UNIVERSITY OF CALIFORNIA, SAN DIEGO

Interleukin-33 signaling occurs through an autocrine loop, and is further augmented by Notch

A Thesis submitted in partial satisfaction of the requirements

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in

Biology

by

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The Thesis of Tzuhan Tsui is approved, and it is acceptable in quality and form for publication on microfilm and electronically:

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I dedicate this thesis to my parents for all the support and telling me to never give up.

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ABSTRACT OF THE THESIS

Interleukin-33 signaling occurs through an autocrine loop, and is further augmented by Notch

by

Tzuhan Tsui

Master of Science in Biology

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Mark Fuster, Chair

Elina Zuniga, Co-Chair

Pulmonary hypertension is a disease with many causes, but no lasting cures. Its defining symptom is the creation of lesions within the pulmonary arteries during advanced stages of this disease. Recently it was discovered that Interleukin-33 (IL-33) was elevated in patients with pulmonary hypertension. Elevated IL-33 levels have been found in other diseases such as asthma, rheumatoid arthritis, and chronic

obstructive pulmonary diseases, but the cause of secretion is still unclear. We wanted to see if IL-33 has an autocrine or paracrine function in human pulmonary arterial endothelial cells (hPAECs) by culturing the cells with exogenous IL-33. We also looked at IL-33 signaling function while under the influence of the notch ligand Dll4, which has shown to increase IL-33 expression. We found that Dll4 or IL-33 alone are capable of increasing IL-33 expression, but a combined treatment significantly raises IL-33 and receptor ST2 expression. Next, we wanted find out if it was possible to mimic the current mouse model of hypoxia in human cells. This was achieved by culturing hPAECs in the vegfr2 receptor antagonist SU5416 or vehicle, and comparing cultures incubated in hypoxia or normoxia. We saw a decrease in IL-33 when the cultures were under SU5416/hypoxia, as well as an increase in the soluble receptor sST2. This could be the result of a hierarchy where VEGF is a more powerful controller of IL-33 than Dll4. Overall these studies shed more light on the possible mechanisms of IL-33 signaling.

INTRODUCTION

Pulmonary hypertension

Pulmonary hypertension (PH) is a rare lung disease, with 15-50 cases per million worldwide. It is characterized by a mean pulmonary artery pressure equal to or over 25 mmHg at rest. If left untreated, the disease leads to right heart failure with circulatory collapse. Pulmonary arterial hypertension (PAH) is a severe form of this disease, and in late stages is characterized by vessel obstructions called plexiform lesions (Tuder 2007). These lesions are composed of proliferating smooth muscle cells growing in the medial layer in combination with endothelial cell growth into the vessel lumen. In idiopathic and familial PAH, this growth is thought to be caused by mutations in the BMPR2 gene (Machado 2006). Current treatment of PAH includes prostaglandins, endothelin-1 receptor antagonists, nitric oxide therapy, and vasodilators in addition to conventional therapy consisting of anticoagulants, oxygen supplementation, and calcium channel blockers (Gabbay 2007; Fetalvero 2006; Pepke-Zaba 1991; Humbert 2004). While there are a plethora of treatments, none have the ability to reverse the remodeling that inevitability occurs, hence the necessity for continued research. One possible venue is the role of the cytokine interleukin-33 (IL-33), and its association with Notch signaling on vascular reconstruction.

Notch signaling

Notch signaling is a highly conserved and regulated system involved in the formation and maintenance of various cell processes **(Kopan 2009).** In humans, the system consists of 4 receptors (Notch1-4) and 5 ligands (Delta-like ligands 1, 3, 4, and Jagged1 and 2) (D’Souza

2010). Notch deficiencies are linked to various conditions such as aortic valve disease, T-Cell acute lymphoblastic leukemia, Alagille syndrome, Hajdu-Cheney syndrome, CADASIL, and Tetralogy of Fallot (Garg 2005; Weng 2004; Li 1997; McDaniell 2006; Isidor 2011; Chabriat 2009; Eldadah 2001). The canonical signaling mechanism for Notch starts with ligand binding from the signaling cell onto the Notch receptor of the receiving cell (Kopan 2009). This leads to cleavage of the Notch extracellular domain by a disintegrin and metalloprotease (ADAM). The remaining Notch extracellular truncation (NEXT) is then cleaved again by ɣ-secretase, resulting in release of the Notch intracellular domain (NICD). Once free, NICD translocates to the nucleus and binds with transcription factor CBF-1/RBPjκ. This recruits the co-activator Mastermind-like (MAML) protein, which induces transcription of downstream targets.

