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Article

RELM α and Pulmonary Vascular Remodeling: A Multi-Strain Murine Model of Antigen and Urban Ambient PM Co-Exposure

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Abstract: Pulmonary hypertension (PH) has a high mortality, and few treatment options. Adaptive immune mediators of PH in mice challenged with antigen/particulate matter (antigen/PM) has been the focus of our prior work. We identified key roles of type 2 and type 17 responses in C57BL/6 mice. Here, we focused on type 2 response related cytokines, specifically Resistin-like-molecule (RELM) α , a critical mediator of hypoxia-induced PH. Because of strain differences in the immune responses to type 2 stimuli, we compared C57BL/6J and BALB/c mice. A model of intraperitoneal antigen sensitization with subsequent, intranasal challenges with antigen/PM (ovalbumin and urban ambient PM_{2.5}) or saline was used in C57BL/6 and BALB/c wild type or RELM α -/- mice. Vascular remodeling was assessed by histology, right ventricular (RV) pressure, RV weights and cytokines were quantified. Upon challenge with antigen/PM_{2.5}, both C57BL/6 and BALB/c mice developed pulmonary vascular remodeling; these changes were more prominent in the C57BL/6 strain. Compared to wild type mice, RELM α -/- had significantly reduced pulmonary vascular remodeling in BALB/c, but not in C57BL/6 mice. RV weights, RV IL-33 and RV ST2-IL-33-receptor were significantly increased in BALB/c wild type mice, but not in BALB/c-RELM α -/- or in C57BL/6-wild type or C57BL/6-RELM α -/- mice. RV systolic pressures (RVSP) were higher in BALB/c compared to C57BL/6J mice, and RELM α -/- were not different from their respective wild type controls. In BALB/c mice, RELM α was a key contributor to antigen/PM_{2.5} induced pulmonary vascular remodeling, RV thickening and RV cytokine responses, highlighting the significance of the genetic background. The RELM α -/- animals demonstrated significantly decreased expression of RELM β and RELM γ which makes these mice comparable to a situation where human RELM α levels would be significantly modified, as humans only have one RELM molecule.

Keywords: resistin like molecule; pulmonary hypertension; type 2 inflammation; adaptive immune response; retnla; retnlb; retnlg; mouse strains; experimental pulmonary hypertension; urban PM; urban fine dust; immune response in pulmonary hypertension

1. Introduction

Pulmonary hypertension (PH) not only has a high mortality but also has limited treatment options [1–9]. Our prior work has shown that pulmonary arterial remodeling and subsequent increased right ventricular (RV) systolic pressure (RVSP) could be induced by Th2 response to soluble antigen, and could be exacerbated by urban particulate matter (PM_{2.5}). [10,11] Specifically, CD4+ T cells, B cells, antigen specific antibody, Interleukin (IL)-13 and IL-17A had critical roles in mediating the severe pulmonary arterial remodeling and PH in our C57BL/6 murine model. [10,12,13]

Resistin like molecule α (RELM α) is an intriguing cytokine at the intersection of response to hypoxia [14–18] and adaptive immune response and belongs to an ancient mediator family, with Resistin being the oldest member. [19,20] In the context of our prior findings, our aim was to identify the mediator down-stream of the adaptive immune response that would induce the PH phenotype.

We focused on RELM α because of the intriguing role of this cytokine at the intersection of the immune and the hypoxia response. RELM α was termed 'found in inflammatory zone 1' (FIZZ1) because it was originally discovered in inflammatory zones associated with an experimental allergic airway disease model [15,21]. Subsequently, RELM α was identified as a biomarker in Th2 asthma models [22], since it is highly upregulated in epithelial cells and alternatively activated macrophages of the M2 type [23,24]. RELM α was also termed hypoxia induced mitogenic factor (HIMF) because it is highly upregulated in lung tissues exposed to hypoxia, specifically in vascular smooth muscle cells and endothelial cells. [15,17,25,26] HIMF induces cell proliferation and chemotaxis in smooth muscle cells [14,18,27–29]. RELM α belongs to the resistin family of cytokines which in mice consists of four members: resistin, RELM α , β , and γ [21,30–32].

Mechanistically, RELM α has a critical role in hypoxia induced PH phenotype [17]. With respect to inflammation induced by an adaptive immune response, RELM α has been reported to have either pro-inflammatory [23,33], anti-inflammatory [24,34], or neutral [35] roles depending on the experimental model used.

