Title: Nuclear IL-33 regulates soluble ST2 receptor and IL-6 expression in primary human arterial endothelial cells and is decreased in idiopathic pulmonary arterial hypertension

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Keywords: IL-33, soluble ST2, pulmonary hypertension, human endothelial cells, nuclear repressor

Corresponding Author: Dr. Stephen John Wort, M.D, Ph.D

Corresponding Author's Institution: Imperial College London

First Author: Dongmin Shao

Order of Authors: Dongmin Shao; Frédéric Perros; Gaetano Caramori; Chao Meng; Peter Dormuller; Pai-Chien Chou; Colin Church; Alberto Papi; Paolo Casolari; David Welsh; Andrew Peacock; Marc Humbert; Ian M Adcock; Stephen John Wort, M.D, Ph.D
Dear Sir,

Re: MS: : BBRC-14-3615 - Nuclear IL-33 regulates soluble ST2 receptor and IL-6 expression in primary human arterial endothelial cells and is decreased in idiopathic pulmonary arterial hypertension by Stephen John Wort and colleagues.

We thank the reviewers for their time spent reading the above manuscript and their detailed and insightful suggestions for how to improve it. We have incorporated their suggestions into the revised manuscript and have provided a point-by-point rebuttal below.

We hope that we have satisfactorily addressed all the Reviewer’s concerns and that the manuscript is now acceptable for publication. All authors have approved submission to your journal and there is no conflict of interest of any authors in relation to the submission.

Yours sincerely,

Dr SJ Wort
Point-by-point response to Reviewer’s comments

The authors found that nuclear IL33 is decreased locally in idiopathic pulmonary arterial hypertension and also that it regulates soluble ST2 receptor and IL-6 expression in primary human arterial endothelial cells which is thought to explain the pathogenesis of IPAH to some extent. The data is new and interesting. Though I think that it is worth being published in this journal, the way how to write the manuscript should be improved and the explanation of the data and conclusion should be more precise (not so broad and vague).

Response to Reviewer comment: We thank the reviewer for his/her comments and appreciate the effort they have made in improving the manuscript.

Major comments

1. It is a new finding that nuclear IL33 decreased locally in IPAH, and the data of "in vitro" study is convincing. However, still I do not think you can conclude that IL-33 has a cause of IPAH. According to your data, in IPAH, nuclear IL33 is reduced in the pulmonary endothelial cells, which lead to the chronic inflammation and thus also lead to IPAH. However, from your data, IL6 was upregulated but CX3CL1/fractalkine is reduced in the cells with siRNA of IL-33, and there are no data on the expression levels of other cytokines written in the 1st page of the introduction. Further, chronic inflammation of pulmonary artery really induces the IPAH? (I think inflammation is observed but this does not mean the causality. Are there any reports on this causality in vivo, or ...). You also wrote in the introduction that nuclear localization of IL33 leads to chronic inflammation in COPD and asthma, and this is confusing...(IL33 nuclear localization leads to inflammation? Anti-inflammation?) Please add some discussion including the limitation of this study.

Response to Reviewer comment: We thank the Reviewer for appreciating the novelty of the results and agree with them that it is too early to conclude if loss of IL-33 is a cause or a consequence of IPAH. We appreciate the Reviewer’s concerns regarding the over-interpretation of the results particularly with respect to the fact that not all the inflammatory mediators measured had enhanced expression following loss of IL-33. In light of this we had modified our conclusions to be more circumspect and emphasise the need for further experiments on primary cells and in animal models. We also agree with the Reviewer regarding the causality of the loss of nuclear IL-33 with respect to disease processes. In light of this we have modified the text to emphasise the association of iPAH with inflammation and that this may not be causal (page 3, para 1; also see answer to point 2 below). In the same vein we have modified the text relating to chronic inflammation in asthma and COPD (page 4, para 1). In addition, we have added a paragraph towards the end of the Discussion section highlighting some of the study limitations (page 12 para 3 and page 14, para 4).

‘...The limitations of the study are centred on the observational nature of the study and the need to define a clear mechanism for the loss of nuclear IL-33 in iPAH tissues. It will also be important to study samples from patients with milder disease and with different types of PAH. The results from these experiments and animal studies using conditional IL-33 nuclear localisation signal deficient mutant mice may also address whether IL-33 is an important driver of disease or merely a consequence of the disease process.....’

2. Please add the discussion on the IL33 KO mice and IPAH. The data on these mice was already reported (Oboki, Keisuke, et al. "IL-33 is a crucial amplifier of innate rather than acquired immunity." Proceedings of the National Academy of Sciences 107.43 (2010): 18581-18586.). Do you think IL-33 could be a cause of IPAH from these data?

