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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

Cover Letter

Dr Naoyuki Taniguchi, MD, PhD
Editor
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16-06-2014

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 you 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 '*….Athough 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 (page1).

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 Shao[1+], Frédéric Perros[2+], Gaetano Caramori[3], Chao Meng[1,5], Peter Dormuller[2], Pai-Chien Chou[4], Colin Church[6], Alberto Papi[3], Paolo Casolari[3], David Welsh[6], Andrew Peacock[6], Marc Humbert[2], Ian M Adcock[4*] and Stephen J Wort[1*]

[1]Section of Vascular Biology, National Heart and Lung Institute, Imperial College London, London, UK; [2]Faculté de Médecine, Université Paris-Sud, Paris, Clamart, France;[3]Dipartimento 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; [4]Airways Disease, National Heart and Lung Institute, and [5]Department of Geriatrics, Renji Hospital, School of Medicine, Shanghai Jiaotong University, Shanghai, China. [6]Scottish 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 $2x10^5$cells/well. After 24 hours, cells were transfected with smart pool siRNA for IL-33 or negative control siRNA (Fisher Scientific/Dhammacon) 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_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

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 Immunoprecipitaion (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^{TM} 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’-TCTGTGCCTCAGTGTCCTTG&5’-TGTCCTCTATGCCAGACACAGT

-744/-621:5’-CATGATAGGGTCATCGCAACT&5’-CCTCAAGGGGAGTGACAAAG

-343/-252:5’-GCCAAATGAGGAGTCAAGGA&5’-ACCCCGATATTGGGACACTT

Distal promoter

-983/-881:5’-TGGATAGCATCCTCCATAGGTT&5’-TCTTCCCAGCTGCTTGACTT

-12/101:5’-TGGGAGGTTTTTAAAGAGAGG&5’-CCTCAACTTTCTGCCCACAG

-636/-521:5’-TTTCCCTTGTACTGGCTGCT&5’-CCAGGCTCTGTGTGCAGTAA

Exon 2:5’-AACTGCCTCATGTGTGGTGA&5’-GATCCAAAACCCCATTCTGTT

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 (Figure1A). 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 Figure1B). 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, $p<0.001$; Figure1D). 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 (Table1 and Figure4A). 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 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 (Figure4B&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 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 (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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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

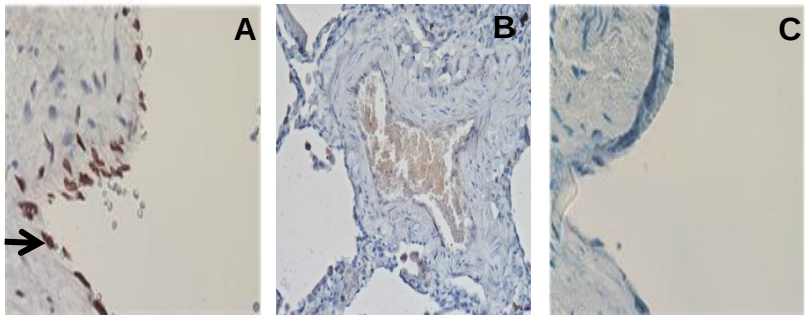
with the repressive co-factor SUV39H1 (D). Data were presented as mean±SEM n=3, *p<0.05, **p< 0.01, ***p<0.001, two-way ANOVA.

Table. 1 Multiple punitive Flat motifs found in genes affected by IL-33 Knockdown

Gene Name (Protein Name)	**Punitive Flat Motif**	**Orientation**	**Accession No.**
IL-6	-298 TGACA -302 -143TTAATA -138 -82 TGATA -78	Reverse Forward Reverse	AF048692
CCL-5 (RANTES)	-825 TGACA -821 -696 TAATTG -701 -221 TGACA -225 -85 TAAGTG -90 -84 TGATA -80 100 TGACA 96	Forward Reverse Reverse Reverse Forward Reverse	AB023652
CX3CL1 (Fractalkine)	-400 TGACA -404 -306 TAAGTG -311 -195 TGACA -191	Reverse Reverse Forward	AC004382 1kb upstream CX3CL1 transcription start (22519)
CTSL (Cathepsin L)	-1785 TGACA -1789 -1184 TAAGTG -1189 -1146 TGATA -1150 -1055 TAATTA -1050 -991 TGACA -987 -947 TAATTA -942 -935 TTAATA -930 -855 TGACA -851 -841 TGATA -845 -792 TAATCC -787 -724 TGACA -728 -628 TGACA -624 -494 TAAGTG-489	Reverse Reverse Reverse Forward Forward Forward Forward Forward Reverse Forward Reverse Forward Forward	AF163338