Notch1 and Notch4, as well as all ligands barring Dll3 are expressed in arterial endothelial cells (Kume 2009). The variety of ligands and receptors present lead to a multitude of functions, but they primarily govern angiogenesis. Jagged1 and Dll4 produce complementary effects on endothelial cells (Benedito 2009). High Jagged1 signaling leads to a “tip” cell phenotype, a filopodia-rich cell that guides new sprouts. Conversely, High Dll4 signaling encourages a “stalk,” cell phenotype, which have fewer filopodia and form the lumen of the new blood vessel.

Notch signaling has been associated with hypoxia induced PH, as Li et al. found an upregulation of Notch3 receptor expression in conjunction with vascular smooth muscle proliferation in rats with PH (Li 2009). This group demonstrated that inhibition of Notch3 prevented this proliferation and reversed pulmonary

hypertension. Furthermore hypoxia has been shown to upregulate the notch ligand Dll4 and direct endothelial progenitor cells towards an arterial fate (Diez 2007). This brings a point of interest as Dll4 is also known to induce IL-33 expression (Sundlisaeter 2012).

IL-33 function and disease roles

Interleukin-33 (IL-33) is a cytokine of the IL-1 superfamily that is expressed in various cell types throughout the human body including epithelial cells, smooth muscle cells, fibroblasts, myofibroblasts, and endothelial cells (Mirchandani 2012). This system signals the same downstream targets as Toll-like Receptors (TLRs) by way of Toll/interleukin-1 receptor homology (TIR) domains found on several adaptor proteins (O'Neill 2007) Cell stimulation with IL-33 begins when IL-33 binds to the receptor known as suppression of tumorigenicity 2 (ST2). This recruits Myeloid differentiation primary response gene 88 (MyD88), an adaptor protein used throughout IL-1 cytokine signaling (Schmitz 2005). This in turn recruits interleukin receptor associated kinase (IRAK), which activates TNF-associated factor 6 (TRAF6). TRAF6 then phosphorylates inhibitor of nuclear factor kappa-B kinase (IKK), releasing it from the transcriptional factor nuclear factor kappa-B (NF-κB). This results in the transcription of other factors, ultimately leading to the expression of IL-4, IL-5, and IL-13 cytokines, and polarization of naive T cells to Type-2 T helper (Th2) cells. IL-4 and IL-13 stimulate B-cells to switch to immunoglobulin-E (IgE) class antibody, specializing them against helminths. IL-5 promotes eosinophil growth and

differentiation. The combined effects help the body fight off extracellular parasites and toxins.

IL-33 has shown various effects depending on the system studied. Exogenous administration of IL-33 in mice results in splenomegaly, with increased eosinophil and mononuclear cell counts (Schmitz 2005). Lung, esophageal, and intestinal tissue display epithelial hyperplasia with IL-33 treatment. IL-33 levels are elevated in both Alternaria alternata as well as house dust mite (HDM) models of asthma, leading to higher levels of inflammation (Boitano 2011; Snelgrove 2014). In contrast, IL-33 has been shown to have protective properties in cardiomyocytes (Sanada 2007), and IL-33 treatment reduces atherosclerotic plaques in ApoE-/- mice (Miller 2008), although IL-33 deficient ApoE-/- mice do not develop any worse (Martin 2015). Recently, it has been shown that IL-33 may be a viable treatment for Alzheimer's Disease as it has anti-inflammatory effects in the brain (Fu 2016). There have been reports showing increased and decreased serum IL-33 from PH patients, which warrants further investigation (Titone 2014; Shao 2014).

Additionally, IL-33 has angiogenic effects (Choi 2009). Administration of IL-33 stimulates angiogenesis in endothelial cells through TNF-receptor associated factor 6 (TRAF6) mediated signaling. IL-33 stimulation also increase cell permeability and decreases stability, but this effect is maintained by IL-4 (Chalubinski 2015). As IL-33 is not secreted by any known pathways, this suggests that it may induce its own release. However, where this initial release remains unknown.

