UNIVERSITY OF CALIFORNIA SAN DIEGO

IL-33/ST2 Dependent Endothelial Cell Hyperproliferation and Vascular Remodeling in Sugen/Hypoxia Mice

A thesis submitted in partial satisfaction of the requirements

for the degree Master of Science

in

Biology

by

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2020

The thesis of Cynthia Sharmini Indralingam is approved, and it is acceptable in quality and form for publication on microfilm and electronically:

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Co-chair

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Chair

University of California San Diego

2020

Dedication

I dedicate this thesis to my parents for all the sacrifices they have made to give me invaluable education opportunities. To my siblings, thank you for your love and support.

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LIST OF ABBREVIATIONS

PH = Pulmonary Hypertension

PAH = Pulmonary Arterial Hypertension

IL-33 = Interleukin-33

C57Bl/6J = C57 Black 6J

ST2 = Suppression of tumorigenicity 2 gene

ST2-/- = Suppressor of tumorigenicity 2 gene ablation

MYD88 = Myeloid differentiation primary response 88 gene

MYD88-/- = Myeloid differentiation primary response 88 gene ablation

WT = Wildtype, C57Bl/6J

SU5416 = Sugen 5416, vascular endothelial growth factor receptor antagonist

SUHX = Sugen5416/Hypoxia

DMSO/RA = Dimethyl sulfoxide/room air

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Figure 2 is co-authored with Scott Johns. The thesis author was co-author of this Figure.

ABSTRACT OF THE THESIS

IL-33/ST2 Dependent Endothelial Cell Hyperproliferation and Vascular Remodeling in Sugen/Hypoxia Mice

by

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Master of Science in Biology

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Pulmonary arterial hypertension (PAH) is a devastating disease that is often misdiagnosed. PAH is characterized by the remodeling of the small resistance arteries in the lungs that leads to elevated right ventricular pressure (RVP) and cardiac failure. Endothelial cells can sense danger or damage to the arterial wall caused by mechanical injury or respiratory pathogens through a subset of cytokines with an alarmin function to signal proliferation and restore barrier integrity. In

this study we hypothesize that under chronic pulmonary hypertension conditions Interleukin-33 (IL-33) signals pulmonary artery endothelial cells to initiate a hyperproliferative response and this cellular event contributes to the remodeling of the vascular wall. To test this hypothesis pulmonary hypertension was induced in C57Bl/6J (wildtype), IL-33 receptor gene deleted (ST2-/-) and the upstream adaptor protein, MYD88 gene deleted (MYD88-/-) mice by exposure to 10% O_2 and weekly SU5416 injections (SUHX). Arterial wall thickness increased in wild-type mice, but not ST2-/- and MYD88-/- mice, under SUHX treatments. The percentage of proliferating endothelial cells increased in wild-type mice under SUHX conditions but, this endothelial proliferative response was absent in ST2-/- and MYD88-/- mice. These responses suggest IL-33 signaling through the ST2 receptor is important for endothelial cell proliferation and vascular remodeling in the SUHX model of pulmonary hypertension.

INTRODUCTION

Pulmonary hypertension (PH) is a disease defined by the development of high blood pressure in the pulmonary arteries which carries blood away from the heart to the lungs (1). PH is classified into five categories by the World Health Organization to facilitate diagnosis and treatment (1). Pulmonary arterial hypertension (PAH) falls into the Group 1 category, which defines the pulmonary hypertension as a result of increased pulmonary vascular resistance (PVR) (1). This is a disease initiated by pulmonary arterial wall thickening that leads to high blood pressures in the lungs and right heart (2). The high blood pressure in the pulmonary vasculature means the right ventricle has to exert a lot of effort to move the blood past the pulmonary valve usually this eventually leads to right heart failure over a fairly rapid time course (2). The mean survival of diagnosed PAH patients is 5 years with current treatment strategies (3). PAH can develop in both males and females of any age but is more prevalent among females (2). Some common treatment options to improve symptoms and slow the progression of PAH for Group 1 patients include pulmonary artery vasodilators, such as phosphodiesterase-5 (PDE-5) inhibitors, endothelin receptor antagonists (ERAs), and prostacyclin analogs, which open narrowed blood vessels to allow blood to flow more easily and maintain lower blood pressures (4). However, as of 2020, PAH is still a potentially fatal disease that effects patients ranging from 30-60 years of age with no effective cure (5). In recent years, researchers have focused on understanding the inflammation and impaired immunity that may underlie PAH in an effort to develop new therapeutics (6).