Figure

Figure 1



Health IPAH Isotype control

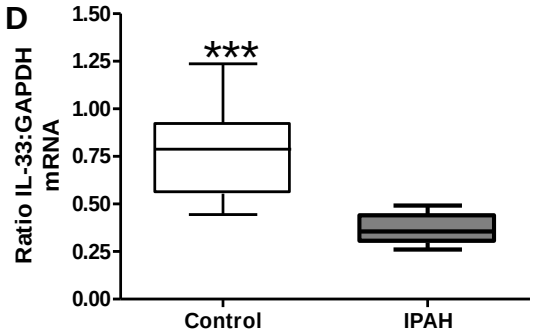


Figure 2

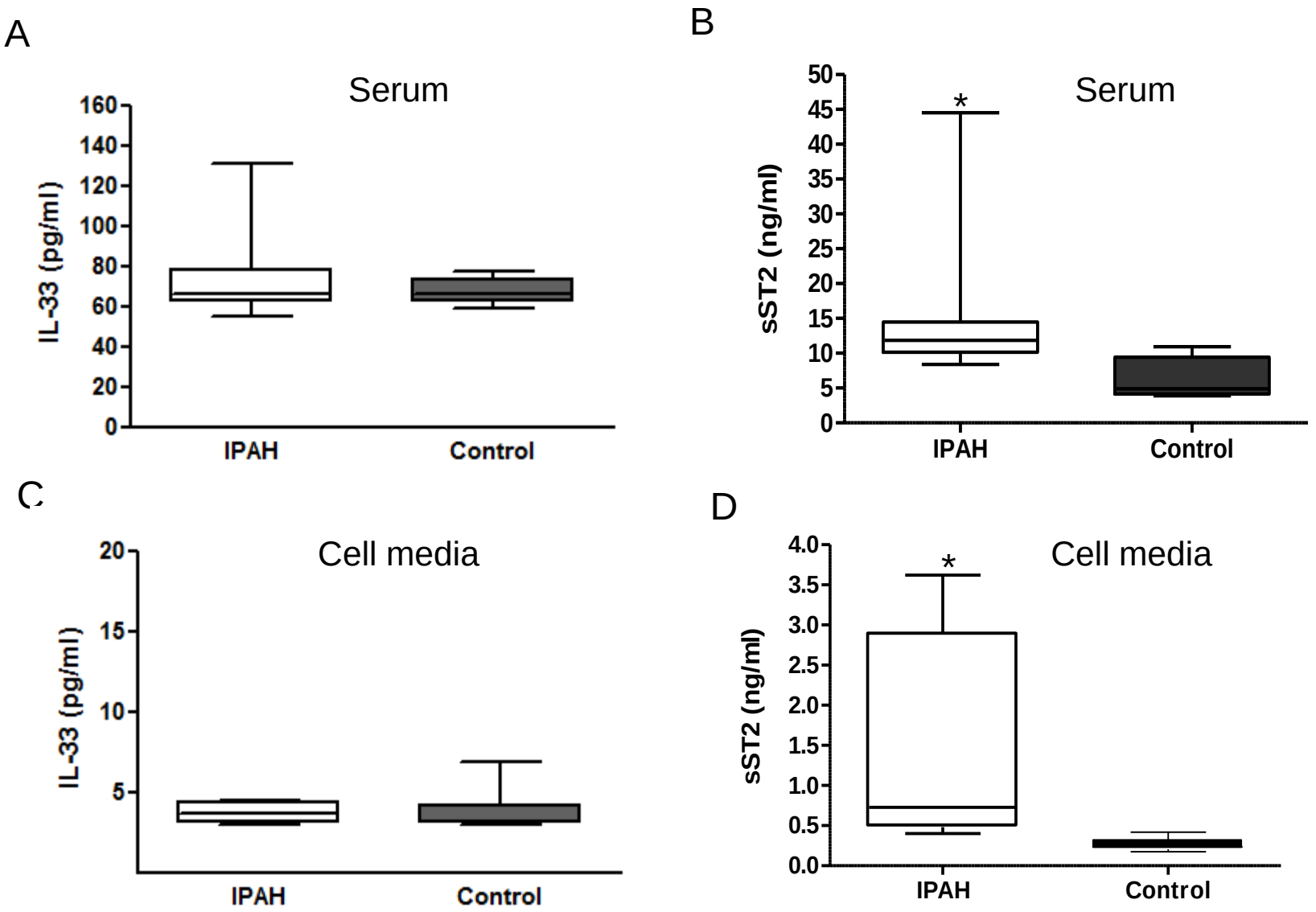

A
Serum
IL-33 (pg/ml)
160
140
120
100
80
60
40
20
0
IPAH
Control
B
Serum
sST2 (ng/ml)
50
45
40
35
30
25
20
15
10
5
0
*
IPAH
Control
C
Cell media
IL-33 (pg/ml)
20
15
10
5
IPAH
Control
D
Cell media
sST2 (ng/ml)
4.0
3.5
3.0
2.5
2.0
1.5
1.0
0.5
0.0
*
IPAH
Control