Notch and IL-33: relationship involving PH?

While the effects of IL-33 activation have been characterized, it is unclear how this cytokine is released. IL-33 was originally thought to be cleaved by caspase-1 into an active form before being released (Schmitz 2005). Subsequent studies found IL-33 to be a direct substrate of the pro- apoptotic enzyme caspase-3, which inactivates the cytokine (Ali 2009; Cayrol 2009). The studies also found IL-33 to be released upon damage or necrosis, leading to the theory that IL-33 is an 'alarmin'. Treatment with prostaglandin E2 and adrenoceptor agonists on murine bone marrow dendritic cells (mBMDCs) has shown to increase IL-33 protein induction upon lipopolysaccharide (LPS) challenge (Yanagawa 2011; Yanagawa 2011). There is also evidence to suggest an autocrine function in BMDCs, though the exact mechanism has yet to be elucidated (Su 2013). Intracellular IL-33 has been shown to be expressed constitutively and under Notch ligand stimulation in endothelial cells, but no mechanism of release has been put forth (Kuchler 2008; Moussion 2008; Sundlisaeter 2012). In this report, we show through RNA and protein measurements that IL-33 signaling is under both its own control and the notch system. We also knock down IL-33 expression to show that Dll4 can signal IL-33 independently. Lastly, we look at IL-33 expression in hPAEC cultures while emulating the rat/mouse model of pulmonary hypertension.

MATERIALS AND METHODS

Pulmonary Artery Endothelial Cell Model

Human Pulmonary Artery Endothelial Cells (hPAECs) were cultured in Endothelial Cell Growth Supplement (EnGS) media (Lifeline Cell Technology, Frederick, MD). Media was prepared per company instructions and supplemented with pencillin (50U/mL) and streptomycin (50μg/mL). For all experiments, cells were used from passage 4 to 5.

Notch ligand (DLL4) and IL-33 stimulation

For experiments, hPAECs were seeded in 6-well plates at a density of $3.0x10^5$ cells/cm^2. Samples were treated for 96 hours (refeeding at 48 hours) with recombinant human (rh) DLL4, IL- 33, or both cytokines. For experiments in which cells were treated with the notch ligand, plates were coated with 0.2% gelatin (w/v) in phosphate buffered saline (PBS) with or without rhDLL4 (1μg/mL) overnight. IL-33 protein (100ng/mL) was introduced into media at the same time the cells were seeded. Cells treated with IL-33 were cultured on plates with and without gelatin coating for comparison.

siRNA knockdown

Forward transfection of hPAECs was conducted using the Lipofectamine RNAiMAX kit with silencer select negative control or pre-designed IL-33 siRNA mixed in opti-MEM low serum media (Life Technologies, Carlsbad, CA).

SU5416/Hypoxia chamber culture

hPAEC cultures were treated with media alone, 1.2µl of vehicle (DMSO), or 1.2ug of the vascular endothelial growth receptor 2 (VEGFR2) inhibitor SU5416 (Pfizer, New York City, NY) and then either placed in room air or a hypoxia chamber (~7% oxygen measured) for 48 hours.

RNA extraction and Real time RTPCR

Total RNA was extracted from hPAECs with a RNeasy mini kit (Qiagen, Valencia, CA). RNA was reverse-transcribed using SuperScript Reverse Transcriptase III with Oligo(dT) primers (Life Technologies, Carlsbad, CA). Transcripts were amplified with gene specific primers and SYBR green master mix (Eurogentech, LIEGE Science Park, Belgium) using a quantitative PCR instrument (StepOnePlus, Life Technologies, Carlsbad, CA). Measurement of the housekeeping gene GAPDH was used as an internal control., Levels of IL-33, sST2, and ST2L were assessed using the 2deltaCT method. Primers specific for each gene are listed in Table 1.

