecular phenotype of endothelial cells and in the structure and function of blood vessels that contribute to the pathophysiology.<sup>5</sup>

Vascular endothelial growth factor (VEGF) and other growth factors drive angiogenesis in tumors and macular degeneration. Less is known about cytokines that promote vascular remodeling in inflammation, where blood vessels can undergo phenotypic changes in the absence of sprouting angiogenesis. VEGF is not involved in the vascular remodeling that occurs soon after infection of the murine respiratory pathogen *Mycoplasma pulmonis*,<sup>6,7</sup> but tumor necrosis factor- $\alpha$  (TNF- $\alpha$ ) and related cytokines are likely contributors.<sup>8,9</sup>

Angiopoietins (Ang1, 2, and 3 in mice and Ang1, 2, and 4 in human) are key regulators of vascular remodeling, maturation, and stability through effects on Tie2 receptor signaling in endothelial cells.<sup>10</sup> Angiopoietins are also important regulators of inflammatory responses,<sup>11</sup> but their precise roles are complex.

**Vascular remodeling is a feature of chronic inflammation during which capillaries transform into venules that expand the region of the vasculature in which leakage and leukocyte emigration both occur. Recently, we found that angiopoietin/Tie2 receptor signaling drives the transformation of capillaries into venules at an early stage of the sustained inflammatory response in the airways of mice infected with *Mycoplasma pulmonis*. However, the precise contributions of both angiopoietin-1 (Ang1) and angiopoietin-2 (Ang2) are not clear. In this study, we sought to determine the contribution of Ang2 to this vascular remodeling. Ang2 mRNA expression levels increased and phosphorylated Tie2 immunoreactivity in mucosal blood vessels decreased, indicative of diminished receptor signaling after infection. Selective inhibition of Ang2 throughout the infection by administration of either of two distinct function-blocking antibodies reduced the suppression of Tie2 phosphorylation and decreased the remodeling of mucosal capillaries into venules, the amount of leukocyte influx, and disease severity. These findings are consistent with Ang2 acting as an antagonist of Tie2 receptors and the reduction of Tie2 phosphorylation in endothelial cells rendering the vasculature more responsive to cytokines that promote both vascular remodeling and the consequences of inflammation after *M. pulmonis* infection. By blocking such changes, Ang2 inhibitors may prove beneficial in the treatment of sustained inflammation in which vascular remodeling, leakage, and leukocyte influx contribute to its patho-**

Supported in part by NIH grants HL-024136, HL-059157, and HL-096511 (D.McD.) from the National Heart, Lung, and Blood Institute. ST was also supported by a fellowship of the Belgian American Educational Foundation and a fellowship of the "Centre Anticancéreux" (University of Liege, Belgium).

Accepted for publication August 19, 2010.

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Supplemental material for this article can be found on <http://ajp.amjpathol.org>.

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The conventional view is that Ang1 has anti-inflammatory properties and Ang2 has pro-inflammatory properties. Ang1 promotes endothelial cell stability by inducing Tie2 phosphorylation (p-Tie2).<sup>12,13</sup> Ang1 protects against airway inflammation and hyperreactivity in asthma,<sup>14,15</sup> reduces thrombin-induced interleukin-8 production and neutrophil adhesion,<sup>16</sup> and decreases VEGF-induced up-regulation of inflammatory adhesion molecules.<sup>17</sup> Ang2, which is dynamically regulated, can be rapidly released from endothelial cell Weibel-Palade bodies and can antagonize the action of Ang1 by binding Tie2 without inducing phosphorylation.<sup>18,19</sup> Ang2 sensitizes endothelial cells to TNF- $\alpha$  and can exaggerate inflammatory responses.<sup>11,20</sup>

Under certain conditions, Ang2 can activate Tie2 like Ang1, instead of opposing the actions of Ang1.<sup>21,22</sup> As an example, overexpression of Ang2 results in microvascular enlargement similar to that found after Ang1 overexpression.<sup>23</sup> Both Ang1 and Ang2 can also have pro-inflammatory effects. Induction of P-selectin translocation in endothelial cells, activation of PAF synthesis in endothelial cells, and promotion of neutrophil activation, adhesion, and chemotaxis are examples.<sup>24–27</sup> The usual explanation of these diverse effects is that the actions of Ang1 and Ang2 on Tie2 are dose- and context-dependent.

In a previous report, we describe that Ang1 protein and Ang2 protein both accumulate in mouse airways after *M. pulmonis* infection and that neutralization of angiopoietins by soluble Tie2 decoy receptors that sequester Tie2 ligands reduces vascular remodeling in this setting of sustained airway inflammation.<sup>28</sup> Because the soluble receptors neutralized all Tie2 ligands, the approach did not distinguish the contributions of Ang1 from those of Ang2 and did not learn whether the angiopoietin proteins measured were intracellular or were free to act on endothelial cell Tie2 receptors.

In the present study we used *M. pulmonis* infection as a model of sustained inflammation to test the concept that Ang2 promotes vascular remodeling by suppressing Tie2 signaling, which thereby supports the pro-inflammatory actions of TNF- $\alpha$  and other cytokines.<sup>7–9</sup> Our approach was to compare, before and after infection, the amounts of Ang1 and Ang2 mRNA expression and Tie2 phosphorylation and to determine the Ang2-dependence of infection-related vascular remodeling, adhesion molecule expression, and leukocyte influx by inhibiting Ang2 with a selective function-blocking antibody.

We found distinctive changes involving Ang1, Ang2, and p-Tie2 after *M. pulmonis* infection: Ang1 mRNA expression decreased, Ang2 mRNA expression increased, and p-Tie2 immunoreactivity decreased in mucosal blood vessels. Importantly, selective inhibition of Ang2 during infection not only blocked the change in p-Tie2, but also reduced the vascular remodeling, leukocyte influx, and infection severity. These findings are consistent with Ang2 having a pro-inflammatory action in this model by promoting vascular remodeling.

## Materials and Methods

### Model of Airway Inflammation in Mice

Male pathogen-free C57BL/6 mice (Charles River, Hollister, CA), housed under barrier conditions, were anesthetized by intramuscular injection of ketamine (10 mg/kg) and xylazine (20 mg/kg) and then infected by intranasal inoculation of 50  $\mu$ l of broth containing 10<sup>6</sup> CFU of strain CT7 *M. pulmonis* organisms (derived from UAB CT<sup>29</sup>), half of the dose into each nostril.<sup>30</sup> Mice were studied 1, 3, 7, or 14 days after inoculation. All experimental procedures were approved by the Institutional Animal Care and Use Committee of the University of California at San Francisco.

