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## Eosinophils are necessary for pulmonary arterial remodeling in a mouse model of eosinophilic inflammation-induced pulmonary hypertension

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<sup>1</sup>Pulmonary and Critical Care Unit, <sup>3</sup>Anesthesia Center for Critical Care Research, Massachusetts General Hospital and Harvard Medical School, Boston; <sup>2</sup>Division of Rheumatology, Allergy, and Immunology, Center for Immunology and Inflammatory Diseases, Massachusetts General Hospital and Harvard Medical School, Charlestown, Massachusetts; and <sup>4</sup>Department of Biochemistry and Molecular Biology, Mayo Clinic, Scottsdale, Arizona

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**Weng M, Baron DM, Bloch KD, Luster AD, Lee JJ, Medoff BD.**

Eosinophils are necessary for pulmonary arterial remodeling in a mouse model of eosinophilic inflammation-induced pulmonary hypertension. *Am J Physiol Lung Cell Mol Physiol* 301: L927–L936, 2011. First published September 9, 2011; doi:10.1152/ajplung.00049.2011.—There is increasing evidence that inflammation plays a pivotal role in the pathogenesis of some forms of pulmonary hypertension (PH). We recently demonstrated that deficiency of adiponectin (APN) in a mouse model of PH induced by eosinophilic inflammation increases pulmonary arterial remodeling, pulmonary pressures, and the accumulation of eosinophils in the lung. Based on these data, we hypothesized that APN deficiency exacerbates PH indirectly by increasing eosinophil recruitment. Herein, we examined the role of eosinophils in the development of inflammation-induced PH. Elimination of eosinophils in APN-deficient mice by treatment with anti-interleukin-5 antibody attenuated pulmonary arterial muscularization and PH. In addition, we observed that transgenic mice that are devoid of eosinophils also do not develop pulmonary arterial muscularization in eosinophilic inflammation-induced PH. To investigate the mechanism by which APN deficiency increased eosinophil accumulation in response to an allergic inflammatory stimulus, we measured expression levels of the eosinophil-specific chemokines in alveolar macrophages isolated from the lungs of mice with eosinophilic inflammation-induced PH. In these experiments, the levels of CCL11 and CCL24 were higher in macrophages isolated from APN-deficient mice than in macrophages from wild-type mice. Finally, we demonstrate that the extracts of eosinophil granules promoted the proliferation of pulmonary arterial smooth muscle cells in vitro. These data suggest that APN deficiency may exacerbate PH, in part, by increasing eosinophil recruitment into the lung and that eosinophils could play an important role in the pathogenesis of inflammation-induced PH. These results may have implications for the pathogenesis and treatment of PH caused by vascular inflammation.

pulmonary hypertension; eosinophils; pulmonary artery smooth muscle cells; adiponectin; mouse model

PULMONARY HYPERTENSION (PH) can occur in association with multiple disorders, many of which share a common pathological appearance characterized by inflammation and remodeling of the pulmonary vasculature (3, 13, 22). Accumulating evidence suggests that pulmonary vascular inflammation is an important stimulus for the pathological changes seen in various types of PH (3, 8, 13, 19, 59). For example, it is now felt that pulmonary vascular eosinophilic inflammation in response to parasite eggs is a primary factor in the development of PH in

schistosomiasis, the most common cause of PH worldwide (4, 6). Similarly, it has been demonstrated that, when pulmonary eosinophilic (allergic) inflammation is induced in mice by sensitization and challenge with high-dose ovalbumin (OVA), mice develop prominent pulmonary vascular remodeling that mimics human disease (8, 45, 61, 62, 65, 66, 71, 75). It is likely that inflammation is a direct stimulus for vascular remodeling possibly via the release of growth factors and other mediators, or via metabolic changes such as focal hypoxia (28). Together, these data suggest that inflammation can be an important pathogenic factor in the development of PH and thus could be an effective therapeutic target for some forms of the disease.

Recent experimental evidence suggests that adipose tissue may contribute to the pathogenesis of inflammatory vascular diseases such as atherosclerosis through the secretion of multiple bioactive mediators (adipokines), which influence energy homeostasis, inflammation, and tissue remodeling (30, 63, 70). One of the most important adipokines is adiponectin (APN), which has a wide range of metabolic, anti-inflammatory, and antiproliferative activities (55). Interestingly, obese individuals have lower amounts of circulating APN compared with lean individuals, suggesting that decreased APN levels may contribute to the increased incidence of vascular diseases associated with obesity. Links between APN and pulmonary vascular disease are not fully defined; however, recent data in mouse models of PH from our group and others suggest that APN deficiency can increase the severity of pulmonary vascular inflammation, pulmonary vascular remodeling, and PH (19, 45, 49, 66). In our prior study (45), prominent vascular remodeling and PH was seen in APN-deficient (APN<sup>−/−</sup>) mice utilized in a low-dose OVA model of pulmonary allergic inflammation, but not in wild-type (WT) control mice. There was also increased pulmonary eosinophil recruitment in the APN<sup>−/−</sup> mice compared with WT mice in this model. Low-dose OVA was used for these experiments, since high-dose OVA overwhelmed the anti-inflammatory activity of APN in the WT control mice. Our data also suggested that APN deficiency increased eosinophil recruitment by enhancing the production of the eosinophil-active chemokine CCL11 (45). Based on these data, we hypothesized that eosinophilic inflammation of the pulmonary vasculature can promote pulmonary artery smooth muscle cell (PASMC) proliferation and, thus, APN could indirectly suppress vascular remodeling by inhibiting eosinophil accumulation in the lung. In the current study, we sought to follow up on our previous findings and investigate this hypothesis to better define the mechanisms

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of APN-mediated suppression of PH. In the data presented here, we demonstrate that inhibition of eosinophil accumulation in the lung prevents the development of pulmonary vascular remodeling induced by inflammation. Additionally, we show that APN deficiency is associated with elevated chemokine production from alveolar macrophages isolated from the lungs of APN<sup>-/-</sup> mice compared with WT mice. Finally, we report in vitro data showing that eosinophil granule extracts can stimulate PASM C proliferation and signaling. These data support an important role for APN in the pathogenesis of PH by modulating pulmonary vascular inflammation.