Table 1: primer sequences

Gene	Primers
GAPDH	Fwd 5’ - ACAGTCAGCCGCATCTTCTT - 3’ Rev 5’ - TGGAAGATGGTGATGGGATT - 3’
IL-33	Fwd 5’ - GGAGTGCTTTGCCTTTGGTA - 3’ Rev 5’ - TCATTTGAGGGGTGTTGAGA - 3’
sST2	Fwd 5’ - CTGTCTGGCCCTGAATTTGC - 3’ Rev 5’ - TGGAACCACACTCCATTCTGC - 3’
ST2L	Fwd 5’ - AGGCTTTTCTCTGTTTCCAGTAATCGG - 3’ Rev 5’ - GGCCTCAATCCAGAACATTTTTAGGATGATAAC - 3’

IL-33 Measurements

To assess for the possibility of released or secreted IL-33 protein, 1 mL of cell media was set aside from each cell culture to be assayed. Then, sample wells were then washed with PBS and lysed with 400 µl of RIPA buffer. All samples were kept on ice shortly after extraction.

IL-33 levels were measured in the medial and cell layer using a human IL-33 ELISA kit from R&D Systems was used (Minneapolis, MN). Capture antibody was set overnight in room temperature. The next day, the plate was washed 3 times and blocked with 1% bovine serum albumin (BSA) in PBS for 1 hour. Plate wells were washed 3 times, then loaded with sample or standards and incubated for 2 hours at room temperature. Standards were prepared using RIPA or hPAEC media, depending on the sample type. The plate was washed 3 times, loaded with detection antibody, and incubated for 2 hours at room temperature. This step was followed with an additional wash (3 times) and incubation with streptavidin-HRP for 25 minutes away from the light at room temperature. The plate was washed 3 times, loaded with substrate A+B (R&D systems), and allowed to sit for 25 minutes at room temperature in the dark. Sulfuric acid (1 M) was used to stop the color reaction, and the plate was immediately read on a plate reader (SpectraMax 190, Molecular Devices, Sunnyvale, CA). Plate was read at 450nm for sample collection, and 540nm for material correction.

RESULTS

To determine whether endothelial cells produced IL-33 in quiescence, hPAECs were cultured in media alone, 100ng/mL of IL-33, 1µg/mL Dll4, or IL-33/Dll4 combined. In resting cultures there was a little production of IL-33(Fig 1B). IL-33 alone was able to upregulate its own message, while Dll4 was also able to signal IL-33 message and protein as previously reported (Sundlisaeter 2012). With both treatments combined there was a significant increase in IL-33 transcript and protein production within the cell ($P<0.0001$). A similar pattern emerges when looking at receptor expression. There are two forms of the receptor, which are alternatively expressed from the same gene (Bergers 1994). The soluble receptor sST2 is a decoy that is secreted to control the bioavailability of IL-33, while the ST2L contains a transmembrane and intracellular domain that allows for activation of the signaling pathway. Overall ST2 protein levels rose under IL-33 alone or IL-33/Dll4 treatment (Fig. 2A). There was no commercial ELISA that could distinguish between the two isoforms, but looking at the mRNA transcripts shows that expression of both receptor forms occurs upon combined treatment, while IL-33 treatment alone upregulates sST2 (Fig 2B, 2C).

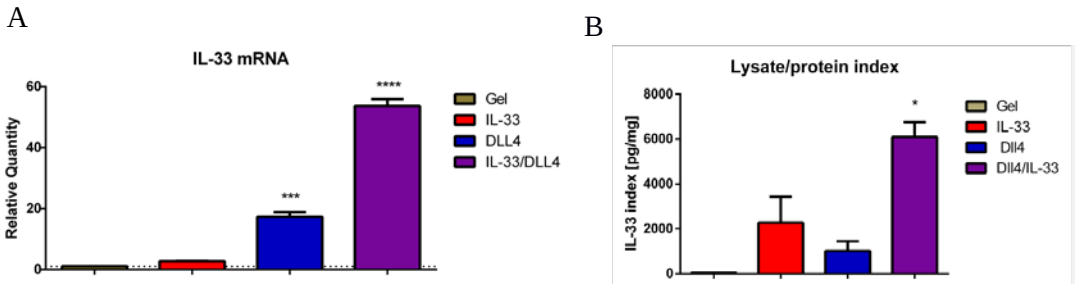

Figure 1: **IL-33 induces its own production, but is highest when combined with Dll4.** IL-33 message (A) and cell lysate protein levels (B) were measured. n = 3, *p<0.05, ***p<0.001, ****p<0.0001.