Response to Reviewer comment: We thank the Reviewer for pointing out this important manuscript. We have now cited this manuscript and discussed it in relation to our data on page 13 para 1. The section now reads as ‘...A role for both the innate and adaptive immune systems has been proposed in the development of PAH (El Chami H & Hassoun PM. Prog Cardiovasc Dis. 2012 Sep-Oct;55(2):218-28). Results using IL-33 knockout mice has emphasised that whilst IL-33 does not
affect the acquired immune response it is a crucial amplifier of mucosal and systemic innate immunity (Oboki, Keisuke, et al. PNAS 2010; 107: 18581-18586). This suggests that loss of nuclear IL-33 has the potential to act as a driver of PAH pathogenesis. Nuclear IL-33 is expressed in blood vessels of healthy tissues but down-regulated at the earliest onset of angiogenesis during wound healing and when cultured endothelial cells begin wound healing, angiogenesis or start to migrate in response to vascular endothelial growth factor (VEGF), IL-1β and TNFα. (Küchler AM et al., Am J Pathol. 2008 Oct;173(4):1229-42). Furthermore, mechanical stress can also cause a loss of nuclear IL-33 due to release from murine fibroblasts in vitro and in vivo in the absence of cellular necrosis (Kakkar R et al., J Biol Chem. 2012 Feb 24;287(9):6941-8) and thereby regulate structural cell activation.

In addition, cell injury or necrosis can also cause loss of nuclear IL-33 and mediate inflammation and some aspects of disease pathogenesis in vivo (Bessa et al., J Autoimmun. 2014). This is highlighted in mutant mice lacking the IL-33 nuclear localisation signal which demonstrate severe non-resolving inflammation (Bessa et al., J Autoimmun. 2014). Nuclear IL-33 can modulate inflammation through actions on NF-κB where it has been reported to both enhance basal and TNFα-stimulated ICAM-1 and VCAM-1 expression (Choi YS et al., BBRC 2012; 421(2):305-11) and attenuate NF-κB activation (Ali S et al., J Immunol. 2011 Aug 15;187(4):1609-16) and the expression of selected inflammatory genes. Finally, acetic acid-induced colitis is associated with enhanced IL-33 expression which is reduced by anti-oxidants suggesting that oxidative stress may be implicated in the loss of nuclear IL-33. Overall, this suggests that VEGF, a potential driver of iPAH, may work in concert with IL-33 to enhance the inflammation associated with the development of the disease (Archer SL et al., Circulation 2010;121:2045–2066). This does not however directly address the issue as to whether the loss of nuclear IL-33 is a causative factor in iPAH or merely a response to disease pathogenesis. Answering this question will require further experimentation using both conditional IL-33 nuclear localisation signal deficient mutants in models of iPAH and primary human cells.

3. IL33 and reduced locally, but how? Please discuss.

Response to Reviewer comment: We thank the Reviewer for giving us the opportunity to add some discussion regarding the potential mechanisms underlying the loss of nuclear IL-33 in iPAH cells. As stated above in the response to Point 2 we have discussed the possible mechanisms by which nuclear IL-33 may be lost from the nucleus in PAH (page 3 and page 14 para 1).

4. Again, do you think decrease of nuclear localization of IL33 is a cause or consequence of IPAH. Please discuss.

Response to Reviewer comment: In light of the comments above we are happy to assert that loss of nuclear IL-33 is associated with a specific type of inflammation in iPAH but further experimentation is required to address the issue of causality. We have discussed this in more detail in the discussion section as detailed above in the responses to point 2 (page 15). We have also amended the final part of the Discussion section to read ‘...Although loss of nuclear IL-33 is associated with exposure to proinflammatory cytokines, VEGF or a loss of cell-cell contacts, it is unclear what precise role IL-33 has in the pathogenesis of disease. The mechanism for this loss of nuclear IL-33 in iPAH requires further investigation....’.

Due to the word limits of the revised manuscript we cannot be as extensive in our discussion of the important points raised by the Reviewer as we would like. As a result we have tried to be as succinct as possible whilst still highlighting the key issues.

Minor comments
1. In the abstract, it is written that sST2 is enhanced in patients with IPAH. Please add the phrase "in the serum"
Response to Reviewer comment: We thank the Reviewer for pointing out this lack of clarity. We have now added this phrase to the abstract (page 1).

2. Please add a brief explanation of "alarmin" to the readers.
Response to Reviewer comment: We have included a brief definition of the term “alarmin” to the Introduction to aid readers in their understanding (page 3 para 2).

3. In page 12 line 3, the data on exon 2 coding region was not shown.
Response to Reviewer comment: We thank the Reviewer for pointing out this omission. We have now added exon 2 data to Figure 5A in the revised manuscript.
**Highlights**
- Nuclear IL-33 expression is reduced in vascular endothelial cells from PAH patients
- Knockdown of IL-33 leads to increased IL-6 and sST2 mRNA expression
- IL-33 binds homeobox motifs in target gene promoters and recruits repressor proteins
Nuclear IL-33 regulates soluble ST2 receptor and IL-6 expression in primary human arterial endothelial cells and is decreased in idiopathic pulmonary arterial hypertension

Dongmin Shao¹⁺, Frédéric Perros²⁺, Gaetano Caramori³, Chao Meng¹,⁵, Peter Dormuller², Pai-Chien Chou⁴, Colin Church⁶, Alberto Papi³, Paolo Casolari³, David Welsh⁶, Andrew Peacock⁶, Marc Humbert², Ian M Adcock⁴⁺ and Stephen J Wort¹⁺