## MATERIALS AND METHODS

**Mice.** APN<sup>-/-</sup> mice (45) and PHIL mice (32) were all backcrossed more than seven generations onto a C57BL/6 background. WT C57BL/6 control mice were obtained from the National Cancer Institute (Bethesda, MD). Mice were used at 6–8 wk of age and were age and sex matched for all experiments. There were no baseline differences in weight between genotypes (data not shown). All protocols for mice experiments were approved by the Institutional Animal Care and Use Committee of Massachusetts General Hospital.

**Murine models of PH.** The low-dose OVA (45) and high-dose OVA (8) models were performed as previously described. Briefly, for the low-dose model, mice were immunized with three intraperitoneal injections of 50 µg of OVA in 0.1 ml PBS on days 1, 4, and 7. Starting on day 12, mice were challenged by intranasal injection with 20 µg OVA in 30 µl PBS or PBS alone (control mice) weekly for 4 wk. For the high-dose model, mice were immunized with intraperitoneal injections of 50 µg OVA (Sigma-Aldrich, St. Louis, MO) bound to 2 mg Alum (Sigma-Aldrich) in 0.5 ml PBS on day 1 and day 14. The mice were then challenged with aerosolized OVA or PBS for 45 min at a concentration of 10 mg/ml on days 28, 29, 30, 35, 36, 37, 41, and 42 and with OVA at a concentration of 25 mg/ml on day 43 only. Mice were analyzed 24 h after the last challenge in both models.

**Administration of antibody directed against interleukin-5.** APN<sup>-/-</sup> mice in the low-dose OVA model were injected intraperitoneally with 1 mg of anti-interleukin (IL)-5 antibody [obtained from the TRFK-5 cell line (ATCC, Manassas, VA), purified by BioXCell (West Lebanon, NH)] or isotype IgG control antibody (Abcam, Cambridge, MA) 1 h before each intranasal injection of OVA.

**Bronchoalveolar lavage.** Bronchoalveolar lavage (BAL) was performed as previously described (46). Mice were anesthetized with a lethal injection of ketamine (100 mg/kg). The cells recovered from the BAL were washed in PBS and enumerated in a hemocytometer. The differential cell count on cells isolated from the BAL were determined by enumerating mononuclear cells (macrophages, monocytes, and lymphocytes), neutrophils, and eosinophils on cytocentrifuge preparations of the cells stained with Diff-Quick (Dade Behring, Newark, DE). At least 200 cells were counted on each slide.

**Histological analyses.** For histopathological examination, lungs were flushed free of blood, inflated with 10% buffered formalin to 25 cmH<sub>2</sub>O of pressure, and prepared and evaluated as previously described (45). Briefly, sections of paraffin-embedded lungs were stained with hematoxylin-eosin. For measurement of vessel wall thickness, sections were stained with an antibody directed against  $\alpha$ -smooth muscle actin (Abcam) according to the manufacturers' recommended protocol. The quantitative analysis of vessel wall thickness was performed as previously described (75). Briefly, the external diameter of the vessel of interest was measured using NIS Elements AR imaging analysis software (Nikon, Melville, NY). The distance between the endothelial and the adventitia components of the vessel wall at two diametrically opposed locations was measured. The vessel

wall thickness was represented as the percentage of the sum of the two endothelia-to-adventitia distances over the external diameter. One hundred to 150 small- and medium-sized preacinar pulmonary arteries per mouse were analyzed. Genotypes of mice were blinded to examiners who performed the measurements.

**Hemodynamic studies.** Right ventricular systolic pressure (RVSP) was measured as previously described (45). In brief, mice were anesthetized, and a PE-10 polyethylene catheter was placed in the left carotid artery for monitoring heart rate and systemic arterial pressure. A 1.2-Fr high-fidelity pressure catheter (FTS-1211B-0018; Scisense, London, ON, Canada) was advanced into the right ventricle via the jugular vein to measure RVSP. All signals were recorded and analyzed using a data acquisition system (AD Instruments, Colorado Springs, CO).

**Isolation of eosinophil granule extracts.** Eosinophil granules were isolated as previously described (37). Briefly, eosinophils were isolated and purified from blood of IL-5 transgenic mice. Heparinized blood was layered on a Percoll E gradient [60% Percoll E, 1× Hanks' balanced salt solution, 15 mM HEPES (pH 7.4), and 0.003 N HCl] and centrifuged (45 min, 3,000 rpm, 4°C). The buffy coat was recovered and washed in PBS plus 2% FCS. Eosinophils were isolated using a magnetic cell separation system (Miltenyi Biotec, Auburn, CA). The isolated eosinophils were lysed with 0.25 M sucrose, 300 U/ml heparin, and 200 U/ml DNase. Granules were recovered by centrifuging the lysate (20 min, 10,000 g, 4°C). The pellet was then resuspended in 0.01 M HCl, sonicated, and recentrifuged at 300 g. The supernatant was removed, and the acid soluble granule proteins were stored at a concentration of protein equivalent to 1 × 10<sup>8</sup> cells/ml by adding 0.01 M HCl as needed.

**In vitro proliferation assay of PASM Cs.** PASM Cs were isolated from the main pulmonary arteries of male C57BL/6 mice and cultured as previously described (75). After three to six passages, cells were used for the proliferation assay. PASM Cs were seeded in 96-well plates at 8,000 cells/well. After starvation in 0.1% BSA media for 24 h, PASM Cs were cultured with an aliquot of eosinophil granule extracts isolated from 10<sup>6</sup> eosinophils or an equivalent volume of vehicle. After 48 h, the number of cells was determined using the CyQUANT NF Cell Proliferation Assay Kit (Invitrogen, Carlsbad, CA).