### Ang2 Function-Blocking Antibodies

Function-blocking monoclonal anti-Ang2 antibody REGN145 (5 mg/kg, Regeneron) or monoclonal antibody (mAb) 3.19.3 (10 mg/kg, MedImmune)<sup>31</sup> or isotype-matched control IgG antibody was injected intraperitoneally into mice every other day for 7 days beginning 1 day before infection. REGN145 is a human monoclonal antibody with an estimated equilibrium dissociation constant (kDa) for native human Ang2 of about 50 pmol/L and inhibits Ang2 with approx 500-fold greater potency than it inhibits Ang1. REGN145 has similar specificity and potency for mouse angiopoietins (Thurston et al, unpublished results). The human monoclonal antibody 3.19.3 has a kDa for human Ang2 of 86 pmol/L and binds Ang2 with 500-fold or greater affinity than for Ang1. The EC50 value of mAb 3.19.3 was 478 pmol/L for human Ang2 and >194 nmol/L for human Ang1 in a cell-based assay. Values for mouse angiopoietins were in the same range.

### Immunohistochemistry

Mice were anesthetized and perfused through the left ventricle for 2 minutes with 1% paraformaldehyde in PBS, pH 7.4. Tracheas were removed, immersed in fixative for an additional 1 hour and stained as whole mounts. The following primary antibodies were used for immunohistochemistry: PECAM-1 (CD31, hamster anti-mouse PECAM-1, clone 2H8, 1:500, Fisher), P-selectin (CD62P, rat anti-mouse, BD Biosciences), CD11b (rat anti-mouse CD11b, clone M1/70; eBioscience), Tie2 (rat anti-mouse, Tek4, eBioscience), p-Tie2 (Y992, rabbit anti-mouse, R&D Systems), and Ang2 (LS-B58, LifeSpan Biosciences). Secondary antibodies were conjugated to fluorescein isothiocyanate, Cy3, or Cy5 (Jackson ImmunoResearch, West Grove, PA). Tracheas from pathogen-free mice were compared to those from mice infected for 7 or 14 days by staining with of the antibodies, except p-Tie2 and CD11b, where the staining was impaired by poor penetration into the thickened tracheal whole mounts.

## Imaging

Digital images were acquired with a Zeiss Axiophot fluorescence microscope equipped with single, dual, and triple fluorescence filters and a low-light, 3-chip, charge-coupled device (CCD) camera (CoolCam, Sci-Measure, Decatur, GA, 640 × 480 pixel RGB-color). This camera also enabled real-time viewing and measuring of blood vessels using custom software. Confocal images were obtained with a Zeiss LSM-510 confocal microscope with Argon, Helium-Neon, and UV lasers (512 × 512 or 1024 × 1024 pixel RGB-color) and AIM 3.2.2 software.

## Measurement of Vascular Remodeling

The diameter of capillaries in the tracheal mucosa was measured at the rostro-caudal midpoint of cartilage rings, which was roughly halfway between the arteriole and venule.<sup>32</sup> Five or more vessels were measured over each of 10 cartilage rings in each trachea while viewing real-time fluorescent images of 960 × 1280 μm regions of tissue (×10 objective, ×1 Optovar).

## Measurement of Immunoreactivity

Amounts of CD11b immunoreactivity of leukocytes and P-selectin immunoreactivity of blood vessels were measured in digital fluorescence images of regions of tracheal mucosa measuring 960 × 1280 μm (×10 objective, ×1 Optovar) over cartilage rings in tracheal whole-mounts. Five regions were assessed in each trachea, beginning with the second cartilage ring caudal to the larynx and continuing consecutively toward the bronchi. Area density of CD11b or P-selectin immunoreactivity (red pixels) was calculated as the proportion of total pixels having a fluorescence intensity greater than background based on a threshold value of 20 in the range 0 < 255 by using ImageJ software (<http://rsb.info.nih.gov/ij>).<sup>33</sup> The overall mean for each group was calculated from the average of all values in each mouse.

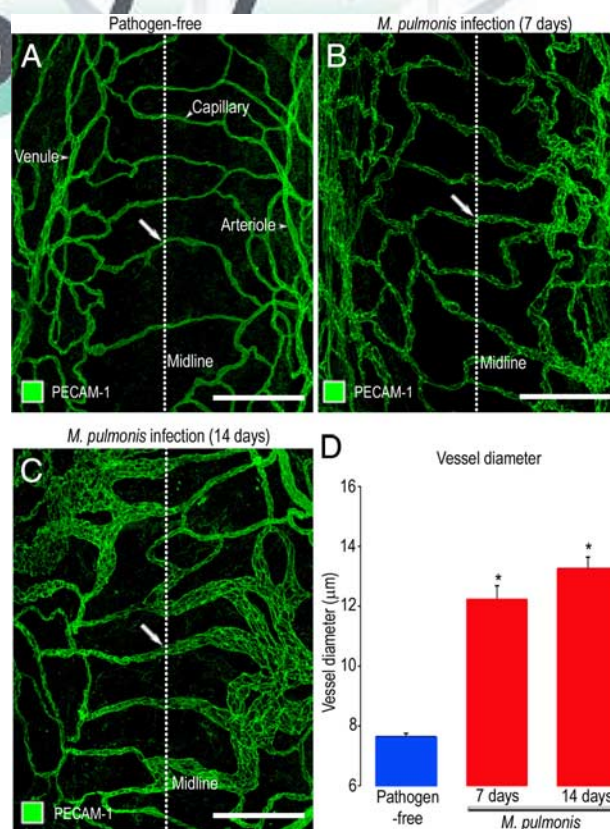
## RNA Extraction and Quantitative Reverse Transcription-PCR

RNA was isolated from tracheas using the RNeasy Plus Mini isolation kit (Qiagen). RNA yield and purity were determined by spectrophotometry. cDNAs were synthesized with 1 μg of total RNA using the cDNA synthesis kit (Roche Applied Science). Quantitative reverse transcription (qRT) PCR was performed with Kapa SYBR Fast qRT-PCR master mix (Kapa Biosystems, Cambridge, MA) using a BioRad MyIQ detection system (Bio-Rad). The relative expression of each target gene was normalized to the expression of β-actin by calculation of the difference between the Ct target and the Ct reference gene, where Ct is the fractional PCR cycle number at which the reporter fluorescence is greater than the threshold. The primers used were: *M. pulmonis* 16S RNA: 5'-CCCTAAGTATGACGGTACCTTGTC-3' and 5'-GCGGCTGCTGGCACAT-3'; *TNF-α*: 5'-GATCTCTCG-