¹Section of Vascular Biology, National Heart and Lung Institute, Imperial College London, London, UK; ²Faculté de Médecine, Université Paris-Sud, Paris, Clamart, France; ³Dipartimento di Scienze Mediche, Sezione di Medicina Interna e Cardiorespiratoria, Centro Interdipartimentale per lo Studio delle Malattie Infiammatorie delle Vie Aeree e Patologie Fumo-Correlate, University of Ferrara, Ferrara, Italy; ⁴Airways Disease, National Heart and Lung Institute, and ⁵Department of Geriatrics, Renji Hospital, School of Medicine, Shanghai Jiaotong University, Shanghai, China. ⁶Scottish Pulmonary Vascular Unit, University of Glasgow

⁺⁺ These authors contributed equally to this manuscript

Corresponding Author:
Stephen J. Wort.
Section of Vascular Biology,
National Heart and Lung Institute,
Imperial College London,
Dovehouse Street.
London SW3 6LY.
UK
E-mail address: s.wort@imperial.ac.uk
Abstract

Idiopathic pulmonary arterial hypertension (IPAH) is an incurable condition leading to right ventricular failure and death and inflammation is postulated to be associated with vascular remodelling. Interleukin (IL)-33, a member of the “alarmin” family can either act on the membrane ST2 receptor or as a nuclear repressor, to regulate inflammation. We show, using immunohistochemistry, that IL-33 expression is nuclear in the vessels of healthy subjects whereas nuclear IL-33 is markedly diminished in the vessels of IPAH patients. This correlates with reduced IL-33 mRNA expression in their lung. In contrast, serum levels of IL-33 are unchanged in IPAH. However, the expression of the soluble form of ST2, sST2, is enhanced in the serum of IPAH patients. Knock-down of IL-33 in human endothelial cells (ECs) using siRNA is associated with selective modulation of inflammatory genes involved in vascular remodelling including IL-6. Additionally, IL-33 knock-down significantly increased sST2 release from ECs. Chromatin immunoprecipitation demonstrated that IL-33 bound multiple putative homeodomain protein binding motifs in the proximal and distal promoters of ST2 genes. IL-33 formed a complex with the histone methyltransferase SUV39H1, a transcriptional repressor. In conclusion, IL-33 regulates the expression of IL-6 and sST2, an endogenous IL-33 inhibitor, in primary human ECs and may play an important role in the pathogenesis of PAH through recruitment of transcriptional repressor proteins.

Keywords

IL-33, soluble ST2, pulmonary hypertension, human endothelial cells, nuclear repressor
Introduction

Idiopathic pulmonary arterial hypertension (IPAH) is an incurable disease characterized by remodelling of peripheral pulmonary arterial resistance vessels (<100µM diameter), which leads to right ventricular failure and premature death[1]. The pathogenesis of PAH is likely a multi-hit phenomenon, similar to that described in cancer biology. There is evidence for an underlying genetic predisposition and known "hits" such as increased blood flow (Eisenmenger Syndrome), auto-antibodies (connective tissue disease), exposure to drugs (such as appetite suppressants), viruses (HIV) and inflammation, although the exact mechanisms are yet to be defined[2]. There are increased circulating levels of cytokines and chemokines, e.g. IL-6, IL-1, TNFα, CCL2/MCP-1, CCL5/RANTES and CX3CL1/fractalkine in IPAH patients compared to control subjects, some of which have prognostic importance[3-6]. Furthermore, we have recently shown an increased NF-κB expression in the nuclei of endothelial cells (ECs) and macrophages in the lungs of IPAH patients who have undergone lung transplantation, suggesting on-going inflammation during the course of PAH[7].

IL-33, a 31KDa cytokine, that lacks a signal peptide, is a recent addition to the “alarmin” family. This family is comprised of structurally diverse and evolutionarily unrelated multifunctional ‘danger signals’ that are released from damaged epithelial or endothelial cells or are secreted by stimulated leukocytes and epithelia to alert the immune system of cell damage during trauma or infection. It plays a key role in the defence against, or warning about, environmental stresses and infections which cause injury and necrosis of epithelial cells and ECs[8-11]. In contrast, IL-33 release via caspase-1 activation, results in an inactive form of IL-33[12]. Upon cellular release, IL-33 binds to its receptor, the suppressor of tumourogenicity 2 (ST2)
receptor[13-14]. ST2 is a member of interleukin-1 receptor family and undergoes alternative splicing to produce ST2L, a transmembrane receptor, and sST2, a soluble decoy receptor, which lacks the transmembrane and intracellular components and exists in the extracellular space and serum. ST2 receptors are expressed by ECs, myocytes and fibroblasts within the cardiovascular system[15]. IL-33 also has a nuclear localisation signal and contains a homeobox Helix-Turn-Helix DNA binding motif found in many transcription factors[16] which may account for its nuclear localization in epithelial cells and ECs. Increased IL-33 expression has been found in several chronic inflammatory diseases such as asthma[17-18] and chronic obstructive pulmonary disease[19-20]. IL-33 treatment reduces cardiac hypertrophy and fibrosis and improves survival following aortic constriction in wild-type but not in ST2−/− mice. Importantly, administration of sST2 blocked the protective, anti-hypertrophic effect of IL-33[21]. IL-33 was also shown to reduce cardiomyocyte apoptosis, infarct size and fibrosis whilst improving left ventricular function[22]. IL-33 has a protective effect, by promoting Th1 to Th2 T-cell cytokine skewing, in ApoE−/− model of atherosclerosis which is reversed by sST2[23]. Interestingly, higher serum sST2 levels are predictive of increased mortality in heart failure[24], various respiratory diseases[25] and PAH[26].

