Interleukin-33 is a regulatory nuclear factor in lung cancer and wound healing.

Breanne Elson

Master of Research

Western Sydney University, 2017
Acknowledgements

I would like to thank Dr Graham Jones from Western Sydney University for his supervisory role in this Master of Research Thesis. I would also like to thank Western Sydney University for funding this research project.
Statement of Authentication

The work presented in this thesis is, to the best of my knowledge and belief, original except as acknowledged in the text. I hereby declare that I have not submitted this material, either in full or in part, for a degree at this or any other institution.

.................................................. (Signature)
Table of Contents:

List of Tables: .................................................................................................................. 6
List of Figures and Illustrations: .......................................................................................... 6
Abbreviations Table: ......................................................................................................... 8
Chapter 1: Abstract ........................................................................................................... 10

Chapter 2: Introduction .................................................................................................... 12
  IL-33 and Th2 .................................................................................................................. 12
  IL-33 as an alarmin ........................................................................................................ 13
  ST2; The IL-33 receptor .................................................................................................. 14
  The ST2 Receptor Gene, IL1RL1 ................................................................................. 16
  IL-33 Isoforms ............................................................................................................... 17
  IL-33 Cleavage and C-Terminal .................................................................................... 17
  IL-33 Cleavage and N-Terminal .................................................................................... 19
  IL-33 as a Nuclear Factor ............................................................................................. 19
  IL-33 and DNA Methylation .......................................................................................... 20
  IL-33 and NF-κB ............................................................................................................ 21
  IL-33 and Apoptosis ...................................................................................................... 22
  IL-33 in lung cancer ...................................................................................................... 23
  IL-33 in wound healing ................................................................................................. 24

Chapter 3: Materials and Methods .................................................................................. 26

Chapter 4: Results .......................................................................................................... 29
  IL-33 translocates into the nucleus in vitro ..................................................................... 29
  Nuclear IL-33 expression increases during wound healing in BEAS-2B cells .......... 31
  Nuclear IL-33 expression and Poly (I:C) ...................................................................... 34
  Nuclear IL-33 increases in low density BEAS-2B cells .............................................. 40
H3K27M3 is upregulated in scratched and Poly (I:C) treated BEAS-2B cells. ..............51

IL-33 Translocates into the nucleus during apoptosis. ...........................................53

ST2 expression decreases when scratched and treated with Poly (I:C). ....................54

Chapter 5: Discussion and Future Implications ..................................................... 59

Nuclear IL-33 is expressed in lung epithelial cells. ..................................................59

Nuclear IL-33 is upregulated in BEAS-2B cells during wound healing. .....................59

Nuclear IL-33 Turns on Apoptosis. ..........................................................................62

Gene targets of nuclear IL-33. ..................................................................................63

Cause of Nuclear IL-33 Expression. ........................................................................64

IL-33 in the tumour microenvironment. .................................................................66

ST2 internalises during IL-33 expression. ...............................................................69

Future Implications and Research Question ..........................................................70

Chapter 6: Conclusion .............................................................................................73

References ..............................................................................................................76
List of Tables:

Table 1: Abbreviations Table

List of Figures and Illustrations:

Figure 2.1: IL-33/ST2 axis ............................................................................................................. 15

Figure 2.2: The structure of full-length IL-33 ................................................................................. 18

Figure 4.1: IL-33 localisation in A549 control cells and BEAS-2B control cells at
  100x magnification; ......................................................................................................................... 30

Figure 4.2: IL-33 localisation in A549 scratched cells and BEAS-2B scratched cells
  at 100x magnification ....................................................................................................................... 32

Figure 4.3: Quantitative real-time PCR analysis of IL-33 expression in A549 cells
  and BEAS-2B cells .............................................................................................................................. 33

Figure 4.4: IL-33 localisation in A549 Poly (I:C) treated cells and BEAS-2B Poly (I:C)
  treated cells at 100x magnification; ............................................................................................... 35

Figure 4.5: Quantitative real-time PCR analysis of IL-33 expression in A549 cells
  and BEAS-2B cells ............................................................................................................................ 36

Figure 4.6: IL-33 localisation in A549 combined scratched and Poly (I:C) treated
  cells and BEAS-2B combined scratched and Poly (I:C) treated cells at 100x
  magnification ..................................................................................................................................... 38

Figure 4.7: Quantitative real-time PCR analysis of IL-33 expression in A549 cells
  and BEAS-2B cells; .......................................................................................................................... 39

Figure 4.8: Nuclear IL-33 expression at the site of a scratch at 10x magnification in
  BEAS-2B cells; ................................................................................................................................. 42

Figure 4.9: IL-33 localisation in BEAS2B cells at 100x magnification ........................................... 43

Figure 4.10: IL-33 expression in BEAS-2B cells at 10x magnification ............................................ 44
Figure 4.11: Quantitative IL-33 expression data of fluorescence microscopy images in control BEAS-2B cells ................................................................. 47

Figure 4.12: Quantitative IL-33 expression data of fluorescence microscopy images in scratched BEAS-2B cells ........................................................................ 48

Figure 4.13: Quantitative IL-33 expression data of fluorescence microscopy images in Poly (I:C) treated BEAS-2B cells ........................................................................ 49

Figure 4.14: Quantitative IL-33 expression data of fluorescence microscopy images in dual scratched and Poly (I:C) treated BEAS-2B cells ........................................... 50

Figure 4.15: H3K27M3 localisation in BEAS2B at 100x magnification ...................... 52

Figure 4.16: IL-33 localisation during apoptosis in A549 cells .................................. 53

Figure 4.17: ST2 localisation in A549 cells at 100x magnification ............................... 55

Figure 4.18: ST2 localisation in BEAS2B at 100x magnification ................................ 56

Figure 4.19: Quantitative real-time PCR data for ST2 expression in A549 cells ...... 57

Figure 4.20: Quantitative real-time PCR data for ST2 expression in BEAS-2B cells 58

Figure 5.1: Proposed IL-33/ST2 pathway ................................................................ 67
### Abbreviations Table:

<table>
<thead>
<tr>
<th>Term</th>
<th>Abbreviation</th>
</tr>
</thead>
<tbody>
<tr>
<td>Antigen Presenting Cell</td>
<td>APC</td>
</tr>
<tr>
<td>Chronic Obstructive Pulmonary Disease</td>
<td>COPD</td>
</tr>
<tr>
<td>Chromatin Binding Motif</td>
<td>CBM</td>
</tr>
<tr>
<td>Focal Adhesion Kinase</td>
<td>FAK</td>
</tr>
<tr>
<td>Full-length IL-33</td>
<td>flIL-33</td>
</tr>
<tr>
<td>Glycogen Synthase Kinase 3β</td>
<td>GSK3β</td>
</tr>
<tr>
<td>Genome Wide Association Study</td>
<td>GWAS</td>
</tr>
<tr>
<td>Helix Turn Helix Motif</td>
<td>HTH Motif</td>
</tr>
<tr>
<td>High-mobility group box 1</td>
<td>HMGB1</td>
</tr>
<tr>
<td>Histone 2A</td>
<td>H2A</td>
</tr>
<tr>
<td>Histone 2B</td>
<td>H2B</td>
</tr>
<tr>
<td>Histone 3 Lysine 27 Methyl 3</td>
<td>H3K27Me3</td>
</tr>
<tr>
<td>Ikβ kinase</td>
<td>IKK</td>
</tr>
<tr>
<td>Immunoglobulin E</td>
<td>IgE</td>
</tr>
<tr>
<td>Interleukin-1 Receptor Associated kinase</td>
<td>IRAK</td>
</tr>
<tr>
<td>Interleukin-1 Receptor Ligand 1</td>
<td>IL1-RL1</td>
</tr>
<tr>
<td>Interleukin-6</td>
<td>IL-6</td>
</tr>
<tr>
<td>Interleukin-4</td>
<td>IL-4</td>
</tr>
<tr>
<td>Interleukin-5</td>
<td>IL-5</td>
</tr>
<tr>
<td>Interleukin-10</td>
<td>IL-10</td>
</tr>
<tr>
<td>Interleukin-12</td>
<td>IL-12</td>
</tr>
<tr>
<td>Term</td>
<td>Abbreviation</td>
</tr>
<tr>
<td>-------------------------------------------</td>
<td>--------------</td>
</tr>
<tr>
<td>Interleukin-13</td>
<td>IL-13</td>
</tr>
<tr>
<td>Interleukin-18</td>
<td>IL-18</td>
</tr>
<tr>
<td>Interleukin-18 Receptor Accessory Protein</td>
<td>IL-18RAP</td>
</tr>
<tr>
<td>Interleukin-33</td>
<td>IL-33</td>
</tr>
<tr>
<td>Interferon Gamma</td>
<td>IFN-γ</td>
</tr>
<tr>
<td>Latency-Associated Nuclear Antigen</td>
<td>LANA</td>
</tr>
<tr>
<td>Mature IL-33</td>
<td>matIL-33</td>
</tr>
<tr>
<td>Nuclear Factor Kappa B</td>
<td>NF-κB</td>
</tr>
<tr>
<td>Rel homology domain</td>
<td>RHD</td>
</tr>
<tr>
<td>Suppression of Tumorigenicity 2</td>
<td>ST2</td>
</tr>
<tr>
<td>T Helper 1</td>
<td>Th1</td>
</tr>
<tr>
<td>T Helper 2</td>
<td>Th2</td>
</tr>
<tr>
<td>TNF Receptor Associated Factor</td>
<td>TRAF</td>
</tr>
<tr>
<td>Toll-interleukin receptor</td>
<td>TIR</td>
</tr>
</tbody>
</table>
**Chapter 1: Abstract**