**Protein kinase B and extracellular signal-regulated kinase phosphorylation.** PASM Cs were cultured in serum-free medium for 24 h. After exposure to eosinophil granule extracts or vehicle for varying durations of time, cells were washed in PBS and lysed in Nonidet P-40 cell lysis buffer (Invitrogen) containing phosphatase inhibitors (Calbiochem, La Jolla, CA) and protease inhibitor cocktail tablets (Roche, Indianapolis, IN). Lysates were cleared by a centrifugation at 12,000 rpm and 4°C for 15 min, and protein concentrations were determined. Cell proteins (15 µg) were subjected to SDS-PAGE on a 4–20% gradient Tris-glycine gel (Invitrogen) under reducing conditions. Proteins were then transferred onto a nitrocellulose membrane (Invitrogen). Membranes were blocked in Tris-buffered saline and Tween 20 (TBST) with 5% nonfat milk for 1 h at room temperature and incubated overnight at 4°C with primary antibodies in TBST containing 5% BSA. Membranes were washed three times in TBST, each for 5 min, and then incubated 1 h at room temperature with horseradish peroxidase-conjugated secondary antibodies (Cell Signaling Technology, Danvers, MA). Bound antibodies were detected with enhanced chemiluminescence reagents (GE Healthcare). The dilution of primary antibodies was 1:1,000 and 1:2,000 for the secondary antibodies. Primary antibodies to protein kinase B (Akt) 1 and phospho-Akt1 (Ser<sup>473</sup>) were obtained from Cell Signaling Technology. Primary antibodies to extracellular signal-regulated kinase (ERK) and phospho-ERK (Tyr<sup>204</sup>) (detects both ERK1 and ERK2 in mice) were obtained from Santa Cruz Biotechnology (Santa Cruz, CA).

**Lung macrophage sorting.** Single-cell suspensions of lungs were freshly obtained from collagenase-digested lung tissues as previously

described (47). CD11c<sup>+</sup> cells were then isolated via magnetic bead separation according to the manufacturer's introduction (Miltenyi Biotec). Alveolar macrophages were isolated using a BD FACSARIA cell sorter (BD Biosciences, Rockville, MD) based on high levels of CD11c (CD11c<sup>high</sup>) and high autofluorescence in the FL1 channel (FL1<sup>high</sup>) as previously described (47, 73).

**Quantification of phosphorylated signal transducer and activator of transcription 6.** RAW 264.7 cells were plated into a 96-well plate ( $2 \times 10^5$  cells/well). The cells were incubated in media alone or media with 10  $\mu$ g/ml of APN for 24 h. The cells were then stimulated with 100 ng/ml IL-4 for 7 min and analyzed for phosphorylated signal transducer and activator of transcription (STAT) 6 using a commercial In-Cell ELISA kit (Thermo Scientific, Rockford, IL) according to the manufacturer's protocol.

**Quantification of gene expression.** RNA was purified from the lung or isolated macrophages and analyzed by quantitative RT-PCR as previously described (46). Primer sequences used were selected using the Massachusetts General Hospital PrimerBank (pga.mgh.harvard.edu/primerbank/).

**Statistical analysis.** Results are shown as mean  $\pm$  SE values. Graphpad Prism software was used to analyze the results. Two groups were compared using a Student's *t*-test. Between-group comparison of means with two independent variables was performed by two-way ANOVA. A value of  $P < 0.05$  was regarded as a significant difference.

## RESULTS

**Anti-IL-5 antibody treatment attenuates pulmonary vascular remodeling and hypertension.** We have previously reported that APN<sup>-/-</sup> mice develop increased pulmonary arterial muscularization, pulmonary eosinophilia, and PH compared with WT mice in a murine model that utilizes low-dose OVA sensitization and challenge to induce eosinophilic pulmonary vascular inflammation (45). In those previous experiments, we used a low-dose OVA model rather than a previously published high-dose OVA model (8) to limit the inflammatory stimuli from overwhelming the activity of APN. To examine if eosinophils play a role in pulmonary vascular remodeling associated with allergic inflammation, we treated APN<sup>-/-</sup> mice with antibodies against IL-5 before mice were challenged with OVA. IL-5 is essential for the proliferation of eosinophil lineage-committed cells in the bone marrow and has direct effects facilitating eosinophil maturation and survival (12, 16). Treatment with anti-IL-5 antibodies reduced the number and percentage of eosinophils in BAL fluid from OVA-challenged APN<sup>-/-</sup> mice compared with APN<sup>-/-</sup> mice treated with isotype control antibodies (Fig. 1, A and B). PBS challenge did not result in inflammation in both groups of mice (data not