Despite these observations IL-33 has never convincingly been detected in the plasma of humans with cardiovascular conditions. The relative importance of signalling via exogenous IL-33/ST2L and intracellular “nuclear” IL-33 signalling is also unclear. However, in conditions where necrosis and direct injury to cells is not present (e.g. vascular remodelling) nuclear signalling may be more important. The aim of this study was to investigate the expression of the IL-33/ST2 nexus in IPAH
lung, plasma and ECs. Furthermore, we investigated whether nuclear IL-33 regulates the expression of key inflammatory mediators known to be involved in pulmonary vascular remodelling and that of sST2.
Materials and methods

Patients with IPAH and control subjects

The clinical details of IPAH patients and the control subjects used in this study have already been described[7].

Cell culture:

Normal human pulmonary arterial endothelial cells (HPAECs) and lung microvascular endothelial cells (HLMVECs) were purchased from Lonza. HLMVECs from IPAH patients were isolated as described previously[6]. Cells were maintained in EGM-2 and used at passages 3-8.

Immunohistochemistry

Paraffin sections of human lung tissue from 10 IPAH patients and 11 healthy donors were prepared and immunohistochemistry performed as described previously[7] using an anti-human IL-33 antibody (Enzo, Nessy-1, 1:100).

Small RNA interference

Small RNA interference (siRNA) was carried out as described previously[27]. Briefly, HPAECs were seeded onto 6-well plates at 2x10⁵ cells/well. After 24 hours, cells were transfected with smart pool siRNA for IL-33 or negative control siRNA (Fisher Scientific/Dharmacon) respective for 5 hours. After transfection, cells were cultured in EGM-2 for a further 72 hours.

Real-time quantitative PCR
Total RNA were isolated from human lung or HPAECs using Qiagen mini-RNA isolation kit according to manufacturer’s instructions (Qiagen). Total RNA (1µg) was transcribed to cDNA using L-AMV reverse transcriptase (Invitrogen) according to manufacturer’s instructions. Real-time quantitative PCR (QPCR) was performed using SYBR green master mix (Qiagen) on a Corbett Rotor-Gene6000 (Corbett). The relative expression of target genes was quantified using the ΔΔCt method normalized to housekeeping genes (β-actin or GAPDH) as described previously[7, 28]. All primers were purchased from Qiagen (QuantiTect Primer Assay):

- Hs_IL6_1_SG     QT00083720
- Hs_IL8_1_SG     QT00000322
- Hs_CCL5_1_SG(RANTES) QT00090083
- Hs_CX3CL1_1_SG(Fractalkine) QT00098490
- Hs_IL1RL1_2_SG QT01742881
- Hs_DEFB4_4_SG QT01852277
- Hs_MMP9_1_SG QT00040040
- Hs_CTSB_1_SG QT00088641
- Hs_CTS1L1_2_SG QT01664978
- Hs_RELA_1_SG QT01007370
- Hs_EDN1_1_SG QT00088235
- Hs_CCL3_2_SG(MIP-1) QT01008063
- Hs_TGFB1_1_SG QT00000728
- Hs_PDGFB_1_SG QT00001260
- Hs_VEGFA_6_SG QT01682072
- Hs_GDF2_1_SG(BMP-9) QT00210462
Western blot

Cell lysates were prepared as described previously[27] and were precipitated using cold acetone, and air-dried. The resulting pellets were dissolved in 50ul 1XSDS sample buffer and cleared by centrifugation. Samples were separated on SDS-PAGE and transferred to nitrocellulose membranes (GE Healthcare). Membranes were probed with Nessy-1 (1:1000) and loading quantified using an antibody against human β-actin (Cell Signaling, 1:2000) to confirm equal protein loading. Membranes were developed using ECL (GE Healthcare).

ELISA

ELISA kits for human IL-33 (Enzo) and sST2 (R&D) were used to measure concentrations within human serum and cell culture medium according to manufacturer’s instructions.

Co-immunoprecipitation

Co-immunoprecipitation of nuclear proteins was performed as described previously[28] using Nessy-1 (1:100) for immunoprecipitation and detection of SUV39H1 (Millipore, 1:5000) by Western blotting.

