Interleukin 13– and interleukin 17A–induced pulmonary hypertension phenotype due to inhalation of antigen and fine particles from air pollution

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Abstract: Pulmonary hypertension has a marked detrimental effect on quality of life and life expectancy. In a mouse model of antigen-induced pulmonary arterial remodeling, we have recently shown that coexposure to urban ambient particulate matter (PM) significantly increased the thickening of the pulmonary arteries and also resulted in significantly increased right ventricular systolic pressures. Here we interrogate the mechanism and show that combined neutralization of interleukin 13 (IL-13) and IL-17A significantly ameliorated the increase in right ventricular systolic pressure, the circumferential muscularization of pulmonary arteries, and the molecular change in the right ventricle. Surprisingly, our data revealed a protective role of IL-17A for the antigen- and PM-induced severe thickening of pulmonary arteries. This protection was due to the inhibition of the effects of IL-13, which drove this response, and the expression of metalloelastase and resistin-like molecule α . However, the latter was redundant for the arterial thickening response. Anti-IL-13 exacerbated airway neutrophilia, which was due to a resulting excess effect of IL-17A, confirming concurrent cross inhibition of IL-13- and IL-17A-dependent responses in the lungs of animals exposed to antigen and PM. Our experiments also identified IL-13/IL-17A-independent molecular reprogramming in the lungs induced by exposure to antigen and PM, which indicates a risk for arterial remodeling and protection from arterial constriction. Our study points to IL-13- and IL-17A-coinduced inflammation as a new template for biomarkers and therapeutic targeting for the management of immune response–induced pulmonary hypertension.

Keywords: pulmonary vasculature, particulate matter 2.5, air pollution, inhaled antigen.

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INTRODUCTION

Pulmonary hypertension has a major effect on quality of life and life expectancy¹⁻³ with impairment of two organ systems, the lungs and the right heart. Remodeling and constriction of the pulmonary arteries are causes of the increase in pressure in the pulmonary circulation.¹ Inflammation with an increase in the activity of pathogenic T cells⁴ and macrophages⁵ is a hallmark of different types of pulmonary hypertension,⁶ particularly when associated with autoimmunity (systemic sclerosis, systemic lupus erythematosus),⁷ chronic obstructive pulmonary disease,⁸ or

helminth parasite infections (schistosomiasis),^{9,10} and also of idiopathic pulmonary hypertension.¹¹ The right heart develops structural changes (hypertrophy, dilation, fibrosis) as well as metabolic and molecular reprogramming.¹²⁻¹⁴

Although often unrecognized, a variety of environmental and occupational exposures are associated with the development of pulmonary hypertension. Prime examples are silicosis and cigarette smoke–induced pulmonary hypertension associated with chronic obstructive pulmonary disease.^{15,16} Our group has recently shown that exposure

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of immunized mice to a weak antigen that induces type 2 T helper (Th2) responses results in severe thickening of approximately one-quarter of the pulmonary arteries.¹⁷ Further studies showed that when the immunized mice were coexposed to the weak antigen and particulate matter (PM) collected from urban air, the abundance of severely thickened arteries in the lungs was significantly increased, and pulmonary hypertension, measured as an increase in right ventricular pressures, was induced.¹⁸

In this study, we sought to determine the molecular mechanism of pulmonary hypertension induced by coexposure to antigen and PM. We focused our studies on interleukin 13 (IL-13) and IL-17A and the mediators controlled by these cytokines as well as on known risk markers of pulmonary arterial remodeling and constriction (Fig. 1). Clinical data point out the significance of IL-13 and IL-17A for pulmonary hypertension associated with chronic inflammation. In particular, studies of pulmonary hypertension associated with systemic sclerosis showed correlation of markers for Th17 and Th2 responses, including IL-17A and IL-13, with disease severity.¹⁹⁻²¹ Additionally, two Th17-associated markers, neutrophils²² and IL-6,²³ have long been implicated in idiopathic pulmonary hypertension. Th17 responses eliciting IL-17A have been identified as pathogenic mechanisms in many autoimmune diseases²⁴⁻²⁷ and in silica particle exposure-induced lung inflammation in mice.²⁸ IL-13 is an important Th2 effector molecule, particularly in asthma.²⁹⁻³² IL-13 and IL-17A are thought to be independent and cross-inhibitory inducers of inflammation and associated tissue destruction/remodeling.^{24,25} Surprisingly, our studies show cooperative effects despite concurrent subtle cross inhibition, pointing to a therapeutic strategy of simultaneously targeting both IL-13 and IL-17A for immune response-induced pulmonary hypertension.

METHODS

Ethics statement

All animal experiments were performed according to guidelines outlined by the US Department of Agriculture and the American Association of Laboratory Animal Care under the supervision and specific approval of the Institutional Animal Care and Use Committees (IACUCs) at New York University Medical Center, New York, New York (IACUC no. 111107-03; most recent approval date, November 18, 2013).

Mice

C57BL/6J (wild-type) mice were purchased from the Jackson Laboratory. Stock *Retnla*^{-/-} BALB/c background mice

Figure 1. Illustration depicting the major pathways being examined in mice exposed to antigen and particulate matter (PM) with respect to responses in the pulmonary artery (severe thickening, circumference muscularization) and the right heart (right ventricular systolic pressure, molecular expression changes). We tested the hypothesis that the interleukin 13 (IL-13)- and the IL-17A-dependent molecular pathways would be independent mechanisms that induce the pulmonary hypertension phenotype. CD11c⁺ cells: bronchoalveolar lavage macrophages or dendritic cells that express CD11c; MHCII: major histocompatibility complex class II; RELM: resistin-like molecule; MMP: matrix metalloproteinase; miR: microRNA; S100a: S100 calcium binding protein A; BNP: brain natriuretic peptide; BMPR: bone morphogenetic protein receptor; VEGF: vascular endothelial growth factor; Kcnk3: potassium channel, subfamily K, member 3. A color version of this figure is available online.

were provided by Dr. Marc E. Rothenberg (Cincinnati Children's Hospital Medical Center).^{33,34} In the Grunig laboratory, the animals were backcrossed to the C57BL/6 strain for six generations, and heterozygous *Retnla*^{+/-} mice were interbred to generate wild-type and knockout littermate mice.

Mostly female mice (90% of the animals were female, 10% male) were used for the study, being 5–7 weeks of age at the start of the experiment. This is an important aspect of the animal model because women are overproportionally affected by pulmonary hypertension. For each experiment, littermate mice were randomized into cages holding up to four mice each. The mice were housed under specific pathogen-free conditions at the Department

of Environmental Medicine, New York University (NYU) Medical Center, Tuxedo, New York.