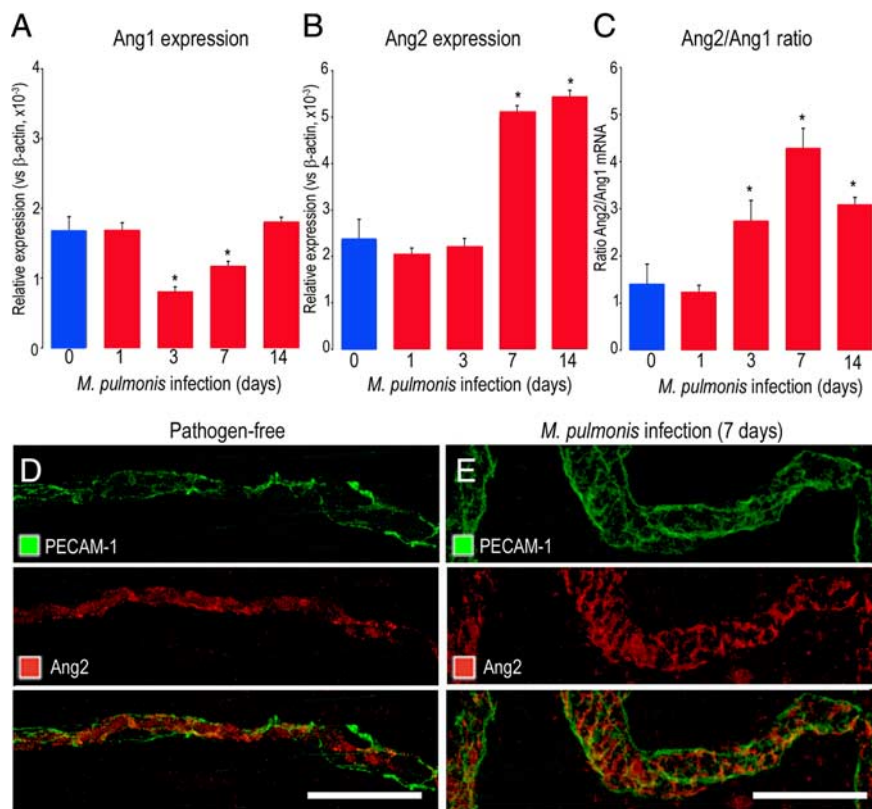
GAACCTCATCG-3' and 5'-GGCTTCTTTGCGACAATCTC-3'; *CD11b*: 5'-ATGGACGCTGATGGCAATACC-3'; *P-selectin*: 5'-GCATACTCATGGAATAACTCACG-3' and 5'-GACGTCATTGAGGTGAGCG-3'; *E-selectin*: 5'-ATGCCTCGCGCTTTCTCTC-3' and 5'-GTAGTCCCCTGACAGTATGC-3'; *ang1*: 5'-GCAAAGGCTGATAAGGTTATGA-3' and 5'-AGCTACCAACAACAACAGCA-3'; *ang2*: 5'-TTCTTCTTACGGATAGCAAC-3' and 5'-AGCCACGGTCAACAACTCGC-3'; *tie-2*: 5'-GTTGACTCTAGCTCGGACTGT-3' and 5'-GAAGTCGAGAGGCGATCCC-3'; *ve-cadherin*: 5'-CACTGCTTTGGGAGCCTC-3' and 5'-GGGGCAGCGATTCATTTTCT-3'.

## Statistical Analysis

Values are expressed as means ± SEM with five mice per group except with indicated otherwise. All of the experiments were repeated twice. The significance of differences among groups was assessed by analysis of variance (analysis of variance) followed by the Dunn-Bonferroni posthoc test for multiple comparisons with differences considered significant where  $P < 0.05$ .



**Figure 1.** Vascular enlargement after *M. pulmonis* infection. **A–C:** Confocal microscopic images of blood vessels (green, PECAM-1) in tracheal whole mounts comparing capillaries in pathogen-free mouse (**A**, arrow) to enlarged, remodeled vessels (arrows) in mice infected with *M. pulmonis* for 7 days (**B**) or 14 days (**C**). **D:** Measurements of vessels at midpoint of cartilage ring (dotted line) show the magnitude of vascular enlargement after infection. \* $P < 0.05$  compared with pathogen-free. Scale bars = 100 μm.



**Figure 2.** Increase in Ang2 expression after *M. pulmonis* infection. **A** and **B**: qRT-PCR measurements of expression of Ang1 mRNA (**A**) and Ang2 mRNA (**B**) in trachea of pathogen-free mice and mice infected with *M. pulmonis* for 1, 3, 7, or 14 days. **C**: Ratio of qRT-PCR measurements of Ang2 mRNA to Ang1 mRNA. \**P* < 0.05 compared to pathogen-free (day 0). **D** and **E**: Confocal micrographs of vessels in tracheal whole mounts showing Ang2 (red) and PECAM-1 (green) immunoreactivities of a capillary in a pathogen-free mouse (**D**) and a remodeled vessel over a cartilage ring in a mouse infected with *M. pulmonis* for 7 days (**E**). Scale bars = 30 μm (**D** and **E**).

**Results**

*Vascular Remodeling after M. pulmonis Infection*

In the trachea of pathogen-free mice, capillaries over cartilaginous rings were organized in a distinctive ladder-like pattern (Figure 1A). Most arterioles and venules were located in regions of the mucosa between cartilage rings (Figure 1A). Conspicuous changes were evident in the tracheal vasculature after *M. pulmonis* infection. At 7 and 14 days after infection, capillaries were conspicuously enlarged but had no evidence of new blood vessel growth by sprouting angiogenesis (Figure 1, B–C). The diameter of the remodeled vessels was on average 60% greater than the pathogen-free value at 7 days after infection and 75% greater at 14 days (Figure 1D).

*Expression of Ang1 and Ang2 after M. pulmonis Infection*

Measurements by qRT-PCR of tracheas of pathogen-free mice revealed roughly equal amounts of Ang1 and Ang2 mRNA when expressed as copy number relative to β-actin (Figure 2, A–C). On days 3 and 7 after *M. pulmonis* infection, Ang1 mRNA expression was significantly less than in pathogen-free mice (Figure 2, A–B). Ang2 expression was unchanged on day 3, but on day 7 was significantly greater than the pathogen-free value. At day 14 of infection, Ang1 expression was back to the baseline, but Ang2 remained increased (Figure 2B). Because

of the early reduction in Ang1 and subsequent sustained increase in Ang2, the mRNA the ratio of Ang2 to Ang1 was significantly greater at 3, 7 and 14 days after infection (Figure 2C).

The distribution of Ang2 immunoreactivity in relation to PECAM-1 staining of blood vessels was assessed by immunohistochemical staining of tracheal whole-mounts before and after *M. pulmonis* infection. Ang2 immunoreactivity was found in capillaries of pathogen-free tracheas (Figure 2D) and in remodeled vessels at 7 days after infection, when capillaries had enlarged and acquired the phenotype of venules (Figure 2E). Because the remodeled vessels had a conical shape (Figure 1, B–C), those sampled near the midline (Figure 2E) were not as large as those downstream.

Ang2 immunoreactivity did not strictly colocalize with PECAM-1. Staining with the hamster anti-mouse PECAM-1 antibody was largely membrane-associated and strongest at endothelial cell borders. However, most Ang2 staining was not in the membrane but was instead cytoplasmic, presumably in Weibel-Palade bodies<sup>19</sup> that could not be individually resolved in tracheal whole mounts. Therefore, PECAM-1 and Ang2 were associated with the same blood vessels but had slightly different distributions within the vessel wall.

