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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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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.

1. Introduction

Idiopathic pulmonary arterial hypertension (IPAH) is an incurable disease characterised by remodelling of peripheral pulmonary arterial resistance vessels (<100 μM 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 31 KDa 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

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'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 localisation 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.

2. Materials and methods

2.1. 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].

2.2. 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.

2.3. 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).

2.4. Small RNA interference

Small RNA interference (siRNA) was carried out as described previously [27]. Briefly, HPAECs were seeded onto 6-well plates at 2×10^5 cells/well. After 24 h, cells were transfected with smart pool siRNA for IL-33 or negative control siRNA (Fisher Scientific/Dhammacon) respective for 5 h. After transfection, cells were cultured in EGM-2 for a further 72 h.

2.5. 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 (1 μg) 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 normalised 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_CTSL1_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

2.6. 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 50 μl 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 Signalling, 1:2000) to confirm equal protein loading. Membranes were developed using ECL (GE Healthcare).

2.7. 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.

2.8. 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.

2.9. Chromatin immunoprecipitation (ChIP) analysis

HPAECs were seeded onto 10 cm tissue culture dishes at 5×10^5/dish and grown to confluence (approximately 2×10^6 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 1 kb from transcriptional starting sites (TSS). The sequences of primers are as follows:

2.9.1. Proximal promoter

-977/-851:5′-TCTGTGCCTCAGTGTCCTTG&5′-TGTCCTCTATGC CAGACACAGT
-744/-621:5′-CATGATAGGGTCATCGCAACT&5′-CCTCAAGGGGA GTGACAAAG
-343/-252:5′-GCCAAATGAGGAGTCAAGGA&5′-ACCCCGATATTG GGACACTT

2.9.2. Distal promoter

-983/-881:5′-TGGATAGCATCCTCCATAGGTT&5′-TCTTCCCAGCT GCTTGACTT
-12/101:5′-TGGGAGGTTTTTAAAGAGAGG&5′-CCTCAACTTTCTG CCCACAG
-636/-521:5′-TTTCCCTTGTACTGGCTGCT&5′-CCAGGCTCTGTGT GCAGTAA
Exon 2:5′-AACTGCCTCATGTGTGGTGA&5′-GATCCAAAACCCCAT TCTGTT

3. Results and discussion

3.1. 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 (Fig. 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 Fig. 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, $N = 14$, $p < 0.001$; Fig. 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].

3.2. 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 ± 8.56 pg/ml, $n = 8$ vs. 67.5 ± 2.8 pg/ml, $n = 6$, $p = 0.476$, two-tailed t-test) (Fig. 2A). In contrast, we observed a significant increase in serum sST2 in IPAH patients (15.7 ± 4.2 ng/ml, $n = 8$ vs. 6.2 ± 1.6 ng/ml, $n = 6$ $p = 0.0485$, two-tailed t-test) (Fig. 2B). 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