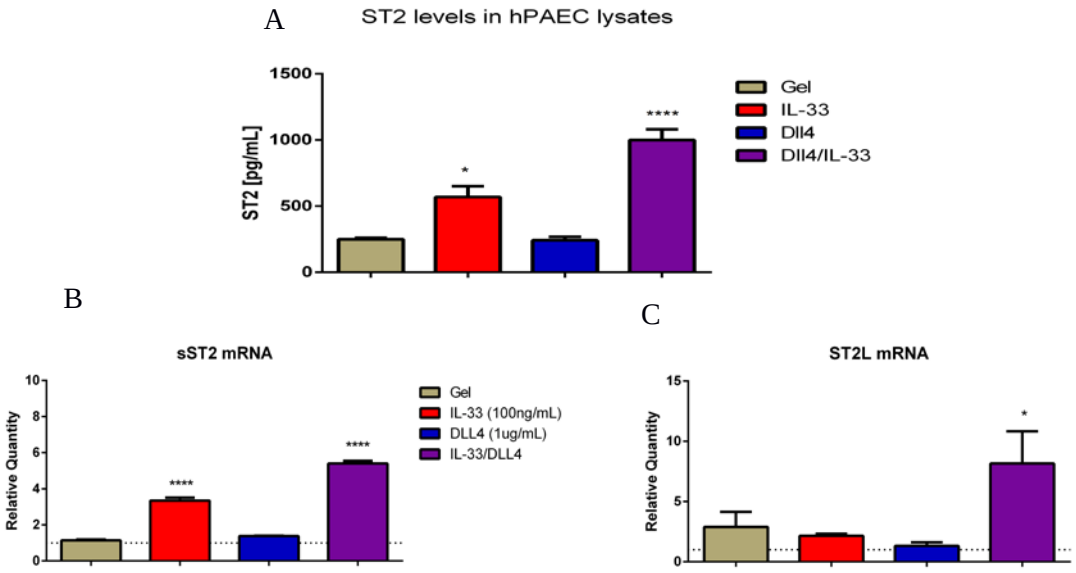

Figure 2: **Soluble and transmembrane ST2 levels are raised upon combined Dll4/IL-33 treatment.** Total ST2 receptor protein (A) as well as soluble (B) and transmembrane (C) receptor message measured from hPAEC cultures. n = 3, *p<0.05, ****p<0.0001.

We tried to emulate the mouse model of PH using the vascular endothelial cell growth factor receptor 2 (vegfr2) antagonist SU5416 and hypoxia in order to observe its effects on endothelial cells alone (Fig. 3). The concentration of SU5416 was used from a previous clinical trial **(Mendel 2000).** DMSO was found to be a suitable vehicle with the exception of ST2L expression. While hypoxia alone didn't affect gene expression of IL-33 or sST2, adding in SU5416 lowered IL-33 expression and raised sST2 (P<0.05).