Figure 3

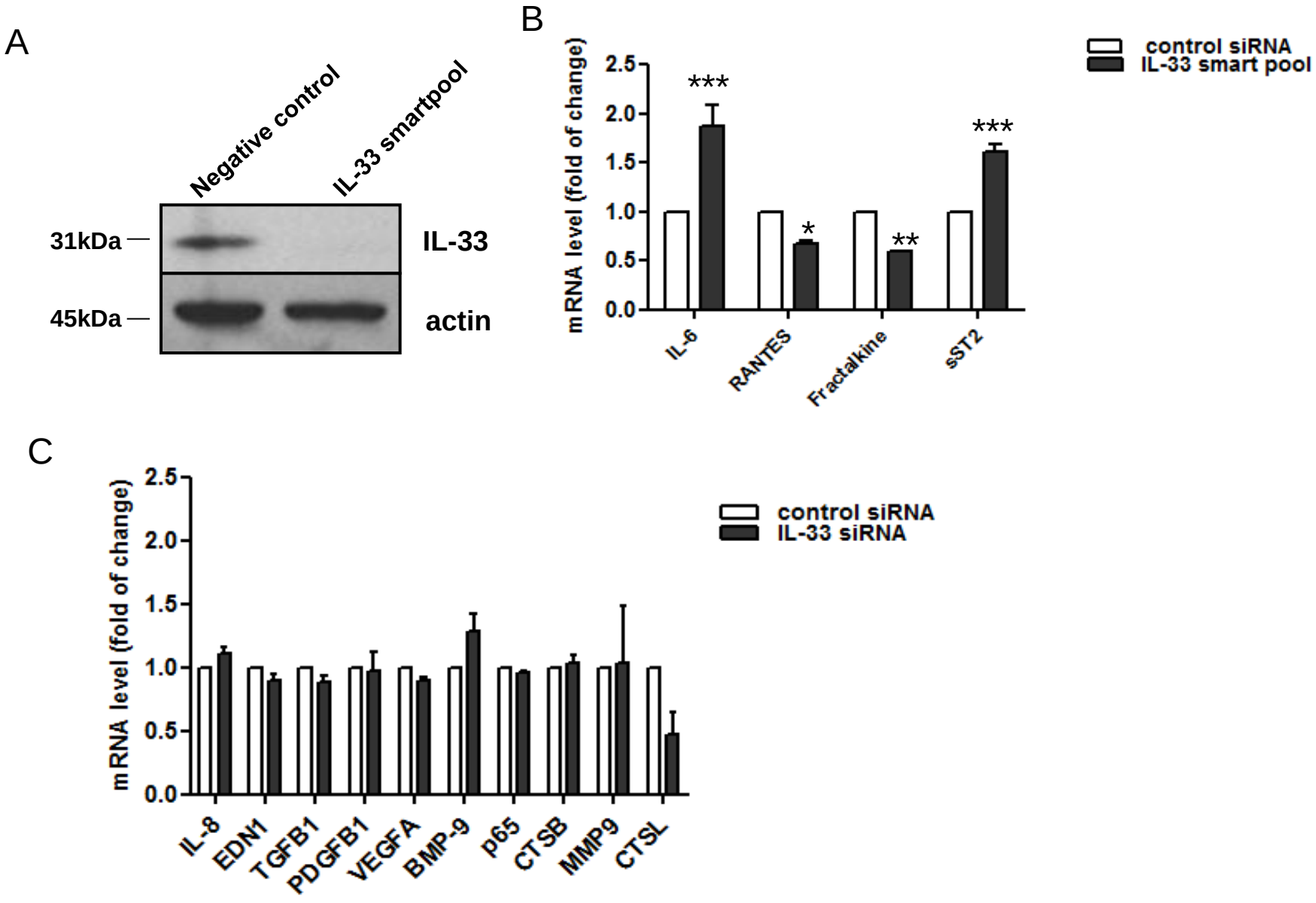

A
Negative control
IL-33 smartpool
31kDa
45kDa
IL-33
actin
B
control siRNA
IL-33 smart pool
mRNA level (fold of change)
2.5
2.0
1.5
1.0
0.5
0.0
***
*
**
***
IL-6
RANTES
Fractalkine
sST2
C
control siRNA
IL-33 siRNA
mRNA level (fold of change)
2.5
2.0
1.5
1.0
0.5
0.0
IL-8
EDN1
TGFB1
PDGFB1
VEGFA
BMP-9
p65
CTSB
MMP9
CTSL

Figure 4

A

Distal promoter

```
-1001 actagtattt catcaatatg gatagcatcc tccataggtt actgatttaa tattgacaac
 -941 aataccctttc acacgagaga gaggttagag aatttgccca aagtcaagca gctgggaaga
 -881 ggaaaactag gctgtgctct ccctaaatct catgctctat tatattaggg aagactctgg
 -821 cagatggcac atcctgatta tttgaaagtc accaatattt ttagaaattg catagataat
 -761 taaggttagt ttatccagaa ttctaataat tgcagcacat gtccataaat ctctattaca
 -701 accacctgag ataggccatc tcgggcatgt ggaaaaagcc caggtataga cactttggat