Chromatin Immunoprecipitation (ChIP) analysis

HPAECs were seeded onto 10cm tissue culture dishes at 5x10^5/dish and grown to confluence (approximately 2x10^6 cells/dish for one ChIP experiment). Cells were fixed and ChIP analysis was performed using EZ-CHIP™ kit (Millipore) according to manufacturer’s instruction. The binding of IL-33 to the distal and proximal promoter was analysed by RT-PCR using primers designed close to the punitive flat motifs up
to 1kb from transcriptional starting sites (TSS). The sequences of primers are as follows:

Proximal promoter
-977/-851: 5’-TCTGTGCCTCAGTGCTTTG&5’-TGTCCTCTATGCCAGACACAGT
-744/-621: 5’-CATGATAGGGGTATCGCAACT&5’-CCTCAAGGGGAGTGACAAAG
-343/-252: 5’-GCCAAATGAGGAGTCAGGGA&5’-ACCCCGATATTGGGACACTT

Distal promoter
-983/-881: 5’-TGGATAGCATTCCCTTACGTT&5’-TCTTCCACGCTTGCTTGACTT
-12/101: 5’-TGGGAGGTTTTAAGAGAGAG&5’-CCTCAACTTTCTGCCACAG
-636/-521: 5’-TTTCCCTTGTACTGGCTGCT&5’-CCAGGCTCTGTGTGGACAGTAA

Exon 2: 5’-AATCGCCTCATGTGTGGTG&5’-GATCCAAAACCCCATTTCTGTT
Results and Discussion

Reduced pro-IL-33 expression is found in lungs from IPAH patients

In human lung tissue obtained from normal non-smoking subjects, IL-33 was found to be predominantly expressed in ECs in pulmonary arterioles (Figure 1A). Consistent with previous reports in other tissues [29-30], IL-33 resided predominantly in the nuclei of endothelial cells. In contrast, nuclear staining was reduced in intensity or absent in the lung samples obtained from IPAH patients (a representative example is shown in Figure 1B). In addition there was a highly significant 50% reduction in IL-33 mRNA expression in IPAH lung tissue compared to control lung (0.369±0.025, N=10 vs. 0.761±0.059, N=14, p<0.001; Figure 1D). During the pathogenesis of IPAH, the pulmonary arterial vasculature undergoes an extensive remodelling process, characterised by proliferation of ECs, smooth muscle cells and fibroblasts. Furthermore, vascular cells from IPAH patients are known to maintain a proliferative phenotype in vitro [31-33]. These results are consistent with previous findings demonstrating that IL-33 expression is known to be inversely related to cellular proliferation. For instance, nuclear expression of IL-33 increases with increasing confluence in cell culture, but is down-regulated at the onset of angiogenesis during wound healing or with migration. In addition, TNFα exposure leads to a rapid loss of nuclear IL-33 and subsequent activation of these cells [29].

Up-regulation of sST2 release in serum and culture medium of HLMVECs from IPAH patients

IL-33 and sST2 have been implicated in several cardiovascular diseases [11, 34] and sST2 is increased in the plasma of PAH patients [26]. We found no significant increase in serum IL-33 concentrations in IPAH patients compared to control
subjects (75.0±8.56pg/ml, n=8 vs. 67.5±2.8pg/ml, n=6, p=0.476, two-tailed t-test) (Figure2A). In contrast, we observed a significant increase in serum sST2 in IPAH patients (15.7±4.2ng/ml, n=8 vs. 6.2±1.6ng/ml, n=6 p=0.0485, two-tailed t-test) (Figure2B). Importantly, the serum levels of sST2 (ng/ml) were a log-fold greater than those of IL-33 (pg/ml). In addition, there was no significant correlation between serum IL-33 and sST2 levels (r= 0.06, p=0.882). Because serum proteins may come from various sources, we isolated HLMVECs from lungs of IPAH patients and compared them to HLMVECs obtained from the control subjects. The release of IL-33 was just above the limit of detection in all samples (3.7±0.4pg/ml, n=4 vs. 3.8±0.6pg/ml, n=6, p=0.9499) (Figure2C). In contrast, sST2 release into the medium of HLMVECs derived from IPAH patients was significantly higher than that seen from control cells (1.4±0.8ng/ml n=4 vs. 0.3±0.03ng/ml n=6, p=0.0190) (Figure2D).

Dogma indicates that in vitro, IL-33 levels need to be at ng/ml level to activate ST2 receptors. However, IL-33 is barely detectable in serum or in cell culture medium in many studies. These observations raise an important question whether sST2 functions solely as a decoy receptor. sST2 can bind to breast cancer cells and enhance ErbB2/HER2-mediated cellular motility[35]. The behaviour of vascular cells in IPAH has been likened to mitogenic cells[36-38] and excessive expression or activity of growth factors including platelet-derived growth factor (PDGF), epidermal growth factor (EGF) and vascular endothelial growth factor (VEGF) contributes to pulmonary vascular remodelling. In keeping with these data, inhibition of EGF and PDGF receptors has beneficial effects on haemodynamic, remodelling, and survival in experimental PAH[39-40]. It is therefore possible that sST2 may also function as a co-factor to so far unrecognised growth factor receptors and, thereby, contribute to
excessive pulmonary vascular and right ventricular remodelling associated with IPAH.