Inflammation is a defense mechanism employed by our immune system in response to infection and injury such as pathogens or damaged cells (7). Acute inflammation leads to vascular remodeling, a predisposing factor to the development of PAH (8). Cytokines and chemokines belong to a protein superfamily that is an essential part of the signaling network between cells that

produce and control the immune system (9). Alarmins are a group of cytokines expressed in endothelial and epithelial cell protective barriers of the lung and function as part of a first line of defense against respiratory infections and injury (10, 11). Circulating levels of alarmins, including interleukin-1β (IL-1β), high-mobility group box 1 (HMGB1), S100A8/9 and interleukin-33 (IL-33), are abnormally elevated in PAH patients as well as PH animal models (12-16). In particular, interleukin-33 was detected in high levels in the serum of patients diagnosed with Group 1 PAH (14). However, the steps leading from tissue damage to alarmin activation to an aberrant hyperproliferative response are unknown.

Interleukin-33 (IL-33) is a member of the IL-1 cytokine family that functions as an alarmin to induce inflammation as a response to cell injury, damage or necrosis (17). IL-33 is primarily expressed in stromal cells like epithelial and endothelial cells (17). Research has shown that pulmonary artery endothelial cells isolated from idiopathic PAH patients have a greater proliferation rate than control cells indicating a hyperproliferative response may be an initiating factor in the pathogenesis of PAH (18). Data collected *in vitro* has also been presented to show that IL-33 can increase the rate of proliferation, migration and formation of endothelial cell tubular networks (19). In addition, Schmitz et al. observed that the in vivo treatment of mice with exogenous IL-33 present pathological lung phenotype which is similar to the remodeling response in PAH (20.

IL-33 is comprised of 2 main domains; an N-terminal nuclear domain and a cytokine domain connected by the central domain (21). Post-translational intracellular and extracellular modifications allow IL-33 to exists in many different forms that differ in their biological properties (21). Like other IL-1 family members, IL-33 is expressed as a full-length precursor (pro-IL-33) upon stimulation by pro-inflammatory cytokines like interferon-gamma (IFN-γ) (22). In an IL-33-

mediated inflammatory response, full-length IL-33 is modified extracellularly to a shorter, "mature" form (18-21 kDa) that acquires amplified biological activity greater than the full-length IL-33 and can be deactivated by structural changes due to oxidation (21). Lefrançis et al. show that neutrophil serine proteases cathepsin G and elastase cleave full-length IL-$33_{1–270}$ into mature forms IL-$33_{95–270}$, IL-$33_{99–270}$, and IL-$33_{109–270}$ (23). This mature form acts as a signaling molecule and as a transcriptional regulator that modulates NF-κB activity (24). IL-33 is the ligand of the ST2 receptor that activates immune responses in many cells like endothelial cells that ultimately is causing the vascular remodeling seen in PH as mentioned earlier (25). Alternative splicing and promoter activation produce ST2L (transmembrane) and sST2 (soluble) isoforms. IL-33 binds to ST2L to signal the presence of tissue damage whereas sST2 acts as a decoy receptor to inhibit IL-33 signaling (25). ST2 forms a heterodimer along with IL-1RAP (26) leading to the dimerization of the TIR domain (27). IL-33/ST2 signal transduction results in the recruitment of adaptor proteins including myeloid differentiation factor 88 (MyD88) that regulate interleukin-1 receptor-associated kinase (IRAK) mediated TNF receptor-associated factor 6 (TRAF6) activation, eventually stimulating the activation of NF-κB and MAP kinases (25,27).

Previous *in vivo* studies in our laboratory indicate that 3 weeks of PH treatments results in right ventricular pressure (RVP) increases of about 50% in both male and female ST2-/- mice but is attenuated in both sexes of MYD88-/- mice when compared to WT mice exposed to PH conditions (28). These suggests that RVP is partially regulated by the IL-33/ST2 pathway (28). Previous research done by our lab show that exogenous IL-33 that was directly administered to wildtype (WT) mice caused pulmonary remodeling (14). However, IL-33 administered to ST2 -/- mice did not show visible remodeling of the small resistance arteries of the lungs indicating exogenous IL-33 through a ST2-dependent pathway is involved is pulmonary remodeling (14).

Despite early observations of increased IL-33 levels with PAH (14, 15), the role of IL-33 in regulating the endothelial cell hyperproliferative response *in vivo* has not been established. Thus, we hypothesized that IL-33 is essential for initiating early endothelial cell hyperproliferation in PH that leads to remodeling of the small resistance arteries of the lungs. This was tested in an established model of PH that combines chronic hypoxia with a vascular endothelial growth factor receptor antagonist, Sugen 5416 (SU5416) (29). SU5416 is shown to induce apoptosis in apoptosis-resistant endothelial cells while hypoxia promotes proliferation of the endothelial cells (29). SU5416/hypoxia conditions (SUHX) were used to model PH in wildtype (WT), IL-33 receptor ST2 gene deleted (ST2 -/-) or its partner MYD88 gene deleted (MYD88 -/-) mice. Lung endothelial proliferation, arterial wall remodeling and mature IL-33 levels were measured. We present data in support of IL-33/ST2 dependent endothelial cell hyperproliferation as an early event in the development of PH.