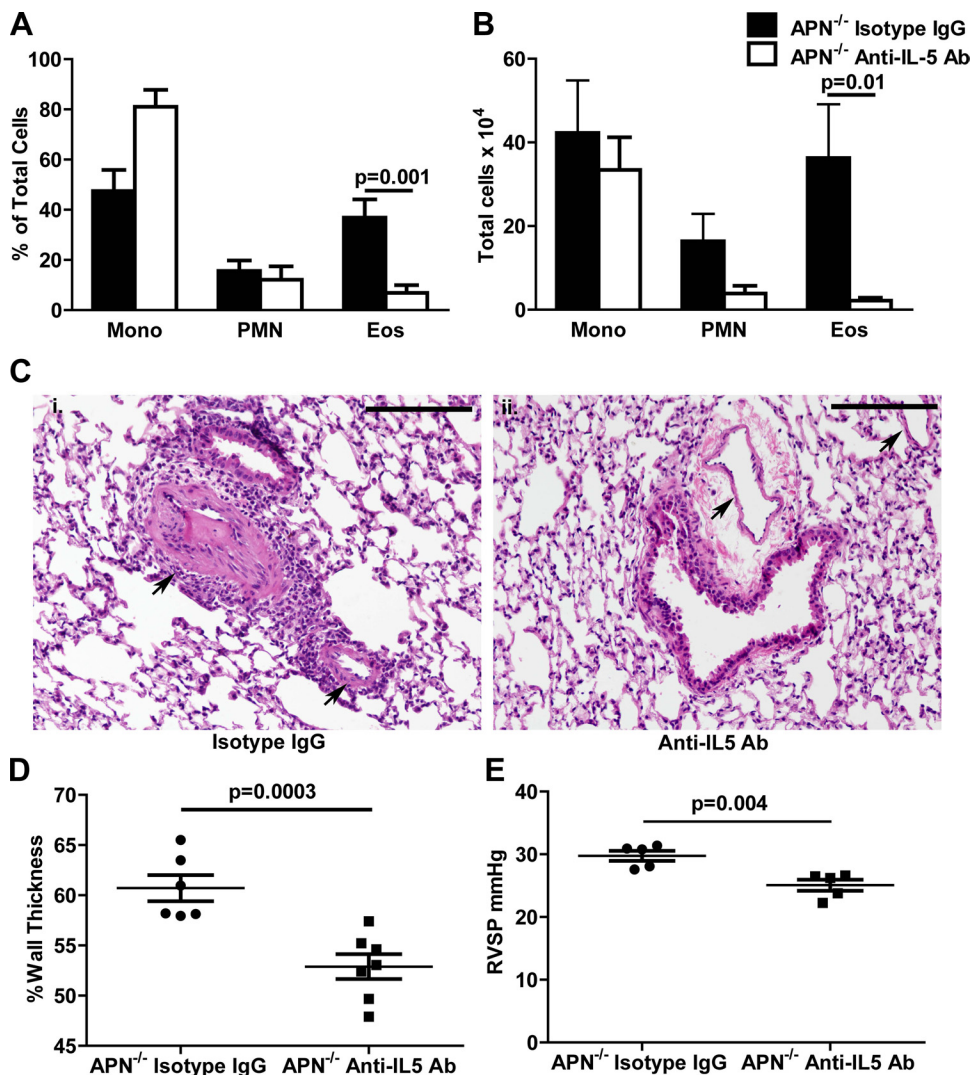


Fig. 1. Eosinophils are necessary for pulmonary vascular remodeling in adiponectin-deficient (APN<sup>-/-</sup>) mice. **A** and **B**: percentage and number of mononuclear cells (Mono), neutrophils (PMN), and eosinophils (Eos) in the bronchoalveolar lavage (BAL) from APN<sup>-/-</sup> mice following ovalbumin (OVA) immunization and challenge and pretreatment with anti-interleukin (IL)-5 or isotype control antibodies ( $n = 6-7$  mice/group). **C**: representative hematoxylin- and eosin-stained lung sections of APN<sup>-/-</sup> mice following OVA immunization and challenge and treatment with isotype control (**i**) or anti-IL-5 (**ii**) antibodies (Ab) ( $\times 100$  magnification). Pulmonary arteries are indicated with black arrows. Black bars are 100  $\mu$ m (representative images from  $n = 6-7$  mice/group). **D**: vessel wall thickness (% of total) in small and medium preacinar blood vessels in lung sections from APN<sup>-/-</sup> mice following OVA immunization and challenge and treatment with isotype or anti-IL-5 control antibodies ( $n = 6-7$  mice/group). **E**: right ventricular systolic pressure (RVSP) measured in APN<sup>-/-</sup> mice following OVA immunization and challenge and treatment with isotype or anti-IL-5 control antibodies ( $n = 5$  mice/group).

shown). Histological assessment of the lung tissue revealed that pulmonary arterial inflammation and muscularization were less in  $APN^{-/-}$  mice given anti-IL-5 antibody than in mice treated with isotype control antibody (Fig. 1C, *i* and *ii*). The cells within the vasculature stained positive for  $\alpha$ -smooth muscle cell (SMC) actin (data not shown) consistent with a mesenchymal origin for the cells, and image analysis indicated a reduction in pulmonary arterial wall thickness in the anti-IL-5-treated mice compared with vessels in control mice (Fig. 1D). We also measured right ventricular pressures in OVA-challenged  $APN^{-/-}$  mice treated with anti-IL-5 antibody or isotype control antibody. Consistent with the remodeling data, anti-IL-5-treated mice had reduced right ventricular pressures compared with isotype control-treated mice (Fig. 1E). Other hemodynamic parameters such as heart rate, systolic blood pressure, and right ventricular diastolic pressure were not different between the two groups (data not shown).

**PHIL mice do not develop pulmonary vascular remodeling.** To confirm the importance of eosinophils for pulmonary vascular remodeling induced with allergic inflammation, we utilized a mouse strain that is specifically devoid of eosinophils without disturbing the production of other hematopoietic lin-

eages (PHIL mice) (32). We used these mice in a recently described model of pulmonary vascular remodeling that utilizes high-dose OVA to induce muscularization of pulmonary arteries in WT mice (8). High-dose OVA was used instead of low-dose OVA because WT mice do not develop significant pulmonary vascular remodeling in the low-dose OVA model (only  $APN^{-/-}$  mice). Following OVA challenge, WT mice developed prominent BAL eosinophilia, but PHIL mice did not have any eosinophils in the BAL (Fig. 2, A and B). PBS challenge did not induce lung inflammation in either group of mice (data not shown). Consistent with our studies in anti-IL-5-treated mice, PHIL mice developed less pulmonary vascular remodeling in response to OVA challenge than did similarly treated WT mice (Fig. 2C, *i* and *ii*) and had less pulmonary arterial wall thickening (Fig. 2D). The high-dose OVA model used in these experiments does not induce PH under normoxic conditions so we did not measure right ventricular pressures in these mice (8). Together these data suggest that recruitment of eosinophils to the lung is necessary for the development of pulmonary vascular remodeling in models of PH induced by eosinophilic lung inflammation. Thus, if APN suppresses eo-