Our results are consistent with the hypothesis that endothelial activation or dysfunction in lung arterial vessels is being associated with a loss of nuclear IL-33 and an increase in sST2. To understand the mechanism of which IL-33 may potentially contribute to the IPAH progression, we examined the effect of reducing nuclear IL-33 using siRNA and determined, using ChIP analysis whether IL-33 could bind to the promoter region of sST2.

**Regulation of gene expression by IL-33**

The role of nuclear IL-33 remains unclear as it can both enhance or reduce NF-κB activity in a gene dependent manner[41-42] and also modulate gene expression via binding to putative homeobox motifs[30]. Knock-down of IL-33 using siRNA in confluent HPAECs was very efficient with almost complete suppression of IL-33 expression (Figure3A). This selectively modulated the expression of NF-κB-dependent genes with significant up-regulation of IL-6 mRNA and down-regulation of CCL5/RANTES and CX3CL1/fractalkine mRNAs whilst other inflammatory genes were not affected (Figure3B&C). The expression of sST2, unknown to be regulated by NF-κB, was significantly increased by IL-33 knock-down. Using a focused RT-PCR array limited the number of IL-33 regulated genes that could be identified in this study. A combination of microarray/deep sequencing and/or ChIP-seq is required to reveal the full scope of IL-33 regulated genes.
Bioinformatic analysis indicated that all the affected genes contain multiple homeobox binding motifs in their promoters (Table 1 and Figure 4A). The human ST2 gene has two TSSs located in exon 1a and exon 1b with some evidence for cell-dependent promoter usage. For example, mast cells predominantly use the distal promoter while fibroblasts and HUVECs predominantly use the proximal promoter [43-45]. Using ChIP analysis, we were able to detect selective binding of IL-33 at homeobox sites in both proximal and distal promoters, but not the exon2 coding region (Figure 4B&C). Homeodomain proteins generally act in complex with other transcription factors such as NF-κB [46] to increase target specificity and also play a key role in innate immunity [47]. A role for both innate and adaptive immune systems has been proposed in the development of PAH [48]. Results using IL-33 knockout mice have emphasised that whilst IL-33 doesn’t affect the acquired immune response, it is a crucial amplifier of mucosal and systemic innate immunity [49]. This suggests that loss of nuclear IL-33 has the potential to act as a driver of PAH pathogenesis. Nuclear IL-33 is expressed in blood vessels of healthy tissues but down-regulated at the onset of angiogenesis, during wound healing and when cultured ECs begin wound healing, angiogenesis, or start to migrate in response to VEGF, IL-1β and TNFα [29]. Furthermore, mechanical stress also cause a loss of nuclear IL-33 due to release from fibroblasts in vitro and in vivo, in the absence of cellular necrosis [50] and thereby regulate structural cell activation. Additionally, cell injury or necrosis can also cause loss of nuclear IL-33 and mediate inflammation and some aspects of disease pathogenesis in vivo [51]. This is highlighted in mutant mice lacking IL-33 nuclear localisation signal which demonstrate severe non-resolving inflammation [51]. Nuclear IL-33 can modulate inflammation through actions on NF-κB where it can both enhance basal and TNFα-
stimulated ICAM-1 and VCAM-1 expression[42] and attenuate NF-κB activation and the expression of selected inflammatory genes[41]. Finally, acetic acid-induced colitis is associated with enhanced IL-33 expression which is reduced by antioxidants suggesting that oxidative stress may be implicated in the loss of nuclear IL-33.

Overall, this suggests that VEGF, a potential driver of IPAH, may work in concert with IL-33 to enhance the inflammation associated with the development of IPAH[52]. This doesn’t however directly address the issue whether the loss of nuclear IL-33 is a causative factor in IPAH or merely a response to disease pathogenesis. Answering this question will require further experimentation using both conditional IL-33 nuclear localisation signal deficient mutants in models of PAH and primary human cells.

Previous reports have suggested that IL-33 has transcriptional repressor properties in HEK293 cells associated with the recruitment of histone methyltransferase SUV39H1[30]. We were able to confirm that IL-33 was in complex with SUV39H1 in HPAECs using co-immunoprecipitation (Figure4D).

The limitations of the study are centred on the observational nature and the need to define a clear mechanism for the loss of nuclear IL-33 in IPAH. It will also be important to study samples from patients with milder and with different types of PAH. These experiments and animal studies using conditional IL-33 nuclear localisation signal deficient mutants may also address whether IL-33 is an important driver of IPAH or merely a consequence of the process.