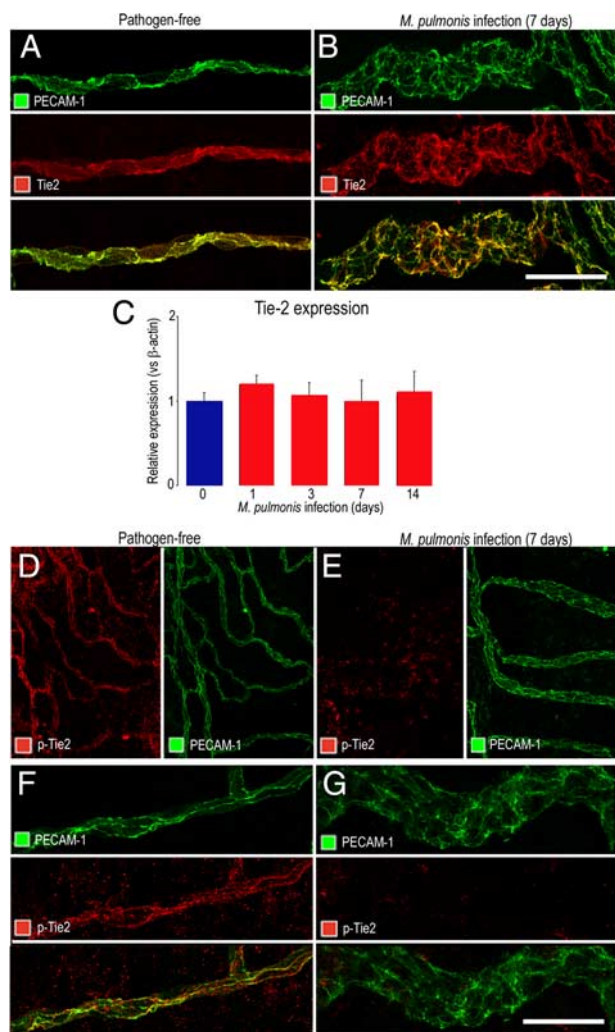
*Distribution of Tie2 in Airway Vasculature*

Tie2 immunoreactivity was present throughout the vasculature of pathogen-free tracheas and largely matched the distribution of PECAM-1 (Figure 3A). The distribution and

F1

F2

F3



**Figure 3.** Decrease in Tie2 expression after *M. pulmonis* infection. **A** and **B**: Confocal micrographs showing the similarity of Tie2 (red) in tracheal capillary of pathogen-free mouse (**A**) and remodeled vessel in mouse infected for 7 days (**B**). PECAM-1, green. **C**: qRT-PCR measurements showing no difference in Tie-2 mRNA, expressed relative to  $\beta$ -actin, in trachea of pathogen-free mice (day 0) and mice infected for 1, 3, 7, or 14 days. **D–G**: Confocal micrographs at lower (**D** and **E**) and higher (**F** and **G**) magnifications showing weaker p-Tie2 immunoreactivity (red) in remodeled vessels after infection for 7 days (**E** and **G**) than in tracheal capillary of pathogen-free mouse (**D** and **F**). Images were acquired with the same fluorescence intensity setting. Scale bars: 30  $\mu$ m (**A** and **B**); 100  $\mu$ m (**D** and **E**); 30  $\mu$ m (**F** and **G**).

intensity of Tie2 immunoreactivity was about the same in remodeled blood vessels after *M. pulmonis* infection for 7 days (Figure 3B) or 14 days. Similarly, overall expression of Tie2 mRNA in the trachea was essentially unchanged after infection for 1 to 14 days (Figure 3C).

p-Tie2 immunoreactivity was clear and uniformly distributed in the vasculature of pathogen-free tracheas (Figure 3, D and F) but was faint or absent in the remodeled vasculature after 7 days of infection (Figure 3, E and G, and Supplemental Figure S1, at <http://ajp.amjpathol.org>), consistent with continued presence of Tie2 receptors in endothelial cells but reduced activation after infection.

### Reduction in Vascular Remodeling by Ang2 Blockade with REGN145

The contribution of Ang2 to vascular remodeling after *M. pulmonis* infection was tested by administration of a function-blocking anti-Ang2 antibody, REGN145, throughout the infection. Tracheal capillaries in mice treated with REGN145 three times a week starting the day before infection were smaller at 7 days after infection than in mice treated with control IgG (Figure 4, A–C). Measurements revealed that the enlargement of blood vessels over cartilage rings after 7 days of infection was 70% less in mice treated with REGN145 than in mice treated with IgG (Figure 4D). Similarly, qRT-PCR measurements of expression of PECAM-1 and the endothelial cell-specific adherens junction protein VE-cadherin showed a significant increase after 7 days of infection in mice treated with control IgG, consistent with vascular enlargement, but not in mice treated with REGN145 (Figure 4, E and F). Similarly, the reduction in p-Tie2 immunoreactivity found after infection was less in mice treated with REGN145 (Figure 4, G and H). Together, these results fit with Ang2 blockade by REGN145 during infection leading to greater Tie2 activation and less vascular remodeling.

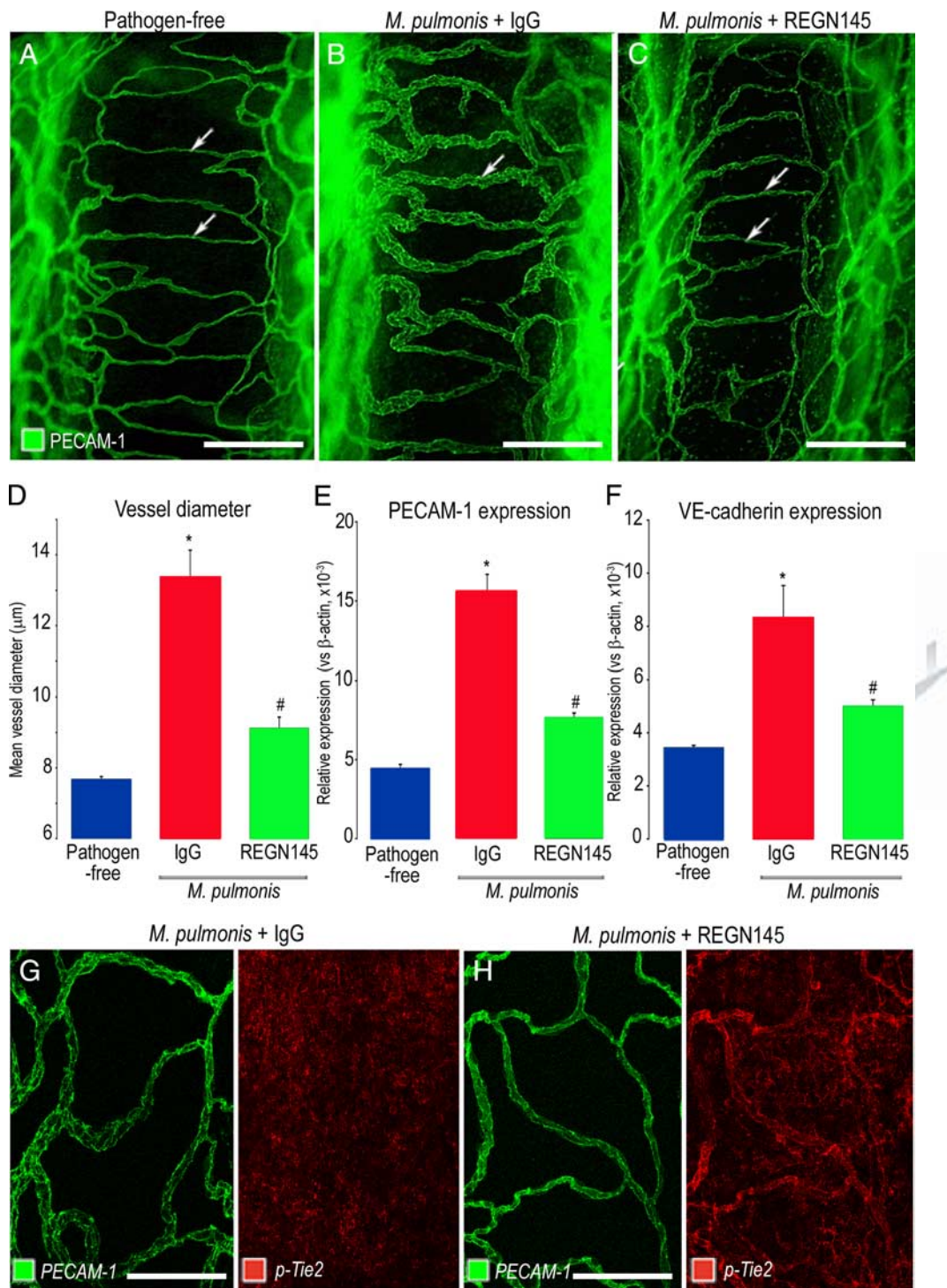
### Reduction in P-Selectin Expression by Ang2 Blockade with REGN145