Interleukin-33 (IL-33) is an IL-1-like alarmin, and is a ligand for the ST2 receptor. IL-33 functions as a dual function cytokine, with the ability to induce Th2 immune cells and translocate into the nucleus and repress gene transcription. However, the role of nuclear IL-33 is not completely understood in lung epithelial cells. The aim of this study was to visualise nuclear IL-33 expression in lung epithelial cells and to compare the role of nuclear IL-33 in healthy lung epithelial cells (BEAS-2B cells) compared to a non-small cell lung cancer (NSCLC) cell line (A549 cells). Human A549 cells and BEAS-2B cells were cultured and analysed for nuclear IL-33 via fluorescence microscopy and quantitative real-time PCR at a controlled state. Additional A549 cells and BEAS-2B cells were cultured and physically scratched to mimic the wound healing process, and analysed for nuclear IL-33 via fluorescence microscopy and quantitative real-time PCR, with nuclear IL-33 shown to increase in comparison to control cells. Additional cells were treated with Poly (I:C) and analysed for nuclear IL-33 via fluorescence microscopy and quantitative real-time PCR. Nuclear IL-33 expression was seen in Poly (I:C) treated cells was equal to control cells in both cell lines. Interestingly, when BEAS-2B cells were treated with a combination of scratches and treated with Poly (I:C), they expressed the highest levels of nuclear IL-33. A549 cells undergoing apoptosis were visualised via fluorescence microscopy, showing that nuclear IL-33 is highly expressed in cells undergoing apoptosis. Although nuclear IL-33 levels are high in cells undergoing apoptosis, apoptosis is not needed to visualise nuclear IL-33 in both A549 cells and BEAS-2B cells. A549 and BEAS-2B cells were cultured and scratched or treated with Poly (I:C) and analysed for H3K27Me3 via fluorescence microscopy. An increase in H3K27Me3 expression was visualised in BEAS-2B cells when cells were combined treated with scratches and Poly (I:C), similar
to that of nuclear IL-33 expression. The expression of ST2 was also analysed via fluorescence microscopy and quantitative real-time PCR. ST2 expression did not differ from control to treated in A549 cells. In BEAS-2B cells, ST2 expression was more cytoplasmic than the control cells. Together, these treatments mimic normal wound healing and infection responses, both of which cause cell stress and damage, and would therefore expect to elicit an alarmin response. Our findings support this with scratched and Poly (I:C) treated cells showing an increase in IL-33 expression.
Chapter 2: Introduction

Interleukin-33 (IL-33) is a protein belonging to the IL-1 family of cytokines (Sims and Smith, 2010). The IL-1 family consists of 11 members, and is associated with the pathogenesis of allergic diseases such as asthma, psoriasis and chronic obstructive pulmonary disease (COPD) (Ballak et al., 2015).

IL-33 and Th2

Th1 and Th2 were the first CD4$^+$ T-cells to be described in literature (Romagnani, 1992) and are examples of cell-mediated and humoral immunity. Th1 and Th2 immune cells are activated by different pathogens in an attempt to stimulate immune responses that specifically attack the pathogen they are activated by (Romagnani, 1992). Th1 immune responses are activated by intracellular bacteria and viruses, and are predominately IFN-γ mediated, while Th2 immune responses are activated by extracellular parasites and are predominately IL-4 mediated, leading to IgE release and therefore allergic inflammation (Yoshimoto et al., 2009). Generally, IL-1 immune responses are Th1 predominant, mediated by the release of IFN-γ by T cells (Chizzolini et al., 1997). The IFN-γ inducing factor and IL-1 family member, IL-18, plays an important role in IFN-γ activation via IL-12 dependant MYD88 signalling (Ballak et al., 2015). In contrast to its respective family, IL-33 has been shown to promote Th2 allergic inflammation via the stimulation of Th2 associated cytokines such as IL-4, IL-5, IL-10 and IL-13 (Santarasi et al., 2013). IL-33 induces Th2 inflammatory responses in both epithelial and endothelial cells (Zhao et al., 2014). Epithelial cells provide the first line of defence against pathogens, forming a physical barrier between the external and internal environments (Oshio et al., 2017). Cell damage, necrosis and inflammation in these cellular barriers stimulates the release of IL-33, that will in turn
activate the appropriate immune response (Liew, Pitman and McInnes, 2010). The recruitment of immune cells is vital to remove pathogens and promote wound healing. As a result of this, the role epithelial cells might play in the regulation of IL-33 and therefore the regulation of inflammation is an important part of this research project. To expand on this, current literature does not describe the role of IL-33 in cancer cells. As a result of this, this research paper aims to expand on the role epithelial cells play in the regulation of IL-33, leading to the metastasis of cancer.

**IL-33 as an alarmin**

IL-33 is defined as an alarmin; alarmins are endogenous molecules that are released from a cell as a result of cellular damage and bind to receptors on innate immune cells and epithelial cells, alerting the immune system of cell death and potential pathogen invasion (Mousson, Ortega and Girard, 2008). IgE is a Th2 antibody produced by the immune system to combat allergies to pathogens (Galli and Tsai, 2012). Studies confirm IL-33 as an innate alarmin by showing an increase in serum levels of IgE when IL-33 expression increases (Prefontaine et al. 2009). High-mobility group box 1 (HMGB1) protein is a well-known alarmin, and like HMGB1, IL-33 functions as a chromatin-associated cytokine, where it is released from damaged cells as an extracellular cytokine but is also able to act as an intracellular nuclear factor, translocating into the nucleus and binding to chromatin (Kim et al., 2012). Previous studies show that IL-33 also possesses this dual function capability, where it is able to regulate inflammation by turning on inflammation as an alarmin, and turn off gene transcription as a nuclear factor (Roussel et al., 2008). Therefore, IL-33 bound to chromatin works as a repressor of gene transcription, turning off inflammation and supporting cellular homeostasis (Zhao et al., 2014). Homeostasis refers to the ability of
the body to maintain a stable internal environment despite changes in the external environment. Fluctuations in the internal environment are common, however, internal conditions must return back to homeostasis for optimum biological functioning. The idea that nuclear IL-33 and IL-33 as a soluble cytokine potentially work together to maintain cellular homeostasis has been proposed, and will be explored later on.

**ST2; The IL-33 receptor**

IL-33 as a soluble cytokine, signals via its receptor ST2 (Fig. 2.1) that exists in four spliced isoforms, ST2L, ST2V, ST2LV, and soluble ST2 (sST2) (Carriere et al., 2006). IL-33 signals through transmembrane ST2 (ST2L) and the spliced transmembrane variants ST2V/ST2LV, which are expressed on epithelial cells (autocrine signalling) and Th2 innate immune cells (paracrine signalling) (Lin et al., 2013). Heterodimerization of ST2L with IL-1RaP occurs upon activation, recruiting MYD88 to its Toll-interleukin receptor (TIR) domain (Schmitz et al., 2005). Downstream signalling from MYD88 recruits IRAK, IRAK4 and TRAF6, leading to NF-κB nuclear translocation and inflammatory transcriptional activation (Jovanovic et al., 2012). Soluble ST2 (sST2) acts as a decoy receptor by inhibiting IL-33 induced pro-inflammatory responses by binding to released IL-33 and producing no immune response (Granne et al., 2011). Serum levels of sST2 are elevated post inflammation, supporting the idea that sST2 acts as a IL-33 inhibitory cytokine receptor, released by cells to diminish inflammatory responses (Oshio et al., 2017).
**Figure 2.1:** *IL-33/ST2 axis*; IL-33 binds to ST2 on the surface of epithelial cells. ST2 will dimerize with IL-1Racon recruiting MYD88 to their intracellular TIR domain. MYD88 initiates an intracellular signalling cascade, recruiting TRAF, IRAK 1 and IRAK 4. IRAK stimulates the IκB dependant activation of NF-κB, prompting NF-κB translocation into the nucleus. NF-κB stimulates transcriptional activation of inflammatory genes.
The ST2 Receptor Gene, IL1RL1

ST2 is encoded by the gene *IL1RL1*, and genetic variants in this gene have been linked to a number of inflammatory diseases (Ho et al., 2013). As a result of this, Ho et al (2013) undertook a genome wide association study (GWAS) to examine the effect of genetic variants in *IL1RL1* on ST2 expression. They show how missense mutations in *IL1RL1* can increase the expression of ST2L, and therefore enhance IL-33 responsiveness. Results indicate that genetic variants in the *IL1RL1* gene increase ST2L expression, and are associated with the development of allergic diseases such as asthma as it increases IL-33 responsiveness. Additionally, Ho et al, 2013 shows that *IL1RL1* resides in a linkage disequilibrium block consisting of IL-18 and IL-18RAP. Polymorphisms within these genes are shown to associate with inflammatory conditions such as asthma (Ho et al., 2013). These experiments are relevant because if the ST2 gene (*IL1RL1*) resides in a linkage disequilibrium block that has already been shown to associate with inflammatory diseases, then it could possibly increase susceptibility to the development of asthma. This could mean that if a linkage disequilibrium lock extends across multiple genes, then it is possible that it indicates the presence of SNP’s in regulatory elements that co-regulate several genes. Definitively, these experiments provide evidence of a dysregulation in ST2 inflammatory pathways caused by genetic mutations, amplifying what are otherwise normal responses (IL-33 hyper responsiveness). Poly (I:C) is an analogue of viral replication intermediates that when introduced to a cell will mimic normal immune responses. As a result of this, this research project will visualise the expression of ST2 under Poly (I:C) induced inflammatory conditions in healthy cells compared to cancerous cell types, to differentiate the role of IL-33/ST2 in both cell types.
**IL-33 Isoforms**

It is currently known that IL-33 exists in various isoforms, differing in length and therefore structure (Ali et al., 2009). Full length IL-33 (flIL-33) is synthesised as a 31-kDa precursor protein (Fig. 2.2) that is 270 base pairs in length and contains two conserved domains; a C-terminal and N-terminal domain (Carriere et al., 2006).

**IL-33 Cleavage and C-Terminal**

The C-terminal domain of flIL-33 is the IL-1-Like domain, and is the site of degradation by caspase-3 and caspase-7 (Schmitz et al., 2005). Caspases are enzymes that serve to breakdown proteins into smaller biologically active molecules or for degradation (Cayrol and Girard, 2009). Similar to its family members IL-1β and IL-18, cleavage of flIL-33 by caspase-1 processes flIL-33 into its mature form (matIL-33) comprising of only the C-terminal domain (Talabot-Ayer et al., 2009). Analysis of serum levels of IL-33 show that matIL-33 is the predominant cytokine form, suggesting that caspase processing of IL-33 may be required to optimise biological activity (Ali et al., 2009). Caspase-3 and caspase-7 are also associated with IL-33, with their cleavage leading to IL-33 inactivation and degradation (Cayrol and Girard, 2009). Proteases are enzymes that assist with the cellular breakdown of proteins into smaller fragments, known as proteolysis (Oshio et al., 2017). Mast cells and neutrophils secrete proteases that cleave IL-33 into matIL-33 of varying lengths, such as 95-270bp, 107-270bp and 109-270bp. This secondary mechanism of creating active forms of IL-33 are 30-fold more potent at activating type 2 innate immune cells. In contrast to this, Cayrol and Girard (2009) saw that cleavage of IL-33 by caspase-1 caused IL-33 inactivation rather than the previously seen biological activation. Cleavage also occurred at a different site than previously seen; in the IL-1-Like domain rather than central domain, similar
to that of caspase-3 and caspase-7. It is important to know that IL-33 exists as a variety of isoforms, as its variety of forms allows IL-33 to possess dual function qualities; as a nuclear factor and as an extracellular cytokine. The role of fIIIL-33 in the nucleus of epithelial cells is an important part of this research project during apoptosis and for the suppression of inflammatory genes.