In summary, we have demonstrated a marked loss of nuclear IL-33 in lung arterial endothelial cells from IPAH patient without significant release from these cells. Knock-down of IL-33 is associated with the induction and release of both IL-6 and
sST2. Our data suggests that IL-33 acts as a nuclear suppressor to reduce sST2 expression by binding to homeobox regions and potentially recruiting transcriptional repressor proteins e.g. SUV39H1. Although loss of nuclear IL-33 is associated with exposure to proinflammatory cytokines, VEGF or loss of cell-cell contacts, it is unclear what precise role IL-33 has in the pathogenesis of IPAH. The mechanism for this loss of nuclear IL-33 in IPAH requires further investigation. Our data also clearly shows that serum sST2 levels should be measured at the same time as IL-33 and that serum sST2 may be a useful biomarker of vascular remodelling in IPAH and other cardiovascular conditions.
Acknowledgements

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References


[22] K. Seki, S. Sanada, A.Y. Kudinova et al., Interleukin-33 prevents apoptosis and improves survival after experimental myocardial infarction through ST2 signaling, Circ Heart Fail 2 (2009) 684-691.


Figure legends:

Figure 1: IL-33 expression in human lung. Immunohistochemical staining of IL-33 in paraffin embedded sections of lung tissue from a human healthy control subjects (A), a patient with IPAH (B), and an isotype control (C). Results are representative of 11 healthy subjects and 10 patients with IPAH. QPCR analysis of IL-33 mRNA levels in healthy controls (n=14) and IPAH (n=10) lung tissues (D). Data are presented as mean±SD, **P< 0.0001, two-tailed t-test.

Figure 2: ELISA detection of IL-33 (A&C) and sST2 (B&D) levels in serum (A&B) and culture medium (C&D) from HLMVEC from healthy donors and IPAH patients. Data are presented as mean±SEM. For IPAH plasma samples, n=8, whereas control plasma samples, n=6. For IPAH cell culture supernatant, n=4, and for control cell culture supernatant, n=6. *p<0.05

Figure 3: Effects of IL-33 knock-down on gene expression in HPAEC. Western blot analysis demonstrated effective knock-down of IL-33 expression by siRNA (A) (representative of three independent experiments). IL-33 knock-down selectively affected gene expression (B&C). Data are presented as mean±SEM n=3, *p<0.05; **p<0.01, ***p<0.001 two-way ANOVA.

Figure 4: IL-33 binds to ST2 promoters and recruits repressive co-factor SUV39H1. The punitive Flat motifs in ST2 promoters (A) are highlighted in red and the sequences used for PCR primers including Exon2 are underlined. ChIP analysis shows IL-33 binding to selective regions of the ST2 distal (B) and proximal (C) promoters. Co-immunoprecipitation experiments indicate that IL-33 forms a complex
with the repressive co-factor SUV39H1 (D). Data were presented as mean±SEM n=3, *p<0.05, **p< 0.01, ***p<0.001, two-way ANOVA.
Table 1: Multiple punitive Flat motifs found in genes affected by IL-33 Knockdown

<table>
<thead>
<tr>
<th>Gene Name (Protein Name)</th>
<th>Punitive Flat Motif</th>
<th>Orientation</th>
<th>Accession No.</th>
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<tr>
<td>IL-6</td>
<td>-298 TGACA -302</td>
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<td>-143TTAATA -138</td>
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<td>CCL-5 (RANTES)</td>
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<td>-696 TAATTG -701</td>
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<td>1kb upstream CX3CL1 transcription start (22519)</td>
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<td>CTSL (Cathepsin L)</td>
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<td>-494 TAAGTG-489</td>
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Figure 1

- **A**: Health
- **B**: IPAH
- **C**: Isotype control

**D**: Box plot showing the ratio of IL-33:GAPDH mRNA. The graph indicates a significant difference between the control and IPAH groups, with a p-value of ***.
Figure 2

(A) IL-33 (pg/ml) in Serum
- IPAH
- Control

(B) sST2 (ng/ml) in Serum
- IPAH
- Control

(C) IL-33 (pg/ml) in Cell media
- IPAH
- Control

(D) sST2 (ng/ml) in Cell media
- IPAH
- Control
Figure 3

A

Negative control  IL-33 smartpool

31kDa  IL-33  actin
45kDa

B

mRNA level (fold of change)

control siRNA  IL-33 smart pool

IL-6  RANTES  Fractalkine  sST2

***  *  **  ***

C

mRNA level (fold of change)

