

The Role of Interleukin-33 in the Development of Idiopathic Pulmonary Fibrosis



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Abstract

Background: Idiopathic pulmonary fibrosis (IPF) is a chronic, progressive interstitial lung disease characterised by the excessive deposition of extracellular matrix by fibroblasts and an irreversible loss of lung function. Recently, the interleukin (IL)-1 family member IL-33 has been implicated in the pathogenesis of IPF. Currently however, the cellular and molecular mechanisms by which IL-33 exerts its reported pro-fibrotic effects are poorly understood.

Objective: The overall aim of this thesis was to investigate the role of IL-33 in the development of IPF.

Methods: To assess the pro-fibrotic activity of IL-33 *in vitro*, primary non-IPF and IPF human lung fibroblasts (HLFs) were stimulated with recombinant IL-33 and the expression of fibrotic genes (*ACTA2* and *COL1A1*) assessed by quantitative real-time PCR (qPCR). To determine the importance of IL-33 during the fibrotic response *in vivo*, the bleomycin (BLM) mouse model of pulmonary fibrosis was used. Specifically, male C57BL/6 mice were exposed to 60 IU of BLM and 10 mg/kg of either isotype control, anti-ST2 antibody (α ST2) or ST2-Fc fusion protein administered during the fibrotic phase of the BLM model. Lung and bronchoalveolar lavage (BAL) samples were collected 28 days post BLM treatment and multiple inflammatory (IL-33 and ST2 levels, IL-4 and IL-5 levels and total and differential BAL cell counts) and fibrotic readouts (lung hydroxyproline (collagen) content, lung weight and modified Ashcroft score) measured. To evaluate the fibrotic potential of IL-33 *ex vivo*, a method to generate and culture human precision cut lung slices (PCLS) was developed. Using this method, PCLS from normal adjacent and IPF lung tissue were stimulated with

recombinant IL-33 and the expression of fibrotic markers (*ACTA2*, *COL1A1*, *FN1* and fibronectin) quantified by qPCR and western blotting. Finally, to assess the importance of the oxidised form of IL-33 in IPF, non-IPF and IPF HLFs were treated with oxidised IL-33 (oxIL-33) *in vitro* and the phosphorylation of multiple proteins measured via phospho-kinase array and western blotting.

Results: IL-33 stimulation had no effect on *ACTA2* and *COL1A1* gene expression by HLFs *in vitro*, with these cells found to lack the ST2 component of the IL-33 receptor. Despite having effects on inflammatory readouts suggestive of target engagement, neither the α ST2 antibody nor the ST2-Fc fusion protein reduced established BLM-induced fibrosis *in vivo*. Likewise, IL-33 stimulation had no effect on *ACTA2*, *COL1A1*, *FN1* and fibronectin expression by human PCLS. Interestingly however, oxIL-33 treatment appeared to induce ST2-independent PI3K/AKT/mTOR signalling in HLFs, with significant increases in phospho-AKT and phospho-PRAS40 observed verses unstimulated control.

Conclusion: The data presented in this thesis suggests that, when signalling via ST2, IL-33 is unlikely to play a key pathophysiological role during the development of IPF. However, as oxIL-33 signals via a novel, potentially pro-fibrotic pathway in HLFs, it is possible that, once oxidised, IL-33 may promote pulmonary fibrosis. Further work investigating the role of oxIL-33 in the development of IPF is warranted.