**Figure 2.2**: The structure of full-length IL-33; Full length IL-33 is 270 base pairs in length, with a molecular mass of 31kDa. The nuclear domain or N-terminal domain contains a conserved region known as the chromatin binding motif (CBM), essential for nuclear translocation. Caspase-1 cleaves IL-33 at a site not yet determined to form mature IL-33, lacking the N-terminal domain and the ability to translocate into the nucleus. The C-terminal domain is the IL-1-Like domain at the end of IL-33. IL-33 is cleaved here by caspase-3 and caspase-7 for degradation.
**IL-33 Cleavage and N-Terminal**

As well as its role as an alarmin, IL-33 also acts as a nuclear factor, where it binds to chromatin and represses inflammatory gene transcription (Carriere et al., 2006). The varying isoforms of IL-33 contain specific structural elements that allow each isoform to function differently (Ali et al., 2009). As previously mentioned, flIL-33 contains all these structural elements, consisting of a C-Terminal and N-terminal (Carriere et al., 2006). The N-terminal domain of IL-33 is comprised of a conserved domain known as the chromatin binding motif (CBM) or the helix-turn helix (HTH) motif, that allows IL-33 to translocate into the nucleus and bind to chromatin (Roussel et al., 2008). Carriere et al (2006) shows that an N-terminal IL-33/GFP fusion protein displayed nuclear localization and heterochromatin association, compared to a C-terminal IL-33/GFP fusion protein that is distributed throughout the cell (Carriere et al., 2006). Loss of CBM/HTH abrogated nuclear localization and heterochromatin association, demonstrating that CBM/HTH within the N-terminal domain is necessary for nuclear translocation and chromatin association.

**IL-33 as a Nuclear Factor**

Previous research confirms the ability of IL-33 to translocate into the nucleus via its N-Terminal domain (Carriere et al., 2006). Analysis by Roussel et al (2008) reveals that the N-Terminal of IL-33 binds to the H2A-H2B acidic pocket on chromatin. Histones are proteins that wrap up DNA into compact, inactive forms, with multiple histones wrapped in DNA known as nucleosomes (Campos and Reinberg, 2009). Nucleosomes spaced repetitively are known as nucleosomal arrays, forming dense, compact, inactive chromatin structures (Kalashnikova et al., 2013). Between histones, they form an ‘acidic patch’, which is a highly negatively charged region on the surface of the
nucleosome (Kalashnikova et al., 2013). H2A and H2B are types of histones that come together to create an acidic patch (H2A-H2B) (Cayrol et al., 2006). Due to its high negativity, H2A-H2B creates the perfect conditions to act as a binding site for specific proteins. IL-33 has been shown to bind to this negatively charged H2A-H2B acidic pocket due to shape and charge complementarity between H2A-H2B and IL-33 (Roussel et al., 2008). When bound, IL-33 regulates higher-order chromatin compaction, causing nucleosomal array compaction and therefore turns off transcription (Roussel et al., 2008). In addition to this, IL-33 is shown to possess similar properties to that of the Kaposi sarcoma herpesvirus LANA (latency-associated nuclear antigen). LANA is also shown to bind to histone acidic pockets to stimulate chromatin compaction (Roussel et al., 2008).

**IL-33 and DNA Methylation**

From each histone core protrudes a N-terminal histone tail, that can be modified by post-translational modification to alter DNA density and therefore turn on/off gene activity (Kalashnikova et al., 2013). Methylation in particular is one type of post translational modification, defined by the addition of a methyl group to DNA histone tails (Razin and Riggs, 1980). Known as methyl marks, they exist in a variety of forms serving different functions for each mark (Kalashnikova et al., 2013). H3K27Me3 (histone 3 lysine 27 methyl 3) is one type of methyl mark that this research project is interested in, as it acts as an inhibitor of DNA transcription by altering histone epigenetic traits (Razin and Riggs, 1980). Carriere et al (2006) show that nuclear IL-33 functions as a gene repressor by binding to chromatin and turning off gene transcription in the same manner as the repressive methyltransferase SUV39H1. SUV28H1 is a methyltransferase that adds repressive methyl marks to DNA (Shao et
There is no current literature that tests for the expression of H3K27Me3 in cells expressing IL-33. However, because H3K7Me3 is also a repressive mark of gene transcription, it is hypothesised that IL-33 is prompting the addition of H3K27Me3 to inflammatory genes, turning inflammatory transcription off. This hypothesis will be tested within this research paper, comparing the expression levels of nuclear IL-33 with H3K27Me3 expression in both healthy and cancer cells lines. Therefore, it is suggested that IL-33 binds to H2A-H2B to promote methylation and gene repression. However, future study is needed to completely understand the role of IL-33 in the nucleus, as specific gene targets of IL-33 are currently unidentified.

**IL-33 and NF-κB**

NF-κB is an important transcription factor involved in the transcriptional activation of inflammation (Perkins, 2012). There are two separate pathways that activate NF-κB (Lawrence, 2009), the pathway of interest sees TNF-α, IL-33 and IL-1 release, active NF-κB consisting of a p50 and p65 subunit (Lawrence, 2009). NF-κB is inhibited in the cytoplasm when bound to the protein IκBα (Israel, 2009). The release of pro-inflammatory cytokines such as IL-1, IL-33 and TNF-α stimulates IκBα phosphorylation and targeted ubiquitination and degradation by proteases (Kaileh and Sen, 2010). NF-κB activated by the loss of IκBα translocates into the nucleus, turning on inflammatory gene transcription (Haraldsen et al., 2009). IL-33 bound to ST2 is an inducer of this downstream signalling cascade (Jovanovic et al., 2011). In contrast to this, it has been suggested that flIL-33 in the nucleus of epithelial cells acts as a direct NF-κB inhibitor (Ali et al., 2011). FlIL-33 has been shown to interact directly with the p65 subunit of NF-κB, with the N-terminal domain of IL-33 from amino acid 66-109 interacting with the N-terminal Rel homology domain (RHD) on the p65 subunit of NF-κB (Ali et al.,
The RHD is a conserved sequence found in NF-κB and NFAT, that is essential for binding to cognate DNA elements (Hoesel and Schmid, 2013). These previous findings build upon the idea that nuclear IL-33 could potentially be playing an inflammatory repressive role when translocated into the nucleus of epithelial cells by turning off NF-kB. The initiating factor resulting in IL-33 translocation into the nucleus is currently unknown and requires further study. The exact molecular mechanism of action of nuclear IL-33 and direct targets is also currently unknown.

**IL-33 and Apoptosis**

Apoptosis, also known as programmed cell death, is the elimination of defective and unwanted cells from the body (Barkett and Gilmore, 1999). Apoptosis is initiated by an intrinsic or an extrinsic pathway, where the activation of ligands and caspases results in the death of unwanted cells (Elmore, 2007). No current research exists in regards to the effect of nuclear IL-33 on apoptosis. However, previous research studies show how NF-κB is an inhibitor of apoptosis in epithelial cells when activated, but an activator of apoptosis when inactivated (Barkett and Gilmore, 1999 and Wullaert, Bonnet and Pasparakis, 2010). As previously mentioned, nuclear IL-33 binds to the p65 subunit of NF-κB via its N-terminal domain, causing NF-κB to turn off (Ali et al., 2011). As a result of this, it is hypothesised that nuclear IL-33 binds to active NF-κB in the nucleus of epithelial cells, causing NF-κB induced apoptosis. It is clear that soluble IL-33 acts as an alarmin that is released from damaged or dying cells to induce an immune response to defend the body against invading pathogens (Mousson, Ortega and Girard, 2008). However, literature describes nuclear IL-33 as portraying the opposite role, turning off immune inflammation, suggesting that nuclear and soluble cytokine work together to play different roles and maintain homeostasis. However, the
role of nuclear IL-33 in regulating apoptosis is unclear and is explored in this research project.

**IL-33 in lung cancer**

Lung cancer is the leading cause of cancer related death worldwide (Siegel, Miller and Jemal, 2016). The tumour microenvironment is the cellular environment created by a tumour, supporting its growth and tumorigenesis (Wasmer and Krebs, 2017). Tumours reprogram non-cancer cells such as epithelial cells, endothelial cells and stromal cells to assist with growth and metastasis of the tumour microenvironment (Maywald et al. 2015). In addition to this, reprogrammed cells assist with angiogenesis, supplying the tumour with infiltrating immune cells (Saranchova et al., 2016). The reprogramming of infiltrating immune cells allows the tumour to undergo immune tolerance and subsequently immune evasion (Kim et al., 2015). Multiple research papers show that IL-33 over expression in the tumour microenvironment limits tumour immune tolerance, allowing for Th1 immune cells to promote cellular immunity, allowing for the influx of NK cells and CD8⁺ T cells that attack and reduce the size of the tumour (Gao et al., 2014). Prior to immune tolerance, Th1 immune responses are predominantly activated in response to the detection of a tumour (Gao et al., 2014). Th1 responses are triggered by the release of IL-12 and IFN-γ, recruiting CD8⁺ and NK T cells (Kim et al., 2015). In contrast to initiating a Th2 predominant immune response, IL-33 has also been shown to enhance IFN-γ production via Th1 cells, CD8⁺ T cells and NK cells (Gao et al., 2014). Several recent studies show IL-33 inducing a CD8⁺ T cell immune response when over expressed in tumour models, showing a reduction in tumour size (Dominguez et al., 2016). These results are important because they show the importance of IL-33/ST2 signalling in the elimination of tumours. However, the
molecular mechanisms of IL-33 in the tumour microenvironment remain unclear. A protective role of the IL-33/ST2 axis is suggested by several studies as the expression of IL-33 in the tumour microenvironment acts to destroy tumour cells (Gao et al., 2014 and Millar et al., 2017).