MATERIALS AND METHODS

Animals

Animal protocols were reviewed and approved by the Veterans Administration San Diego Healthcare System (VASDHS) Institutional Animal Care and Use Committee (IACUC). This study used adult, age 2-4 months wildtype (WT) male and female mice on a C57BL/6J background purchased from Jackson Laboratory (Bar Harbor, ME, USA). ST2-/- mice were provided by Dr. Kenji Nakanishi (Hyogo College of Medicine, Hyogo, Japan) (19) and MYD88-/- mice were developed by Dr. Shizuo Akira (Osaka University, Osaka, Japan) (20). All mice were housed in cages containing up to 5 animals, according to standard laboratory conditions with an ambient temperature of 24°C and 12hr of light per day. Mice had free access to water and standard chow.

Hypoxia/Sugen 5416 (SUHX) mouse model

WT, ST2-/- and MYD88-/- mice were exposed to the combination of weekly subcutaneous injections of Sugen 5416 (20 mg/kg in DMSO; Cayman Chemical, Ann Arbor, MI, USA) with three weeks of normobaric hypoxia (10% O_2) to induce pulmonary hypertension. Additional mice in each genotype received weekly subcutaneous injections of the vehicle, DMSO, and were exposed to room air conditions (21% O_2) for three weeks.

Lung Histology and Small Artery Wall Morphometry

The right lung was perfused with phosphate-buffered saline (PBS) and airway-fixed with 0.75 mL buffered aqueous zinc formalin (Z-fix, Anatech Ltd, Battle Creek, MI, USA). Lungs were then dehydrated and embedded in paraffin. To quantitate the remodeling response, wall thickness of the small arteries was measured in 5 μm paraffin- embedded lungs sections stained with hematoxylin and eosin (H&E) (Thermo Fisher Scientific, Waltham, MA, USA). Prepared slides

were digitally imaged with a Hamamatsu 2.0-HT Nanozoomer Slide Scanning System at the UCSD Microscopy Core (La Jolla, CA). The total vascular area at the adventitial border and the lumen area at the basement membrane were outlined and measured using the Nanozoomer Digital Pathology NDP.view2 software. The wall thickness in arteries with a diameter less than 50 μm was calculated as follows: wall thickness = (total vascular area – lumen area)/total vascular area. Data are presented as changes as a ratio of wall area to total vessel area (22).

Immunofluorescent Detection of Ki67+ Proliferating Cells

Immunostaining analysis was performed on paraffin imbedded lung sections mounted on Superfrost Plus slides. Briefly, slides were washed several times in a d-Limonene-based solvent (CitroSolv, Deacon Laboratories Inc., King of Prussia, PA, USA) heated to 60°C followed by a PBS rinse. Sections were then dehydrated for 5 mins each in graded ethanol (100%, 95%, 90%, 70%, and 50% ethanol) and washed with 0.1% PBS-Tween-20. Serial sections were then washed in antigen retrieval solution (Dako Target Retrieval Solution Agilent, Santa Clara, CA, USA) for 5 mins at 95°C. Sections were then incubated for 1 hr at room temperature in a blocking buffer containing 1.5% normal goat serum (NGS)/1.5% bovine serum albumin (BSA) in PBS. Samples were then incubated overnight at 4°C with primary antibodies directed against Ki-67 (Thermo Fisher Cat #50245564, 10:1000) and αSMA-eFluor660 (eBioscience 50976080, 1:1000), appropriately diluted in blocking solution. The following day, sections were rinsed in PBS, then incubated for 30 min at RT with the Alexa Fluor 546 diluted 1:1000 in blocking buffer, washed and mounted with Pro-long Gold with DAPI (Thermo Fisher). Digital images were acquired using Olympus FV100 Confocal with SIM Scanner and the final composites were processed using ImageJ.

Flow Cytometer Measure of Endothelial Cell Mitotic Index

Mice were injected intraperitoneally with 5-bromo-2′deoxyuridine (BrdU, B5002 Sigma) at a dose of 50 mg/kg body mass for seven consecutive days prior to harvest. The left pulmonary lobe was removed, minced, and digested with 0.2% Collagenase I (Sigma-Aldrich) and 100 ug/ml DNase type I (Sigma-Aldrich) in DMEM (GIBCO, Waltham, MA, USA) at 37°C for 30-45 minutes, passaged over 40 μm cell strainer (VMR International, Radnor, PA, USA), washed and centrifuged. Cells were incubated with the following antibodies: anti-CD31 (dilution 1:100, Cat. No. 550274, BD Pharmingen, Franklin Lakes, NJ), CD31-FITC (dilution 1:100, clone 390, 11-0311-82, eBioscience, San Diego, CA, USA) and anti-BrdU-PE (dilution 1:100, clone: BU20A, eBioscience), detected with a Cytoflex (Brea, CA, USA) and analyzed using FlowJo Software (Tri Star Inc., Ashland, OR, USA).