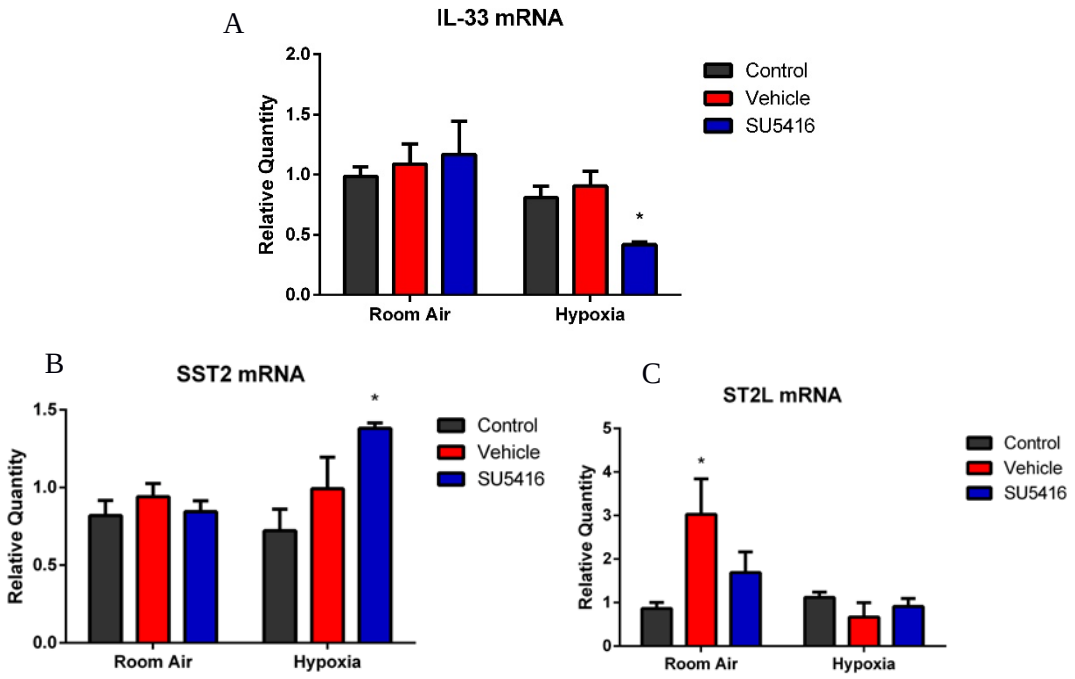

Figure 3: **hPAECs exhibit lower IL-33 expression levels when emulating a mouse model of PH.** IL-33 (A) and ST2 (B, C) gene transcript levels measured in room air and SU5416/Hx cultures. Cultures were treated with vehicle or SU5416 and incubated for 48 hours before extraction. n = 1, *p<0.05.

In order to determine if IL-33 was being signaled independently by Dll4-Notch signaling, cultures were incubated with scrambled siRNA or IL-33 siRNA under treatments stated earlier (Fig. 4). IL-33 expression still increased, though to only 20% and 6% of previous readings (Fig. 4A). Any synergistic effects that combined Dll4/Il-33 treatment showed were also not present. sST2 and ST2L expression unexpectedly increased when IL-33 was knocked down (Fig 4B-C, Gel/IL-33 siRNA). Furthermore, this result was not seen with knockdown of the combined IL-33/Dll4 culture (Fig 4B-C, Dll4/IL-33/IL-33 siRNA).