We used mice deficient in RELM $\alpha^{-/-}$ and measured right ventricular systolic pressure, right heart weight as measure of hypertrophy, pulmonary arterial remodeling and several mediators of immune and vascular responses in the lungs and right ventricle. We compared C57BL/6 and BALB/c mice because BALB/c mice have relatively larger constrictive responses in the airways compared with C57BL/6 mice [36,37]. Further, the lung's immune response in C57BL/6 mice is more of pleiotropic T helper type, while BALB/c mice have a more polarized, type 2 dominant response to antigen exposure [38,39]. Taken together, this design allowed us to study the function of RELM α for the PH phenotype induced by an adaptive immune response in the lungs.

2. Materials and Methods

Ethics Statement. All animal experiments were performed according to guidelines outlined by the United States 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 at New York University, Grossman School of Medicine (IACUC #140812-01).

Mice. RELM $\alpha^{-/-}$ on a C57BL/6 background (Regeneron Pharmaceuticals, Inc., Tarrytown, NY) were backcrossed to BALB/c (Dr. Marc E. Rothenberg's laboratory, Cincinnati Children's Hospital Medical Center) and a breeding pair was kindly provided [23,33]. RELM $\alpha^{-/-}$ mice (KO) were then backcrossed to C57BL/6J for 6 generations. C57BL/6J and BALB/c wild type (WT) mice were purchased (Jackson Laboratory) and adjusted to the environment in our colony for 10 days. Either these mice, or littermates of the backcross strain were used for comparison with RELM $\alpha^{-/-}$. Male and female mice 5-7 weeks of age at the start of the experiment were randomized into cages holding up to 4 mice. All mice were housed under pathogen free conditions.

RELM $\alpha^{-/-}$ Genotyping. PCR-based method using ear tissues was used for genotyping. Briefly, for pretreatment of ear tissues, 180 μ l of 50 mM NaOH was added into a tube containing ear tissues and incubated at 95°C for 13 min, and stayed at 22°C until use. 20 μ l of 1M Tris-HCl (pH 8.0) was added, mixed with vortexing. 5 μ l of pretreated sample was used for total 25 μ l of PCR reaction. TERRA™ PCR Direct Polymerase mix kit (Clontech, Mountain View, CA) was used with primers 5-GTCAGCAATCCCATGGCGTA-3 (forward) and 5-GTCTGTCCTAGCTTCCTCACTG-3 (reverse); 400 bp for KO allele or primers 5-GTCAGCAATCCCATGGCGTA-3 (forward) and 5-ACTTCCCTACCC ACCCATTC-3 (reverse); 800 bp for WT allele. A gradient PCR method was used with the following conditions: 98°C for 2 min, followed by 2 cycles of 94°C for 10 s, 64°C for 30 s, 72°C for 50 s, followed every 2 cycles with 1°C reduced annealing temperature until annealing temperature go to 58°C, followed 27 cycles of 94 °C for 35 s, 58°C for 30 s and 72 °C for 50 s, followed by 1 cycle of 72°C for 10 min, and followed by a hold at 4°C. The product was run on a gel to distinguish WT, heterozygous, KO mice.

Urban PM (PM_{2.5}) (<2.5 mm in aerodynamic diameter) was collected from New York ambient air and resuspended as previously described [40,41]. PM_{2.5} was diluted in phosphate buffered

saline(PBS), ultrasonicated before use and mixed with OVA solution so that the final concentration was 25 µg PM_{2.5} / 50 µl intranasal dose.

Antigen priming and antigen & PM_{2.5} challenge (OVA&PM). Animals were primed and challenged with antigen as previously published [3,11–13,42]. Briefly, mice were injected intraperitoneally with Ovalbumin (OVA) (grade V; Sigma-Aldrich, St. Louis, MO; 50 µg/dose) adsorbed to Alum (Imject Alum; Thermo Fisher Scientific, Rockford, IL; 2 mg/dose) at a two-week interval. Two weeks later, the mice were intranasally challenged with either PBS, or combined OVA (100 µg/dose) & PM_{2.5} (25 µg/dose) for two times each week, for a total of 6 doses given over a three-week period. The mice were analyzed one day following the last intranasal exposure, **Figure 1**. This OVA preparation contains lipopolysaccharide (LPS) that contributes to the responses in the airways and lungs to intranasal OVA challenge in this mouse model [43].