**IL-33 in wound healing**

IL-33 is abundant in cutaneous and lung epithelial cells that provide a physical barrier between the external and internal environment (Oshio et al., 2017). Wounds disrupt barrier defences allowing for the invasion of foreign pathogens (Rak et al., 2016). As previously mentioned, IL-33 is released from damaged epithelial cells to induce a Th2 immune response, targeting pathogen invasion (Cayrol and Girard, unclear). Wound healing consists of four stages; hemostasis, inflammation, proliferation and maturation/remodelling (Braiman-Wiksman et al., 2007). The deposition of connective tissue and reepithelialisation of wounds is a critical component of wound healing, as it is at this stage where chronically inflamed tissues impair tissue repair mechanisms, leading to over epithelialization and collagen induced fibrosis (Millar et al., 2017). Studies suggest that IL-33 is involved in the wound healing process by activating group 2 innate lymphoid cells (ILC2) to promote wound healing (Rak et al., 2016). Rak et al (2016) shows that a depletion in ILC2’s delayed epithelial wound closure in mice, and that IL-33 knock-out mice show diminished ILC2 responses. These results show that IL-33 activated ILC2 regulate cutaneous wound healing. Oshio et al (2017) also visualised delayed wound healing in mice with knockout IL-33 compared to wild-type mice. They show that IL-33 has beneficial effects on wound healing as the loss of IL-33 slowed down the wound healing process. In contrast to these results, Küchler et al (2008) uses endothelial cells to demonstrate the rapid down-regulation of IL-33 in the
pro-inflammatory environment of wound healing. They show high levels of IL-33 expression when endothelial cells become highly confluent and stop proliferating, and low levels of IL-33 expression when the cells become motile. In addition to this, they show that inhibiting VE-cadherin expression (an endothelial cadherin for cell-cell contact) did not alter IL-33 expression, and knock-down of IL-33 did not alter VE-cadherin, showing that endothelial cells needed cell-cell contact to express nuclear IL-33. These results differ from those seen in Rak et al (2016) and Oshio et al (2017) as different cell lines are used in each project (Rak and Oshio used epithelial cells and Kuchler used endothelial cells). More research is needed to completely understand the mechanism of action of IL-33 in wound healing of multiple cell types, as clinical treatment determined by research may only be applicable in specific cells.

To further understand the role of IL-33 in lung epithelial cells, this research project uses fluorescence microscopy and quantitative real-time PCR to observe IL-33 in the nucleus of NSCLC (A549) cells and healthy lung epithelial (BEAS-2B) cells. By comparing A549 cells with BEAS-2B cells, it is anticipated that we will have a clearer understanding of the role nuclear IL-33 plays in cancerous lung epithelial cells compared to healthy lung epithelial cells. We also physically scratch each cells line in an attempt to show the expression of nuclear IL-33 during natural wound healing processes.
Chapter 3: Materials and Methods

Cell Culture

Human non-small cell lung cancer (NSLC) A549 and healthy lung epithelial BEAS-2B cell lines were cultured in DMEM supplemented with 10% foetal calf serum (FCS) and 1% PSF. Cells were passaged every 3 days via trypsinization and seeded at a density of 1x10^6 in T75 flasks. Cells were incubated at 37°C in 5% CO₂.

Wound Assay

Cells were seeded at a density of 4x10^5 in 6-well plates or 5 x10^4 in 8-well chamber slides. After 24 hours of growth, the cells were starved in 1% FCS for an additional 24 hours (24 hours of growth in 10% plus 24 hours of growth in 1%). Cells were wounded using a knit comb (scratched horizontally and vertically) in PBS at 46 hours of growth time for the remaining 2 hours. The cells were washed with additional PBS and the original media was placed back into the wells. Cells were either fluorescently stained or the RNA was extracted.

Poly (I:C) Treatment

Cells were grown as previously stated and after 24 hours of growth time, varying concentrations of Poly (I:C) were added into the appropriate wells, diluted in DMEM supplemented with 1% FCS. The concentrations used are 1 ug/mL, 5 ug/mL, and 10 ug/mL of Poly (I:C).
**Fluorescence Microscopy**

Cells were seeded and treated as above. Once growth and treatment was complete, the cells were fixed in 4% formaldehyde and washed in 0.01% Triton x100/PBS. IL-33 anti-rabbit, ST2 anti-rabbit, E-Cadherin anti-mouse and H3K27M3 anti-rabbit primary antibodies were diluted at a ratio of 1:200 and incubated on the cells overnight. Cells were stained with the secondary antibody Alexa Flour 488 anti-rabbit and Alexa Flour 555 anti-mouse for 1 hour in the dark and 33342 Hoechst DNA Dye for 10 minutes in the dark. Slides were prepared with Antifade and viewed under the Leica fluorescence microscope.

**RNA Extraction**

Cells were lysed and RNA was extracted using the Bioline Isolate II Mini RNA Kit protocol. Total RNA concentration was determined via the Thermo Scientific Nanodrop 2000c and RNA was stored at -70°C.

**cDNA Synthesis and Quantitative real-time PCR**

cDNA was synthesised using the Sensifast cDNA Synthesis Kit at a RNA concentration of 500ng/mL. Mixes were run in the Applied Biosystems 96-well Thermal Cycler and stored at -20°C. Quantitative real-time PCR was prepared using the SensiMix SYBR Low-ROX Kit and run in the Applied Biosystems 7500 Fast Real-Time PCR System. Results were analysed using Microsoft Excel.
Image J Analysis

The Image J cell counter tool is used to conduct a quantitative study of fluorescence microscopy images. Cell totals were counted from each image and compared where necessary.
Chapter 4: Results

IL-33 translocates into the nucleus in vitro.

Previous studies show that IL-33 localises in the nucleus of endothelial cells during wound healing and infection (Oshio et al., 2017). To test whether this is also the case in lung epithelial cells, A549 cells and BEAS-2B cells were analysed via fluorescence microscopy under four different conditions; control cells, scratched cells, Poly (I:C) treated cells, and a combination of scratched and Poly (I:C) treated cells. All IL-33 fluorescence microscopy images were taken at an exposure time of 200 ms (all four treatment types and both cell lines) to ensure consistency when visualising differences in IL-33 expression. Control A549 cells (Fig. 4.1A) showed low IL-33 expression, with some of the cells expressing nuclear IL-33. Control BEAS-2B cells (Fig. 4.1B) expressed nuclear IL-33 at a higher expression than A549 cells, however, expression was not completely condensed to the nucleus. There is a slight expression difference between A549 control cells and BEAS-2B control cells, with the expression levels of IL-33 slightly lower in A549 cells.
Figure 4.1: IL-33 localisation in A549 control cells and BEAS-2B control cells at 100x magnification; A) A549 control cells. A549 cells were grown for 24 hours in 10% FCS then 24 hours in 1% FCS with no treatment. B) BEAS-2B control cells. BEAS-2B cells were grown for 24 hours in 10% FCS then 24 hours in 1% FCS with no treatment. White arrows indicate nuclear localisation and red arrows indicate IL-33 cytoplasmic localisation.
Nuclear IL-33 expression increases during wound healing in BEAS-2B cells.

As previously mentioned, nuclear IL-33 expression plays an important role in wound healing in mice (Oshio et al., 2017). However, little is known about the role of nuclear IL-33 in lung epithelial cell wound healing. To examine this, scratch assays were observed via fluorescence microscopy and Quantitative real-time PCR. By physically scratching cell monolayers, we are able to visualise epithelial cell natural wound healing responses to epithelial cell barrier damage. In this research project, we hypothesise that nuclear IL-33 expression will increase when the cells are physically scratched. We also compared this wound healing response in cancerous A549 epithelial cells to healthy BEAS-2B lung epithelial cells, to see how natural wound healing responses differ in cancer cells. Compared to control A549 cells (Fig. 4.2A), results showed that scratched A549 cells (Fig. 4.2B) expressed nuclear IL-33 to the same extent as control A549 cells, with little to no increase in nuclear IL-33 fluorescence. In contrast to this, compared to control BEAS-2B cells (Fig. 4.2C), scratched BEAS-2B cells (Fig. 4.2D) showed an upregulation of nuclear IL-33 expression, showing condensing of nuclear IL-33 in the nucleus of BEAS-2B cells. Fig. 4.3 shows quantitative real-time PCR data for IL-33 expression in control A549 cells, scratched A549 cells, control BEAS-2B cells and scratched BEAS-2B cells. We show that there is a 1-fold difference in IL-33 expression between control and scratched A549 cells. BEAS-2B cell analysis shows that there is a 3-fold difference between control and scratched BEAS-2B cells. These results also show that BEAS-2B scratched cells express IL-33 2-folds higher than scratched A549 cells. Therefore, we can see that there is a significant IL-33 expression difference between A549 cells and BEAS-2B cells, with IL-33 expression clearly peaking in scratched BEAS-2B cells.
Figure 4.2: IL-33 localisation in A549 scratched cells and BEAS-2B scratched cells at 100x magnification; Cells were grown as previously described for 24 hours in 10% FCS plus 24 hours in 1% FCS, and left untreated, or scratched for a 2-hour incubation. A) A549 control cells. B) A549 scratched cells. C) BEAS-2B control cells. D) BEAS-2B scratched cells. White arrows indicate nuclear IL-33 localisation, and red arrows indicate cytoplasmic IL-33 expression. The white dotted lines indicate the sites of the scratches.
**Figure 4.3:** Quantitative real-time PCR analysis of IL-33 expression in A549 cells and BEAS-2B cells; This graph describes control A549 cells, scratched A549 cells, control BEAS-2B cells and scratched BEAS-2B cells respectively. Cells were cultured as previously described and analysed via QPCR. Error bars describe standard error from several experiments.
Nuclear IL-33 expression and Poly (I:C).