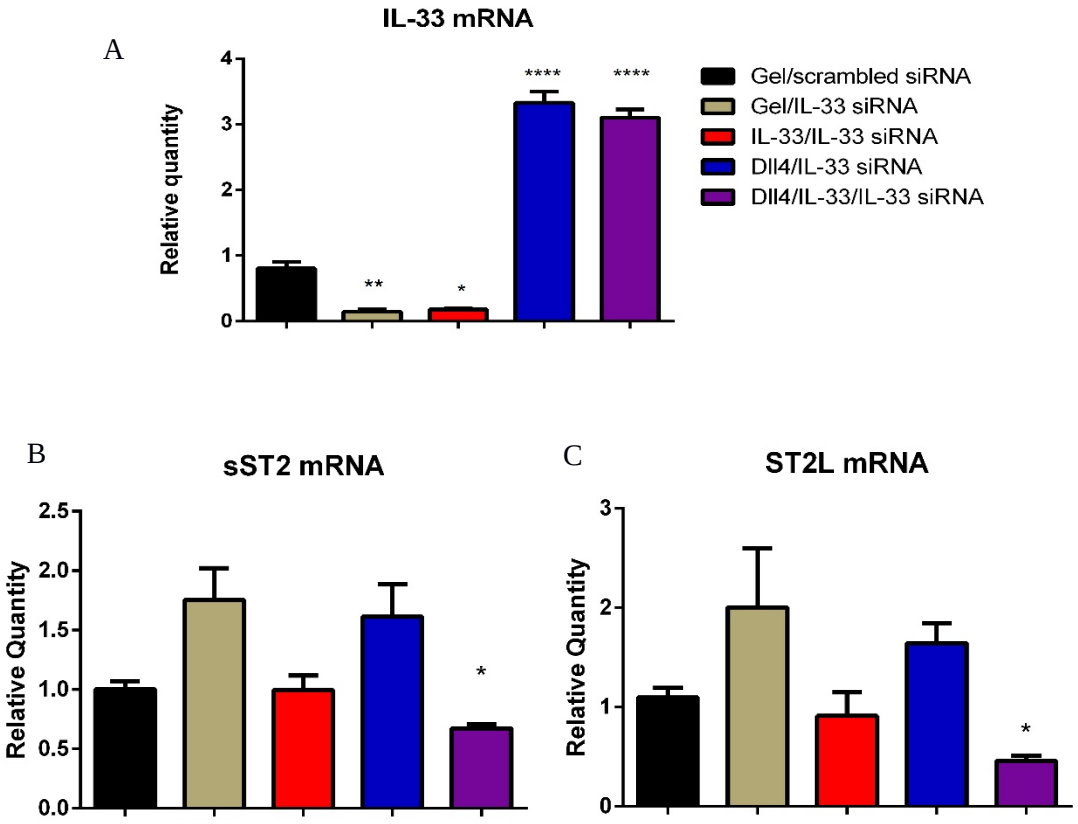

Figure 4: **Notch signaling still capable of inducing IL-33 gene expression despite IL-33 silencing.** hPAEC cultures were given siRNA and stated treatment simultaneously, then refreshed with media after 4 hours. Samples were collected after 48 hours. n = 2, *p<0.05, **p<0.01, ****p<0.0001.

DISCUSSION

IL-33 is a cytokine with a wide range of functions that depend on the cell type. Its active form and regulation are still not well known. We decided to see if IL-33 can control its own gene expression in endothelial cells. Upon stimulation with IL-33, there was an observed upregulation of IL-33 gene and protein expression. This was also similar when looking at receptor expression levels. There was an upregulation of IL-33 under Dll4 treatment, but there was also a marked increase in IL-33 and ST2 levels under a combined Dll4 and IL-33 treatment, more than the single treatments alone. Furthermore, ST2L was only upregulated with the combined therapy. This would suggest that endothelial cells become more receptive when both factors are present.

We then wanted to see if Notch signaling is sufficient to target IL-33 production alone without needing to signal through IL-33. Knocking out IL-33 was not enough to stop IL-33 signaling in Dll4 treated cultures, but the synergistic effects that the combined treatment showed previously were not present. Interestingly, both soluble and transmembrane ST2 levels were upregulated when IL-33 was knocked down, but these same mRNA readings went back to baseline once there was exogenous IL-33 present. This would suggest that extracellular IL-33 detection helps stabilize ST2 readings.

Lastly, we wanted to see if this was the mechanism being activated in the animal models of PH. Currently, there are several animal models of PH, with the most widely used method being to house the animals in a hypoxic environment for 3 weeks, coupled with a weekly administration of SU5416. In rat and mice models, this is sufficient enough to give irreversible PH. Treatment of hPAECs with SU416/hypoxia

resulted in IL-33 expression reduction, which was not expected. Furthermore, sST2 expression was still upregulated. This could possibly mean that there are lower IL-33 protein levels in the SU5416/hypoxia model of PH, which is consistent with Shao et al.'s findings (Shao 2014). Furthermore, Shao et al. was able to detect higher levels of soluble ST2 in the patient sera, consistent with the elevated levels of sST2 found from our study (Shao 2014). On the other hand, the elevated sST2 levels lead to the decline of IL-33 expression. Traditionally, sST2 has been known as a decoy receptor, only being upregulated in order to bind IL-33 and prevent signaling on cells. This upregulation of sST2 despite lower IL-33 levels suggests that the receptor is binding free IL-33 that is being released from the endothelial cells, limiting the cytokine's autocrine effects.

Future experiments can further uncover the relationship between Il-33 and Notch. IL-33 has shown to require additional signaling in order to stimulate production. Th2 cells express low levels of the receptor ST2, and will only upregulate ST2 when cultured in a combination of IL-33 and IL-2, IL-7, or TSLP (Guo 2009). Given the synergistic effects of IL-33 production due to Dll4/IL-33 treatments, a screen of downstream targets from both of these signaling systems could be conducted. There is also a question of how the VEGF system affects Notch signaling as well, as that may have implications on IL-33 production. Protein quantitation of ST2 would also need to be performed

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