P-selectin immunoreactivity was not present in capillaries (arrows) in the trachea of pathogen-free mice (Figure 5A), and was faint in venules (arrowheads) of these mice. However, after *M. pulmonis* infection for 7 days, P-selectin was unusually strong in venules, including the remodeled vessels (arrow) over a cartilage ring where capillaries were normally located (Figure 5B). The change in vessel phenotype from capillary to venule fits with published evidence showing that the remodeled vessels acquire multiple markers of a venous identity (P-selectin, E-selectin, ICAM-1, EphB4).<sup>28</sup>

P-selectin staining was much weaker in the vasculature of infected mice treated with REGN145 (Figure 5C). Measurements revealed that the 20-fold increase in P-selectin immunofluorescence after infection was reduced by 70% when the infection was accompanied by REGN145 (Figure 5D). Similarly, tracheal expression of P-selectin mRNA was 10-fold greater after infection for 7 days and was reduced by 70% in infected mice treated with REGN145 (Figure 5E). Changes in expression of E-selectin (Figure 5F) were similar in magnitude.

### Reduction in Vascular Remodeling by Ang2 Blockade with mAb 3.19.3

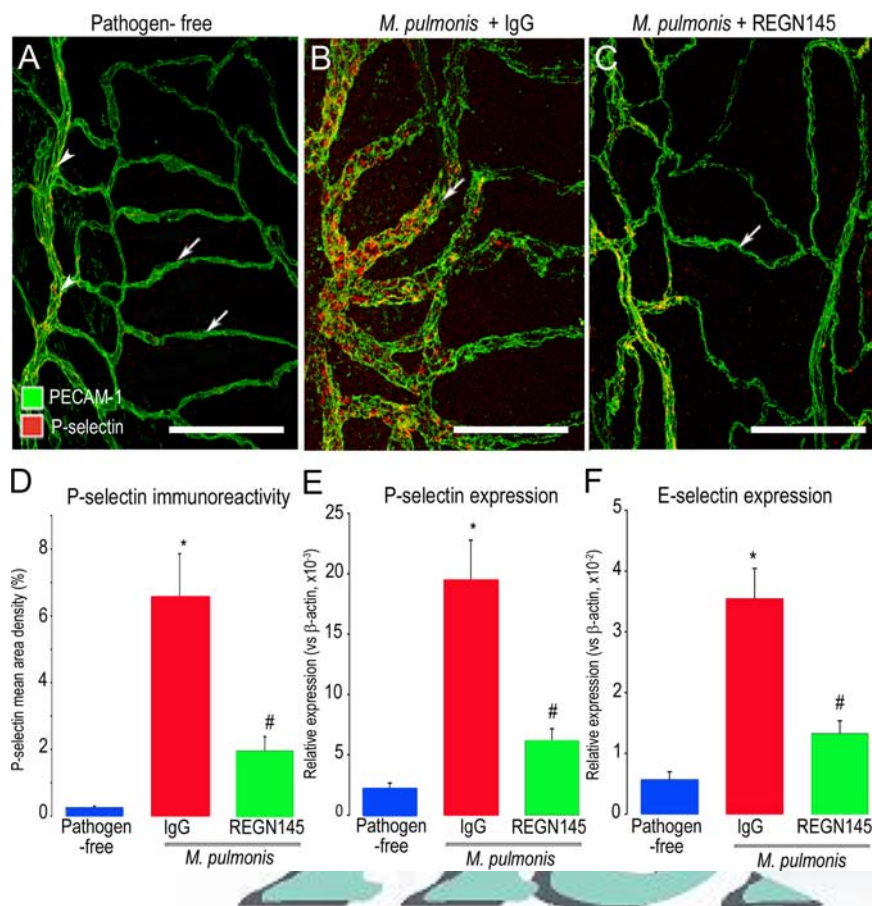
A second function-blocking anti-Ang2 antibody (mAb 3.19.3) gave results consistent with the effects of REGN145 and provided further support for the contribution of Ang2 to vascular remodeling after *M. pulmonis* infection. Vascular remodeling, reflected by increase in



**Figure 4.** Reduction in vascular enlargement and p-Tie2 suppression by Ang2 blockade with REGN145. **A–C:** Confocal micrographs comparing the size of blood vessels (green, PECAM-1) in tracheal whole mounts from a pathogen-free mouse (**A**) and mice infected with *M. pulmonis* for 7 days with concurrent treatment with normal IgG (**B**) or REGN145 5 mg/kg (**C**). **Arrows** mark blood vessel measurement site over midpoint of cartilage ring. **D:** Contrasting mean vessel diameters in tracheas of pathogen-free mice and infected mice treated with IgG or REGN145 for 7 days. **E and F:** qRT-PCR measurements of PECAM-1 (**E**) and VE-cadherin (**F**) in tracheas of pathogen-free mice and infected mice treated with IgG or REGN145 for 7 days. \**P* < 0.05 compared to pathogen-free; #*P* < 0.05 compared to infected mice treated with IgG. **G and H:** Pairs of confocal micrographs showing PECAM-1 (green) and p-Tie2 (red) immunoreactivities of tracheal blood vessels after infection for 7 days. p-Tie2 is faint or absent on vessels in infected mouse that received IgG (**G**) but is stronger on vessels in infected mouse treated with REGN145 (**H**). Scale bars: 150 µm (**A–C**) or 100 µm (**G and H**).