Western Blot Analysis

Left lung tissue extracts prepared in RIPA Buffer with Protease Inhibitor (Sigma-Aldrich) were fractionated by sodium dodecyl sulphate-polyacrylamide gel (SDS-PAGE) electrophoresis and transferred to a Fluorescence PVDF membrane (MilliporeSigma, Burlington, MA, USA) using a transfer apparatus according to the manufacturer's protocols (Bio-Rad). After incubation with blocking buffer (LI-COR Biosciences, Lincoln, NE, USA) for 60 min, the membrane was incubated with antibodies against IL-33 (mouse mAb, 1:200 dilution, Nessy-1 Enzo ALX-804-840/1), ST2 (goat pAb, 1:1000, Human ST2/IL-33R AF523; R&D Systems, Minneapolis, MN, USA), MYD88 (goat pAb, 1:1000, Mouse/Rat MyD88 AF3109, R&D Systems) and α-tubulin (rabbit Ab, 1:2000 dilution, Sigma-Aldrich, USA) overnight at 4 °C. The membrane was then washed in PBST (0.1% Tween 20-PBS, Sigma-Aldrich) thrice for 10 min and incubated with

IRDye800-conjugated secondary antibody (mouse or rabbit mAb, 1:10000, Odyssey LI-COR, USA) for 1 h. After washing in PBST 10 times for 5 min again, the membrane staining was visualized using the LI-COR Odyssey imaging system. The protein signals were quantified using Image J and their relative expression normalized to that of α-tubulin as ratios of their respective maximal expression.

Statistical Analysis

All data were analyzed using GraphPad Prism 8.0 (GraphPad Software, USA) and expressed as the mean ± standard deviation (SD). T-tests were used to compare conditions within each genotype (Fig.1, 2). Two-way (genotype, treatment) analysis of variance (ANOVA) was used to detect variation between the IL-33 levels in the lungs. Statistical significance was defined as $p < 0.05$.