Previously Gao et al 2012 showed that overexpression of IL-33 correlated with an increase in Th1 immune responses and therefore a reduction in tumour size. Poly (I:C) is an immune stimulant that causes Th1 immune responses when exposed to cultured cells. As a result of this, A549 and BEAS-2B cells were treated with the immune stimulant Poly (I:C) in an attempt to induce an increase in IL-33 expression. Cells were also dual treated with Poly (I:C) and scratches to test for IL-33 expression in cells with multiple inflammatory triggers. Compared to control A549 images (Fig. 4.4A), our results showed that A549 cells treated with Poly (I:C) (Fig. 4.4B) showed little to no expression difference. BEAS-2B control cells (Fig. 4.4C) compared to cells treated with Poly (I:C) (Fig. 4.4D) showed that treated cells expressed nuclear IL-33 slightly higher than the control images, but slightly lower than scratched images, as nuclear IL-33 had a higher density in scratched cells. Fig. 4.5 shows quantitative real-time PCR data for IL-33 expression in control A549 cells, Poly (I:C) treated A549 cells, control BEAS-2B cells and Poly (I:C) treated BEAS-2B cells. We show that there is a 0-fold difference between control A549 cells and A549 cells treated with Poly (I:C). We were also able to show that there is a 1-fold difference between control BEAS-2B cells and BEAS-2B cells treated with Poly (I:C). Therefore, our quantitative real-time PCR data supports our fluorescence microscopy data, where scratched BEAS-2B cells expressed higher levels of IL-33 compared to BEAS-2B cells treated with Poly (I:C). We also support the idea that IL-33 expression in A549 cells shows little change between treatment types.
Figure 4.4: IL-33 localisation in A549 Poly (I:C) treated cells and BEAS-2B Poly (I:C) treated cells at 100x magnification; Cells were grown as previously described for 24 hours in 10% FCS plus 24 hours in 1% FCS, and left untreated, or treated with Poly (I:C) for a 24-hour incubation. A) A549 control cells. B) A549 Poly (I:C) treated cells. C) BEAS-2B control cells. D) BEAS-2B Poly (I:C) treated cells. White arrows indicate nuclear IL-33 localisation, and red arrows indicate cytoplasmic IL-33 expression.
Figure 4.5: Quantitative real-time PCR analysis of IL-33 expression in A549 cells and BEAS-2B cells; This graph describes control A549 cells, Poly (I:C) treated A549 cells, control BEAS-2B cells and Poly (I:C) treated BEAS-2B cells respectively. Cells were cultured as previously described and analysed via QPCR. Error bars describe standard error from several experiments.
We then went on to test both cell lines under a combination of conditions (scratched and treated with Poly (I:C)) as it was hypothesised that nuclear IL-33 will further increase when the cells are under higher amounts of stress. Control A549 cells (Fig. 4.96) were compared to A549 cells dual scratched and treated with Poly (I:C) (Fig. 4.96), showing no increase in nuclear IL-33 expression levels compared to the other treatment types. Control BEAS-2B cells (Fig. 4.6C) compared to BEAS-2B cells scratched and treated with Poly (I:C) (Fig. 4.6D) expressed the highest amount of nuclear IL-33 seen in the gathered fluorescence microscopy images. Interestingly, we saw the highest expression levels of nuclear IL-33 in BEAS-2B dual treated cells, however, scratched only cells showed a higher amount of nuclear IL-33 than Poly (I:C) only cells. These results suggest that nuclear IL-33 plays a role in wound healing in BEAS-2B cells, as nuclear IL-33 was highly expressed in scratch assays in BEAS-2B. However, Poly (I:C) (infection) is needed with the scratch to induce nuclear IL-33 expression. This may be as cell damage is inducing nuclear IL-33 expression and the presence of an infection entering the cells in further inducing nuclear IL-33 expression. We saw no evidence of an increase in nuclear IL-33 in A549 cells. Fig. 4.7 shows quantitative real-time PCR data for IL-33 expression in control A549 cells, scratched and Poly (I:C) treated A549 cells, control BEAS-2B cells and scratched and Poly (I:C) treated BEAS-2B cells. We show that there is a 1-fold difference between control A549 cells and dual treated A549 cells. We also show a 5-fold difference between control BEAS-2B cells and dual treated BEAS-2B cells. This data supports our fluorescence microscopy data, where dual treated BEAS-2B cells expressed the highest levels of IL-33 of all treatment types. It also supports our A549 data that showed minimal expression difference between control and treated cells.
Figure 4.6: IL-33 localisation in A549 combined scratched and Poly (I:C) treated cells and BEAS-2B combined scratched and Poly (I:C) treated cells at 100x magnification. Cells were grown as previously described and left untreated, or treated with Poly (I:C) for a 24-hour incubation, as well as scratched and incubated for 2-hours. A) A549 control cells. B) A549 scratched and Poly (I:C) treated cells. C) BEAS-2B control cells. D) BEAS-2B scratched and Poly (I:C) treated cells. White arrows indicate nuclear IL-33 localisation, and red arrows indicate cytoplasmic IL-33 expression. White dotted lines indicate the site of the scratch.
Figure 4.7: Quantitative real-time PCR analysis of IL-33 expression in A549 cells and BEAS-2B cells; This graph describes control A549 cells, scratched/Poly (I:C) treated A549 cells, control BEAS-2B cells and scratched/Poly (I:C) treated BEAS-2B cells respectively. Cells were cultured as previously described and analysed via QPCR. Error bars describe standard error from several experiments.
**Nuclear IL-33 increases in low density BEAS-2B cells.**

Through fluorescence image analysis, we showed that high nuclear expression of IL-33 in BEAS-2B was not specific to the site of the scratch. We noticed that in patches of the well where cell confluency was lower, nuclear IL-33 expression was higher. To rule out experimental error, fluorescence experiments were replicated several times to ensure cells were incubated with adequate media throughout the entire well, and incubated with the same concentration of antibody and fluorescence dye throughout the entire well. DAPI stains did not reveal fluorescence patches similar to IL-33. To determine the cause of this nuclear IL-33 expression pattern, we analysed chamber slides with different cell densities to see if cell density affected nuclear IL-33 expression. We dropped the cell concentration of some wells by 50% and analysed the cells via fluorescence microscopy. Fig. 4.8 shows nuclear IL-33 expression at the site of two different scratches, in varying cell densities at 10x magnification. Fig. 4.8A shows low density cells at 50,000 cells per well, expressing high levels of nuclear IL-33. We can see that nuclear IL-33 expression isn’t located around the site of the scratch, however it is spread out through the entire 10x image. Fig. 4.8B shows high density cells at 100,000 cells per well expressing low levels of nuclear IL-33. Fig. 4.9 shows differing cell densities at a high magnification (100x). Fig. 4.9A shows low density cells at 50,000 cells per well with high nuclear IL-33 expression. Fig. 4.9B shows high density cells at 100,000 cells per well expressing low levels of nuclear IL-33. Both Fig. 4.8 and Fig. 4.9 showed that in low density cells, nuclear IL-33 expression was higher. To compare all treatment types, the same analysis was performed on Poly (I:C) treated cells (Fig 4.10). We showed that in low density BEAS-2B cells treated with Poly (I:C) (Fig. 4.10A), nuclear IL-33 expression increased. In high density BEAS-2B cells treated with Poly (I:C) (Fig. 4.10B), nuclear IL-33 expression was low.
Therefore, we were able to show that cell density plays a role in nuclear IL-33 expression in BEAS-2B cells. Cells of a lower density expressed higher levels of nuclear IL-33 and cells of a higher density expressed lower levels of nuclear IL-33. We did not perform the same analysis on A549 cells as we previously showed that no expression difference was seen between each treatment type, regardless of cell density.
**Figure 4.8:** Nuclear IL-33 expression at the site of a scratch at 10x magnification in BEAS-2B cells; Cells were grown as previously described and scratched for 2-hours. The site of the scratch was analysed under the fluorescence microscope during two different experiments showing IL-33 expression, DAPI and IL-33 expression and DAPI respectively.

A) The white dotted lines indicate the site of the scratches, seen at the bottom of the images and on the left side of the images. Cells are not very confluent and highly express nuclear IL-33.

B) The white dotted lines indicate the site of the scratches, seen on either side of the image (left and right). Cells are highly confluent and express lower levels of nuclear IL-33 than A. Red squares zoom into images for closer observation.
**Figure 4.9:** IL-33 localisation in BEAS2B cells at 100x magnification; All cells are grown for 24 hours in 10% FCS then 24 hours in 1% FCS and 10 ug/mL of Poly (I:C). Cells were scratched for 2-hours during treatment. Images were taken from the same chamber well in different sections. White arrows point to the nucleus of opposing cells. A) Shows IL-33 translocated into the nucleus. B) Shows little evidence of IL-33 in the nucleus. The white dotted lines represent the location of the scratch in each image.
**Figure 4.10:** IL-33 expression in BEAS-2B cells at 10x magnification; Images are taken from the same well at different locations and the cells are treated with Poly (I:C). A) Low density cells with high nuclear IL-33 expression. B) High density cells with low nuclear IL-33 expression. The red squares zoom in for a clearer picture.
To further support our fluorescence microscopy results, images were categorised into two separate groups; cells of low density and cells of high density. For each, 6 images were quantified and the averages were calculated for the total number of cells in each image, the total number of cells that expressed nuclear IL-33, and the total number of cells that expressed cytoplasmic IL-33. Fig. 4.11 shows quantitative expression of IL-33 in control BEAS-2B cells, comparing cells in low density images and cells in high-density images. We showed that in a well with low cell density, more cells express nuclear IL-33 than cytoplasmic IL-33. In wells with a higher cell density, cells expressing cytoplasmic IL-33 peaked. 76% cells in low density images expressed nuclear IL-33, whereas 34% of cells in high density images expressed nuclear IL-33. Fig. 4.12 shows quantitative expression of IL-33 in scratched BEAS-2B cells, comparing cells in low density images and cells in high-density images. We showed that in a well with low cell density, more cells express nuclear IL-33 than cytoplasmic IL-33. In wells with a higher cell density, cells expressing cytoplasmic IL-33 peaked. 81% of cells in low density images expressed nuclear IL-33, whereas 39% of cells in high density images expressed nuclear IL-33. These percentages are slightly higher than those seen in control BEAS-2B cells. Fig. 4.13 shows quantitative expression of IL-33 in Poly (I:C) treated BEAS-2B cells, comparing cells in low density images and cells in high-density images. We showed that in a well with low cell density, more cells express nuclear IL-33 than cytoplasmic IL-33. In wells with a higher cell density, cells expressing cytoplasmic IL-33 peaked. 58% of cells in low density images expressed nuclear IL-33, whereas 31% of cells in high density images expressed nuclear IL-33. These percentages are significantly lower than those seen in scratched BEAS-2B cells. Fig. 4.14 shows quantitative expression of IL-33 in dual scratched and Poly (I:C) treated BEAS-2B cells, comparing cells in low density images and cells in high-density
images. We showed that in a well with low cell density, more cells express nuclear IL-33 than cytoplasmic IL-33. In wells with a higher cell density, cells expressing cytoplasmic IL-33 peaked. 82% of cells in low density images expressed nuclear IL-33, whereas 39% of cells in high density images expressed nuclear IL-33. These percentages are the highest percentage of nuclear IL-33 seen in low density BEAS-2B cells. This data supports our previous data that showed that cell density affects nuclear IL-33 expression, with all graphs providing evidence of high nuclear IL-33 expression in low density cells.
Figure 4.11: Quantitative IL-33 expression data of fluorescence microscopy images in control BEAS-2B cells; Images were separated into two groups; low density or high density. Averages were calculated for total cells, cells expressing nuclear IL-33, and cells expressing cytoplasmic IL-33 respectively. Error bars show standard error from several experiments.
Figure 4.12: Quantitative IL-33 expression data of fluorescence microscopy images in scratched BEAS-2B cells; Images were separated into two groups; low density or high density. Averages were calculated for total cells, cells expressing nuclear IL-33, and cells expressing cytoplasmic IL-33 respectively. Error bars show standard error from several experiments.
**Figure 4.13:** Quantitative IL-33 expression data of fluorescence microscopy images in Poly (I:C) treated BEAS-2B cells; Images were separated into two groups; low density or high density. Averages were calculated for total cells, cells expressing nuclear IL-33, and cells expressing cytoplasmic IL-33 respectively. Error bars show standard error from several experiments.
Figure 4.14: Quantitative IL-33 expression data of fluorescence microscopy images in dual scratched and Poly (I:C) treated BEAS-2B cells; Images were separated into two groups; low density or high density. Averages were calculated for total cells, cells expressing nuclear IL-33, and cells expressing cytoplasmic IL-33 respectively. Error bars show standard error from several experiments.
**H3K27M3 is upregulated in scratched and Poly (I:C) treated BEAS-2B cells.**