Urban PM¹⁸

Urban PM was captured from a roof of a low-rise building in New York City.³⁵ A large batch of PM_{2.5} from New York ambient air has been prepared and is available to all interested investigators on request. This is size fractionated, and only the PM_{2.5} fraction was used. This PM_{2.5} was provided by Dr. Terry Gordon's laboratory at the NYU Medical Center, resuspended, and ultrasonicated before use.³⁵ This fraction was chosen because the size of <2.5 μ m in diameter allows the particles to travel via the airways into the alveoli. The dose chosen was at half the concentration of the dose reported to induce significant airway inflammation using PM_{2.5} sampled in New York City,³⁵ Beijing,³⁶ and Baltimore.³⁷⁻³⁹ PM_{2.5} was given intranasally at a dose of 25 μ g/50 μ L in phosphate-buffered saline (PBS) combined with ovalbumin (OVA; see below). When given without OVA, PM_{2.5} at the 25- μ g/50- μ L dose

did not elicit significant airway inflammation or vascular remodeling.¹⁸

Antigen immunization and exposure to antigen and PM

Animals were primed and challenged with antigen as described elsewhere^{17,18} and schematically presented in Figure 2A. In brief, mice were injected intraperitoneally with OVA (grade 5; Sigma, St. Louis, MO; 50 μ g/dose) adsorbed to alum (Imject Alum; Thermo Fisher Scientific, Rockford, IL; 2 mg/dose) to prime the immune response at a 2-week interval. Two weeks later, the mice were intranasally challenged with either PBS or combined OVA (100 μ g/dose) and PM_{2.5} (25 μ g/dose) two times each week, for a total of six doses given over a 3-week period. This exposure regimen has been shown to induce severe arterial remodeling^{17,18} (just like antigen exposure by nebulization¹⁷), and when antigen exposure was combined with PM, significant increases in right ventricular systolic pressures (RVSPs) were measured.¹⁸ To verify that the anti-

Figure 2. Experimental schedule and inflammation (airway/interstitial) induced by exposure to antigen and particulate matter (PM). A, Timeline showing the timing of the priming injections with ovalbumin (OVA)/alum, the dosing of intranasal challenges with saline or antigen and PM (OVA&PM), and the antibody injections administered intraperitoneally. B, C, Bar graphs showing scores for airway (peribronchial/perivascular) inflammation (B) and interstitial (alveolar) inflammation (C). Groups of mice were exposed to saline, PM, or OVA&PM and given isotype control or neutralizing antibodies. A score of 1 was given for normal morphology, 2 for mild infiltrates, and 3 for severe infiltrates of inflammatory cells. Data were pooled from 2–3 experiments, $n = 5-20$ per group. $P < 0.0001$ for group comparisons with saline (a). IL: interleukin. A color version of this figure is available online.

gen and PM delivery elicited the expected inflammation and that the PM dose used for the study did not induce microscopic signs of inflammation when given alone, the lungs were scored for airway and interstitial (alveolar) inflammation (Fig. 2B, 2C) as described elsewhere.^{17,40-42} Airway (peribronchial, perivascular) inflammation was scored on 20 or more consecutive view fields, as follows: 1, normal with very few inflammatory cells; 2, scattered inflammatory cells up to two rings in depth; and 3, cuffs of inflammatory cells measuring three rings or more in depth. Interstitial (alveolar) inflammation was scored on 20 or more consecutive view fields, as follows: 1, normal; 2, increased numbers of cells within the alveoli; and 3, consistent increase in the numbers of cells within the alveoli, appearance of multinucleated giant cells, and thickening of the alveolar septa. All measurements were performed without prior knowledge of the group designation of the lungs.

Antibody injections

Wild-type mice were injected intraperitoneally with 150 μ g of anti-mouse-IL-13 (Ebioscience, San Diego, CA; clone eBio1316H), anti-mouse-IL-17A (BioXCell, West Lebanon, NH; clone MM17F3⁴³), or isotype control (rat immunoglobulin G [IgG] 1, mouse IgG1; Biolegend, San Diego, CA, or BioXCell, respectively) monoclonal antibodies prior to each of the intranasal challenges. The antibody preparations were of cell culture/experimental animal usage grade (<2 EU lipopolysaccharide/mg). The dose of monoclonal antibody used (6.8 mg/kg body weight) is within the dose range used clinically in human patients (2–3 mg/kg body weight).

Functional evaluation and tissue collection

RVSP was measured after inserting a catheter via the jugular vein in anaesthetized, spontaneously breathing mice.^{40,44} Right ventricular hypertrophy was determined as right ventricular weight relative to the weight of the left ventricle and septum.⁴⁴

Bronchoalveolar lavage (BAL) and tissue harvest were performed after right heart catheterization and euthanasia of animals.^{17,40,44} BAL was performed by gently washing with three 1-mL aliquots of Hank's balanced salt solution. Following BAL, lungs and ventricles were recovered. The right lung lobe was snap-frozen in liquid nitrogen, and the remainder of the lungs were inflated with buffered formaldehyde and placed in formaldehyde for histology. In our laboratory, BAL does not wash out all inflammatory cells from the lungs and is performed gently to minimize the potential for mechanical damage of the tissue. The above protocol allows us to reduce the number of

animals used for each study. All measurements were performed without prior knowledge of the group designation of mice.

Circumferential muscularization of lung blood vessels was measured following immunohistochemical staining of lung sections with anti-von Willebrand factor and anti-smooth muscle actin, as described elsewhere.⁴⁰ Blood vessels <100 μ m in diameter were analyzed for the circumference length of smooth muscle actin staining relative to the length of the vessel circumference. For each lung, at least 20 vessels (mean, 128) were analyzed. Dual immunohistochemistry was performed with a rabbit anti-von Willebrand factor antibody (Dako, Glostrup, Denmark) recognizing endothelial cells and goat anti-smooth muscle actin antibody (Everest Biotech, Upper Heyford, United Kingdom) to detect muscularization, as described elsewhere.^{40,45,46} Slides were scanned using an Aperio XX slide scanner, and the images were analyzed using VisiopharmDP software (Visiopharm, Hoersholm, Denmark). From each slide at least 20 vessels (mean, 128) were identified and analyzed for muscularization by calculating the length of smooth muscle actin staining relative to the length of the vessel circumference for vessels <100 μ m in diameter.

The vessels were categorized into three groups—non-muscularized (<0.3), partially muscularized (0.3–0.8), and fully muscularized (>0.8)—and for each lung the percentage of vessels per group was calculated (sum of nonmuscularized + partially muscularized + fully muscularized was 100). For each lung, the ratio of nonmuscularized to fully muscularized blood vessels was also calculated, using the formula (% nonmuscularized)/(% fully muscularized).

Severe arterial thickening was determined on lung sections stained with hematoxylin and eosin. The severe arterial thickening change induced by prolonged exposure to antigen has been described in detail by our group.¹⁷ The number of arteries (blood vessels adjacent to airways) at 200 $\times$ magnification that showed severely thickened walls characterized by disorganized layers of cells (cells in the blood vessel wall assume a pattern that differs from that of the lumen)¹⁷ were counted relative to all arteries scored per lung. All arteries from at least 20 consecutive view fields comprised the sum of arteries with normal (thin) walls, mildly thickened walls, or the above-mentioned severely thickened walls. For each lung, severe arterial thickening was calculated using the following formula: 100/number of all arteries examined $\times$ number of severely remodeled arteries. The muscularized nature of the severe thickening lesions¹⁷ was confirmed by immunohistochem-

istry with anti-smooth muscle actin staining. Digital photomicrographs were obtained from images captured by scanning the lung slides using the Leica SCN400F whole-slide scanning system at the Histopathology Core of the NYU Medical Center and capturing images via SlidePath's Digital Image Hub (Leica Biosystems, Buffalo Grove, IL).

Messenger RNA (mRNA) and microRNA (miRNA) expression

Total RNA from lung or right heart tissue was isolated using the RNeasy Mini Kit (Qiagen, Valencia, CA). Reverse transcription was performed using the High-Capacity cDNA Reverse Transcription Kit (Applied Biosystems/Life Technologies, Grand Island, NY) for mRNA and the Universal RT cDNA Synthesis Kit (Exiqon, Woburn, MA) for miRNA, with locked nucleic acid-modified primers (Exiqon). Quantitative polymerase chain reaction (qPCR) was performed in duplicate with 20 ng of complementary DNA (cDNA) for mRNA or 0.1 ng of cDNA for miRNA, using the 7900HT Fast Real-Time PCR System (Applied Biosystems).

qPCR for the detection of mRNA expression was performed with SYBR Green (Invitrogen, Grand Island, NY). For the specific detection of the homologous genes *Retnla* and *Retnlb*, we used the TaqMan Gene Expression Assay (Applied Biosystems) based on a FAM-labeled probe and the corresponding TaqMan Gene Expression Assay for β -actin. The sequences for primers and probes are indicated in Table 1. The following conditions were used: 95°C for 10 minutes, followed by 45 cycles of 95°C for 15 seconds and 60°C for 1 minute, followed by a hold at 4°C. The *miR-135a* expression level was determined by using *miR-135a* primer (Exiqon) and SYBR Green Master Mix (Exiqon) with these conditions: 95°C for 10 min, followed by 45 cycles of 95°C for 10 seconds and 60°C for 1 minute, followed by a hold at 4°C.