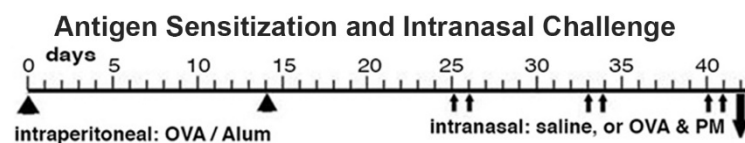


Figure 1. Schematic representation of the sensitization and challenge protocol.

Group comparisons. In a pilot study, we compared sensitized C57BL/6 or BALB/c wild type or KO mice intranasally exposed to either saline, PM_{2.5}, OVA, or OVA&PM_{2.5}. The data of the pilot study showed that as expected [11] all groups of saline or PM_{2.5} exposed mice showed no significant difference in any of our measurements. Therefore, we pooled these two groups. Further, the pilot study showed that the comparison of the responses of WT and KO mice to OVA alone were the same as when WT and KO mice were exposed to OVA& PM_{2.5}. For these reasons, the data from these two exposure groups were pooled to increase statistical power for the WT vs KO comparisons.

RV Systolic Pressure was measured by inserting a catheter via the jugular vein in anaesthetized, spontaneously breathing mice [11,42,44]. Mice were analyzed without prior knowledge of group identity, and we alternated mice from different cages to eliminate 'cage effects'. Briefly, mice were anaesthetized by intraperitoneal injection of Avertin (Sigma-Aldrich) made by mixing 5 ml of 2-Methyl-2-butanol and 5 g of 2,2,2-Tribromoethanol; 0.25 ml of the stock solution was diluted with 10 ml of saline solution; the mice were injected according to their weight, 10 µl / g mouse. Once surgical plane of anesthesia was reached, the right jugular vein was isolated. The pressure catheter (F1.4, Millar Instruments, Inc. Houston, TX) was inserted and advanced into the RV to measure the pressure. Then the mice were euthanized with an overdose of barbiturate and lung and heart tissues were harvested. The RV pressure data were analyzed using the LabChart 7 program (ADInstruments, Colorado Springs, CO).

RV Hypertrophy, Fulton's Index [right/(left + septum) ventricular weight]. The RV, and left ventricle plus septum were removed and weighed. The data were used to calculate the right ventricular weight relative to the weight of the left ventricle and septum.

Pulmonary vascular remodeling. The approach was as previously described [3,45–48] and the analysis was performed without knowledge of the group identity of the sample. Briefly, the lungs were placed into formaldehyde for fixation, paraffin embedded and sections stained with H&E (hematoxylin and eosin) or with Periodic Acid Schiff (PAS). The slides were scanned using the Leica Biosystems SlidePath Gateway and the Leica SCN400 (Leica Microsystems Inc., Buffalo Grove, IL) whole slide scanning system at the Histopathology Core of NYU Medical Center. The digital dynamic slide images were analyzed at 200x magnification.

Examples for the pulmonary vascular scoring are shown in **Figure 2**: 1- normal, 2 – thickened, but regular, 3- thickened and irregular layers of smooth muscle cells, 4- further thickening and more irregular arrangement of smooth muscle cells. At least 20 consecutive view fields randomly selected in all available lung lobes were evaluated at 200x magnification. All arteries with a diameter of

<100µm were scored. For each lung, the remodeling score was calculated as the mean of all scores collected per lung.

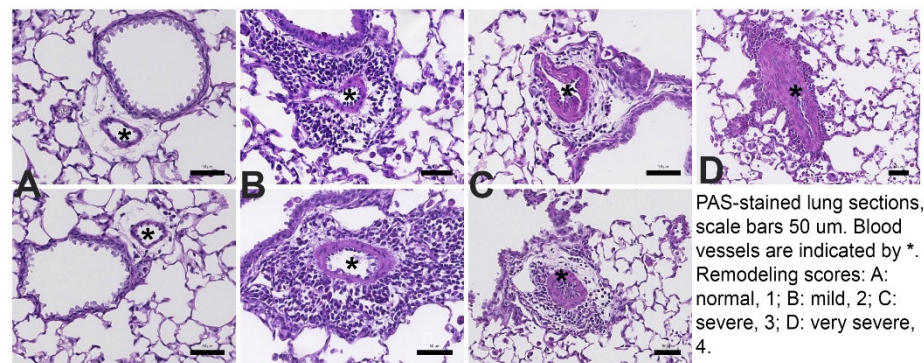


Figure 2. Photomicrographs show examples of small <100µm pulmonary arteries (*).