vessel diameter and in P-selectin immunoreactivity after infection, was greater in mice treated with the control IgG than in corresponding mice treated with the Ang2 inhibitor mAb 3.19.3 (Figure 6, A–C). Measurements showed

that the amount of vessel enlargement was 50% less when the infection was accompanied by mAb 3.19.3 (Figure 6D). Similarly, qRT-PCR measurements of expression of PECAM-1, VE-cadherin, P-selectin, and E-



**Figure 5.** Reduction in adhesion molecule expression by Ang2 blockade with REGN145. **A–C:** Confocal micrographs of P-selectin immunoreactivity (red) in blood vessels (green, PECAM-1) of tracheal whole mounts. P-selectin immunoreactivity in pathogen-free trachea (**A**) is absent in capillaries (**arrows**) and faint in venules (**arrowhead**). After *M. pulmonis* infection, P-selectin is strong in remodeled vessels (**arrow**) of mouse treated with IgG (**B**) but weak in vessels (**arrow**) of mouse treated with REGN145 at a dose of 5 mg/kg (**C**). **D:** Fractional area of P-selectin immunoreactivity in regions of vessels over cartilage in pathogen-free mice and in infected mice treated concurrently with IgG or REGN145. **E and F:** qRT-PCR measurements comparing expression of P-selectin (**E**), and E-selectin (**F**) mRNA in tracheas of pathogen-free and infected mice treated with IgG or REGN145 for 7 days. \* $P < 0.05$  compared to pathogen-free; # $P < 0.05$  compared to infected mice treated with IgG. Scale bars = 150  $\mu\text{m}$ .

selectin were about 75% lower in infected mice treated with mAb 3.19.3 than with IgG (Figure 6, E and F).

### Reduction in Leukocyte Influx and Infection Severity by Ang2 Blockade

The number of CD11b-positive cells in the tracheal mucosa was used to determine whether increased expression of P-selectin and E-selectin on remodeled blood vessels after *M. pulmonis* infection was accompanied by greater leukocyte influx. In pathogen-free tracheas, CD11b immunoreactive cells were sparse, and most around venules in intercartilaginous regions (Figure 7A). By comparison, at 7 days after infection, CD11b-positive cells were abundant throughout the trachea (Figure 7B, control IgG), but when Ang2 was inhibited by REGN145 during the infection, fewer leukocytes were located in the mucosa over cartilage rings (Figure 7C). CD11b immunoreactivity in these regions was 70% less in infected mice treated with REGN145 than in those treated with IgG (Figure 7D). CD11b RNA expression measured by qRT-PCR was significantly lower in infected mice treated with REGN145 than in mice treated with the control IgG (Figure 7E). Expression of TNF- $\alpha$  was also significantly lower in REGN145-treated mice (Figure 7F).

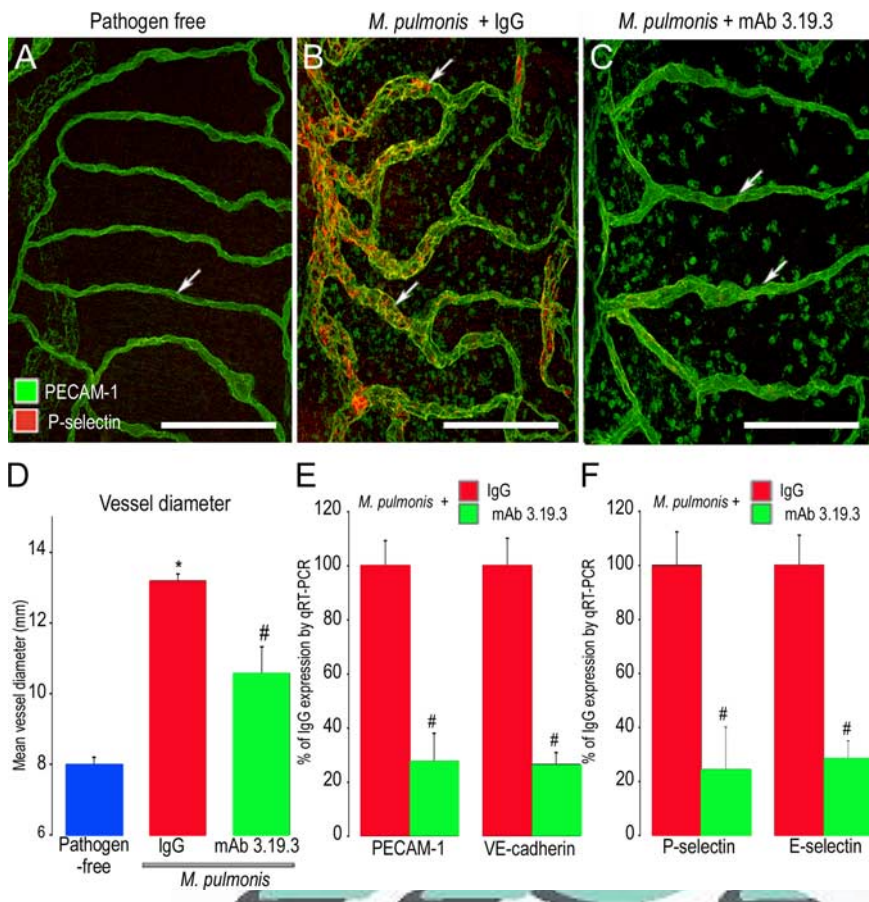
Infection severity was also assessed by qRT-PCR measurements of expression of *M. pulmonis* 16S rRNA in the trachea (Figure 7G). The amount of *M. pulmonis* 16S rRNA was strongly correlated with P-selectin expression

as an index of vascular remodeling ( $R^2 = 0.894$ ,  $P < 0.01$ ). Amount of *M. pulmonis* 16S rRNA was significantly less in infected mice treated with REGN145 or with mAb 3.19.3 than with IgG, consistent with reduced overall severity of infection (Figure 7H).

### Discussion

The present study sought to determine whether Ang2 drives vascular remodeling in the respiratory tract after *M. pulmonis* infection. The remodeling results from endothelial cell proliferation—not vasodilatation or sprouting angiogenesis—and leads to circumferential enlargement of capillaries.<sup>30,34</sup> In the process, capillaries acquire a venular phenotype, reflected by increased immunoreactivity for multiple molecules typical of venules, including P-selectin, E-selectin, ICAM-1, and EphB4, that supports leukocyte adherence and migration and other venule-specific features of the inflammatory response.<sup>28,34,35</sup> Gene expression arrays have documented the increase in expression of leukocyte adhesion molecules at 1 and 12 weeks after infection (at 1 week: P-selectin, 10-fold; E-selectin, 43-fold; and ICAM-1 fourfold compared to pathogen-free; McDonald and Thurston, unpublished data).