To determine whether nuclear IL-33 plays a role in gene repression, the repressive methyl mark H3K27M3 was analysed under conditions where nuclear IL-33 was highly expressed (Fig. 4.15). Control BEAS-2B cells (Fig. 4.15A) showed low levels of H3K27M3 expression when overlapped with DAPI. H3K27M3 expression was upregulated in cells treated with Poly (I:C) (Fig. 4.15B) and then highly upregulated in scratched cells (Fig. 4.15C and Fig. 4.15D). These results mimic the results seen in nuclear IL-33 stains, where the highest levels of nuclear IL-33 was seen in scratched cells. These results suggest that nuclear IL-33 correlates with the expression of H3K27M3. No H3K27M3 expression was seen in A549 cells (data not shown).
Figure 4.15: H3K27M3 localisation in BEAS2B at 100x magnification; A) Control cells. Cells were grown for 24 hours in 10% FCS then 24 hours in 1% FCS with no treatment. B) Scratched cells. Cells were grown for 24 hours in 10% FCS then 24 hours in 1% FCS and scratched for the remaining 2 hours. C) Treated cells. Cells were grown for 24 hours in 10% FCS then 24 hours in 1% FCS and 10 ug/mL of Poly (I:C). D) Treated and scratched cells. Cells were grown for 24 hours in 10% FCS then 24 hours in 1% FCS and 10 ug/mL of Poly (I:C). Cells were then scratched for 2 hours to induce cell stress. White arrows indicate H3K27M3 expression. Red arrows indicate minimal H3K27M3 expression.
**IL-33 Translocates into the nucleus during apoptosis.**

Apoptosis, also known as programmed cell death, can be identified by the condensation of chromatin and membrane blebbing of chromatin caused by cell membrane rupture. Chromatin will condense into small balls once the cell has ruptured and become degraded by caspases and proteases. The role of nuclear IL-33 in apoptosis in epithelial cells is yet to be explored in literature. High nuclear IL-33 expression is visualised in apoptotic A549 cells in this research project. In Fig. 4.16, we show clear evidence of cells undergoing apoptosis as chromatin membrane blebbing is occurring. We can see an increase in nuclear IL-33 expression and chromatin co-localisation during apoptosis. There is no evidence of cytoplasmic IL-33.

![Figure 4.16: IL-33 localisation during apoptosis in A549 cells](image)

*Figure 4.16: IL-33 localisation during apoptosis in A549 cells; cells were grown as previously described and left untreated. It was later seen that the cells were in fact undergoing apoptosis. Cells were stained for IL-33, DAPI and IL-33 and DAPI respectively, and viewed under the fluorescence microscope at 100x magnification.*
**ST2 expression decreases when scratched and treated with Poly (I:C).**

ST2 expression during high nuclear IL-33 expression is analysed via fluorescence microscopy in A549 and BEAS-2B cells. Cell treatments are identical to IL-33 treatments, as we want to see how an increase in nuclear IL-33 expression affects ST2 expression. A549 control cells (Fig. 4.17A) showed no ST2 cell bordering in both control and treated cells. There were also high amounts of ST2 speckles in the cytoplasm. There were no differentiating factors seen between control and treated cells (Fig. 4.17B). BEAS-2B control cells (Fig. 4.18A) expressed ST2 differently to A549 cells under the fluorescence microscope, with no evidence of ST2 speckling in the cytoplasm of BEAS-2B cells. ST2 localised in large cytoplasmic patches in control cells, and when BEAS-2B were scratched (Fig. 4.18B), ST2 expression condensed in the cytoplasm, with the patches becoming more compact than control cells. However, there was minimal expression difference in both cell lines. Quantitative real-time PCR data for ST2 expression supports these results. ST2 expression in A549 cells slightly increased when the cells were scratched and treated with Poly (I:C) (Fig. 4.19). ST2 expression in BEAS-2B cells (Fig. 4.20) decreased when the cells were scratched and treated with Poly (I:C), compared to control cells.
Figure 4.17: ST2 localisation in A549 cells at 100x magnification; N=2 A) Control cells. Cells were grown for 24 hours in 10% FCS then 24 hours in 1% FCS with no treatment. White arrows indicate nuclear/cellular ST2 bordering. Red arrows indicate ST2 speckling in the cytoplasm. B) Dual treated cells. Cells were grown for 24 hours in 10% FCS then 24 hours in 1% FCS and 10 ug/mL of Poly (I:C). Cells were scratched for 2 hours to induce cell stress. White arrows indicate nuclear/cellular ST2 bordering. Red arrows indicate ST2 speckling in the cytoplasm. Cells are stained and viewed for ST2, DAPI and a combination of ST2 and DAPI respectively.
**Figure 4.18**: *ST2 localisation in BEAS2B at 100x magnification; N=2*  
A) Control cells. Cells were grown for 24 hours in 10% FCS then 24 hours in 1% FCS with no treatment. White arrows indicate nuclear/cellular ST2 bordering. Red arrows indicate ST2 speckling in the cytoplasm.  
B) Treated cells. Cells were grown for 24 hours in 10% FCS then 24 hours in 1% FCS and 10 ug/mL of Poly (I:C). Cells were scratched for 2 hours to induce cell stress. White arrows indicate ST2 clustering in the cytoplasm. Red arrows indicate ST2 clustering around the edge of the cell. Cells are stained and viewed for ST2, DAPI and a combination of ST2 and DAPI respectively.
**Figure 4.19**: Quantitative real-time PCR data for ST2 expression in A549 cells; Cells were grown as previously described and left untreated, treated with Poly (I:C), scratched for 2-hours, or treated and scratched respectively. Error bars display standard error from several experiments.
Figure 4.20: Quantitative real-time PCR data for ST2 expression in BEAS-2B cells; Cells were grown as previously described and left untreated, treated with Poly (I:C), scratched for 2-hours, or treated and scratched respectively. Error bars display standard error from several experiments.
Chapter 5: Discussion and Future Implications

Nuclear IL-33 is expressed in lung epithelial cells.

IL-33 is a recently described member of the IL-1 family of cytokines, that is found to induce Th2 immune responses by activating its orphan receptor ST2. Recent studies identify IL-33 as a nuclear factor in epithelial cells and endothelial cells, with the ability to bind to chromatin and stimulate gene repression via the methyltransferase SUV39H1 (Roussel et al., 2008). However, the role of nuclear IL-33 in epithelial cells and the dysregulation of nuclear IL-33 in epithelial cells, causing the development of cancer and chronic inflammatory diseases is unclear and will be explored in this research paper. It is unclear whether lung epithelial cells in particular express nuclear IL-33, and whether or not expression levels differ in cancerous epithelial cells. Using healthy lung epithelial cells (BEAS-2B cells) and NSCLC cells (A549 cells), we showed that nuclear IL-33 is expressed in both A549 control epithelial cells and BEAS-2B control epithelial cells. By confirming the nuclear localisation of IL-33, we can study the effect of a dysregulation in the IL-33 pathway on the development of asthma and cancer for future treatment purposes.

Nuclear IL-33 is upregulated in BEAS-2B cells during wound healing.

Recently, Oshio et al (2017) shows that nuclear IL-33 is involved in wound healing by showing a delay in wound healing in IL-33 knock-out mice. They also show that sST2 over-expression had no effect on the results, meaning that it was nuclear IL-33 causing the results and not cytokine IL-33, as sST2 did not abrogate the wound healing effects of cytokine IL-33. However, the role of nuclear IL-33 in wound healing remains unclear and will be explored in this research paper. Using A549 cancerous epithelial cells and
BEAS-2B healthy epithelial cells, cell monolayers were physically scratched to stimulate normal cellular responses to epithelial barrier damage, and nuclear IL-33 expression was analysed via fluorescence microscopy during cell wound healing. We showed that nuclear IL-33 was upregulated in scratched BEAS-2B cells compared to control samples, with no evidence of nuclear IL-33 upregulation in scratched A549 cells. Barrera et al (2014) previously showed that IL-33 is downregulated in NSCLCs. As a result of this, we compared A549 cells with BEAS-2B cells in an attempt to understand whether or not expression of nuclear IL-33 differs in cancerous epithelial cells for potential clinical use. We provide evidence that nuclear IL-33 expression is lower in A549 cancerous epithelial cells compared to BEAS-2B healthy epithelial cells. Future research should focus on why A549 cells express lower levels of nuclear IL-33, as it could potentially be used in a clinical setting for the treatment of lung cancer. It could possibly mean that the normal functioning of nuclear IL-33 is compromised in A549 cancer cells. To reiterate, it is suggested by Carriere et al (2006) that nuclear IL-33 binds to chromatin to turn off inflammatory gene transcription, acting as a regulatory mechanism in maintaining cellular homeostasis. Cellular homeostasis is defined by the turning on of inflammation when there is a threat identified by the body, and the turning off inflammation when the threat is eliminated to avoid over stimulation of the immune system. Here, we see that A549 cells express lower levels of nuclear IL-33 compared to BEAS-2B cells, which could quite possibly mean that A549 cells lack the ability to upregulate nuclear IL-33 and therefore maintain cellular homeostasis when stimulated by cell damage. Barrera et al (2014) recently underwent a study to determine what immune regulatory cytokines are increased in NSCLC cells, as these cytokines may play a role in tumour growth and metastasis. They show that IL-33 is lower in NSCLC cells compared to controls, and aim to use these results as potential
biomarkers for the prognosis of cancer. In this project, our data showed the same results as Barrera et al (2014), as we showed that nuclear IL-33 expression is lower in A549 cells compared to BEAS-2B cells. In all, these results are significant because by confirming the upregulation of nuclear IL-33 when the cells were scratched, means that nuclear IL-33 might play a vital role in wound healing. By visualising an increase in nuclear IL-33 during wound healing means that in chronically inflamed tissue nuclear IL-33 expression should be elevated, acting as a novel biological marker and therapeutic target for chronic inflammatory conditions such as asthma.