Severe remodeling was also determined as percentage of all scored blood vessels per lung, characterized by having severely thickened walls and disorganized layers of smooth muscle cells (smooth muscle cells in the blood vessel wall that assumed a pattern that differs from the lumen) [10]. For each lung, severe arterial thickening was calculated by the following formula: $100 \div \text{number of all scored blood vessels} \times \text{number of severely remodeled vessels}$.

mRNA expression: Total RNA from lung tissue was isolated (RNeasy Mini Kit; QIAGEN Inc, Valencia CA) and reverse transcribed using the High-Capacity cDNA Reverse Transcription kit (Applied Biosystems, Foster City, CA). Real time PCR was performed in duplicate with 20 ng of cDNA using the 7900HT Fast Real-Time PCR system (Applied Biosystems). The qPCR for the detection of the gene expression was performed with SYBR Green (Invitrogen, Grand Island, NY) and primers purchased from Origene (Rockville, MD). RELM α (Retnl α), RELM β (Retnl β), RELM γ (Retnl γ), were assayed using 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 the primers or probes, respectively, are listed in Table 1. The following conditions were used: 95 °C for 10 min, followed by 40 cycles of 95 °C for 15 s and 60 °C for 1 min, followed by a hold at 4 °C. Raw data were analyzed with SDS Relative Quantification Software version 2.3 (Applied Biosystems) to determine cycle threshold (Δ Ct). For each sample, Δ Ct values were standardized to the housekeeping gene β -actin by calculating $1.98^{\Delta\text{Ct}} \times 10,000$. Data were expressed as β -actin standardized Δ Ct values, or as percent-fold-difference from the mean of the wild type OVA&PM_{2.5} group (set at 100U).

Table 1. List of primers and probes.

ANP-F	nppa	TACAGTGC GGTGTCCAACACAG
ANP-R	nppa	TGCTTCCTCAGTCTGCTCACTC
IL-33-F	il33	ACTGCATGAGACTCCGTTCTG
IL-33-R	il33	CCTAGAATCCCGTGGATAGGC
ST2 rtF	il1rl1	GGATTGAGGTTGCTCTGTTCTGG
ST2 rtR	il1rl1	TCGGGCAGAGTGTGGTGAACAA
β -actin-F	actb	GGCTGTATTCCCCTCCATCG
β -actin-R	actb	CCAGTTGGTAACAATGCCATGT
RELM α (TaqMan)	retnl α	CTTGCCAATCCAGCTAACTATCCCT
RELM β (TaqMan)	retnl β	GGAAGCTCTCAGTCGTCAGAGCCT
RELM γ (TaqMan)	retnl γ	AAACCTGGCTCATATCCCATTGATG
Actin, β (TaqMan)	actb	ACTGAGCTGCGTTTACACCCTTTC

Statistical analysis: Statistical analysis and graphs were generated with Prism version 9.2 (Graphpad). Data from multiple groups were analyzed for significant differences using the Kruskal

Wallis test (tie corrected). Pair-wise comparisons were conducted with the unpaired, two-tailed Mann-Whitney U test. $P < 0.05$ was considered to be statistically significant.

3. Results

Expression of RELM β and RELM γ in RELM $\alpha^{-/-}$ mice. All three members of the resistin like molecule family are located on mouse chromosome 16 in the gene-order Retnlb, Retnla, Retnlg. To test the idea that the gene-deletion manipulation in the RELM $\alpha^{-/-}$ mice could have changed the expression of RELM β and RELM γ , we determined the expression of all three resistin like molecules in the lung and right heart tissues of wild type and KO mice. **Figure 3A** shows that antigen and PM_{2.5} exposure significantly increased the expression of RELM α , β and γ in the lungs of WT mice, and that this increase occurred to a larger fold-degree in BALB/C strain mice. **Figure 3A** also shows that RELM $\alpha^{-/-}$ mice of both strains had no RELM α expression and significantly decreased expression of RELM β and RELM γ in the lungs compared to wild type mice.