Raw data were analyzed with SDS Relative Quantification Software (ver. 2.3; Applied Biosystems) to determine cycle threshold (C_t). Mean C_t values were standardized by calculating ΔC_t using the housekeeping gene β -actin (mRNA) or 5S (miRNA) and calculating $1.98^{\Delta C_t} \times 10,000$.

Immune responses

BAL cells were analyzed by flow cytometry as described elsewhere,^{17,41,47} using a MACS Quant instrument (Miltenyi Biotec, Auburn, CA) and FloJo software (TreeStar, Ashland, OR). Cell counts were performed using the feature provided by the MACS Quant flow cytometer. All cell analysis was performed without prior knowledge of the group designation of the samples. BAL samples were analyzed as described elsewhere^{40,41} for the presence of eosin-

ophils (CD11b^{high}, CCR3^{high}, GR1^{low}, CD11c^{low-intermediate}, MHCII^{low-intermediate}), neutrophils (CD11b^{high}, GR1^{high}, CD11c^{low-intermediate}, MHCII^{low-intermediate}), lymphocytes (cells expressing CD4 or CD8 and falling into the lymphocyte gate by forward- and side-scatter profile), and CD11c⁺ cells that coexpress CD205. The mean fluorescence intensity of CD11c⁺ cells stained with anti-MHCII antibody was determined as a measure of the capacity of these airway dendritic cells/airway macrophages to present antigen.^{41,47} The cell populations were analyzed using monoclonal antibodies tagged with Pacific Blue, AlexaFluor, fluorescein isothiocyanate, phycoerythrin, peridinin-chlorophyll, allophycocyanin, or cyanine tandem dyes that were purchased from BD Biosciences (San Jose, CA), Ebioscience (San Diego, CA), or Biolegend (San Diego, CA).

Statistical analysis

Statistical analysis was performed with Aable (ver. 3; Gigawiz, Tulsa, OK) or Prism (ver. 6; Graphpad, San Diego, CA) software. Two-group comparisons were conducted with the unpaired two-tailed Mann-Whitney *U* test or the unpaired two-tailed *t* test with Welch's correction for unequal variances. Data sets were analyzed for correlation using the Spearman rank correlation test. Contingency tables were analyzed with the χ^2 test for multigroup comparisons and the two-tailed Fisher exact test for two-group comparisons. Differences with $P < 0.05$ were considered significant.

RESULTS AND DISCUSSION

Exposure to antigen and PM induces increased RVSP and circumferential pulmonary arterial muscularization via IL-13 and IL-17A

To understand the mechanism by which exposure to a weak antigen (OVA) and ambient PM_{2.5} induced this phenotype,¹⁸ groups of mice were given neutralizing anti-IL-13 and/or anti-IL-17A antibodies or control antibodies. RVSPs are correlated with pulmonary arterial pressures. We analyzed the medians and data spread by box plot (Fig. 3A) as well as the distribution of data within each group of mice by showing the number of animals with RVSP values below or above a 26-mmHg threshold (Fig. 3B). Both types of analysis showed that the increase in RVSP induced by exposure to OVA and PM was significantly attenuated by coneutralization of IL-13 and IL-17A. Blocking either IL-13 or IL-17A (Fig. 3A, 3B) had no significant effects. Furthermore, the circumferential muscularization of blood vessels in the lungs (Fig. 3C) was significantly attenuated by coneutralization of IL-13 and IL-17A, as indi-

Table 1. Sequences of primers and probes

Name	Gene symbol	Sequence (5'→3')
BMPR2-F	<i>Bmpr2</i>	AGAGGCCCAATTCTCTGGAT
BMPR2-R	<i>Bmpr2</i>	GGAGATGACCCAGGTGGAC
VEGFa-F	<i>Vegfa</i>	CTGCTGTAACGATGAAGCCCTG
VEGFa-R	<i>Vegfa</i>	GCTGTAGGAAGCTCATCTCTCC
Kcnk3/TASK-1-F	<i>Kcnk3</i>	GGCTCCTTCTACTTCGCCATCA
Kcnk3/TASK-1-R	<i>Kcnk3</i>	TGTTGATGCGTTCACCCAGGCT
Endothelin 1-F	<i>Edn1</i>	CTACTTCTGCCACCTGGACATC
Endothelin 1-R	<i>Edn1</i>	CGCACTGACATCTAACTGCCTG
Endothelin receptor a-F	<i>Ednra</i>	TCACCGTCTTGAACCTCTGTGC
Endothelin receptor a-R	<i>Ednra</i>	GATGGAGACGATTTCAATGGCGG
Endothelin receptor b-F	<i>Ednrb</i>	TCTACTTCTGCTTGCCGCTAGC
Endothelin receptor b-R	<i>Ednrb</i>	CAAACACGAGGACCAGGCAGAA
IL-6-F	<i>Il6</i>	TACCACTTCACAAGTCGGAGGC
IL-6-R	<i>Il6</i>	CTGCAAGTGCATCATCGTTGTTC
IFN- γ -F	<i>Ifng</i>	CAGCAACAGCAAGGCGAAAAAGG
IFN- γ -R	<i>Ifng</i>	TTTCCGCTTCCTGAGGCTGGAT
MMP12-F	<i>Mmp12</i>	CACACTTCCCAGGAATCAAGCC
MMP12-R	<i>Mmp12</i>	TTTGGTGACACGACGGAACAGG
Angiotensin receptor 1a-F	<i>Agtr1a</i>	GCCATTGTCCACCCGATGAAGT
Angiotensin receptor 1a-R	<i>Agtr1a</i>	ACACATTTCCGTGGATGACGGC
Angiotensin-converting enzyme-F	<i>Ace</i>	TCAGCGGAATGGCGAAGTCCTA
Angiotensin-converting enzyme-R	<i>Ace</i>	CGGTCATACTCTTCCACGAACC
BNP-F	<i>Nppb</i>	TCCTAGCCAGTCTCCAGAGCAA
BNP-R	<i>Nppb</i>	GGTCCTTCAAGAGCTGTCTCTG
S100a8-F	<i>S100a8</i>	CAAGGAAATCACCATGCCCTCTA
S100a8-R	<i>S100a8</i>	ACCATCGCAAGGAACCTCTCGA
S100a9-F	<i>S100a9</i>	TGGTGGAAGCACAGTTGGCAAC
S100a9-R	<i>S100a9</i>	CAGCATCATACACTCCTCAAAGC
β -actin-F	<i>Actb</i>	GGCTGTATTCCCCTCCATCG
β -actin-R	<i>Actb</i>	CCAGTTGGTAACAATGCCATGT
RELM α (TaqMan)	<i>Retnla</i>	CTTGCCAATCCAGCTAACTATCCCT
RELM β (TaqMan)	<i>Retnlb</i>	GGAAGCTCTCAGTCGTCAAGAGCCT
Actin, β (TaqMan)	<i>Actb</i>	ACTGAGCTGCGTTTTACACCCTTTC
miR-135a (Exiqon)	<i>miR-135a</i>	UAUGGCUUUUAUUCUAUGUGA

Note: BMPR: bone morphogenetic protein receptor; F: forward; R: reverse; VEGF: vascular endothelial growth factor; IL: interleukin; IFN: interferon; MMP: matrix metalloproteinase; BNP: brain natriuretic peptide; RELM: resistin-like molecule.

cated by the restoration of the predominance of nonmuscularized vessels over fully muscularized vessels (Fig. 3D).