 -641 cctggatttc ccttgtactg gctgctacct tgggtaaggt aattctcctc tctgaagctc
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 -521 gtgtacactt aagcacacga gatgtgtcaa agttttcaaa aacattgcca acgaggcatc
 -461 agttacaaaa cttgctgcag agtgagctga tattgtgcca ctgcactcca gcctgggtga
 -401 cacagtgagg ctttgcctca aaaaaaaaaa aaaaaaagaa agaaacacca aataagcaac
 -341 ttgctgcaga aatgggtact cttgttctag aaatgtgact atagggaagt tacaactacc
 -281 aactcgcgtt aagggaaatg agtgacctgc cacctacatg gtgttaggga ggttttgctg
 -221 agaaagtcac tcatgaagaa ggcaaaatat agttaagaca aaatgtaact atctatagag
 -161 ataaggtaaa aattggaaat agaacttcat taaagatctt tcaaataggg agaatgtggt
 -101 gaaaactgca gttaacattt gttaacagtg tgatcatcgg gttcagctta tcagtaacct
  -41 ggttcctgtc tcttaactga taaagaaaat gggaggtttt tAaagagagg ctggctgttg
   19 tatttagtaa agctataaag ctgtaagaga aattggcttt ctgagttgtg aaactgtggg
   79 cagaaagttg aggaagaaag aactcaagta caacccaatg agg
```