Expression of RELM α and RELM γ , was readily detected in the right ventricle (RV), **Figure 3B**. RELM β was not detectable in the right ventricle (data not shown). Exposure with antigen/PM_{2.5} significantly increased the expression of RELM α in the RV of BALB/c WT mice and RELM γ in the RV of WT mice from both strains. As in the lungs, the right ventricles of RELM $\alpha^{-/-}$ mice of both strains had no RELM α expression and significantly decreased expression of RELM γ when compared to WT mice, **Figure 3**.

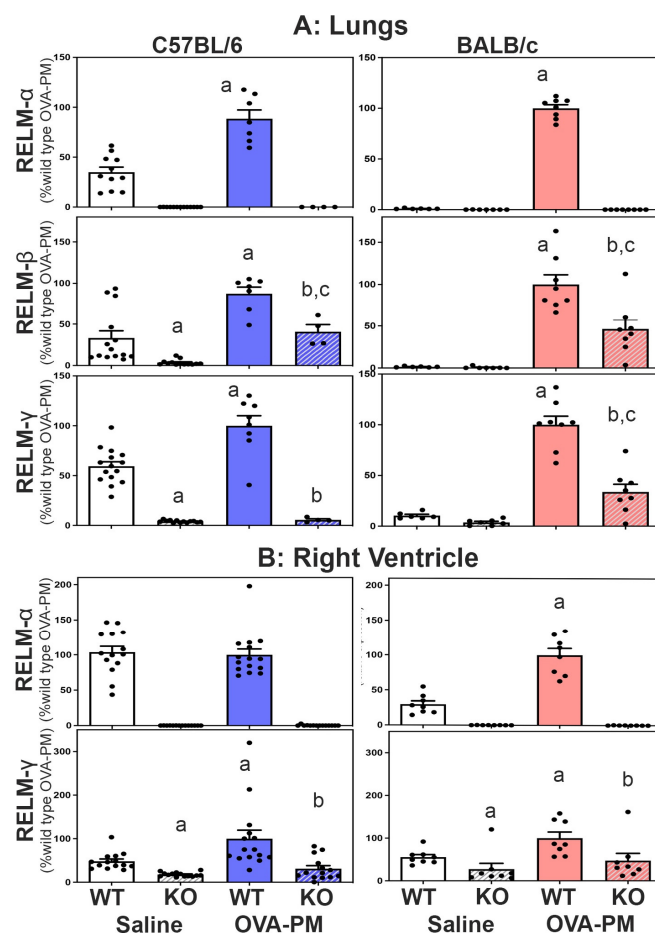


Figure 3. Expression of RELM α , β , γ in the lungs (A) or the right ventricle (B). RELM β was not detected in the right ventricle (data not shown). Bars show means, SEM and individual data points of relative CT values measured to b-actin and calculated relative to the mean of the OVA-PM wild type groups at 100U. Groups were compared by independent, 2-tailed Mann Whitney test, $p < 0.05$ for comparison with a) WT-PBS, b) WT-OVA-PM, c) KO-PBS.

In BALB/c KO mice, decreased RELM expression attenuated pulmonary vascular remodeling induced by exposure to antigen and PM_{2.5}. Groups of WT and KO mice were given antigen and ambient PM_{2.5} (OVA&PM). As expected, [10–13,42] WT C57BL/6 and BALB/c mice developed significantly pulmonary vascular remodeling compared to saline, Figure 4A. Figure 2 shows the vascular pathology corresponding to the scores. RELMα KO mice of the BALB/c strain had significantly ameliorated vascular remodeling compared to wild type, Figure 4A. In contrast, RELMα^{-/-} mice of the C56BL/6 strain developed pulmonary vascular remodeling to the same extent as WT, Figure 4A.

BALB/c RELMα KO mice did not develop severe pulmonary arterial remodeling upon exposure to antigenPM_{2.5}. Following exposure to antigen/PM_{2.5}, the frequency of severely remodeled arteries was higher in C57BL/6 mice when compared to BALB/c mice, **Table 2**. In keeping with the data shown in **Figure 4A**, the percentage of severely remodeled pulmonary arteries was significantly lower in antigen/PM_{2.5} exposed RELMα^{-/-} BALB/c mice when compared to WT, **Table 2**. In contrast, WT and RELMα^{-/-} C57BL/6 mice had a similar frequency of severely remodeled arteries in the lungs, **Table 2**.

Table 2. Percentage of severely remodeled pulmonary arteries following exposure to OVA-PM¹.