Exposure to antigen and PM induces molecular change in the right ventricle that is dependent on IL-13 and IL-17A

The data in Figure 3 show that coexposure to antigen and PM induced modest but significant increases in RVSP.

This was similar to that reported for other mouse models of pulmonary hypertension induced by hypoxia,⁴⁸ a strong antigen,⁴⁹ or a weak antigen combined with the presence of a transgene with a hypomorphic mutation in bone morphogenetic protein receptor type 2 (BMPR2).⁴⁰ To understand the biological significance, we examined right ventricles for signs of molecular change because right ventricular remodeling and ultimately failure are the cause of

Figure 3. Coblockade of interleukin 13 (IL-13) and IL-17A inhibits the increase in right ventricular systolic pressure (RVSP) and the circumferential muscularization of lung blood vessels in response to exposure to antigen and particulate matter (PM). RVSP data are shown as a box plot with circles indicating individual data points (A) or as a bar graph showing the number of observations of RVSP less than or greater than 26 mmHg (B); $n = 7$ –18 per group. C, Photomicrographs of representative sections of lungs stained with anti-smooth muscle actin (brown; scale bars: 50 μ m; v: blood vessel). D, Bar graphs showing mean and SEM values ($n = 5$ –12 per group) for circumferential muscularization as a percentage of blood vessels that were in the category none (<30% of the circumference positive for smooth muscle actin staining) or full (>80% of the circumference positive) as well as for the ratio of none to fully muscularized arteries. Mean and SEM values for the category of partially muscularized arteries were as follows: 50.08 ± 2.47 (saline), 51.2 ± 4.65 (ovalbumin [OVA] and PM [OVA&PM], isotype), 56.8 ± 3.91 (OVA&PM, anti-IL-13), 26.6 ± 7.64 (OVA&PM, anti-IL-17A), and 52.7 ± 3.62 (OVA&PM, anti-IL-13 and anti-IL-17A). Data from groups of mice exposed to saline or antigen and PM and given neutralizing or isotype control antibodies were pooled from 2–3 experiments. $P < 0.05$ for group comparisons with saline (a) or OVA&PM–control antibody (b). ns: not significant for comparison with the saline group.

the impairment of physical activity and mortality associated with pulmonary hypertension in human patients.^{12,13} We examined the expression of brain natriuretic peptide (BNP, *Nppb*) because it is recognized as a marker of right ventricular strain in humans.³ Compared with the saline-exposed group, the mice exposed to antigen and PM had significantly increased expression of *Nppb* in the right ventricle, and this was inhibited by conneutralization of IL-13 and IL-17A (Fig. 4A). Right ventricular *Nppb* expression was significantly associated with right ventricular weight, a measure of right heart hypertrophy ($P = 0.003$), and RVSP ($P = 0.029$; Fig. 4B, 4C). The tightest correlation

was between right ventricular *Nppb* and the numbers of BAL neutrophils ($P = 0.0002$; Fig. 4D), while there was no correlation with BAL eosinophils (Fig. 4E). Right ventricular weights were also significantly correlated with RVSP ($P = 0.016$; Fig. 4F). These correlations suggest that the right ventricular molecular change in *Nppb* expression was associated, as expected, with the increased pressures in the pulmonary circulation and independently with inflammation in the lungs. The latter association has also been suggested by studies in human patients.¹² Right ventricular weights (weight of right ventricle/weight of the left ventricle + septum) were not significantly increased by expo-

Figure 4. Molecular change is induced in the right ventricle (RV) by exposure to antigen and particulate matter (PM) via interleukin 13 (IL-13) and IL-17A and is correlated with airway neutrophils. A, Bar graph of expression of the brain natriuretic peptide (BNP) gene *Nppb* in the RV in mice exposed to saline or to antigen (ovalbumin [OVA]) and PM (OVA&PM) and given neutralizing or isotype control antibodies. Data are means and SEM. $P < 0.05$ for group comparisons with saline (a) or OVA&PM–control antibody (b). B–E, *Nppb* expression in the RV plotted against RV weight (RV/left ventricle plus septum [LV+S]; B), right ventricular systolic pressure (RVSP; C), numbers of neutrophils in bronchoalveolar lavage (BAL) fluid (D), and numbers of eosinophils in BAL fluid (E). F, RV weight (RV/LV+S) plotted against RVSP. P and r_s (tie corrected) values for Spearman's rank correlation test are shown (ns: not significant). Data were pooled from 2–3 experiments, $n = 5$ –20 per group. *Nppb* expression is given relative to that of β -actin (set at 10,000 U).

sure to OVA and PM (mean $\pm$ SD, 0.204 ± 0.019 for saline vs. 0.222 ± 0.028 for OVA and PM). This is likely due to the ability of the mice to adapt to the pulmonary arterial remodeling and the increase in right ventricular pressure or to the insensitivity of this measure for mild changes.

Neutralization of IL-13 and IL-17A reveals their concurrent activity in the lungs of mice exposed to antigen and PM

The cooperative activity of IL-13 and IL-17A for right ventricular pressure, circumferential pulmonary vascular muscularization, and right ventricular strain was surprising because of the cross inhibition of Th17 and Th2 development and the reported metastability and adaptability of Th17 cells.^{24,50} Therefore, we wanted to know

whether IL-13- and IL-17A-dependent changes would occur at the same time (concurrently). BAL cells and lung RNA were probed for direct downstream effectors in the Th2 (Fig. 5A–5E) and Th17 (Fig. 5F–5I) pathways, respectively. The levels of MHCII expression by CD11c⁺ cells in BAL fluid, as a marker of activation of macrophages and dendritic cells,⁴⁷ and expression of *Retnla*, *Retnlb*, and matrix metalloproteinase 12 (*Mmp12*) were decreased by IL-13 neutralization (Fig. 5A–5D). The numbers of BAL eosinophils remained unaffected (Fig. 5E). The numbers of BAL neutrophils and expression of the myeloid cell effector molecules⁵¹ S100 calcium binding protein A8 (*S100a8*), *S100a9*, and *Il6* were decreased by IL-17A neutralization (Fig. 5F–5I).