*Nuclear IL-33 turns off inflammation*

Previous studies provide evidence of a repressive role of nuclear IL-33, binding to chromatin and stimulating the addition of methyl marks by the methyltransferase SUV39H1 (Roussel et al., 2008). However, the role of nuclear IL-33 in A549 cells and BEAS-2B epithelial cells currently remains unclear. On the grounds that previous research studies show that nuclear IL-33 plays a role in gene repression (Roussel et al., 2008), the expression level of the repressive methyl mark H3K2Me3 in A549 cancer cells and BEAS-2B epithelial cells were analysed via fluorescence microscopy. We provide evidence of H3K27Me3 expression in BEAS-2B cells, in an upregulated pattern similar to that seen in nuclear IL-33 stains (both H3K27Me3 and nuclear IL-33 are expressed in control cells and highly expressed in scratched cells). Further staining revealed that the loss of nuclear IL-33 expression caused H3K27Me3 expression loss in BEAS-2B cells (data not shown). In these experiments, nuclear IL-33 loss was not caused by knock-down experiments, but by the loss of the nuclear IL-33 stimulating factor suggested as cytokine IL-33. We were able to show an increase in H3K27Me3 expression at the same time as an increase in nuclear IL-33 expression, suggesting that they correlate. Therefore, by visualising the expression increase in
both nuclear IL-33 and H3K27Me3, these results could mean that nuclear IL-33 is potentially stimulating the addition of H3K27Me3 to chromatin and turning off gene transcription. To further confirm nuclear IL-33’s association with H3K27Me3, future research should test H3K27Me3 expression in knock down IL-33 cells and should co-localise the stains to test for a confirmed relationship between H3K27Me3 and nuclear IL-33. If H3K27Me3 expression diminishes when IL-33 is lost in knock-down experiments, then it is highly possible that they are associated. Due to the time limit in this research project, the proposed experiments were unable to be performed. However, we have currently shown a correlation between H3K27Me3 and nuclear IL-33. These experiments are important because it will confirm the function of nuclear IL-33, allowing for gene targeting in clinical practices to turn off over stimulated inflammatory genes and allow for the relief of chronic illnesses such as asthma.

*Nuclear IL-33 Turns on Apoptosis.*

The role of IL-33 in apoptosis of epithelial cells is yet to be explored in literature. However, we recognise that previous studies show inactive NF-κB as a regulator of apoptosis (Barkett and Gilmore, 1999). In this research project, we confirmed nuclear IL-33 expression in both A549 cells and BEAS-2B cells. We also showed that H3K27Me3 expression increased at the same time as nuclear IL-33 expression. Therefore, by suggesting that IL-33 is a repressive nuclear factor in lung epithelial cells, and that nuclear IL-33 is highly expressed in cells undergoing apoptosis, we hypothesise that nuclear IL-33 binds to active NF-κB to inactive NF-κB and initiate apoptosis. The low expression levels of nuclear IL-33 that we see in A549 cancer cells suggests that the loss of nuclear IL-33 functioning is inhibiting normal IL-33 mediated apoptotic processes, leading to the growth of unwanted cancer cells; no nuclear IL-33
means that cancer cells are not targeted by nuclear IL-33 mediated apoptosis, which means that cancer cells spread and metastasise. As we mentioned earlier, nuclear IL-33 could be controlled by the release of cytokine IL-33. Therefore, the ability for cancer cells to grow and metastasise might relate back to the overall expression levels of all forms of IL-33, as cytokine IL-33 might target cancer by activating immune defences and causing nuclear IL-33 expression, and nuclear IL-33 might target cancer by stimulating apoptosis. Due to the short time span of this research project and the fact that our IL-33 antibody and NF-κB antibody were both rabbit, direct binding and co-localisation of nuclear IL-33 to NF-κB was unable to be researched. However, we were able to show an increase of nuclear IL-33 during apoptosis. Future research on the regulation of apoptosis by nuclear IL-33 and NF-κB serve as important potential cancer treatment directions. The over-expression of nuclear IL-33 in patients suffering from lung cancer will potentially bind to NF-κB and stimulate apoptosis of cancer cells.

**Gene targets of nuclear IL-33.**

To further expand on the role of IL-33 in the nucleus of epithelial cells, a data base search for known genes that are associated with Th2 responses and apoptosis as potential targets of nuclear IL-33 were identified, including STAT6, GATA3 and BCL2L1 (Lund et al., 2005). Due to the short time span of this research project, direct target genes of IL-33 were unable to be identified through practical research such as ChIP, however, they serve as a future research direction for IL-33 research. By determining the specific genes that nuclear IL-33 binds to, the role of nuclear IL-33 can be confirmed and used for clinical treatment of chronic inflammatory diseases.
Cause of Nuclear IL-33 Expression.

The discovery of IL-33 translocation into the nucleus of lung epithelial cells, and the potential of nuclear IL-33 to regulate H3K27Me3 encouraged further experiments to determine the cause of nuclear IL-33 translocation. Our results showed nuclear IL-33 expression in a pattern throughout fluorescence microscopy stains (some cells expressed nuclear IL-33 and some cells did not). As a result of this, we are suggesting that a cytokine is being released from these damaged cells and spreading to a majority of the cells in the same well, not just cells located at the site of a scratch, causing nuclear IL-33 expression. IL-6 and IL-1β levels were therefore analysed via flow cytometry (data not shown) in an attempt to locate the IL-33 translocating inducing factor. It was hypothesised that the serum levels of one of these cytokines will increase synonymously with nuclear IL-33 expression, suggesting that the cytokine is influencing nuclear IL-33 expression. However, we were unable provide evidence of an increase in IL-6 or IL-1β when nuclear IL-33 was expressed.

Lin et al (2013) uses corneal epithelial cells to demonstrate that ST2 is located on epithelial cells, and plays an important role in mediating IL-33 induced Th2 inflammation. These results provide evidence of autocrine functioning of cytokine IL-33; IL-33 is released from an epithelial cell and binds to the ST2 on the same epithelial cell to induce a response. As a result of this, we suggest that cytokine IL-33 is functioning in an autocrine and paracrine manner on epithelial cells to regulate the expression of nuclear IL-33. Due to the time span of this research project we were unable to replicate experiments and test the expression levels of cytokine IL-33, however, we hypothesise that IL-33 itself is controlling the expression levels of nuclear IL-33. As mentioned earlier, nuclear IL-33 is expressed in low density cells. As a result
of this, we suggest that cytokine IL-33 binds to epithelial cells that lack cell-cell contact markers such as E-cadherin, causing nuclear IL-33 expression. Cells that lack these cell-cell contact markers tell cytokine IL-33 that they are damaged, therefore they are targeted by cytokine IL-33 for IL-33 induced apoptosis. In all, we suggest that cytokine IL-33 released by epithelial cells binds to ST2 on epithelial cells that lack E-cadherin, a mark that tells IL-33 that they are damaged, and causes nuclear IL-33 expression. Future research should focus on confirming this hypothesis as diseases caused by the lack of nuclear IL-33 expression could potentially be treated by over-expressing nuclear IL-33. This is seen as a regulatory mechanism of inflammation, preventing over stimulation of immune responses and chronic inflammation. Therefore, it is suggested that IL-33 released by epithelial cells controls nuclear IL-33 translocation. Recently, Küchler et al (2008) demonstrated that IL-33 is expressed in cells of high confluence and lost when cells started to migrate and proliferate. Our results showed the opposite, with cells of low density expressing high nuclear IL-33. The discrepancy between results may be explained by the use of different cell lines, as this project used cancerous and healthy lung epithelial cells where as Küchler et al (2008) used epidermal keratinocytes. The role of nuclear IL-33 might differ in these two types of cells, therefore, in order to completely understand the expression and role of nuclear IL-33 in all cell types, more research is needed in multiple cell lines, as treatments for cancer and chronic inflammatory illnesses by targeting IL-33 may only be applicable in specific cell types.

As we showed earlier, A549 cells express lower levels of nuclear IL-33 compared to BEAS-2B cells. From these results, we suggested that A549 cells might potentially lack proper functioning of the IL-33/ST2 axis and therefore IL-33 cannot translocate
into the nucleus of A549 cells. The ability of the IL-33/ST2 axis to regulate nuclear IL-33 expression in epithelial cells and potentially regulate H3K27Me3 expression is an important pathway in the regulation of multiple diseases such as cancer, as seen by A549 cells lacking this regulatory pathway (Fig. 5.1). The visualisation of this regulatory pathway in BEAS-2B cells, but not A549 cells, could be due to A549 cells containing mutations that prevents these cells from utilising this regulatory pathway. These results are significant as treatment by IL-33 over-expression may potentially provide relief for patients suffering from chronic inflammatory conditions, as it will turn off the effects of chronic inflammation where the uncontrollable release of IL-33 causes over activation of the immune system. IL-33 over-expression could also be utilised in tumours, where the introduction of nuclear IL-33 into cancer cells could aid in apoptosis of cancer cells and the destruction of tumours. Therefore, by over-expressing nuclear IL-33 into chronically inflamed tissues and cancer cells, chronic inflammation caused by the over expression of IL-33 can be controlled to relieve chronically inflammation, and tumour size can potentially be reduced.

*IL-33 in the tumour microenvironment.*

The loss of IL-33 expression in A549 cells suggests that IL-33 may play an important role against cancer. As mentioned earlier, the initial recognition of tumours by immune cells causes a Th1 mediated immune response, with Th1 responses causing an increase of IFN-Y, IL-1, IL-12 and IL-18, leading to the activation of NK cells and CD8⁺ cells. The tumour microenvironment undergoes immune evasion by reprogramming infiltrating Th1 immune cells to believe the tumour is part of the healthy tissue. Cytokine IL-33 and nuclear IL-33 work closely in the tumour microenvironment, with cytokine IL-33 activating cytokine IL-33 and immune responses for the destruction of
**Figure 5.1:** Proposed IL-33/ST2 pathway; healthy epithelial cells are damaged causing IL-33 release from the cytoplasm. IL-33 binds to ST2 on the same cells it was released from, and causes cytoplasmic full-length IL-33 to translocate into the nucleus and turn off inflammatory gene transcription.
tumours and nuclear IL-33 turning on apoptosis to remove cancer cells. The tumour microenvironment could potentially be turning off cytokine and therefore nuclear IL-33 functioning, which allows the tumour cells to avoid apoptosis by nuclear IL-33 and avoid the over stimulation of inflammatory responses by the release of cytokine IL-33. This is a clear example of how cytokine IL-33 and nuclear IL-33 work together to maintain cellular homeostasis.