Severely Remodeled Pulmonary Artery (OVA-PM exposed)	C57BL/6				BALB/c			
	Median	Quartiles	Group n	P-value	Median	Quartiles	Group n	P-value
Wild Type	12.03	1.315, 50.460	8	0.1919	7.275	0.00, 10.390	10	0.0126
RELMα ^{-/-}	39.13	19.790, 41.800	9		0.000	0.00, 3.846	10	

¹ Data represent the percent of severely remodeled arteries (score of 3 and more) in each lung calculated based on the analysis of at least 20 view fields visualized at 200x magnification.

RELMα is not necessary for increases in RV systolic pressure induced by exposure to antigen and PM_{2.5}: To understand the role of RELMα for the increase in RV systolic pressures (RVSP), groups of WT and KO mice were given antigen and ambient PM_{2.5} (OVA-PM). As expected, [10–13,42] OVA-PM exposed WT C57BL/6 and BALB/c mice developed significantly increased RVSP compared to control, **Figure 4A,B**. Unexpectedly, RELMα^{-/-} mice of both strains showed increases in RV systolic pressure upon challenge with OVA&PM similar to the WT controls, **Figure 4B**.

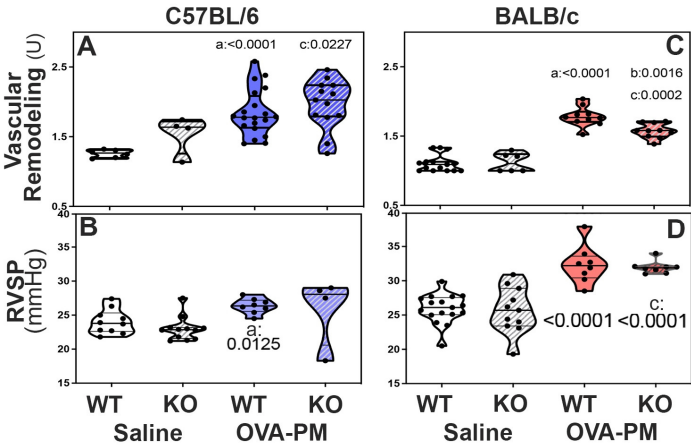


Figure 4. Pulmonary Vascular Responses to exposure with OVA-PM in C57BL/6 strain (A,B) or BALB/c (C,D) strain mice measured by pulmonary arterial remodeling scores (A,C) and right

ventricular systolic pressure (RVSP, B,D) . Violin plots show individual data points and data distribution. Pairwise comparisons were with independent, 2-tailed Mann Whitney test. $P < 0.05$ for comparison with a) WT-PBS, b) WT-OVA-PM, c) KO-PBS.

Right heart weights and expression of ANP, IL-33 and IL-33-receptor (ST2) in the ventricles of the right heart of WT and RELM $\alpha^{-/-}$ mice: In PBS treated mice right heart weights were similar in wild type and RELM α KO mice in both C57BL/6 and BALB/c strains, Figure 5A-B. However, the right heart weight was higher in BALB/c strain control mice when compared to C57BL/6 control mice. Upon exposure to antigen and PM_{2.5}, only BALB/c WT mice developed significantly increased right heart weights Figure 5B. Antigen and PM_{2.5} exposed C57BL/6 strain wild type mice showed a trend to increased right heart weights, Figure 4A. Importantly, RELM $\alpha^{-/-}$ BALB/c mice did not develop significant increases in right heart weight following exposure to antigen and PM_{2.5}, Figure 5B, while RELM $\alpha^{-/-}$ C57BL/6 mice were not different from wild type, Figure 5A.

The mRNA expression of ANP (natriuretic peptide type A) was measured as an indicator of RV stress, [49] Figure 5C,D. Only BALB/c WT mice, but not RELM $\alpha^{-/-}$ BALB/c mice developed increased ANP expression in the RV compared to control groups, Figure 5D. The mRNA expression of IL-33 and the IL-33-receptor ST2 was measured in right heart tissue because IL-33 and ST2 are thought to have important roles in regulating right ventricular homeostasis [50]. Antigen and PM_{2.5} exposure did not change right ventricular IL-33 or ST2 expression in C57BL/6 mice, Figure 5E,G. In contrast, BALB/c WT but not BALB/c RELM $\alpha^{-/-}$ mice had significantly increased right ventricular IL-33 expression following antigen/PM_{2.5} exposure, Figure 5F. The IL-33 receptor, ST2, was expressed at lower levels in the right ventricles of RELM $\alpha^{-/-}$ BALB/c mice, which reached statistical significance in antigen and PM_{2.5} challenged RELM $\alpha^{-/-}$ BALB/c mice relative to WT, Figure 5H.