RESULTS

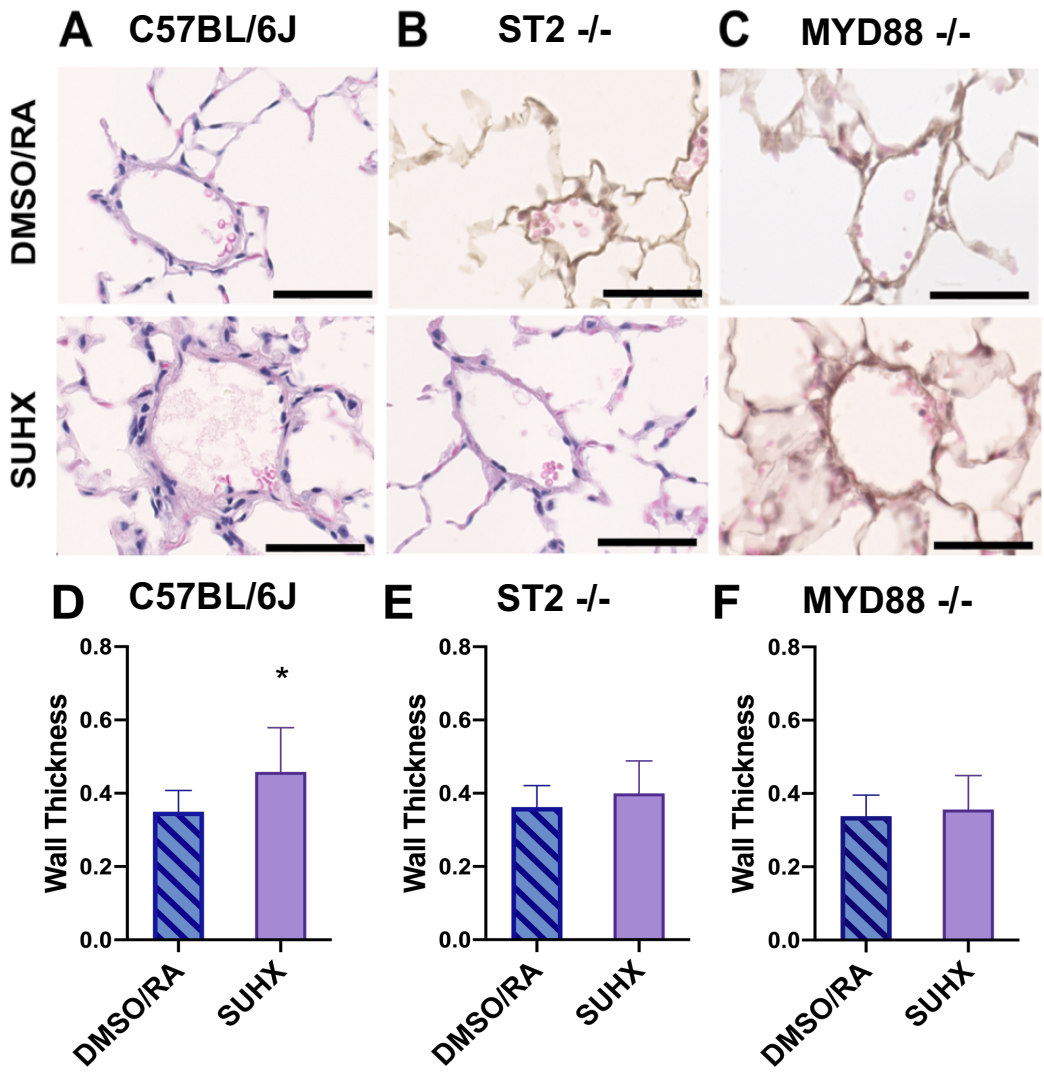
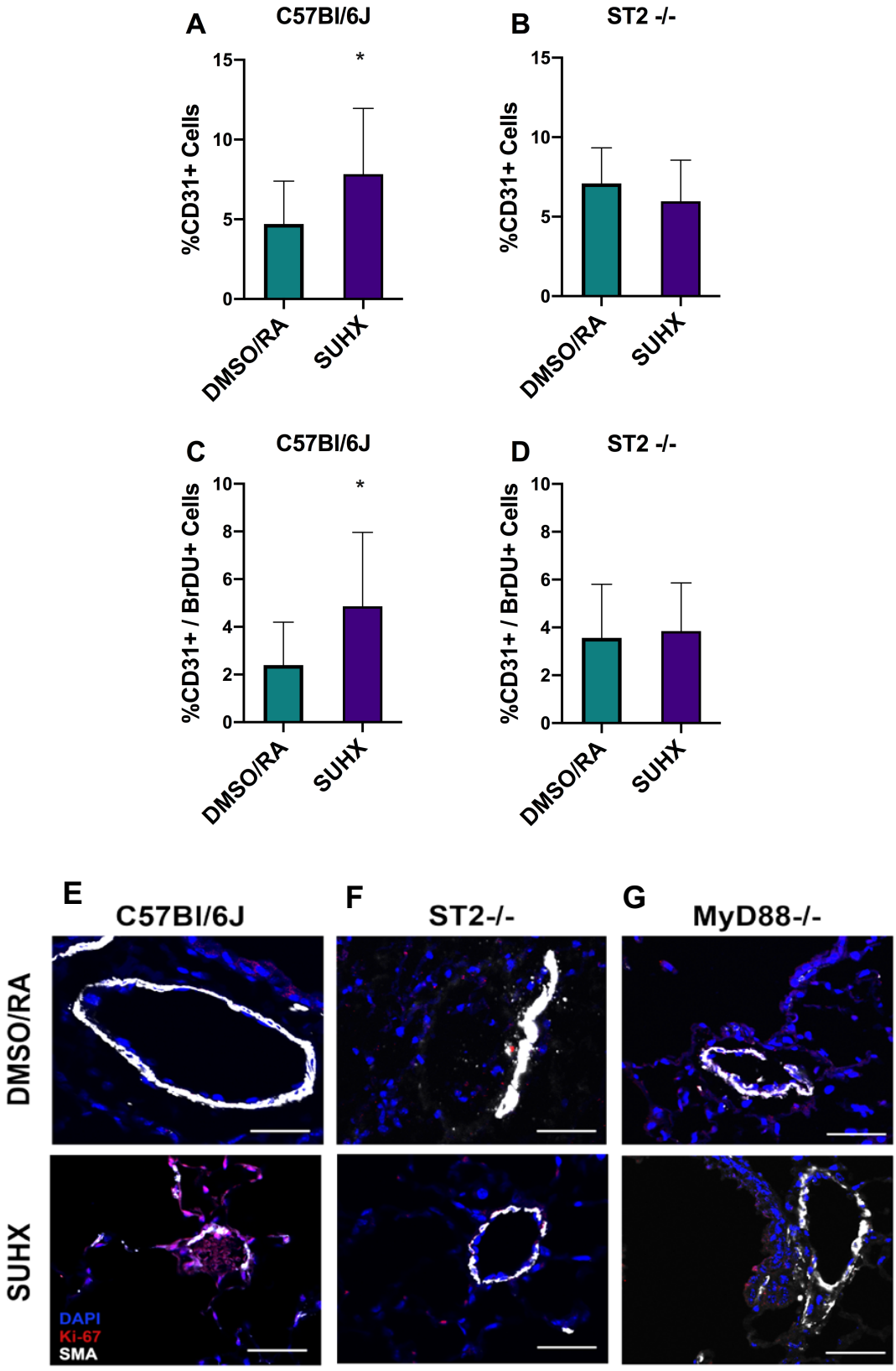


Figure 1. Increased pulmonary vascular remodeling in C57Bl/6J mice treated with SUHX. Wall thickness in the small resistance arteries (< 50 µm in diameter) is shown in H&E-stained lung sections of (A) C57Bl/6J, (B) ST2-/- and (C) MYD88-/- mice under DMSO/RA and SUHX treatments. Quantitation of wall thickness in (D) C57Bl/6J, (E) ST2-/- and (F) MYD88-/- under DMSO/RA and SUHX conditions. Values are presented as mean ± SD (n = 9-14 DMSO; n = 14-17 SUHX). *Represents statistical significance between DMSO/RA and SUHX treatments within each genotype, $p < 0.05$.