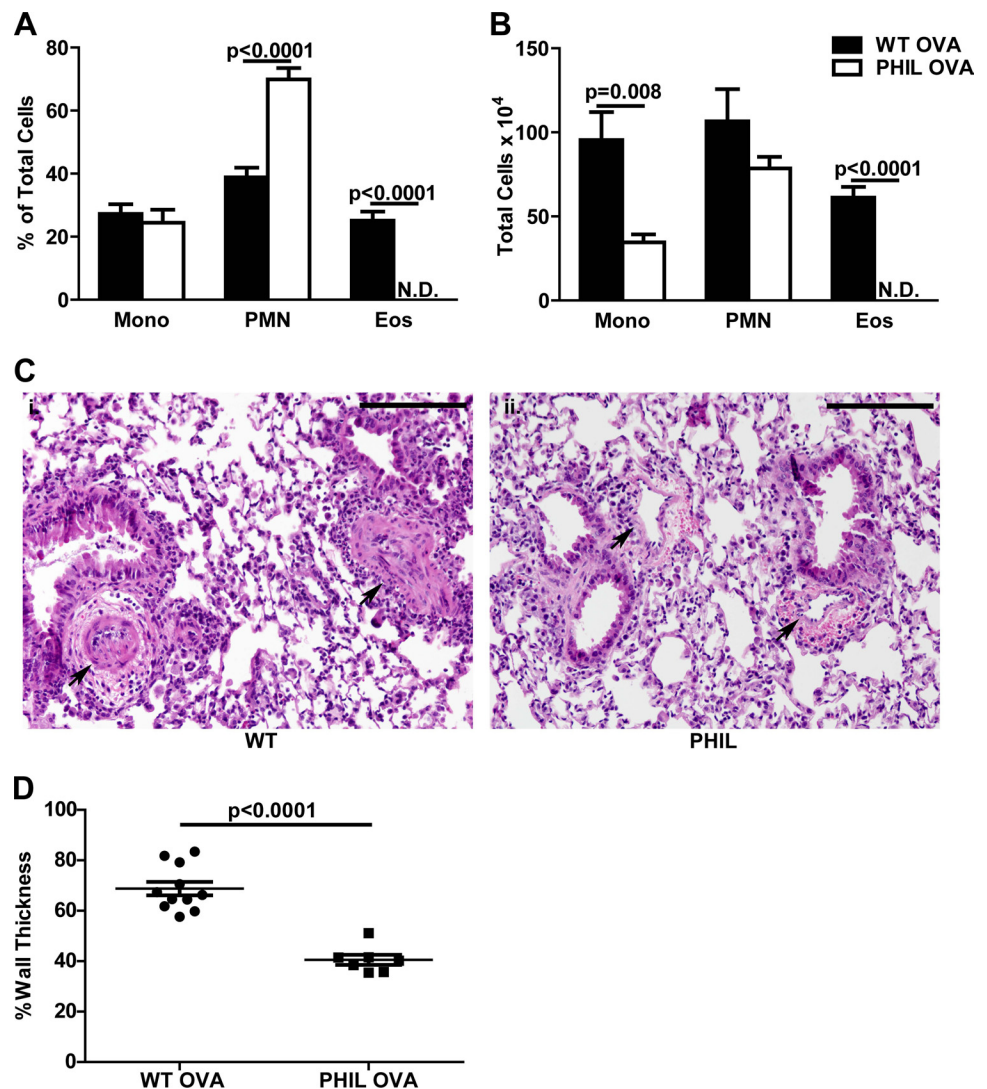


Fig. 2. PHIL mice have attenuated pulmonary vascular remodeling. A and B: percentage and number of mononuclear cells, neutrophils, and eosinophils in the BAL of wild-type (WT) and PHIL mice following OVA immunization and challenge ( $n = 8-11$  mice/group). C: representative hematoxylin- and eosin-stained lung sections of WT (*i*) and PHIL (*ii*) mice ( $\times 100$  magnification). Pulmonary arteries are indicated with black arrows. Black bars are 100  $\mu$ m (representative images from  $n = 8-11$  mice/group). D: vessel wall thickness (% of total) in pre-acinar blood vessels in lung sections from WT and PHIL mice after OVA immunization and challenge ( $n = 8-11$  mice/group). N.D., not detected.

sinophil accumulation in the lung, it could indirectly suppress vascular remodeling.

*Macrophages from APN<sup>-/-</sup> mice secrete more CCL11 and CCL24.* Our previous work has suggested that APN may suppress eosinophil recruitment into the lung via inhibition of eosinophil-active chemokine secretion in the lung (45). We hypothesized that APN could decrease chemokine secretion largely through its effects on lung macrophages. To better define the effects of APN on lung macrophages in vivo, we isolated lung macrophages using cell sorting from single cell suspensions of the lungs from WT and APN<sup>-/-</sup> mice immunized with OVA and challenged with OVA or PBS. As seen in previous studies (47, 73), examination of the sorted cells

demonstrated that they were >95% pure based on their morphology on cytopins (Fig. 3A). We then measured RNA expression levels of a panel of chemokines, cytokines, and growth factors. In addition, we quantified the levels of arginase-I and YM1, markers of alternatively activated (M2-type) macrophages, and inducible nitric oxide synthase (iNOS), a marker of M1-type macrophages. Consistent with our prior results, the levels of the eosinophil-active chemokines CCL11 and CCL24 were upregulated with OVA exposure in WT and APN<sup>-/-</sup> mice, but the mRNA levels were greater in macrophages from APN<sup>-/-</sup> mice (Fig. 3B). The mRNA levels of CCL2, IL-4, IL-6, IL-10, tumor necrosis factor, interferon (IFN)- $\gamma$ , vascular endothelial growth factor, platelet-derived