In addition to this, Wang et al (2016) shows that IL-33 levels are high in slow growing tumours and low in tumours with high metastasis rates. This is because in slow growing tumours there is still evidence of a functioning IL-33/ST2 axis that can target tumour cells, whereas in fast growing tumours IL-33/ST2 expression was lost, allowing the tumour microenvironment to undergo immune evasion. Therefore, IL-33 reintroduction in tumours could reactivate the immune system, initiating the infiltration of CD8⁺ cells and NK cells that will act to destroy the tumour. As suggested earlier, the introduction of IL-33 in tumours could also promote the translocation of IL-33 into the nucleus of cancer cells, helping with immune regulation and the rid of tumours by promoting apoptosis of cancerous cells. However, more research is needed to understand whether IL-33 can aid in the destruction of tumours. By growing 3D spheroid cells in vitro and comparing control cells with knock out IL-33 cells, over expression of cytokine IL-33 cells, nuclear IL-33 cells and a combination of cytokine IL-33 and nuclear IL-33 cells, we will have a clearer understanding of the role of both forms of IL-33 in tumours. This research project attempted to grow 3D cells in vitro on matrigel (data not shown), however, due to the time span of this research project the technique was able to be perfected to form perfect spheroids. This may be due to over seeding of cells in the protocol used or incorrect use of matrigel concentrations.
Through further research, it is suggested that growing spheroids by the hanging drop method may result in better spheroid formation.

**ST2 internalises during IL-33 expression.**

IL-33 is an alarmin shown to exacerbate Th2 inflammatory responses by binding to its receptor ST2. Recent studies show that the ST2 gene, *IL1RL1*, resides in a linkage disequilibrium block where genetic mutations cause the development of asthma (Ho et al., 2013). However, the role of ST2 in the development of chronic illnesses and cancer remains unclear. A previous study by Zhao et al (2014) shows that IL-33 treatment activates focal adhesion kinase (FAK), which in turn activates the glycogen synthase kinase 3β (GSK3β), causing ST2 internalisation. Therefore, it is suggested that over-expression of IL-33 should cause ST2 internalisation, and therefore turn off inflammation. Knock-down of FAK stopped IL-33 mediated ST2 internalisation (Zhao et al., 2014), therefore mutations in signalling proteins associated with FAK could potentially stop regulatory ST2 internalisation and therefore cause IL-33 hyper responsiveness by over-expression of ST2 on cell surfaces. This ST2 over-expression could potentially lead to the development of chronic inflammatory conditions such as asthma. With these results in mind, we compared the expression of ST2 in A549 cells and BEAS-2B cells, in control cells, scratched cells and Poly (I:C) treated cells. Our results show no obvious increase in the expression levels of ST2 via fluorescence microscopy and QRTPCR data, of control compared to scratched and Poly (I:C) treated cells. However, the localisation of ST2 in the cells changed in BEAS-2B between treatments, with ST2 concentrated through the cytoplasm in scratched cells and less around the boarder compared to control cells. These results support the idea that IL-33 expression causes ST2 internalisation and inflammatory control. A549 cells
showed no obvious difference of ST2 expression in control cells compared to scratched cells, with ST2 cytoplasmic speckling seen in both images. As we already know, A549 cells contain multiple mutations that make them cancerous. Mutations in FAK as suggested earlier could potentially be dysregulating the expression of ST2 where minimal ST2 is expressed on epithelial cell surfaces. This in turn means that IL-33 cannot bind to ST2 and therefore cannot cause nuclear IL-33 translocation, meaning no apoptosis is stimulated. Future research should focus on the expression of ST2 and the growth of cancer and development of chronic inflammatory diseases, as future clinical treatments could treat inflammatory conditions and stop the growth of tumours.

**Future Implications and Research Question**

Several research studies confirm nuclear localisation of IL-33 in endothelial and epithelial cells. However, little is known about the difference between nuclear IL-33 expression in healthy lung epithelial cells (BEAS-2B) compared to cancerous lung epithelial cells (A549 cells). Studies indicate that the normal functioning of nuclear IL-33 controls cellular homeostasis by repressing gene transcription and turning off inflammation. As A549 cancer cells are highly malignant and consist of many genetic mutations, it is hypothesised that A549 cells will express lower levels of nuclear IL-33 compared to BEAS-2B cells due to genetic mutations that stop the normal functioning of nuclear IL-33. This research project visualised nuclear IL-33 expression in A549 cells and BEAS-2B cells via fluorescence microscopy and quantitative real-time PCR (QRTPCR), with nuclear IL-33 expression in BEAS-2B cells higher than A549 cell nuclear IL-33 expression.
We visualised the expression of nuclear IL-33 during wound healing by growing cell monolayers and physically scratching them to induce epithelial wounds. Wound closure was examined at the 2-hour time point and showed an increase in nuclear IL-33 expression. IL-33 expression was seen in patches throughout the entire well and not just around the site of the scratch, suggesting that a cytokine is being released from the damaged cells, causing IL-33 to translocate into the nucleus of cells.

Previous studies show that nuclear IL-33 turns off inflammatory gene transcription by directly binding to chromatin and stimulating the addition of methyl marks by methyltransferases. As a result of this, this research project analysed the expression levels of the methyl mark H3K27Me3 and showed that when nuclear IL-33 expression is upregulated, HEK27Me3 expression is also upregulated. When nuclear IL-33 expression was lost, H3K27Me3 expression was also lost.

ST2 expression is also analysed in this research project. It is unconfirmed whether ST2 expression increases when nuclear IL-33 expression increases. As a result of this, ST2 expression is examined under conditions where nuclear IL-33 was highly expressed (scratched cells and cells treated with Poly (I:C)). We showed that ST2 expression did not change in response to nuclear IL-33 upregulation.

Nuclear IL-33 expression during apoptosis is also analysed in this research project. We showed that nuclear IL-33 is highly expressed in cells undergoing apoptosis. As previously mentioned, cancer cells have the ability to evade immune cells allowing the uncontrollable growth of tumours, and therefore should lack IL-33/ST2 mediated apoptosis. By researching IL-33 and ST2, it is anticipated that a clearer understanding
of the role of the IL-33/ST2 axis will be identified in healthy and cancerous epithelial cells, for future treatment and research implications.

In this study, we visualised nuclear IL-33 in both A549 cells and BEAS-2B cells, and showed that nuclear IL-33 is upregulated in BEAS-2B cells, but not A549 cells, that are scratched and treated with Poly (I:C). We also showed that nuclear IL-33 is upregulated in cells going through apoptosis, and that H3K27Me3 and nuclear IL-33 expression increased synonymously with each other. Our findings suggest that nuclear IL-33 is a regulatory factor in lung cancer and wound healing, by promoting the addition of H3K27Me3 to chromatin, and promoting apoptosis in unwanted cells, particular damaged cells in wounds. The regulatory mechanisms of nuclear IL-33 are lost in lung cancer cells, causing outgrowth and metastasis.
Chapter 6: Conclusion

In summary, IL-33 is an IL-1-like alarmin released by epithelial cells to induce Th2 immune responses. This research project provides evidence of IL-33 exerting dual functions, visualising IL-33 translocation into the nucleus of NSCLC A549 cells and healthy lung epithelial BEAS-2B cells under resting conditions. When the cells are scratched and treated with Poly (I:C), nuclear IL-33 rapidly increases in BEAS-2B cells, but not A549 cells. This data suggests that A549 cells lack the ability to utilise normal IL-33 functioning.

This research project provides a clearer understanding of the role that nuclear IL-33 plays in epithelial cells. We showed that nuclear IL-33 increased in response to being physically scratched, suggesting that IL-33 plays a role in wound healing. We also showed that when nuclear IL-33 increased, the repressive methyl mark H3K27Me3 increased synonymously. We did not visualise co-localisation of IL-33 and H3K27Me3 as both antibodies were rabbit, however, we did see a direct correlation between the expression levels of each, suggesting that nuclear IL-33 regulates chromatin compaction via the addition of the repressive methyl mark H3K27Me3.

We also examined the expression level of nuclear IL-33 in cells undergoing apoptosis, and saw that during apoptosis, nuclear IL-33 is upregulated. Therefore, we visualised that nuclear IL-33 is turning on apoptosis to help maintain cellular homeostasis. We previously stated that A549 cells lack the ability to utilise nuclear IL-33 functioning, suggesting that in cancer cells, nuclear IL-33 is unable to stimulate normal apoptotic processes and remove unwanted cancer cells from the body. Unable to remove cancer cells due to nuclear IL-33 inactivity, tumours grow and metastasise.
As an additional response to cancer, cytokine IL-33 is shown as highly expressed in slow growing cancers and as not expressed in fast growing cancers, suggesting that cytokine IL-33 acts defensively against cancers by activating immune responses. This suggests that both forms of IL-33 work together to destroy cancer cells, with cytokine IL-33 expression preventing tumours from undergoing immune tolerance by activating CD8\(^+\) and NK cells, while nuclear IL-33 identifies non-self-cells and initiates apoptosis. It is suggested that nuclear IL-33 is stimulated by an increase in the levels of cytokine IL-33, causing IL-33 itself to translocate into the nucleus of the same cell and either turn off inflammation if the cause has been rectified, or turn on apoptosis if the cells cannot return back to homeostasis. A similar role of nuclear IL-33 in cancer cells is described in wound healing, with IL-33 mediated apoptosis potentially used to eliminate damaged epithelial cells. Even though this research project is suggesting an inflammatory repressive role of nuclear IL-33 via the addition of H3K27Me3, it is also suggesting that nuclear IL-33 binds to chromatin to turn on apoptosis. It is highly possible that it is doing both, however, future research is needed to understand the complete role of nuclear IL-33 in lung epithelial cells.

In all, a protective role of IL-33 has been identified in healthy lung epithelial cells, with IL-33 mediated protection lost in cancer cells. We suggest that when cells are damaged by physical scratches, they release cytokine IL-33 that turns on inflammation in an attempt to battle cancer or invading pathogens. This cytokine can spread in serum and affect cells not only locally, but cells that are further away, binding to ST2 on epithelial cells causing residual IL-33 to translocate into the nucleus. Nuclear IL-33 will either turn off inflammation via the addition of H3K27Me3 if there is no longer a
threat or it will stimulate apoptosis if the cell is too damaged to return back to a homeostatic state. A549 cells lack this normal regulatory mechanism of cytokine IL-33 and nuclear IL-33, causing over growth and metastasis of tumours. A549 cells also lack proper ST2 functioning, which inhibits cytokine IL-33 binding and therefore functioning. Targeted treatment of this IL-33 regulatory mechanism in a clinical setting will potentially benefit patients suffering from cancer or inflammatory illnesses such as asthma. Apoptosis mediated by nuclear IL-33 could potentially be a therapeutic target treatment in the future for cancer, with supplemented nuclear IL-33 turning on apoptosis in cancer cells, leading to a decrease in tumour size. The importance of nuclear IL-33 is highlighted in this paper, providing evidence to support the treatment of asthma and cancer; IL-33 causes nuclear IL-33 expression which turns off inflammation and turns on apoptosis.
References


58. Shao, D., Perros, F., Caramori, G., Meng, C., Dormuller, P., Chou, P., Church, C., Papi, A., Casolari, P., Welsh, D., Peacock, A., Humbert, M.,