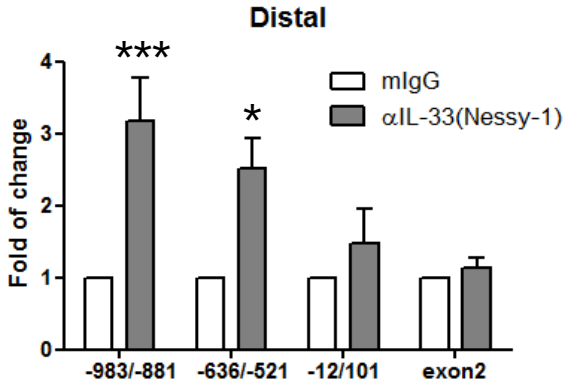
Proximal promoter

```
-1035 gttcaaattc tgacttcacc ccttaatgtg aagtgacatg ggcaagttgc ttaatctctc
 -975 tgtgcctcag tgtccttgtc tgtaaaatgg gcatcataat aatagcgcct gccacattgg
 -915 gtgagtgtga gaatgaagga attaatacat gtaaatcact tagactgtgt ctggcataga
 -855 ggacattcta aagaaaagtt agctattatc attatattat tatatgggtc tggaattagt
 -795 tcctgaatcc ttctgagatg tgatgactta taaacgtagg ttgagtttac tcatgtatagg
 -735 gtcatcgcaa ctatgcatag ctaaaatcaa attttgcttt tcaagtttgt tttacctgga
 -675 gccctagagt tcagggttat ggttttcttt gtcactcccc ttgagggaag cttcttagtc
 -615 acactctcct tctctttctc tgcactctat gcactctaga aaagctcctt tttttttttc
 -555 ttcatccagg cagagaggcc tactgggact taaatccaag gagctgaaat ctgttttggg
 -495 atggggtgga gtcacattct ggaacctaga cagagaattt ctaagttcca gaaagtgctg
 -435 cttacttcgc atttcctctc ccccaccttt gcttttgaaa ctcctggcac caatgctgcc
 -375 aaggctggcg gagctttcct gagtggtgtc tgccaaatga ggagtcaagg aatatctgga
 -315 aaggcagcct ccaggtcccc gatgtcaaga ccatttagaa ctgaaagtgt cccaatatcg
 -255 gggtacaggc aataagcatt agttattaat cagcctgaga agttgattct aaaataggag
 -195 gaaatgattc aattatttcc tctcaaggga ttactcaatg ttgtttttat gtttaaatat
 -135 ttatttgtca acatcaagaa ttcttagtac atgatgcacc agcatttttg aacaagtcat
  -75 agatttggcc acaaatcaaa tttcaggatg ggaggagtgt ctccccttta aaatagaaga
  -15 gagtgagtag tctatGagga
```

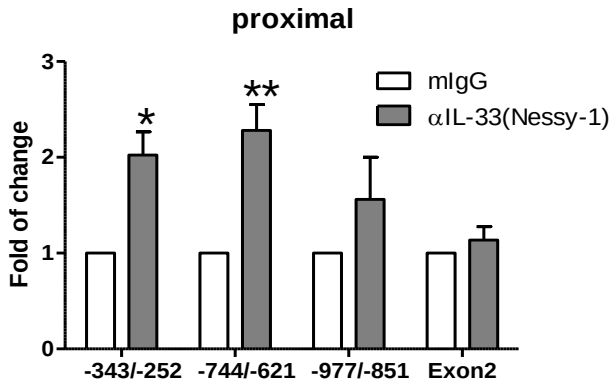
Exon2 (NM_016232.4)

```
121 ggttgagata taggctactc ttcccaactc agtcttgaag agtatcacca actgcctcat
181 gtgtggtgac cttcactgtc gtatgccagt gactcatctg gagtaatctc aacaacgagt
241 taccaatact tgctcttgat tgataaacag aatggggttt tggatcttag caattctcac
```

B



C



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