Pulmonary vascular remodeling observed in C57Bl/6J mice, but not ST2 -/- or MYD88 -/- mice, under SUHX condition. Small pulmonary arteries and arterioles revealed thicker vascular walls in C57BL/6J mouse lungs from the SUHX group than C57BL/6J mice in

the DMSO/RA group ($p = 0.01$) (Fig. 1D). These changes in wall thickness were attenuated in ST2-/- and the MYD88-/- arteries which did not show a difference in vascular remodeling in response to SUHX (Fig. 2E-F) (ST2-/-, DMSO/RA vs. SUHX, $p = 0.20$ and MYD88 -/-, DMSO/RA vs. SUHX, $p = 0.60$, respectively). Figure 1A-C shows representative images of H&E-stained lung sections from mice in each group.

Figure 2. Vascular cell hyperproliferation in small arteries and arterioles of SUHX exposed C57Bl/6J mice. Vascular endothelial cell hyperproliferation was evaluated by immunohistochemical and flow cytometry analysis of proliferating CD31+ cells. The percentage of endothelial cells (%CD31+) in (A) C57Bl/6J and (B) ST2-/- mice under DMSO/RA and SUHX conditions. Percentage of proliferating endothelial cells (% CD31+/BrDU+) under DMSO/RA and SUHX treatments in (C) C57Bl/6J and (D) ST2-/- mice. Localization of proliferating (Ki-67) cells detected in SMA positive small arteries of (E) C57Bl/6J, (F) ST2-/- and (G) MYD88-/- mice under DMSO/RA and SUHX conditions. Scale bars represent 50 μm. Values are presented as the means ± SD (n= 5-12 DMSO/RA, n= 12-18 SUHX). * Represents statistical significance between DMSO/RA and SUHX treatments, $p < 0.05$. Figure 2 is co-authored with Scott Johns. The thesis author was co-author of this Figure.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ST2 -/-
%CD31+ Cells
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DMSO/RA
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ST2-/-
G
MyD88-/-
DMSO/RA
SUHX
DAPI
Ki-67
SMA

ST2 dependent endothelial cell hyperproliferation in response to SUHX. Endothelial cell ST2 dependent hyperproliferation was observed in SUHX treated WT mice but not in DMSO treated WT mice. The total number of $CD31^+$ cells detected by flow cytometry was higher in the lungs of C57BL/67 mice treated with SUHX than DMSO/RA treated wildtype mice (Fig. 2D, $p = 0.02$). The number of $CD31^+$ cells in the lungs of ST2-/- mice do not show a difference in response to treatment conditions (Fig. 2E, $p = 0.80$). An increase in proliferating endothelial cells (CD31+/BrDU+) is seen only in wildtype C57Bl/6J mice exposed to SUHX (Fig. 2F-G, $p = 0.02$). Representative images of lung sections with immunohistochemical staining for α-SMA and Ki-67, a proliferation marker, show the localization of proliferating (Ki-67+) cells located proximally to the SMA positive small arteries of C57Bl/6J but not ST2-/- and MYD88-/- mice under SUHX conditions (Fig. 2A-C).