Th2 and IL-17A responses to *Aspergillus conidia* challenges⁵² or interferon γ (IFN- γ)- and IL-17A-producing T

Figure 5. Concurrent cellular and molecular activity of interleukin 13 (IL-13) and IL-17A in the lungs of mice exposed to antigen and particulate matter (PM). Bar graphs show mean and SEM values for numbers of eosinophils in bronchoalveolar lavage (BAL) fluid (A); major histocompatibility complex class II (MHCII) expression by CD11c⁺ cells from BAL fluid (mean fluorescent intensity [MFI]; B); RNA expression in the lungs for the resistin-like molecule α (RELMA) gene (*Retnla*; C), the RELM β gene (*Retnlb*; D), and the matrix metalloproteinase 12 (*Mmp12*) gene (E); numbers of neutrophils in BAL fluid (F); and RNA expression in the lungs for *S100a8* (G), *S100a9* (H), the IL-6 gene (*Il6*; I), the interferon γ gene (*Ifng*; J), and *miR-135a* (K). Groups of mice were exposed to saline or to antigen (ovalbumin [OVA]) and PM (OVA&PM) and given neutralizing or isotype control antibodies ($n = 5-20$ per group, pooled from 2-3 experiments). $P < 0.05$ for group comparisons with saline (a) or OVA&PM-control antibody (b). RNA expression levels were calculated relative to the means of the saline or OVA&PM group, as indicated. A color version of this figure is available online.

cells from atherosclerotic coronary arteries in humans⁵³ and inflamed mouse tissue⁵⁴ have been reported to coexist consecutively or concurrently. To distinguish between consecutive and concurrent activity of IL-13 and IL-17A, we looked for cross inhibition of effector responses. Our data showed significant increases in (a) MHCII expression by BAL CD11c⁺ cells in mice given anti-IL-17A (Fig. 5B) and (b) numbers of BAL neutrophils in mice given anti-IL-13 (Fig. 5F). In both cases, conneutralization of IL-13 and IL-17A showed that the exacerbated response elicited by

single-cytokine blockade was due to enhanced IL-17A or IL-13 effects, respectively (Fig. 5B, 5F). Conneutralization of IL-13 and IL-17A significantly enhanced the expression of *Ifng* (Fig. 5J), suggesting the independent activation of the Th1 pathway in mice exposed to antigen and PM, and significantly inhibited the expression of *miR-135a* (Fig. 5K). *miR-135a* is upregulated in the allergen-challenged lung,⁵⁵ targets several components of cytokine signaling, and may participate in the control of the IL-13/IL-17A-dependent response to coexposure to antigen and PM.

Severe pulmonary arterial thickening induced by antigen and PM has an independent mechanism

Severe thickening and irregular pattern formation by smooth muscle cells is induced in pulmonary arteries in mice exposed to only antigen via IL-13,¹⁷ and this response occurs in IL-17A-deficient mice.⁵² IL-13 has also been shown to be a critical mediator in experimental *Schistosoma* infection-induced pulmonary arterial remodeling.⁵⁶ As shown schematically in Figure 1, this type of remodeling is phenotypically different from circumferential muscularization. Circumferential muscularization is typically induced by hypoxia,⁴⁸ and in our studies of mice exposed to antigen and PM it was inhibited by IL-13 and IL-17A coneutralization (Fig. 3). The simultaneously occurring arterial wall thickening was quantified by determining for each lung the percentage of pulmonary arteries that were severely thickened and also showed irregular sheets of smooth muscle cells (Fig. 6A).

To our surprise, injection with neutralizing anti-IL-17A antibody significantly exacerbated the severe remodeling response (Fig. 6B). This was reminiscent of findings in a mouse model of atherosclerosis.⁴³ In our mice, the IL-17A-mediated protection was due to the inhibition of IL-13-dependent effects because coinjection of neutralizing anti-IL-17A and anti-IL-13 antibodies resulted in profound inhibition of the severe arterial thickening response, just like anti-IL-13 antibody alone (Fig. 6B). Resistin-like molecule α (RELM α) was not necessary for the severe remodeling response that was fully present in knockout mice (Fig. 6C). This was unexpected because *Retnla* levels in the lungs of antigen- and PM-exposed mice were controlled by IL-13 (Fig. 5C). Furthermore, mouse RELM α and its human homologue, RELM β , are known as hypoxia-induced mitogenic factors and are smooth muscle cell mitogens.^{48,57-60} As an explanation for our unexpected data, the role of RELM α in arterial thickening and remodeling could vary depending on the type of challenge (e.g., hypoxia vs. antigen).^{48,61}

Comparing the data shown in Figures 5 and 6 suggests that airway eosinophils, neutrophils, myeloid cell activation markers S100a8 and S100a9, and IL-6 are not critical promoters of the severe arterial remodeling response and that IFN- γ is not a critical inhibitor. With respect to the role of IL-6 in severe pulmonary arterial thickening, it is of great interest that in experimental schistosomiasis the IL-6 pathway has recently been shown to have a protective function.⁶² In contrast, the IL-13-dependent upregulation of MMP12 metalloelastase expression (Fig. 5E) could represent a critical step because the fragmentation of the internal elastin barrier of the vasculature allows smooth

muscle cells to invade.¹ The ability of IL-13 to directly induce proliferation of smooth muscle cells is controversial;^{20,63} therefore, the effects could be indirect by inactivating a suppressor of the response to a mitogen that may be present ubiquitously or that may be upregulated due to exposure to antigen and PM.

Comparing Figures 3 and 6, our study suggests that circumferential blood vessel muscularization and severe pulmonary arterial thickening have different control mechanisms in mice exposed to antigen and PM (IL-13 and IL-17A induced vs. IL-17A protected via inhibition of IL-13 effects, respectively). This is surprising because one may assume that arterial thickening is a more severe form of circumferential muscularization. Our study refutes this notion in the lungs of mice exposed to antigen and PM. Instead, circumferential muscularization that organizes smooth muscle cells in a chain-like configuration versus the severe muscularization resulting in irregular sheets of smooth muscle cells could recapitulate distinct ancestral responses. For example, choanoflagellates, the closest living eukaryotic relative of animals, use distinct molecular mechanisms to form chain-shaped or rosette-shaped multicellular colonies.^{64,65}

Exposure to antigen and PM induces a molecular risk profile in the lungs for pulmonary arterial remodeling and a protection profile for pulmonary arterial constriction

Our data left two questions open. The first question was, is the role of IL-13 in controlling severe pulmonary arterial thickening supported by molecular reprogramming in the lungs of mice exposed to antigen and PM? To address this, we looked for expression in the lungs of key molecules known to predispose for the development of pulmonary arterial remodeling by controlling smooth muscle and endothelial cells in humans and animals when under-expressed.⁶⁶⁻⁷⁰ These key molecules are BMPR2, vascular endothelial growth factor a (VEGFa), and the potassium channel Kcnk3/TASK-1.^{40,71-74} Here we show significantly decreased expression of all three molecules in the lungs of animals exposed to antigen and PM (Fig. 7A–7C), constituting a molecular risk profile for pulmonary arterial remodeling.

The second question was, why do mice exposed to antigen and PM not develop a higher degree of pulmonary hypertension despite the widespread circumferential muscularization and severe thickening of pulmonary arteries? To address this, we determined the expression of endothelin 1, angiotensin, and their receptors/enzymes

Figure 6. Interleukin 17A (IL-17A) protects against widespread severe thickening of the pulmonary arteries in response to exposure to antigen and particulate matter (PM) by inhibiting IL-13-controlled effects with a redundant role of resistin-like molecule α (RELM α). **A**, Representative lung sections from a saline-exposed mouse (i) and a mouse exposed to antigen (ovalbumin [OVA]) and PM (ii, iii), stained with hematoxylin and eosin (i, ii) or with anti-smooth muscle actin (brown, iii). Vessels (v), airways, and scale bars (100 μ m) are indicated. Bar graphs show mean and SEM values for percentages of severely thickened arteries per lung in groups of wild-type mice exposed to saline or OVA&PM and given neutralizing or isotype control antibodies ($n = 10$ –27 per group, pooled from 2–3 experiments; **B**) or in groups of wild-type and *Relm1* knockout (KO) mice exposed to saline or OVA&PM ($n = 7$ –10 per group, pooled from 2 experiments; **C**). $P < 0.05$ for group comparisons with saline (a) or OVA&PM–control antibody (b). ns: not significant.

needed for activation. Endothelin 1 and angiotensin are major arterial constrictors and play significant roles in pulmonary hypertension.^{2,75,76} Our data showed a significant downregulation of these molecules (Fig. 7D–7H), constituting a molecular protection profile for pulmonary arterial constriction.