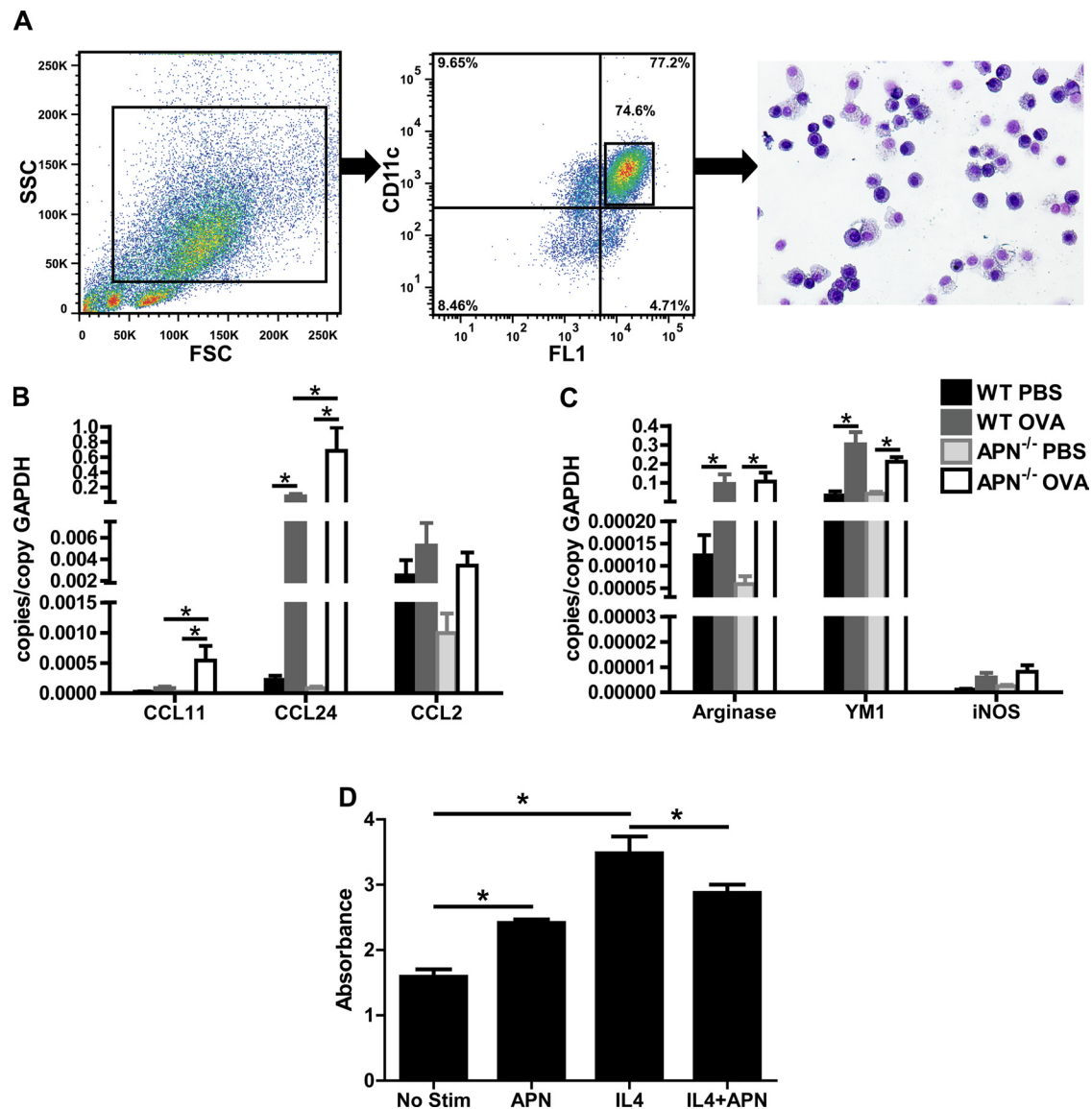


Fig. 3. Increased levels of CCL11 and CCL24 RNA in macrophages from OVA-immunized and -challenged APN<sup>-/-</sup> and WT mice. A: representative flow sorting method for isolation of pulmonary macrophages. Last panel shows a cytopsin of the isolated cells. B: levels of RNA encoding CCL11, CCL24, and CCL2 in lung macrophages isolated from the lungs of WT and APN<sup>-/-</sup> mice following OVA immunization and OVA or PBS challenge ( $n = 7-8$  mice/group,  $P < 0.05$  by 2-way ANOVA for genotype and OVA vs. PBS,  $*P < 0.05$  by  $t$ -test). C: levels of RNA encoding arginase-I, YM1, and inducible nitric oxide synthase (iNOS) in lung macrophages isolated from the lungs of WT and APN<sup>-/-</sup> mice following OVA immunization and OVA or PBS challenge ( $n = 7-8$  mice/group,  $P < 0.05$  by 2-way ANOVA for OVA vs. PBS but not for genotype,  $*P < 0.05$  by  $t$ -test). D: levels of phosphorylated signal transducer and activator of transcription (STAT) 6 in RAW 264.7 macrophages following pretreatment with APN (10  $\mu$ g/ml) and IL-4 (100 ng/ml) (each condition was done in triplicate,  $*P < 0.05$  by  $t$ -test). No Stim, no stimulation.

growth factor (PDGF), and epidermal growth factor in these cells were also induced by OVA challenge, but there were no differences in the expression levels of these genes in macrophages from WT and APN<sup>-/-</sup> mice (Fig. 3B and data not shown). There was also increased expression of arginase-1, YM1, and iNOS with OVA challenge in WT and APN<sup>-/-</sup> mice, but again there was no difference in the levels between the genotypes (Fig. 3C). Interestingly, work from others has suggested that APN could prime vascular macrophages into an M2-type phenotype; however, we did not observe a similar effect in the lung in this model (40, 53). To explore this further, we stimulated a murine macrophage cell line (RAW 264.7) with IL-4 after pretreatment with APN and analyzed STAT6 phosphorylation (activated STAT6). Interestingly, APN treatment of these cells alone increased STAT6 phosphorylation relative to baseline (Fig. 3D). However, when the cells were stimulated with IL-4, APN limited the increase in phosphorylated STAT6 compared with cells that were stimulated with IL-4 without APN pretreatment (Fig. 3D). Taken together, these data suggest that APN modulates eosinophil recruitment into the lung in response to an allergic inflammatory stimulus by suppressing expression of macrophage-derived chemokines. This suppression may occur through effects on nuclear factor (NF)- $\kappa$ B activation and STAT6 activation.