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Publications

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Contents

Abstract.....	1
Acknowledgements	3
Publications	5
Abstracts.....	5
Abbreviations	15
List of Tables.....	21
List of Figures	21
Chapter 1: Introduction.....	24
1. Introduction	25
1.1. Idiopathic pulmonary fibrosis (IPF).....	25
1.1.1. Introduction to IPF	25
1.1.2. The classification and epidemiology of IPF.....	25
1.1.3. The clinical presentation of IPF	28
1.1.4. The progression of IPF and acute exacerbations.....	29
1.1.5. The prognosis for IPF patients	31
1.1.6. The pathogenesis of IPF	32
1.1.6.1. Risk factors for the development of IPF	34
1.1.6.1.1. Non-genetic risk factors	34
1.1.6.1.1.1. Smoking	34
1.1.6.1.1.2. Environmental/occupational exposures.....	34
1.1.6.1.1.3. Gastroesophageal reflux.....	35
1.1.6.1.1.4. Viral Infection	35
1.1.6.1.1.5. Bacterial Infection.....	36
1.1.6.1.2. Genetic risk factors.....	36
1.1.6.1.2.1. <i>TERT</i> and <i>TERC</i>	36
1.1.6.1.2.2. <i>MUC5B</i>	37
1.1.6.1.2.3. <i>SFTPC</i>	37
1.1.6.1.2.4. <i>TOLLIP</i>	38
1.1.6.1.2.5. <i>AKAP13</i>	38
1.1.6.1.2.6. <i>DSP</i> , <i>KIF15</i> and <i>DEPTOR</i>	39
1.1.6.2. Key cell types in the pathogenesis of IPF	39
1.1.6.2.1. Epithelial cells	39

1.1.6.2.2.	Fibroblasts	41
1.1.6.2.3.	Immune cells	44
1.1.6.2.3.1.	Macrophages	44
1.1.6.2.3.2.	Innate lymphoid cells (ILCs)	45
1.1.6.3.	Pro-fibrotic mediators in IPF	46
1.1.6.4.	Signalling pathways in IPF	47
1.1.7.	The treatment of IPF	50
1.2.	Interleukin-33 (IL-33)	53
1.2.1.	The discovery of IL-33.....	53
1.2.2.	The molecular biology of IL-33.....	54
1.2.2.1.	The IL-33 gene	54
1.2.2.2.	IL-33 protein	54
1.2.3.	The expression of IL-33.....	56
1.2.4.	The intracellular role of IL-33.....	57
1.2.5.	The release of IL-33	58
1.2.6.	The forms of IL-33.....	60
1.2.6.1.	Full length IL-33 (fIL-33).....	60
1.2.6.2.	Mature IL-33 (mIL-33)	61
1.2.6.3.	IL-33 splice variants.....	62
1.2.6.4.	Oxidised IL-33 (oxIL-33)	62
1.2.7.	ST2-dependent IL-33 signalling	63
1.2.8.	The biological effects of IL-33	65
1.2.9.	The regulation of IL-33 activity	68
1.2.9.1.	The regulation of IL-33 gene, mRNA and protein expression.....	68
1.2.9.2.	Mechanisms regulating the biological activity of IL-33.....	69
1.2.9.2.1	Intracellular mechanisms	69
1.2.9.2.2	Extracellular mechanisms	70
1.2.9.3.	The regulation of IL-33 signalling	70
1.2.10.	IL-33 in inflammatory diseases, infectious diseases and cancer.....	72
1.2.11.	IL-33 in organ fibrosis	75
1.2.11.1	IL-33 in liver fibrosis	75
1.2.11.2	IL-33 in renal fibrosis.....	75
1.2.11.3	IL-33 in skin fibrosis.....	76
1.2.12.	IL-33 in IPF.....	76

1.3.	Aims and Hypothesis	78
Chapter 2: Materials and Methods.....		79
2.	Materials and Methods.....	80
2.1.	Cell culture	80
2.1.1.	Primary human lung fibroblasts (HLFs)	80
2.1.2.	Human umbilical vein endothelial cells (HUVECs)	80
2.1.3.	A549 cells.....	81
2.2.	Cell counting using a haemocytometer	81
2.3.	RNA extraction	82
2.3.1.	NucleoSpin® RNA extraction.....	82
2.3.1.1.	Principle	82
2.3.1.2.	Method	82
2.3.2.	TRIzol RNA extraction	83
2.3.2.1.	Principle	83
2.3.2.2.	Method	84
2.3.3.	Direct-Zol RNA extraction.....	84
2.3.3.1.	Principle	84
2.3.3.2.	Method	84
2.4.	Determination of RNA concentration	86
2.5.	Reverse transcription.....	86
2.5.1.	Principle	86
2.5.2.	Methods.....	87
2.5.2.1.	M-MLV Reverse transcriptase	87
2.5.2.2.	SuperScript® IV Reverse Transcriptase	87
2.5.2.3.	High-Capacity RNA-to-cDNA™ kit.....	88
2.6.	Quantitative real-time PCR (qPCR).....	88
2.6.1.	qPCR using SYBR® Green	89
2.6.1.1.	Principle	89
2.6.1.2.	Method	89
2.6.2.	qPCR using TaqMan™ probes	90
2.6.2.1.	Principle	90
2.6.2.2.	Method	90
2.6.3.	Analysis of qPCR data	91
2.7.	Protein extraction	91