Our data showed clearly that the concerted molecular profiles of risk for arterial wall thickening and protection from arterial constriction elicited in the lungs of animals exposed to antigen and PM are not controlled by IL-13 and/or IL-17A (Fig. 7). The transcriptional control mechanism is currently under intense inquiry because this molecular profile may enable the right ventricle to adapt to the changes in the pulmonary vasculature.

Relevance and limitations

Because of the inherent limitations of animal experimentation, our study will require follow-up work to understand its relevance for the human condition. Our study has clinically translatable implications by identifying a therapeutic target of two distinct cytokine-controlled pathways, IL-13 and IL-17A, for antigen- and PM-induced increases in RVSP, circumferential muscularization, and right ventricle molecular changes and a single gene target (IL-13) for the severe thickening of pulmonary arteries. This is exciting because of the emerging clinical success of neutralizing IL-13/IL-13 signaling^{30–32} and IL-17A/IL-17 signaling^{26,27} in diseases associated with chronic inflammation (asthma and psoriasis, respectively). Careful

Figure 7. Molecular at-risk profile for arterial remodeling and protection profile for vasoconstriction in the lungs of mice exposed to antigen (ovalbumin [OVA]) and particulate matter (PM). Bar graphs show mean and SEM values for bone morphogenetic protein receptor type 2 (BMPR2; A), vascular endothelial growth factor a (VEGFa; B), the potassium channel Kcnk3/TASK-1 (C), endothelin 1 (D), endothelin receptor (R) a (E), endothelin Rb (F), angiotensin-converting enzyme (ACE; G), and angiotensin R1 (H) RNA expression in the lungs (calculated relative to the means for the saline group). Groups of mice were exposed to saline or to antigen and PM (OVA&PM) and given neutralizing or isotype control antibodies ($n = 5-12$ per group, pooled from 2-3 experiments). $P < 0.05$ for group comparisons with saline (a) or OVA&PM-control antibody (b). A color version of this figure is available online.

studies in human patients have shown increased IL-13-dependent, IL-17A-dependent, or IFN-dependent markers in pulmonary hypertension.¹⁹⁻²³ Further translation of the findings from our study is dependent on the identification of biomarkers for an immune response that elaborates

coexisting IL-13- and IL-17A-driven inflammatory pathways in pulmonary hypertension patients. This would be a necessary step toward evaluating reagents that neutralize IL-13 or IL-17A as potential therapeutic options for the clinical management of pulmonary hypertension. In addi-

tion, this would help to design studies to further understand how fine-dust exposure from air pollution could affect exacerbations of pulmonary hypertension.

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REFERENCES

1. Rabinovitch M. Molecular pathogenesis of pulmonary arterial hypertension. *J Clin Invest* 2012;122:4306–4313.
2. Barst RJ, Gibbs JS, Ghofrani HA, Hoeper MM, McLaughlin VV, Rubin LJ, Sitbon O, Tapson VF, Galie N. Updated evidence-based treatment algorithm in pulmonary arterial hypertension. *J Am Coll Cardiol* 2009;54:S78–S84.
3. Fritz JS, Blair C, Oudiz RJ, Dufton C, Olschewski H, Despain D, Gillies H, Kawut SM. Baseline and follow-up 6-min walk distance and brain natriuretic peptide predict 2-year mortality in pulmonary arterial hypertension. *Chest* 2013; 143:315–323.
4. Tamosiuniene R, Tian W, Dhillon G, Wang L, Sung YK, Gera L, Patterson AJ, et al. Regulatory T cells limit vascular endothelial injury and prevent pulmonary hypertension. *Circ Res* 2011;109:867–879.
5. Tian W, Jiang X, Tamosiuniene R, Sung YK, Qian J, Dhillon G, Gera L, et al. Blocking macrophage leukotriene B₄ prevents endothelial injury and reverses pulmonary hypertension. *Sci Transl Med* 2013;5:200ra117.
6. Price LC, Wort SJ, Perros F, Dorfmueller P, Huertas A, Montani D, Cohen-Kaminsky S, Humbert M. Inflammation in pulmonary arterial hypertension. *Chest* 2012;141:210–221.
7. Chaisson NF, Hassoun PM. Systemic sclerosis-associated pulmonary arterial hypertension. *Chest* 2013;144:1346–1356.
8. Seeger W, Adir Y, Barbera JA, Champion H, Coghlan JG, Cottin V, De Marco T, et al. Pulmonary hypertension in chronic lung diseases. *J Am Coll Cardiol* 2013;62:D109–D116.
9. Kolosionek E, Graham BB, Tudor RM, Butrous G. Pulmonary vascular disease associated with parasitic infection—the role of schistosomiasis. *Clin Microbiol Infect* 2011;17:15–24.
10. Graham BB, Bandeira AP, Morrell NW, Butrous G, Tudor RM. Schistosomiasis-associated pulmonary hypertension: pulmonary vascular disease: the global perspective. *Chest* 2010;137:20S–29S.
11. Savai R, Pullamsetti SS, Kolbe J, Bieniek E, Voswinckel R, Fink L, Scheed A, et al. Immune and inflammatory cell involvement in the pathology of idiopathic pulmonary arterial hypertension. *Am J Respir Crit Care Med* 2012;186: 897–908.
12. Bogaard HJ, Natarajan R, Henderson SC, Long CS, Krasauskas D, Smithson L, Ockaili R, McCord JM, Voelkel NF. Chronic pulmonary artery pressure elevation is insufficient to explain right heart failure. *Circulation* 2009;120:1951–1960.
13. Voelkel NF, Gomez-Arroyo J, Abbate A, Bogaard HJ. Mechanisms of right heart failure—a work in progress and a plea for failure prevention. *Pulm Circ* 2013;3:137–143.
14. Vonk-Noordegraaf A, Haddad F, Chin KM, Forfia PR, Kawut SM, Lumens J, Naeije R, et al. Right heart adaptation to pulmonary arterial hypertension: physiology and pathobiology. *J Am Coll Cardiol* 2013;62:D22–D33.
15. Scano G, Garcia-Herreros P, Stendardi D, Degre S, De Coster A, Sergysels R. Cardiopulmonary adaptation to exercise in coal miners. *Arch Environ Health* 1980;35:360–366.
16. Seimetz M, Parajuli N, Pichl A, Veit F, Kwapiszewska G, Weisel FC, Milger K, et al. Inducible NOS inhibition reverses tobacco-smoke-induced emphysema and pulmonary hypertension in mice. *Cell* 2011;147:293–305.
17. Daley E, Emson C, Guignabert C, de Waal Malefyt R, Louten J, Kurup VP, Hogaboam C, et al. Pulmonary arterial remodeling induced by a Th2 immune response. *J Exp Med* 2008;205:361–372.
18. Grunig G, Marsh LM, Esmaeil N, Jackson K, Gordon T, Reibman J, Kwapiszewska G, Park SH. Perspective: ambient air pollution: inflammatory response and effects on the lung's vasculature. *Pulm Circ* 2014;4:25–35.
19. Christmann RB, Hayes E, Pendergrass S, Padilla C, Farina G, Affandi AJ, Whitfield ML, Farber HW, Lafyatis R. Interferon and alternative activation of monocyte/macrophages in systemic sclerosis-associated pulmonary arterial hypertension. *Arthritis Rheum* 2011;63:1718–1728.
20. Hecker M, Zaslona Z, Kwapiszewska G, Niess G, Zakrzewicz A, Hergenreider E, Wilhelm J, et al. Dysregulation of the IL-13 receptor system: a novel pathomechanism in pulmonary arterial hypertension. *Am J Respir Crit Care Med* 2010; 182:805–818.
21. Radstake TR, van Bon L, Broen J, Hussiani A, Hesselstrand R, Wuttge DM, Deng Y, Simms R, Lubberts E, Lafyatis R. The pronounced Th17 profile in systemic sclerosis (SSc) together with intracellular expression of TGFβ and IFNγ distinguishes SSc phenotypes. *PLoS ONE* 2009;4:e5903.
22. Rose F, Hattar K, Gakis S, Grimminger F, Olschewski H, Seeger W, Tschuschner A, et al. Increased neutrophil mediator release in patients with pulmonary hypertension—suppression by inhaled iloprost. *Thromb Haemost* 2003;90: 1141–1149.
23. Humbert M, Monti G, Brenot F, Sitbon O, Portier A, Grangeot-Keros L, Duroux P, Galanaud P, Simonneau G, Emilie D. Increased interleukin-1 and interleukin-6 serum concentrations in severe primary pulmonary hypertension. *Am J Respir Crit Care Med* 1995;151:1628–1631.
24. Littman DR, Rudensky AY. Th17 and regulatory T cells in mediating and restraining inflammation. *Cell* 2010;140: 845–858.
25. Zhou L, Chong MM, Littman DR. Plasticity of CD4⁺ T cell lineage differentiation. *Immunity* 2009;30:646–655.
26. Leonardi C, Matheson R, Zachariae C, Cameron G, Li L, Edson-Heredia E, Braun D, Banerjee S. Anti-interleukin-17 monoclonal antibody ixekizumab in chronic plaque psoriasis. *N Engl J Med* 2012;366:1190–1199.