control siRNA  IL-33 siRNA

IL-8  EDN1  TGFβ1  PDGFβ1  VEGFA  BMP-9  p65  CTSB  MMP9  CTSL
Figure 4

**A**

**Distal promoter**

-1001 actagtttgt catcaatacg tagacactcc tccataggtt actgtatttaa tattgacaac
-941 aataccttt caacagagaga gaggttagag aatgtgcaac aagtcaagaag gctgggaagaag
-881 ggaaaactag gctgtgctct cccctaaacct tgtcctcttt tatattagg gagaaccttg
-821 ctagttggtactttgtattagttg aagattagttct ctaataaat ttagataat ttagttaggt ttaatcagaaat ttcctaatgt ggctcataat ccctattaca
-761 ttaataggtgt ttaatcagaaat ttcctaatgt ggctcataat ccctattaca
-701 accaccttag ataggctatc tccggccagt ggaahagacc caggtataga cactttggtat
-641 cctggaatctctggtctct tgggtaaggt aatctctcttc tccatgcctctc
tgatggtgga tggaggtggag cagacacact cagagtctgctgc gctgtctgtg
-581 ctgatactgtctgatctatgcgtc aggagagtgt tttttttaaa aatcctcttc
-521 ggtcatactt taagcactatc gatgtgctctct gatagtctctct ctaataaat ttagataat ttagttaggt ttaatcagaaat ttcctaatgt ggctcataat ccctattaca
-461 agttacacttc atgtgattgtag aatgtgcaac aagtcaagaag gctgggaagaag
-401 caggtgagtag tctatgaggtggag cagagactt ccagctaggt gcaagactta cctggaatctctggtctct
-341 tgggtaaggt aatctctcttc tccatgcctctc
tglygggttagt t Tataggttttattt ctaataaat ttagataat ttagttaggt ttaatcagaaat ttcctaatgt ggctcataat ccctattaca
-281 aatcctcttc aatgctgagag caacatctag gtttagagga gtttgtgctctg
-221 aggagactt ccagctaggt gcaagactta cctggaatctctggtctct
-161 ataaattttt ttagaagag aatgtgcaac aagtcaagaag gctgggaagaag
-101 gaaactgca gtaaactttt tttttttttt ctaaatgcct cctggaatctctggtctct
-41 ggttggtgctgc gatgtgctctct gatagtctctct ctaataaat ttagataat ttagttaggt ttaatcagaaat ttcctaatgt ggctcataat ccctattaca
-19 ttagatctttt caggtgagtag tctatgaggtggag cagagactt ccagctaggt gcaagactta cctggaatctctggtctct
-9 cagaaagttg aggaaagtcac tcatgaa

**Proximal promoter**

-1035 gttgcaattcc tagcttcccc ccttaatgag taaatcctc tccataaat tttttttttt cctgtctttc tccctcattc
ggtgagata tagagtagtt ctaataaat ttagataat ttagttaggt ttaatcagaaat ttcctaatgt ggctcataat ccctattaca
-975 tccctcattc tctggctttc tccataaat tttttttttt cctgtctttc tccctcattc
ggtgagata tagagtagtt ctaataaat ttagataat ttagttaggt ttaatcagaaat ttcctaatgt ggctcataat ccctattaca
-915 ggtgagata tagagtagtt ctaataaat ttagataat ttagttaggt ttaatcagaaat ttcctaatgt ggctcataat ccctattaca
-855 ggtgagata tagagtagtt ctaataaat ttagataat ttagttaggt ttaatcagaaat ttcctaatgt ggctcataat ccctattaca
-795 ggtgagata tagagtagtt ctaataaat ttagataat ttagttaggt ttaatcagaaat ttcctaatgt ggctcataat ccctattaca
-735 ggtgagata tagagtagtt ctaataaat ttagataat ttagttaggt ttaatcagaaat ttcctaatgt ggctcataat ccctattaca
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-75 ggtgagata tagagtagtt ctaataaat ttagataat ttagttaggt ttaatcagaaat ttcctaatgt ggctcataat ccctattaca
-15 ggtgagata tagagtagtt ctaataaat ttagataat ttagttaggt ttaatcagaaat ttcctaatgt ggctcataat ccctattaca

**B**

**Distal**

<table>
<thead>
<tr>
<th>Fold of change</th>
<th>mlG</th>
<th>αIL-33(Nessy-1)</th>
</tr>
</thead>
<tbody>
<tr>
<td>-983/-881</td>
<td>3.0</td>
<td>2.0</td>
</tr>
<tr>
<td>-635/-521</td>
<td>2.5</td>
<td>2.0</td>
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<tr>
<td>-12/-101</td>
<td>2.0</td>
<td>2.0</td>
</tr>
<tr>
<td>exon2</td>
<td>1.0</td>
<td>1.0</td>
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**C**

**Proximal**

<table>
<thead>
<tr>
<th>Fold of change</th>
<th>mlG</th>
<th>αIL-33(Nessy-1)</th>
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<tbody>
<tr>
<td>-343/-252</td>
<td>3.0</td>
<td>3.0</td>
</tr>
<tr>
<td>-744/-621</td>
<td>2.5</td>
<td>2.0</td>
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<tr>
<td>-977/-851</td>
<td>2.0</td>
<td>2.0</td>
</tr>
<tr>
<td>Exon2</td>
<td>1.0</td>
<td>1.0</td>
</tr>
</tbody>
</table>

**Exon2 (NM_016232.4)**

121 ggtgagata tagagtagtt ctaataaat ttagataat ttagttaggt ttaatcagaaat ttcctaatgt ggctcataat ccctattaca
181 gttggtgat ttgctctgtc tctggaatctctggtctct
241 taccataactc tgcttttgat tgaataagat ttagggtttt ttagcttttag cattttcac

**D**

-75 mlG1 Nessy-1
IP: IL-33
WB: Suv39H1