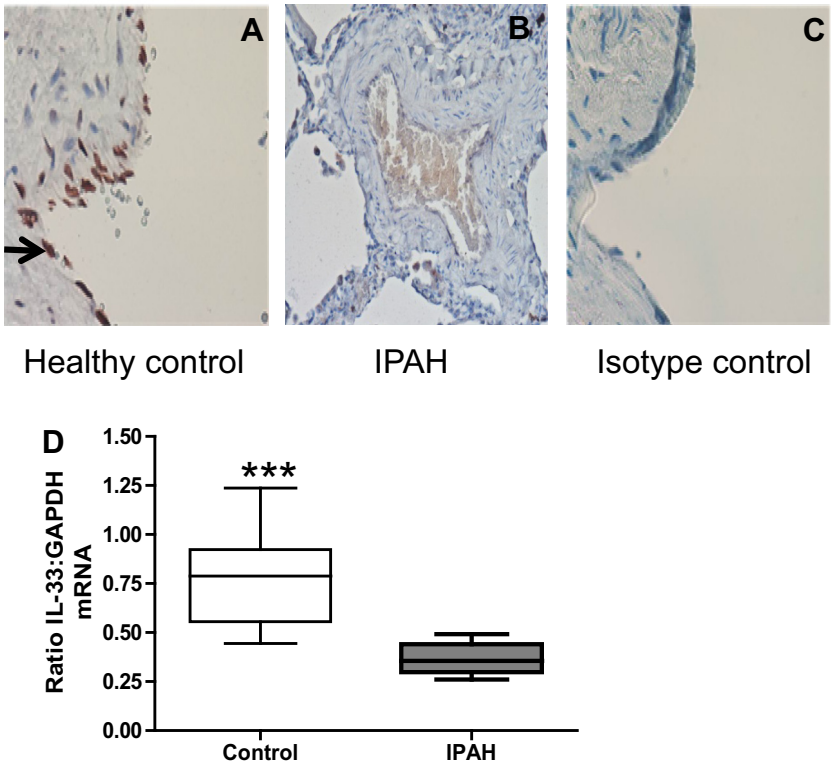

Fig. 1. IL-33 expression in human lung. Immunohistochemical staining of IL-33 in paraffin embedded sections of lung tissue from a human healthy control subjects (A), a patient with IPAH (B), and an isotype control (C). Results are representative of 11 healthy subjects and 10 patients with IPAH. QPCR 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.

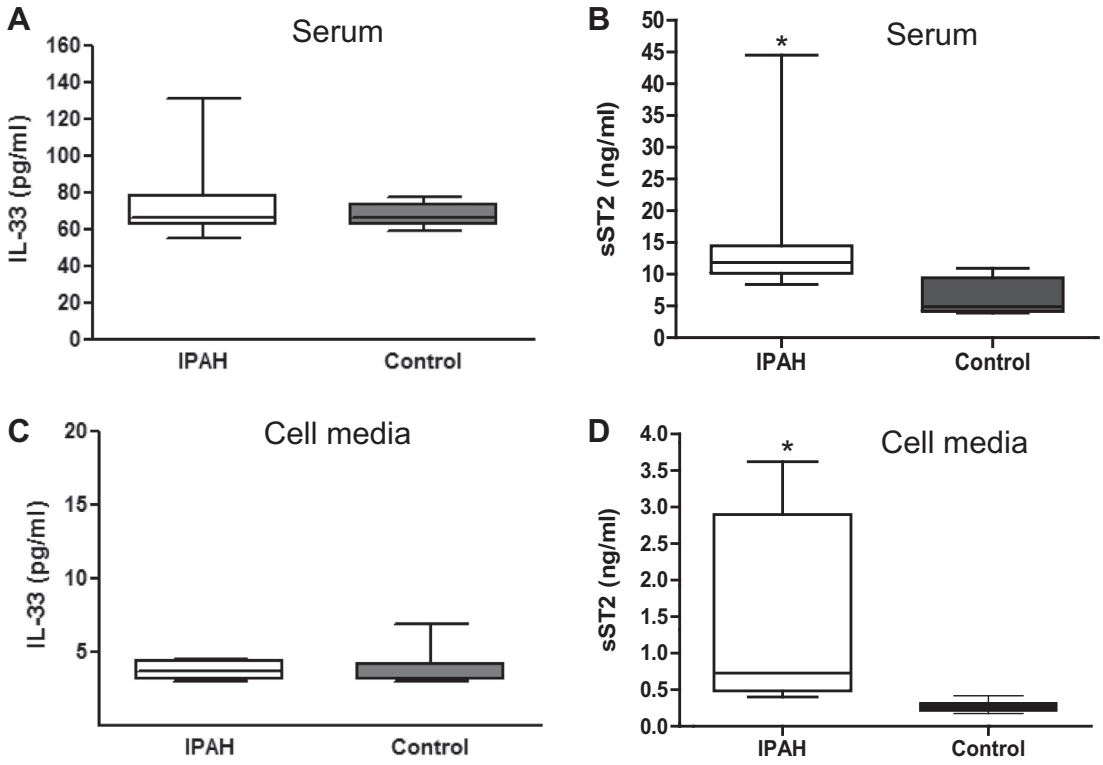

Fig. 2. ELISA detection of IL-33 (A and C) and sST2 (B and D) levels in serum (A and B) and culture medium (C and D) from HLMVEC from healthy donors and IPAH patients. Data are presented as mean ± SEM. For IPAH plasma samples, $n = 8$, whereas control plasma samples, $n = 6$. For IPAH cell culture supernatant,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

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 ± 0.4 pg/ml, $n = 4$ vs. 3.8 ± 0.6 pg/ml, $n = 6$, $p = 0.9499$) (Fig. 2C). In contrast, sST2 release into the medium of HLMVECs derived from IPAH patients was significantly higher than that seen from control cells (1.4 ± 0.8 ng/ml $n = 4$ vs. 0.3 ± 0.03 ng/ml $n = 6$, $p = 0.0190$) (Fig. 2D).