27. Patel DD, Lee DM, Kolbinger F, Antoni C. Effect of IL-17A blockade with secukinumab in autoimmune diseases. *Ann Rheum Dis* 2013;72(suppl. 2):ii116–ii123.
28. Lo Re S, Dumoutier L, Couillin I, Van Vyve C, Yakoub Y, Uwambayinema F, Marien B, et al. IL-17A-producing $\gamma\delta$ T and Th17 lymphocytes mediate lung inflammation but not fibrosis in experimental silicosis. *J Immunol* 2010;184:6367–6377.
29. Grunig G, Corry DB, Reibman J, Wills-Karp M. Interleukin 13 and the evolution of asthma therapy. *Am J Clin Exp Immunol* 2012;1:20–27.
30. Ingram JL, Kraft M. IL-13 in asthma and allergic disease: asthma phenotypes and targeted therapies. *J Allergy Clin Immunol* 2012;130:829–842.
31. Corren J, Lemanske RF, Hanania NA, Korenblat PE, Parsey MV, Arron JR, Harris JM, et al. Lebrikizumab treatment in adults with asthma. *N Engl J Med* 2011;365:1088–1098.
32. Wenzel S, Ford L, Pearlman D, Spector S, Sher L, Skobieranda F, Wang L, et al. Dupilumab in persistent asthma with elevated eosinophil levels. *N Engl J Med* 2013;368:2455–2466.
33. Munitz A, Cole ET, Karo-Atar D, Finkelman FD, Rothenberg ME. Resistin-like molecule- α regulates IL-13-induced chemokine production but not allergen-induced airway responses. *Am J Respir Cell Mol Biol* 2012;46:703–713.
34. Munitz A, Waddell A, Seidu L, Cole ET, Ahrens R, Hogan SP, Rothenberg ME. Resistin-like molecule α enhances myeloid cell activation and promotes colitis. *J Allergy Clin Immunol* 2008;122:1200.e1–1207.e1.
35. Gilmour MI, McGee J, Duvall RM, Dailey L, Daniels M, Boykin E, Cho SH, Doerfler D, Gordon T, Devlin RB. Comparative toxicity of size-fractionated airborne particulate matter obtained from different cities in the United States. *Inhal Toxicol* 2007;19(suppl. 1):7–16.
36. Zhao C, Liao J, Chu W, Wang S, Yang T, Tao Y, Wang G. Involvement of TLR2 and TLR4 and Th1/Th2 shift in inflammatory responses induced by fine ambient particulate matter in mice. *Inhal Toxicol* 2012;24:918–927.
37. Saunders V, Breyse P, Clark J, Sproles A, Davila M, Wills-Karp M. Particulate matter-induced airway hyperresponsiveness is lymphocyte dependent. *Environ Health Perspect* 2010;118:640–646.
38. Walters DM, Breyse PN, Schofield B, Wills-Karp M. Complement factor 3 mediates particulate matter-induced airway hyperresponsiveness. *Am J Respir Cell Mol Biol* 2002;27:413–418.
39. Walters DM, Breyse PN, Wills-Karp M. Ambient urban Baltimore particulate-induced airway hyperresponsiveness and inflammation in mice. *Am J Respir Crit Care Med* 2001;164:1438–1443.
40. Park SH, Chen WC, Hoffman C, Marsh LM, West J, Grunig G. Modification of hemodynamic and immune responses to exposure with a weak antigen by the expression of a hypomorphic BMPR2 gene. *PLoS ONE* 2013;8:e55180.
41. Hoffman C, Park SH, Daley E, Emson C, Louten J, Sisco M, de Waal Malefyt R, Grunig G. Interleukin-19: a constituent of the regulome that controls antigen presenting cells in the lungs and airway responses to microbial products. *PLoS ONE* 2011;6:e27629.
42. Padilla J, Daley E, Chow A, Robinson K, Parthasarathi K, McKenzie AN, Tschernig T, Kurup VP, Donaldson DD, Grunig G. IL-13 regulates the immune response to inhaled antigens. *J Immunol* 2005;174:8097–8105.
43. Taleb S, Romain M, Ramkhalawon B, Uyttenhove C, Pasterkamp G, Herbin O, Esposito B, et al. Loss of SOCS3 expression in T cells reveals a regulatory role for interleukin-17 in atherosclerosis. *J Exp Med* 2009;206:2067–2077.
44. Chen WC, Park SH, Hoffman C, Philip C, Robinson L, West J, Grunig G. Right ventricular systolic pressure measurements in combination with harvest of lung and immune tissue samples in mice. *J Vis Exp* 2013:e50023.
45. Schermuly RT, Dony E, Ghofrani HA, Pullamsetti S, Savai R, Roth M, Sydykov A, et al. Reversal of experimental pulmonary hypertension by PDGF inhibition. *J Clin Invest* 2005;115:2811–2821.
46. Crnkovic S, Hrzenjak A, Marsh LM, Olschewski A, Kwapiszewska G. Origin of neomuscularized vessels in mice exposed to chronic hypoxia. *Respir Physiol Neurobiol* 2011;179:342–345.
47. Padilla J, Daley E, Chow A, Robinson K, Parthasarathi K, McKenzie AN, Tschernig T, Kurup VP, Donaldson DD, Grunig G. IL-13 regulates the immune response to inhaled antigens. *J Immunol* 2005;174:8097–8105.
48. Angelini DJ, Su Q, Yamaji-Kegan K, Fan C, Skinner JT, Poloczek A, El-Haddad H, Cheadle C, Johns RA. Hypoxia-induced mitogenic factor (HIMF/FIZZ1/RELM α) in chronic hypoxia- and antigen-mediated pulmonary vascular remodeling. *Respir Res* 2013;14:1.
49. Mushaben EM, Hershey GK, Pauculo MW, Nichols WC, Le Cras TD. Chronic allergic inflammation causes vascular remodeling and pulmonary hypertension in BMPR2 hypomorphic and wild-type mice. *PLoS ONE* 2012;7:e32468.
50. Mukasa R, Balasubramani A, Lee YK, Whitley SK, Weaver BT, Shibata Y, Crawford GE, Hatton RD, Weaver CT. Epigenetic instability of cytokine and transcription factor gene loci underlies plasticity of the T helper 17 cell lineage. *Immunity* 2010;32:616–627.
51. Gopal R, Monin L, Torres D, Slight S, Mehra S, McKenna K, Fallert Junecko BA, et al. S100A8/A9 proteins mediate neutrophilic inflammation and lung pathology during tuberculosis. *Am J Respir Crit Care Med* 2013;188:1137–1146.
52. Murdock BJ, Falkowski NR, Shreiner AB, Sadighi Akha AA, McDonald RA, White ES, Toews GB, Huffnagle GB. Interleukin-17 drives pulmonary eosinophilia following repeated exposure to *Aspergillus fumigatus* conidia. *Infect Immun* 2012;80:1424–1436.
53. Eid RE, Rao DA, Zhou J, Lo SF, Ranjbaran H, Gallo A, Sokol SI, Pfau S, Poher JS, Tellides G. Interleukin-17 and interferon- γ are produced concomitantly by human coronary artery-infiltrating T cells and act synergistically on vascular smooth muscle cells. *Circulation* 2009;119:1424–1432.
54. Duhon R, Glatigny S, Arbelaez CA, Blair TC, Oukka M, Bettelli E. Cutting edge: the pathogenicity of IFN- γ -producing Th17 cells is independent of T-bet. *J Immunol* 2013;190:4478–4482.
55. Polikepahad S, Knight JM, Naghavi AO, Opllt T, Creighton CJ, Shaw C, Benham AL, et al. Proinflammatory role for *let-7* microRNAs in experimental asthma. *J Biol Chem* 2010;285:30139–30149.
56. Graham BB, Mentink-Kane MM, El-Haddad H, Purnell S, Zhang L, Zaiman A, Redente EF, et al. Schistosomiasis-