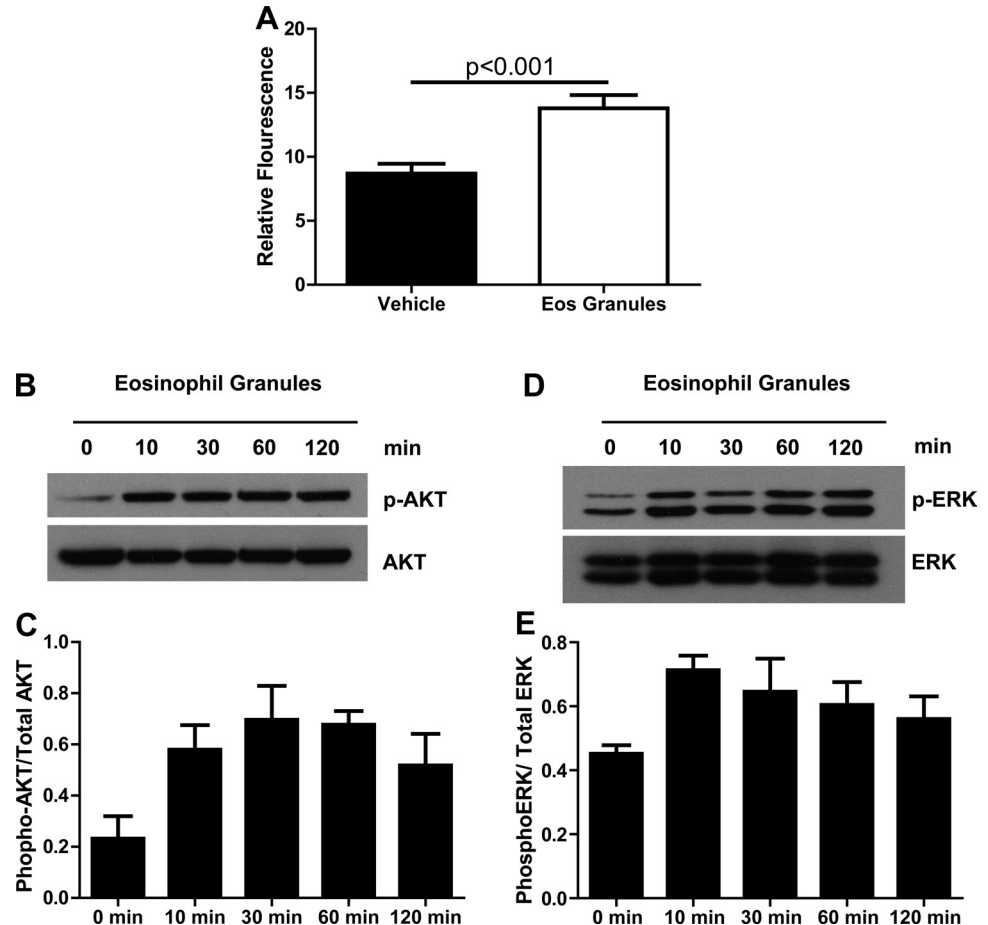
*Eosinophil granule extracts are mitogenic for PASMCs.* To explore the mechanism linking eosinophils to pulmonary vascular remodeling, we cultured PASMCs in the absence and presence of eosinophil granule extracts. PASMCs were cul-

tured as previously described (75) and serum starved. Cells were then incubated with media containing the granule contents from 10<sup>6</sup> eosinophils/culture dish well or vehicle (37). PASMC proliferation was twofold greater in PASMC incubated with eosinophil granules than in cells incubated with vehicle (Fig. 4A). To further explore the mechanism of the mitogenic effect of eosinophil granule proteins, we examined the effects of the granule extracts on phosphorylation of Akt1 and ERK, downstream mediators known to be important in PASMC proliferation (1, 38, 39, 60). Consistent with the proliferation data, treatment with eosinophil granules increased the levels of phosphorylated Akt and ERK in PASMCs (Fig. 4, B–E). These data suggest that eosinophils could stimulate pulmonary vascular remodeling by releasing their granule contents and directly stimulating PASMC proliferation in the vasculature.

## DISCUSSION

In a previous study, we demonstrated that, in response to allergic inflammation, APN<sup>-/-</sup> mice have increased eosinophil recruitment into the lung associated with increased pulmonary artery remodeling and elevated pulmonary pressures (45). Based on these data, we hypothesized that the increased remodeling seen in APN<sup>-/-</sup> mice could result from two different mechanisms: 1) APN deficiency leads to loss of APN-mediated direct suppression of PASMC proliferation; and/or 2) increased eosinophil accumulation around the pulmonary vasculature in

Fig. 4. Eosinophil granules are mitogenic for pulmonary artery smooth muscle cells (PASMCs). **A**: number of PASMCs after 48 h of stimulation with eosinophil granule extract or vehicle control as measured by a fluorescent assay ( $n = 3$  for both groups). PASMCs were prepared from 5 WT mice and pooled for analysis. **B**: representative Western blot of PASMC protein extracts following incubation with eosinophil granule extract for the indicated times and reacted with antibody to phosphorylated protein kinase B (Akt) and total Akt. The experiment was repeated 3 times. **C**: relative quantification of the ratio of phosphorylated Akt (p-AKT) and total Akt levels in PASMC protein extracts after stimulation with eosinophil granule extracts for the indicated times (data pooled from 3 separate experiments). **D**: representative Western blot of PASMC protein extracts following incubation with eosinophil granule extract for the indicated times and reacted with antibody to phosphorylated ERK and total ERK. The experiment was repeated 3 times. **E**: relative quantification of the ratio of phosphorylated ERK and total ERK levels in PASMC protein extracts after stimulation with eosinophil granule extracts for the indicated times (data pooled from 3 separate experiments).



APN<sup>-/-</sup> mice indirectly leads to increased stimulation of the remodeling response. We have recently demonstrated that APN can suppress pulmonary vascular remodeling via direct antiproliferative effects on PASMCs (75). In the studies presented here, we test the other hypothesis, namely that APN may indirectly modulate pulmonary vascular remodeling through effects on eosinophil recruitment. We provide evidence that eosinophils are necessary for the development of pulmonary vascular remodeling in murine models of PH induced by allergic pulmonary vascular inflammation. We also demonstrate that APN may control eosinophil recruitment into the lung via effects on macrophage-mediated chemokine secretion. Finally, we show that eosinophil granule contents are mitogenic for PASMCs and induce proliferative signaling in these cells. Overall, these studies add more support to the growing body of literature linking metabolism, inflammation, and pulmonary vascular disease (8, 18–20, 22, 59) and suggest a potential therapeutic role for manipulation of inflammatory mediators and adipokine activity in the treatment of PH.

Although most of the evidence linking metabolism to vascular inflammation and remodeling is derived from studies of systemic vascular processes (29), there is an increasing appreciation of effects of metabolism on the development of pulmonary vascular disease. Studies in human samples and from animal models have supported a mechanistic role for insulin resistance, apoE deficiency, and peroxisome proliferator-activated receptor- $\gamma$  (PPAR $\gamma$ ) activity in the pathogenesis of PH (2, 15, 18–20). Furthermore, treatment with PPAR $\gamma$  agonists has been shown to mitigate PH and pulmonary vascular remodeling in animal models, similar to effects of these agents on systemic vascular remodeling (7, 27, 52). There also appears to be an increased incidence of PH and pulmonary vascular remodeling in obesity (21, 44, 69). In light of the obesity-associated downregulation of APN expression, these data suggest that APN could play a mechanistic role connecting obesity and metabolism with increased pulmonary vascular inflammation and remodeling.