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.

3.3. 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 (Fig. 3A). 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 (Fig. 3B and 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 Fig. 4A). The human ST2 gene has two TSSs located in exon1a and exon1b with some evidence for cell-dependent promoter usage. For example, mast cells predominantly use the distal promoter whilst 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 (Fig. 4B and 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

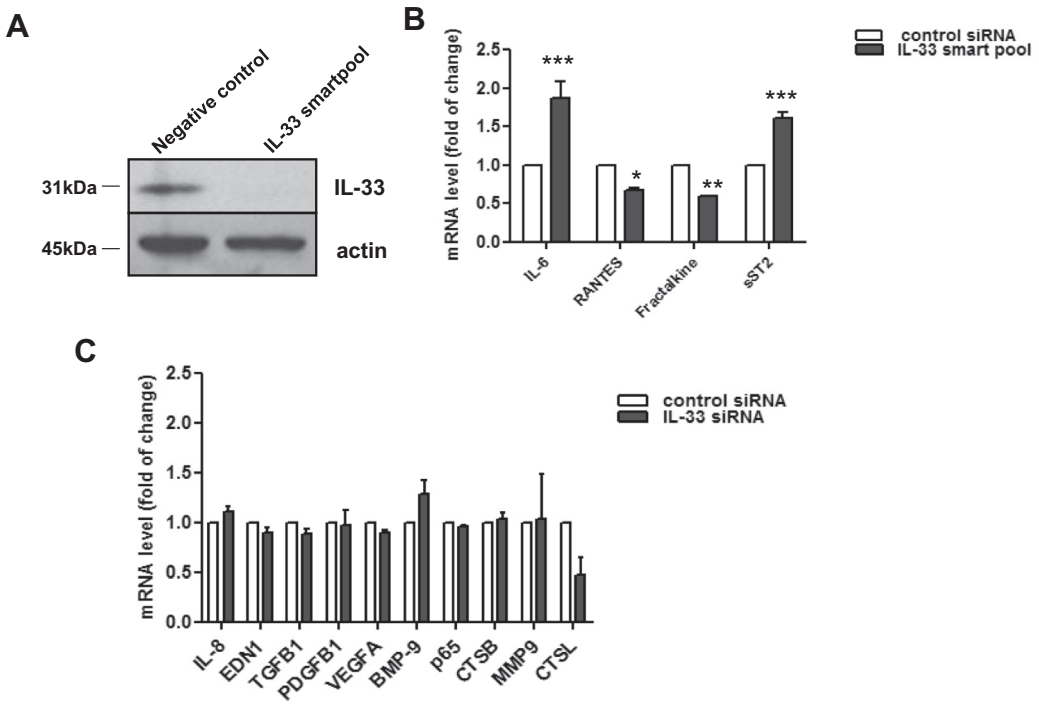

Fig. 3. Effects of IL-33 knock-down on gene expression in HPAEC. Western blot analysis demonstrated effective knock-down of IL-33 expression by siRNA (A) (representative of three independent experiments). IL-33 knock-down selectively affected gene expression (B and C). Data are presented as mean ± SEM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

Table 1
Multiple punitive flat motifs found in genes affected by IL-33 knockdown.

Gene name	Putative flat motif	Orientation	Accession no.
IL-6	-298 TGACA -302	Reverse	AF048692
	-143 TTAATA -138	Forward	
	-82 TGATA -78	Reverse	
CCL-5/RANTES	-825 TGACA -821	Forward	AB023652
	-696 TAATTG -701	Reverse	
	-221 TGACA -225	Reverse	
	-85 TAAGTG -90	Reverse	
	-84 TGATA -80	Forward	
	-100 TGACA 96	Reverse	
CX3CL1/fractalkine	-400 TGACA -404	Reverse	AC004382
	-306 TAAGTG -311	Reverse	1 kB upstream CX3CL1
	-195 TGACA -191	Forward	Transcription start site (22519)
CTSL/cathepsin L	-1785 TGACA -1789	Reverse	AF163338
	-1184 TAAGTG -1189	Reverse	
	-1146 TGATA -1150	Reverse	
	-1055 TAATTA -1050	Forward	
	-991 TGACA -987	Forward	
	-947 TAATTA -942	Forward	
	-935 TTAATA -930	Forward	
	-855 TGACA -851	Forward	
	-841 TGATA -845	Reverse	
	-792 TAATCC -787	Forward	
	-724 TGACA -728	Reverse	
	-628 TGACA -624	Forward	
	-494 TAAGTG -489	Forward	