- induced experimental pulmonary hypertension: role of interleukin-13 signaling. *Am J Pathol* 2010;177:1549–1561.
57. Angelini DJ, Su Q, Yamaji-Kegan K, Fan C, Teng X, Hassoun PM, Yang SC, Champion HC, Tudor RM, Johns RA. Resistin-like molecule- β in scleroderma-associated pulmonary hypertension. *Am J Respir Cell Mol Biol* 2009;41:553–561.
 58. Angelini DJ, Su Q, Yamaji-Kegan K, Fan C, Skinner JT, Champion HC, Crow MT, Johns RA. Hypoxia-induced mitogenic factor (HIMF/FIZZ1/RELM α) induces the vascular and hemodynamic changes of pulmonary hypertension. *Am J Physiol Lung Cell Mol Physiol* 2009;296:L582–L593.
 59. Teng X, Li D, Champion HC, Johns RA. FIZZ1/RELM α , a novel hypoxia-induced mitogenic factor in lung with vasoconstrictive and angiogenic properties. *Circ Res* 2003;92:1065–1067.
 60. Renigunta A, Hild C, Rose F, Klepetko W, Grimminger F, Seeger W, Hanze J. Human RELM β is a mitogenic factor in lung cells and induced in hypoxia. *FEBS Lett* 2006;580:900–903.
 61. Yamaji-Kegan K, Su Q, Angelini DJ, Myers AC, Cheadle C, Johns RA. Hypoxia-induced mitogenic factor (HIMF/FIZZ1/RELM α) increases lung inflammation and activates pulmonary microvascular endothelial cells via an IL-4-dependent mechanism. *J Immunol* 2010;185:5539–5548.
 62. Graham BB, Chabon J, Kumar R, Kolosionek E, Gebreab L, Debella E, Edwards M, et al. Protective role of IL-6 in vascular remodeling in *Schistosoma* pulmonary hypertension. *Am J Respir Cell Mol Biol* 2013;49:951–959.
 63. Cho W, Chen N, Tang C, Elias JA, Lee C. IL-13-induces vascular remodeling and pulmonary arterial hypertension via arginase 2-dependent pathway. *Am J Respir Crit Care Med* 2010;181:A6323.
 64. Alegado RA, Brown LW, Cao S, Dermenjian RK, Zuzow R, Fairclough SR, Clardy J, King N. A bacterial sulfonolipid triggers multicellular development in the closest living relatives of animals. *eLife* 2012;1:e00013.
 65. Dayel MJ, Alegado RA, Fairclough SR, Levin TC, Nichols SA, McDonald K, King N. Cell differentiation and morphogenesis in the colony-forming choanoflagellate *Salpingoeca rosetta*. *Dev Biol* 2011;357:73–82.
 66. Davies RJ, Holmes AM, Deighton J, Long L, Yang X, Barker L, Walker C, Budd DC, Upton PD, Morrell NW. BMP type II receptor deficiency confers resistance to growth inhibition by TGF- β in pulmonary artery smooth muscle cells: role of proinflammatory cytokines. *Am J Physiol Lung Cell Mol Physiol* 2012;302:L604–L615.
 67. Ma L, Roman-Campos D, Austin ED, Eyries M, Sampson KS, Soubrier F, Germain M, et al. A novel channelopathy in pulmonary arterial hypertension. *N Engl J Med* 2013;369:351–361.
 68. Olschewski A, Li Y, Tang B, Hanze J, Eul B, Bohle RM, Wilhelm J, et al. Impact of TASK-1 in human pulmonary artery smooth muscle cells. *Circ Res* 2006;98:1072–1080.
 69. Taraseviciene-Stewart L, Kasahara Y, Alger L, Hirth P, McMahon G, Waltenberger J, Voelkel NF, Tudor RM. Inhibition of the VEGF receptor 2 combined with chronic hypoxia causes cell death-dependent pulmonary endothelial cell proliferation and severe pulmonary hypertension. *FASEB J* 2001;15:427–438.
 70. Atkinson C, Stewart S, Upton PD, Machado R, Thomson JR, Trembath RC, Morrell NW. Primary pulmonary hypertension is associated with reduced pulmonary vascular expression of type II bone morphogenetic protein receptor. *Circulation* 2002;105:1672–1678.
 71. Morty RE, Nejman B, Kwapiszewska G, Hecker M, Zakrzewicz A, Kouri FM, Peters DM, et al. Dysregulated bone morphogenetic protein signaling in monocrotaline-induced pulmonary arterial hypertension. *Arterioscler Thromb Vasc Biol* 2007;27:1072–1078.
 72. Spiekerkoetter E, Tian X, Cai J, Hopper RK, Sudheendra D, Li CG, El-Bizri N, et al. FK506 activates BMPR2, rescues endothelial dysfunction, and reverses pulmonary hypertension. *J Clin Invest* 2013;123:3600–3613.
 73. Takahashi H, Goto N, Kojima Y, Tsuda Y, Morio Y, Muramatsu M, Fukuchi Y. Downregulation of type II bone morphogenetic protein receptor in hypoxic pulmonary hypertension. *Am J Physiol Lung Cell Mol Physiol* 2006;290:L450–L458.
 74. Fujita M, Mason RJ, Cool C, Shannon JM, Hara N, Fagan KA. Pulmonary hypertension in TNF- α -overexpressing mice is associated with decreased VEGF gene expression. *J Appl Physiol* 2002;93:2162–2170.
 75. Chung WK, Deng L, Carroll JS, Mallory N, Diamond B, Rosenzweig EB, Barst RJ, Morse JH. Polymorphism in the angiotensin II type 1 receptor (AGTR1) is associated with age at diagnosis in pulmonary arterial hypertension. *J Heart Lung Transplant* 2009;28:373–379.
 76. McLaughlin VV, Sitbon O, Badesch DB, Barst RJ, Black C, Galiè N, Rainisio M, Simonneau G, Rubin LJ. Survival with first-line bosentan in patients with primary pulmonary hypertension. *Eur Respir J* 2005;25:244–249.