Previous reports have shown the importance of Tie2 signaling in blood vessel remodeling and stabilization<sup>13,36</sup> and have implicated angiopoietins as factors



**Figure 6.** Reduction in vascular remodeling by Ang2 blockade with mAb 3.19.3. **A–C:** Confocal micrographs of P-selectin immunoreactivity (red) in blood vessels (green, PECAM-1) of tracheal whole mount of pathogen-free mouse and mice infected with *M. pulmonis* for 7 days with concurrent treatment with 10 mg/kg of isotype control IgG (**B**) or mAb 3.19.3 (**C**). **Arrows** mark regions where vessel measurements were made. **D:** Diameter of tracheal blood vessels measured over midpoint of cartilage rings in pathogen-free mice and mice treated with IgG or mAb 3.19.3 during 7-day infection. **E and F:** qRT-PCR measurements of expression of PECAM-1 and VE-cadherin (**E**) or P-selectin and E-selectin (**F**) in tracheas of infected mice treated with IgG or mAb 3.19.3 for 7 days. Values after Ang2 blockade by mAb 3.19.3 were significantly less. \**P* < 0.05 compared to pathogen-free; #*P* < 0.05 compared to infected mice treated with IgG. Scale bars = 100 μm.

that drive remodeling of blood vessels and lymphatics during development<sup>37,38</sup> and after *M. pulmonis* infection.<sup>5,28,39</sup> Angiopoietins are also thought to play a role in other models of injury and inflammation.<sup>14,20,40</sup> However, uncertainties regarding the relative contributions of Ang1 and Ang2 to vascular remodeling in inflammation warranted further studies and led to our use of multiple readouts to assess changes in angiopoietins and Tie2 signaling in mouse airways after *M. pulmonis* infection.

Our experiments revealed that the vascular remodeling present after *M. pulmonis* infection for 7 days was accompanied by increased Ang2 mRNA expression, when Ang2 immunoreactivity was largely restricted to endothelial cells. Immunoreactivity for p-Tie2 decreased in remodeled vessels, but overall Tie2 mRNA expression did not change. Selective inhibition of Ang2 significantly reduced the decrease in p-Tie2 immunoreactivity and the vascular remodeling during the infection.

Although the amount of Ang2 immunoreactivity per endothelial cell was not noticeably increased, *M. pulmonis* infection is followed by rapid proliferation of endothelial cells, which results in an increase in the population of endothelial cells and in enlargement of remodeled capillaries and venules.<sup>30</sup> The estimated twofold increase in number of endothelial cells after infection is in scale with the increase in Ang2 mRNA and is consistent with the similar increase in VE-cadherin shown in Figure 4F.

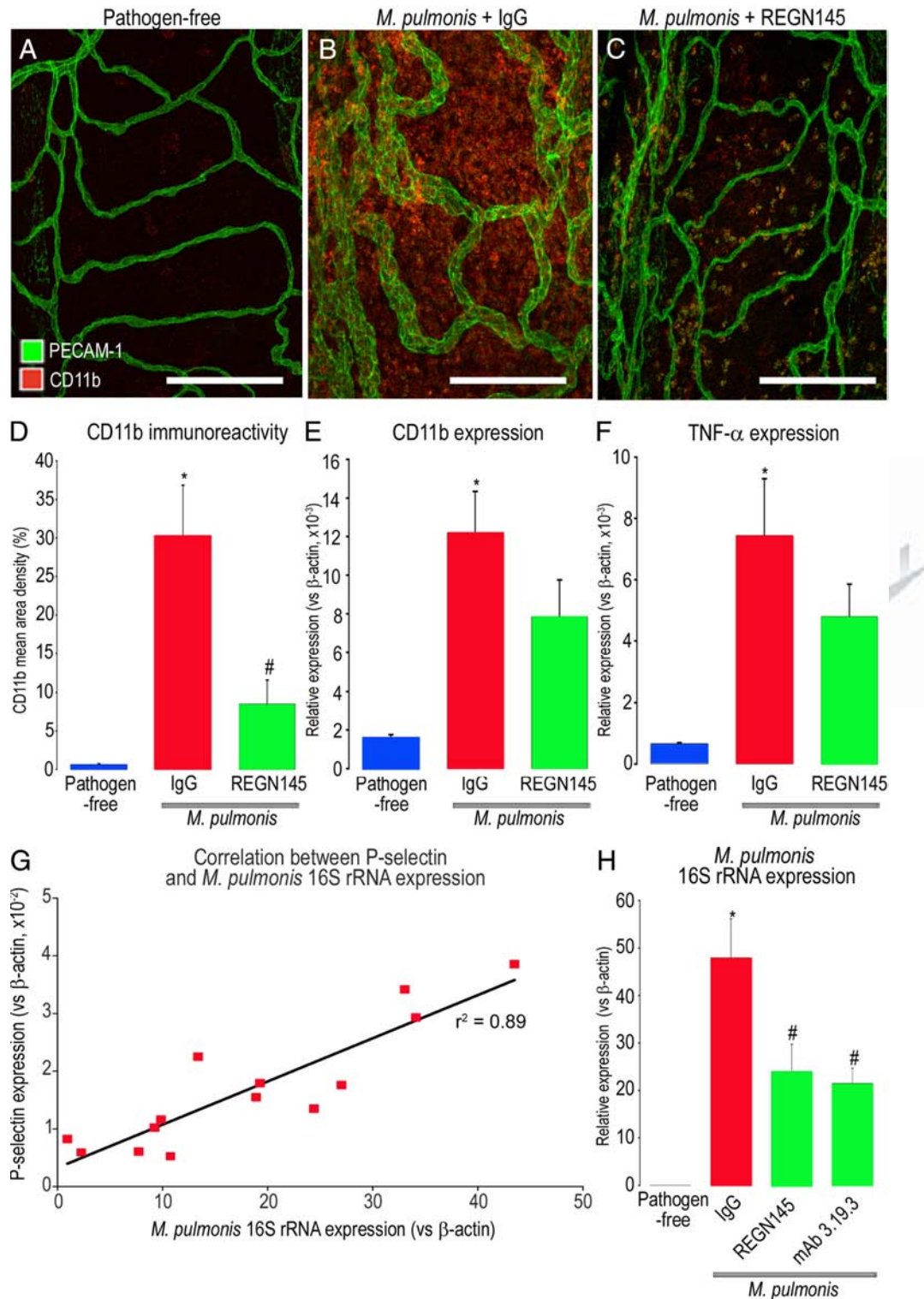
The early increase in Ang2 expression and reduction in p-Tie2 immunoreactivity after *M. pulmonis* infection are

consistent with the contribution of Ang2 to the suppression of Tie2 phosphorylation during the remodeling process. In support of this, we found that inhibition of Ang2 by two different Ang2 function-blocking antibodies reduced vascular remodeling after infection and accompanying changes in expression of PECAM-1, VE-cadherin, P-selectin, and E-selectin, and in leukocyte influx.

The angiopoietins have been implicated in the regulation of vascular permeability in inflammation.<sup>5,41,42</sup> Mucosal edema is a feature of chronic inflammatory airway diseases in general and in *M. pulmonis* infection in particular.<sup>30,43</sup> The rate of leakage after *M. pulmonis* infection is small compared to that after bradykinin, histamine, or substance P, and therefore difficult to measure.<sup>30</sup> However, the impact of leakage after infection is potentially much greater because it is continuous and does not resolve spontaneously, whereas the half-life of leakage after bradykinin, histamine, or substance P is only a few minutes.<sup>42</sup> The reversibility of the leakage in sustained inflammation by manipulation of angiopoietin/Tie2 signaling is a promising direction for future research.