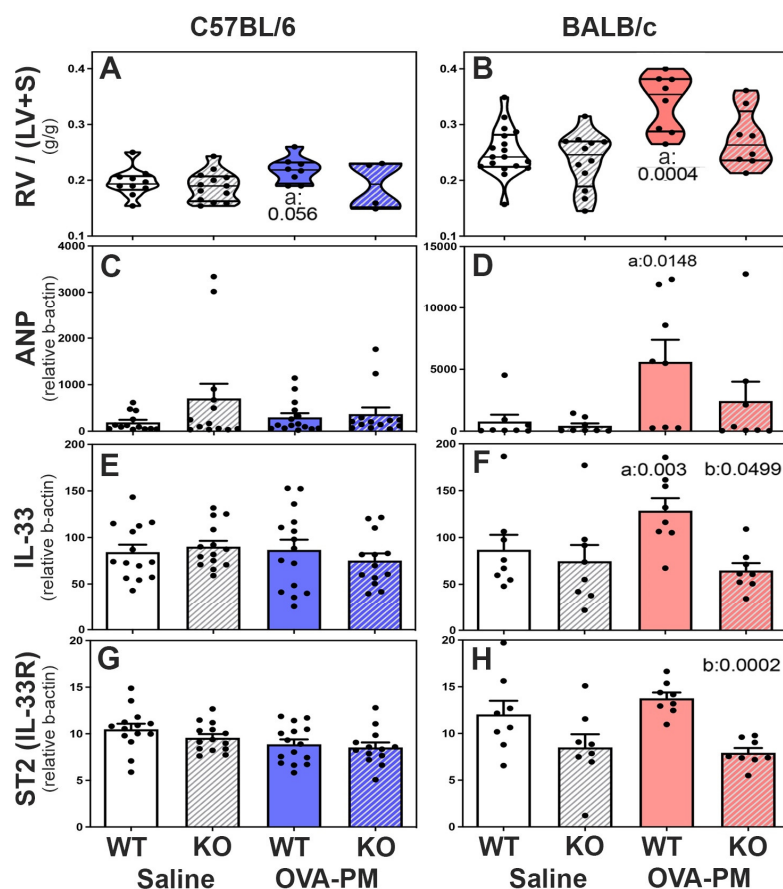


Figure 5. Right Ventricular Responses in C57BL/6 strain (A,C,E,G) and BALB/c (B,D,F,H) strain mice measured by right ventricular weight (RV/LV+S) (A,B) and expression of mediators that reflect RV stress (ANP-natriuretic peptide type A, IL-33, ST2-IL1RL1-IL-33 receptor). Violin plots (A,B) show individual data points and data distribution. Bars (C-H) show means, SEM, and individual data

points. C-H: CT values were calculated relative to b-actin (x1,000). Pairwise comparisons were with the independent, 2-tailed Mann Whitney test. $P < 0.05$ for comparison with a) WT-PBS, b) WT-OVA-PM, c) KO-PBS.

4. Discussion

Our data showed that endogenous RELM α was not necessary for the development of increased right ventricular systolic pressures in mice exposed to antigen and PM_{2.5}. This conclusion is based on our studies using mice of two different strain backgrounds, C57BL/6 and BALB/c. Based on previous reports showing that RELM α , also called HIMF, was both necessary and sufficient to cause the hypoxia induced increase in right ventricular systolic pressure [15–17,25], our data were unexpected. However, the discrepancy can be explained by the very different experimental systems used.

In our studies comparing WT and RELM $\alpha^{-/-}$ mice, we found distinct differences between C57BL/6 and BALB/c strains. In the C57BL/6 strain, RELM α was redundant. In the BALB/c strain relative to WT, RELM $\alpha^{-/-}$ mice challenged with antigen and PM_{2.5} did not develop increased right ventricle weight, had ameliorated pulmonary vascular remodeling, and no increase in right ventricle ANP expression together with decreased IL-33 and ST2 expression in the right ventricle.