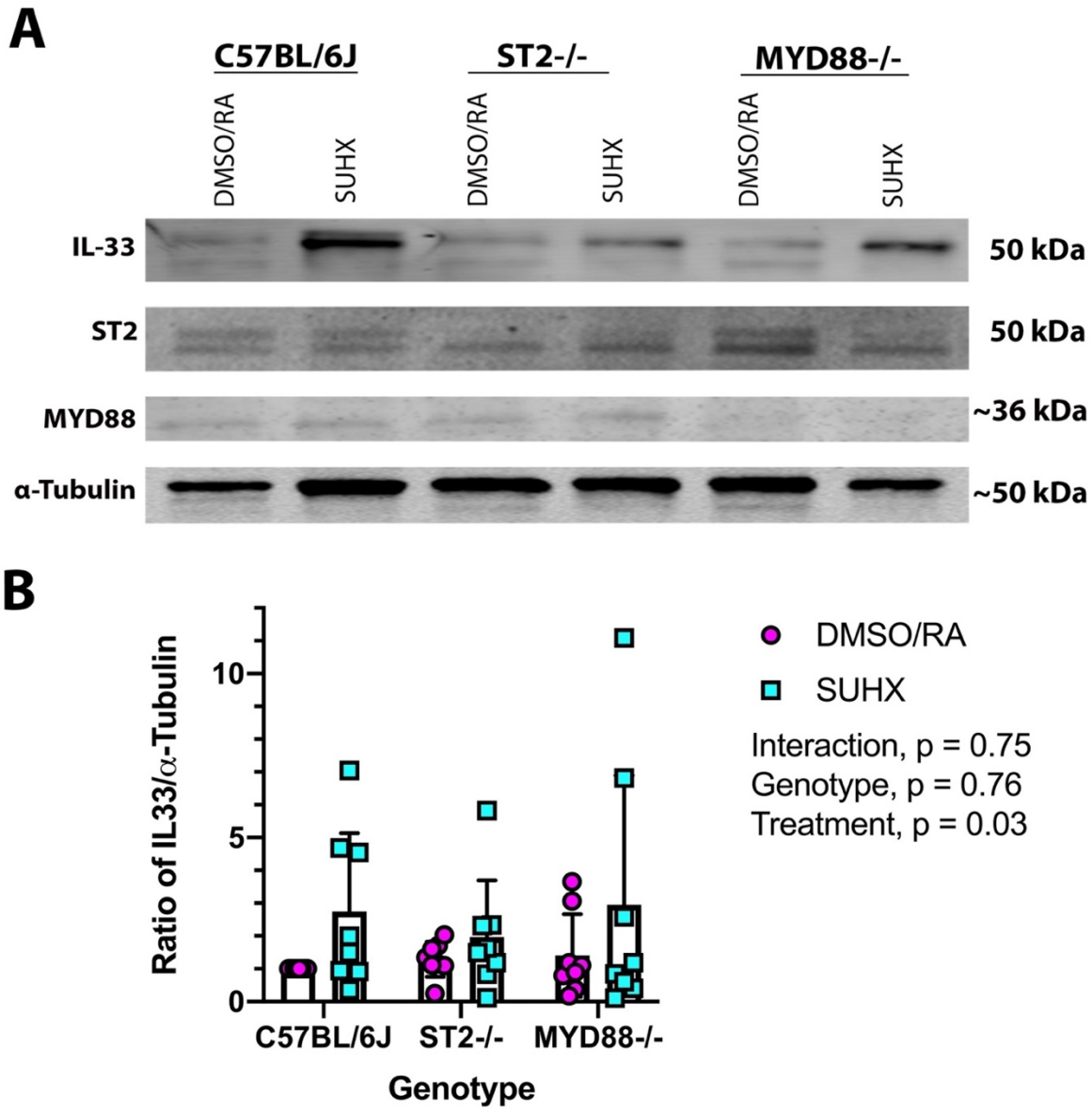

Figure 3. Increased pulmonary IL-33 protein levels in mice exposed to SUHX. (A) Western Blot representation of IL-33, ST2 and MYD88 protein in lungs of C57BL/6J, ST2-/-, and MYD88-/- mice exposed to either SUHX or DMSO/RA conditions (All n=8). Equal loading of protein was confirmed using α-Tubulin. (B) Quantitative analysis of IL-33 in whole lungs. Values are presented as the means ± SD.

Increased IL-33 protein in mice exposed to SUHX independent of ST2 and MYD88. IL-33 protein was detected in lung tissue of C57BL/6J, ST2-/- and MYD88-/- mice exposed to either DMSO/RA or SUHX treatments. The representative western blot image shows an increased in IL-33 levels in WT, ST2 and MYD88 groups (Fig. 3A). Western blots showed that IL-33 levels

were elevated in following exposure to 3 weeks of SUHX (Fig. 3A and B, Treatment effect p=0.03). The increased in IL-33 with SUHX was not different between genotypes, C57Bl/6J, ST2-/- or MYD88-/- (Genotype effect, p=0.76). There was also no interaction between treatment condition and genotype (Interaction, p=0.76).

DISCUSSION

IL-33 has been implicated in the progression of PAH (13,14), but the functional role of IL-33-ST2 signaling in the early cell proliferative response and subsequent vascular remodeling have not been explained. Our data indicate that the IL-33/ST2 signaling pathways regulates the hyperproliferative response in pulmonary artery endothelial cells and contributes to the vascular wall remodeling that develops after just three weeks under SUHX-induced PH conditions. Inhibiting the ST2 receptor and the MYD88 adaptor protein expression completely prevented the SUHX-induced hyperproliferation and wall remodeling responses. Mature, processed IL-33 levels increased in response to SUHX conditions indicating IL-33 activation in this SUHX model.