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

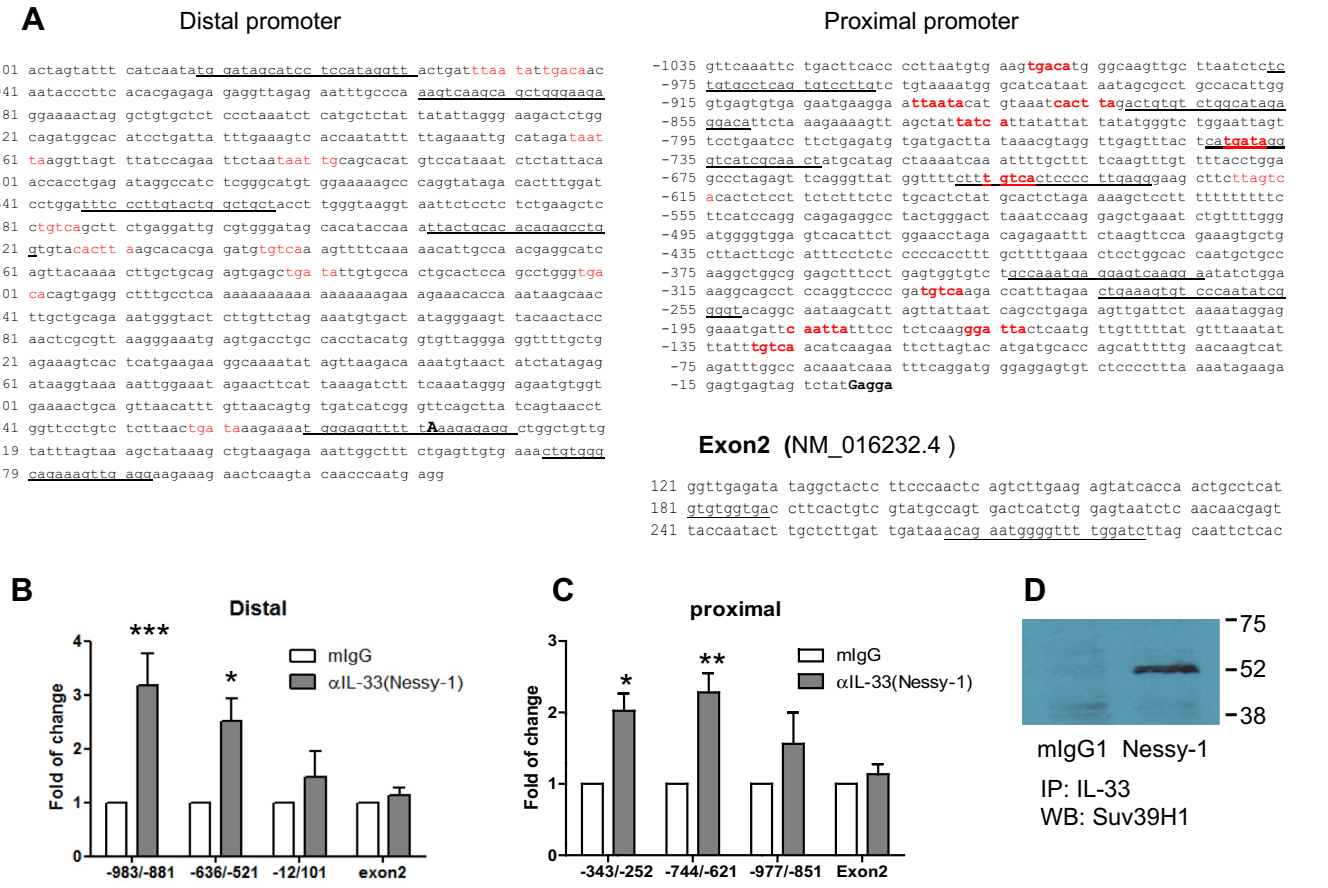

Fig. 4. IL-33 binds to ST2 promoters and recruits repressive co-factor SUV39H1. The punitive Flat motifs in ST2 promoters (A) are highlighted in red and the sequences used for PCR primers including Exon2 are underlined. ChIP analysis shows IL-33 binding to selective regions of the ST2 distal (B) and 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. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

[41]. 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 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 (Fig. 4D).

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 pro-inflammatory 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.

Acknowledgments

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