Cardiac myocytes, fibroblasts, and endothelial cells express IL-33 constitutively [50]. IL-33 expression is further increased by inflammatory stimuli in cardiac myocytes and fibroblasts. Cardiac endothelial cells express the IL-33-receptor, ST2, while cardiac myocytes and fibroblasts have relatively low level of surface ST2-expression. [50] The significantly lower expression of these mediators of inter-cellular communication in the right ventricle of RELM $\alpha^{-/-}$ BALB/c mice may have prevented an increase in right ventricular weight in response to antigen and PM_{2.5} exposure despite the right ventricular systolic pressures that were increased to wild type levels.

The RELM $\alpha^{-/-}$ mice that we studied also had significantly decreased RELMb and RELMg expression in the lungs and right ventricle. Therefore, our study indicates a critical role of these mediators in BALB/c mice for the responses to antigen and PM_{2.5}, while they were redundant in C57BL/6 mice. The strain difference could be due to differences in the responsiveness of the pulmonary vasculature to proliferative and constrictive cues. In this respect, it is remarkable that C57BL/6 mice have polymorphisms (SNPs) in the 3-prime region of the BMPR2 (bone morphogenetic protein receptor, type II) gene that are not found in BALB/c mice (<https://www.informatics.jax.org/snp/marker/MGI:1095407>). Decreased function of the BMPR2 gene is associated with a high risk to develop pulmonary hypertension [51]. Another possibility is that potential receptors for the RELM molecules, among them toll like receptor (TLR4) or RAGE (advanced glycosylation end-product) [52,53] are polymorphic and differ between C57BL/6 and BALB/c mice. There is strong evidence that resistin binds TLR4 [54,55], however, little is known about the binding of RELM molecules to TLR4. However, TLR4 is polymorphic between BALB/c and C57BL/6 mice [56], and this is particularly important because the antigen (OVA) that we used contains lipopolysaccharide which induces inflammation via TLR4. The RAGE gene is located in the MHC locus, H2K, and this locus is highly polymorphic between BALB/c (H2Kd) and C57BL/6 (H2Kb) mice.

There are some limitations of our study: 1) First of all, humans only have one resistin like molecule, RELM β , and human RELM β is induced in hypoxia and is mitogenic for vascular smooth muscle cells [57]. It is thought that mouse RELM α is the homologue of human RELM β but mouse RELM α may not have the same function as human RELM β . 2) Secondly, the RELM $\alpha^{-/-}$ mice of both strain backgrounds that were studied by us have a compound KO phenotype with a deletion of RELM α , and significantly depressed RELM β and RELM γ . In mice, the three resistin like molecules developed by gene-duplication on chromosome 16 and the compound deficiency is likely due to the deletion of a master regulator of all three resistin like molecules in the KO animals. The compound KO phenotype of our mice may partially explain the different data with respect to the role of RELM α for the pulmonary hypertension phenotype that has been reported in the hypoxia system [16]. Those studies were conducted with RELM α specific inhibitors in wild type animals. 3) Lastly, our studies used a very low dose of PM_{2.5} that does not induce a response intranasally which is different from

mice given saline intranasally [11]. Therefore, the RELM α -dependent responses identified in our studies (e.g. BALB/c strain mice pulmonary arterial remodeling, increased right ventricular weights and mediator expression) were driven by the adaptive immune response to OVA and perhaps the associated lipopolysaccharide. Future dose-response studies are necessary to understand the role of RELM α for cardiovascular and lung responses to PM_{2.5} exposure.

5. Conclusions

In conclusion, our study demonstrates that the role of RELM α for the pulmonary hypertension phenotype and molecular markers of right ventricular stress is dependent on the mouse background strain that in turn determines clearly distinct adaptive immune response types in the lungs.

Author Contributions: Conceptualization, ND, AN, GG; Data curation, GG; Formal analysis, ND, WCC, SHP, GG; Funding acquisition, GG; Investigation, WCC, SHP, GG; Methodology, WCC, SHP, LM, AN, GG; Project administration, WCC, AN, GG; Resources, ND, SHP, AN, GG; Supervision, GG; Validation, WCC, SK, GG; Visualization, ND, SK, GG; Writing – original draft, ND, SHP, AN, GG; Writing – review & editing, ND, WCC, LM, SK, AN, GG. All authors have read and agreed to the published version of the manuscript.

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Conflicts of Interest: The authors declare no conflict of interest. The funders had no role in the design of the study; in the collection, analyses, or interpretation of data; in the writing of the manuscript; or in the decision to publish the results.

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