Although our recently published results suggest that APN can directly suppress vascular remodeling via effects on PASMCs, we were also interested in examining the effects of APN on pulmonary vascular inflammation. A role for inflammation in the pathogenesis of PH is suggested by studies demonstrating the presence of increased levels of cytokines in patients with PH (14, 25) and the presence of leukocytes in and around the remodeled vasculature of the lung (10, 51, 72). Clinical studies of idiopathic forms of PH have demonstrated that proinflammatory cytokines and chemokines are biomarkers for PH (14, 25, 64) and have better prognostic value than hemodynamic measurements (64). Furthermore, in animal models, pulmonary vascular inflammation induces vascular remodeling and PH (8, 45, 61, 62, 65, 66, 71). This may be most relevant for forms of PH with prominent vascular inflammation such as PH related to schistosomiasis infection (the most common form of PH worldwide) (6) and autoimmunity, but may also be important in other forms of PH (17). It has been suggested that inflammatory cells release mediators that stimulate remodeling of the vessel wall in part by directly promoting PASMC proliferation (22, 48, 59). Eosinophils, in particular, are known to be especially potent sources of mitogenic mediators (42). Alternatively, inflammation could induce met-

abolic changes such as focal hypoxia that could induce remodeling (28).

In our prior work, we demonstrated that the increased number of eosinophils in the lung of APN<sup>-/-</sup> mice in a model of PH induced by allergic inflammation was associated with elevated levels of the eosinophil-active chemokine CCL11 in the lung (45). These data are consistent with human data which revealed an inverse correlation between serum APN levels and CCL11 levels in patients with obesity (24). In the studies presented here, we now show that levels of the eosinophil-active chemokine CCL24 are also modulated by APN and that APN may control chemokine secretion via effects on lung macrophages. CCL11 and CCL24 have been shown to be key mediators of allergen-induced pulmonary eosinophilia in mice (58) and are produced by multiple cell types in the lung during allergic inflammation. However, it appears that macrophages are the primary source in the lung (31, 34, 35, 56–58, 78). Previous data indicate that APN can influence immune responses through actions on NF- $\kappa$ B signaling in macrophages (76, 77, 79), a cellular pathway that has been linked to the development of allergic inflammation and CCL11 production (9, 36, 43). Furthermore, in vitro and in vivo studies on macrophages have demonstrated that APN inhibits the expression of NF- $\kappa$ B-responsive genes such as IFN- $\gamma$  and CXCL10/IP-10 (54, 67, 68). Studying ex vivo macrophages, we demonstrate that CCL11 and CCL24 expression was greater in macrophages isolated from the lungs of OVA-challenged APN<sup>-/-</sup> mice than in those from WT mice. We did not find differences in the expression of growth factors or other cytokines from these cells. Studies have demonstrated that APN can promote macrophage polarization toward the M2 phenotype in vitro (40, 53), and, consistent with this, we demonstrate enhanced STAT6 activity in a macrophage cell line exposed to APN alone. However, APN limited STAT6 activation when these cells were stimulated with IL-4, suggesting that APN may also reduce STAT6 activity in macrophages in response to inflammatory stimuli. Interestingly, we did not see differences in the expression of a panel of markers for the M2 phenotype on ex vivo macrophages isolated from the lungs of WT and APN<sup>-/-</sup> mice used in the models of PH, suggesting that the effects of APN on macrophage phenotype may be less important in vivo during allergic inflammation. Overall, these data suggest that APN directly suppresses chemokine expression in macrophages, possibly via effects on NF- $\kappa$ B and STAT6 signaling.

Eosinophils are known to be fibrogenic and potent sources of growth factors, cytokines, and other mediators that are mitogenic for SMCs (42, 50, 74). In the OVA-induced chronic model of allergic airway inflammation, eosinophils have been shown to be necessary for airway remodeling (5, 26). Similarly, in our model of allergic inflammation-induced PH, eosinophils are necessary for the development of pulmonary vascular remodeling. Given that APN<sup>-/-</sup> mice have increased vascular inflammation with enhanced eosinophil recruitment into the lung, it follows that some of the increased remodeling seen in these mice may result from the increased inflammation. Together, these data suggest that inflammation can be an important pathogenic factor in the development of PH and could be an effective therapeutic target for forms of the disease induced by inflammation. Our results may be most relevant to PH associated with schistosomiasis infection, where eosino-

philic pulmonary vascular inflammation is very prominent and is thought to stimulate pulmonary vascular remodeling.

It is clear that, in response to growth factors, such as PDGF, and other mediators, such as serotonin, multiple pathways that mediate mitogenic activity in PASMCs are activated in parallel (23, 33, 39). For example, the 3-phosphoinositide-dependent kinase/Akt1 pathway is an important regulator of SMC survival and proliferation (11). Akt is activated via phosphorylation by a number of receptor tyrosine kinases following the binding of growth factors or hormones. Most notably, inhibition of Akt activation reduces proliferation of PASMCs in vitro (38, 41). ERK1 and ERK2 are mitogen-activated protein kinases that have also been shown to be principal mediators of PASMC proliferation in response to PDGF and serotonin stimulation (33, 39, 60). Our data demonstrate that incubation of PASMCs with secretory granule proteins isolated from eosinophils leads to phosphorylation of Akt and ERK and that this activity is associated with increased PASMC proliferation. These data suggest that eosinophilic inflammation could stimulate pulmonary vascular remodeling via direct activation of proliferative signaling pathways in PASMCs by mediators contained in eosinophil granules. Given that eosinophilic granules are a rich source of growth factors, cytokines, and other mediators that can stimulate proliferation, it is likely that multiple pathways in PASMCs are activated in response to eosinophilic inflammation and exposure to granule contents (50, 74). Future studies are necessary to identify the exact components in eosinophil granules that mediate the stimulatory effects on the growth of PASMCs.

In summary, we have established a critical role of eosinophils in the pathogenesis of vascular remodeling in models of PH induced by allergic inflammation. Our data suggest that eosinophils may be directly mitogenic for PASMCs via degranulation. Furthermore, APN may indirectly modulate pulmonary vascular remodeling by inhibiting the recruitment of eosinophils into the lung by decreasing the secretion of CCL11 and CCL24 by macrophages.

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#### DISCLOSURES

No conflicts of interest are declared by the authors.

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