Both function-blocking antibodies bind Ang2 with high affinity (Thurston et al, unpublished data and Brown et al, 2010<sup>31</sup>), but attributing this effect exclusively to Ang2 blockade is dependent on the selectivity of the antibodies. Because the assays used to test antibody specificity have limitations, a minor contribution of inhibitory activity on Ang1 or other angiopoietin cannot be excluded. The two blocking antibodies reduced expression of adhesion





**Figure 7.** Reduction in leukocyte influx by Ang2 blockade during infection. **A–C:** Confocal micrographs of leukocytes (red, CD11b) and blood vessels (green, PECAM-1) in regions over cartilage in tracheal whole mount of pathogen-free mouse and mice with *M. pulmonis* infection for 7 days and concurrent treatment with 5 mg/kg of isotype control IgG (**B**) or REGN145 (**C**). **D:** Fractional area of CD11b immunoreactivity in mucosa over cartilages of pathogen-free mice and infected mice treated with IgG or REGN145. **E and F:** qRT-PCR measurements of expression of CD11b (**E**), TNF- $\alpha$  (**F**) in tracheas of pathogen-free mice and infected mice treated with IgG or REGN145 for 7 days, showing significantly lower values after REGN145 ( $N = 15$ ). **G:** Linear regression ( $r^2 = 0.89$ ) of P-selectin expression and *M. pulmonis* 16S rRNA expression measured by qRT-PCR in tracheas of mice infected for 7 days. **H:** qRT-PCR measurements of *M. pulmonis* 16S rRNA expression in tracheas of pathogen-free mice and mice infected and treated for 7 days with control IgG, REGN145, or mAb 3.19.3. \* $P < 0.05$  compared to pathogen-free; # $P < 0.05$  compared to infected mice treated with IgG. Scale bars = 150  $\mu$ m.

molecules and infection severity to a similar extent. REGN145 led to somewhat greater reduction in capillary diameter than mAb 3.19.3, perhaps because of differences in affinity, pharmacokinetics, or selectivity. Nonetheless, the striking similarity of the effects of the two antibodies during *M. pulmonis* infection argue that Ang2 promotes vascular remodeling in this model.

Our finding of uniform p-Tie2 immunoreactivity on blood vessels of normal tracheas supports the concept that Tie2 is phosphorylated in normal, stable endothelial cells of adult blood vessels.<sup>44</sup> The results linking increased Ang2 expression to reduced Tie2 phosphorylation after *M. pulmonis* infection, and conversely, linking Ang2 blockade to less reduction of p-Tie2, suggest that Ang2 acts as a Tie2 antagonist and that loss of Tie2 phosphorylation renders the vasculature more responsive to inflammatory cytokines in infected airways. This mechanism would explain how the actions of TNF- $\alpha$  and Ang2 cooperate in promoting the remodeling of capillaries into venules after *M. pulmonis* infection.<sup>8</sup>

How can the decrease in Ang1 mRNA found at 7 days after infection be reconciled with the increase in Ang1 protein in a previous study?<sup>28</sup> Several lines of evidence help to explain the difference. *M. pulmonis* infection leads to a massive influx of leukocytes into the airways and lungs.<sup>30</sup> Most of the Ang1 protein detected as immunoreactivity at 7 days is associated with neutrophils, whereas Ang2 immunoreactivity is in endothelial cells.<sup>28</sup> Similarly, purified circulating human neutrophils contain Ang1—but not Ang2.<sup>45</sup>

The earlier study found an increase in Ang1 protein assessed in Western blots,<sup>28</sup> and the present study found a decrease in Ang1 mRNA expression measured by qRT-PCR. Ang1 is likely to be stored in neutrophils as previously synthesized protein and not accompanied by a corresponding level of expression of Ang1 mRNA. Ang1 and VEGF in circulating human neutrophils are released in equal amounts by phorbol myristate acetate stimulation, but expression of Ang1 mRNA is 92% lower than expression of VEGF mRNA.<sup>45</sup> Neutrophil  $\beta$ -granule-associated proteins elastase and myeloperoxidase are synthesized during differentiation and stored until secretion at sites of inflammation.<sup>46,47</sup> Ang1 mRNA expression may be similarly down-regulated during the final steps of neutrophil maturation. The release of Ang1 protein from neutrophils differs from secretion of VEGF and other proteins in  $\beta$ -granules, because Ang1 is cytosolic instead of in granules and its release is neither calcium dependent nor evoked by TNF- $\alpha$  or other neutrophil-activating cytokines.<sup>45</sup> Vascular remodeling after infection was associated with increased Ang2 expression and decreased Tie2 phosphorylation, which are consistent with Ang2-mediated inactivation of Tie2 signaling as a trigger for the vascular remodeling. If, when, where, and how much Ang1 is released and the contribution of neutrophil Ang1 to the response remain to be determined.

TNF- $\alpha$  may promote the increase in Ang2 expression after *M. pulmonis* infection. TNF- $\alpha$  is known to up-regulate Ang2 expression in endothelial cells<sup>48,49</sup> and to increase rapidly in expression after *M. pulmonis* infection.<sup>8</sup> TNF- $\alpha$

can also induce expression of adhesion molecules involved in leukocyte influx via Ang2 activation.<sup>11,50</sup>

Levels of Ang2 in blood or other body fluids increase in multiple diseases. Higher levels of Ang2 in airway fluid or blood are associated with more severe disease in patients with acute lung injury and in infants that develop bronchopulmonary dysplasia.<sup>40,51</sup> Higher levels of Ang2 in sputum correlate with more airway vascular leakiness in asthma.<sup>52,53</sup> High blood levels of Ang2 correlate with disease severity or poor outcome in patients with sepsis,<sup>54,55</sup> systemic lupus erythematosus,<sup>56</sup> inflammatory bowel disease,<sup>57</sup> and malaria.<sup>58</sup>

Ang2 blockade reduced the load of *M. pulmonis* organisms. The anti-inflammatory action of dexamethasone similarly reduces the load of *M. pulmonis* in the airways.<sup>59</sup> Both of these actions reflect the link between conditions that influence the growth of the organisms and the magnitude of the inflammatory response. They also fit with the reciprocal interdependence of the severity of *M. pulmonis* infection and magnitude of the inflammatory response.<sup>60,61</sup> Pro-inflammatory factors exacerbate the infection, and anti-inflammatory factors reduce it.

In conclusion, our results show that inhibition of Ang2 stabilizes Tie2 phosphorylation in endothelial cells, reduces vascular remodeling and expansion of venules that support leukocyte influx, and reduces the overall severity of *M. pulmonis* infection. These findings warrant further studies to develop a better understanding of the favorable actions of Ang2 inhibitors in airway inflammation.

### Acknowledgments

We thank Dongji Zhang and Nigel Killeen for production of the *M. pulmonis* stock and Peter Baluk for helpful discussions.

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