IL-33 regulates the endothelial cell hyperproliferative response in SUHX-induced PH

Our experiments show that IL-33 is essential for the hyperproliferative response in PH induced by SUHX. A hyperproliferative response was detected early in the development of PH (30-32). Endothelial cells isolated from idiopathic PAH patients have a greater proliferative rate that is linked to increased cell survival and an amplified cell cycle progression (33). IL-33 is released extracellularly after endothelial cell damage due to injury or mechanical strain (34). Thus, our studies suggest that early damage to the endothelium and subsequent increased tension as the pulmonary artery remodel leads to a persistent proliferative response which is dependent on IL-33/ST2 signaling. This is supported by our finding that ST2 gene deficient mice are protected from the endothelial cell hyperproliferative response that occurs in the lungs of SUHX exposed WT mice (Fig. 2). This observation complements existing data collected in mice deficient in MYD88, an adaptor protein upstream in the IL-33 signaling pathway, which show that following exposure to chronic hypoxia that there are fewer proliferating cells in the pulmonary vessels of in MYD88 null mice than WT (36). Exogenous IL-33 has also been shown to directly increase human

pulmonary artery endothelial cell (hPAEC) proliferation cultured under normoxic conditions (15), and gene deletion of IL-33 in cultured hPAECs attenuates proliferation under both normoxia and hypoxia conditions (15). Taken together, these *in vitro* studies support an IL-33 regulated mechanism in the proliferation of endothelial cells in PH. We now show the importance of IL-33/ST2 signaling in regulating this key hyperproliferative response in the SuHx-induce mouse model of PH.

Arterial wall remodeling is dependent on IL-33

Acute inflammation associated with increased TNFα and IL-6 levels has been suggested to be a key factor to the development of PAH (8). Our experiments suggest that PH vascular remodeling is dependent on the alarmin IL-33 located in the pulmonary endothelium. We show that ablation of ST2 or MYD88 prevented an increase in the overall thickness of the resistance arteries (< 50 μm) after inducing PH in our model (SUHX) (Fig.1). This suggests that IL-33 contributes to pathogenesis of PH as in the absence of the ST2 receptor as arterial wall remodeling was completely prevented. Similarly, Liu and colleagues observed that pulmonary vascular remodeling in PH induced by hypoxia alone is attenuated in ST2 null mice (15). Our study is focused on the early endothelial hyperproliferative response that could initiate vascular remodeling through the activation of IL-33 and potentially through additional alarmins such as HMGB-1, IL-1ß, and S100A8/A9 (12, 13, 16).

IL-33 is upregulated across all genotypes treated with SUHX

Western blots of the lung tissue of WT mice under PH conditions (SUHX) show an increase of the processed form of IL-33 (Fig. 3). Interestingly, the western blot of lung tissues of the ST2-/- mice in our experiment show that IL-33 levels are increased by SUHX conditions, independent

of the expression of ST2 or MYD88 (Fig. 3). This is interesting because our data shows that the PH condition of SUHX does indeed cause IL-33 signaling to be upregulated (Fig. 3) and IL-33 is upregulated in PH patients (13, 14). However, the upregulation of the IL-33 in SUHX exposed ST2-/- mice did not result in a hyperproliferative response or vascular remodeling (Fig. 1, Fig. 2). Thus, our data suggest that IL-33/ST2 signaling is essential for the early endothelial proliferative response but additional IL-33 signaling pathways or signals initiated but additional alarmins or growth factors may contribute to a remodeling response in non-proliferating cells with in the small pulmonary arteries.

One pathway that is activated in PAH is the Notch signaling pathway. In particular Notch3-HES signaling has been implicated in the smooth muscle cells proliferation in PAH (30, 31). Data by a previous member of the laboratory showed that exogenous IL-33 together with the notch ligand (DLL4) added to hPAECs cultured on gelatin alone increased IL-33 transcripts and protein levels indicating a potential autocrine mechanism initiated in endothelial cells (36). Previous reports in the literature support Notch signaling as a known driver of IL-33 expression and, the notch ligand, DLL4 has been shown to be a strong inducer of IL-33 transcripts in other endothelial cells, i.e. human umbilical vein-derived endothelial cells (HUVECs), human dermal microvascular endothelial cells from juvenile foreskin (HDMECs) and human pulmonary microvascular endothelial cells (HPMECs) (34). Taken together these experiments suggest that multiple signaling pathways are activated in SUHX-induced PH to increase IL-33 expression. Importantly, we show that the IL-33/ST2 signaling axis is essential for *in vivo* endothelial cell proliferation in SUHX-induced PH.

Summary

Understanding the role of inflammatory processes in the development of PH is an area of increasing importance. We investigated the role of the IL-33/ST2/MYD88 axis in the progression of the disease. We find in our mouse model that IL-33/ST2 is essential for the early endothelial cell hyperproliferative response induced by SUHX that leads to remodeling. In addition, we find that the intracellular toll-like receptor adaptor protein, MYD88 synergizes with the IL-33/ST2 receptor complex to regulate remodeling. DLL4 mechanism that activates IL-33 in endothelial cells could possibly be amplified by an IL-33 autocrine response and warrants proper investigation.

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