2.7.1.	Protein extraction from cells	91
2.7.2.	Protein extraction from murine lung tissue.....	92
2.7.3.	Protein extraction from human lung tissue	92
2.7.4.	Protein extraction from human PCLS	93
2.8.	Protein quantification	93
2.8.1.	Principle	93
2.8.2.	Method	93
2.9.	Western blotting	94
2.9.1.	Principle	94
2.9.2.	Methods.....	94
2.9.2.1.	Western blotting at the University of Nottingham.....	94
2.9.2.1.1.	Sample preparation.....	94
2.9.2.1.2.	SDS-PAGE.....	95
2.9.2.1.3.	Protein transfer	95
2.9.2.1.4.	Protein detection.....	96
2.9.2.2.	Western blotting at AstraZeneca.....	96
2.9.2.2.1.	Sample preparation.....	96
2.9.2.2.2.	SDS-PAGE.....	97
2.9.2.2.3.	Protein transfer	97
2.9.2.2.4.	Protein detection.....	97
2.10.	Enzyme-linked immunosorbent assays (ELISAs)	99
2.10.1.	Principle	99
2.10.2.	Method	99
2.11.	MSD assays.....	100
2.11.1.	Principle	100
2.11.2.	Methods.....	101
2.11.2.1.	Commercial MSD assays	101
2.11.2.2.	‘In-house’ reduced and oxidised IL-33 specific MSD assays.....	102
2.12.	Statistical analysis	103
Chapter 3: The effects of IL-33 on the pro-fibrotic activity of fibroblasts.....		104
3.	The effects of IL-33 on the pro-fibrotic activity of fibroblasts.....	105
3.1.	Introduction	105
3.2.	Aims	105
3.3.	Methods.....	106

3.3.1.	IL-33 Immunohistochemistry (IHC)	106
3.3.1.1.	Principle	106
3.3.1.2.	Method	106
3.3.2.	Transfection of HLFs with small interfering RNA (siRNA)	108
3.3.2.1.	Principle	108
3.3.2.2.	Method	108
3.4.	Results	109
3.4.1.	IL-33 is expressed by fibroblasts in IPF.....	109
3.4.2.	TGF β increases IL-33 expression by fibroblasts.....	111
3.4.3.	IL-33 cannot be detected in supernatants collected from TGF β -treated fibroblasts.....	113
3.4.4.	Extracellular IL-33 fails to increase the pro-fibrotic activity of fibroblasts.....	114
3.4.5.	Exploring the intracellular role of TGF β -induced IL-33	120
3.5.	Discussion	123
Chapter 4: The effects of blocking IL-33 signalling in the bleomycin (BLM) mouse model of pulmonary fibrosis.....		129
4.	The effects of blocking IL-33 signalling in the BLM mouse model of pulmonary fibrosis.....	130
4.1.	Introduction	130
4.2.	Aims	133
4.3.	Methods.....	134
4.3.1.	Mice.....	134
4.3.2.	BLM-induced pulmonary fibrosis.....	134
4.3.3.	Bronchoalveolar lavage (BAL)	136
4.3.4.	Total and differential BAL cell counts.....	136
4.3.5.	Processing of lung tissue for biochemical analysis.....	136
4.3.6.	Hydroxyproline assay.....	137
4.3.6.1.	Principle	137
4.3.6.2.	Method	137
4.3.7.	Processing of lung tissue for histological analysis.....	138
4.3.8.	Histological analysis of tissue sections	138
4.3.8.1.	H&E staining.....	139
4.3.8.1.1.	Principle	139
4.3.8.1.2.	Method	139
4.3.8.2.	Sirius red staining.....	140

4.3.8.2.1.	Principle	140
4.3.8.2.2.	Method	140
4.3.8.3.	Masson's trichrome staining	140
4.3.8.3.1.	Principle	140
4.3.8.3.2.	Method	141
4.3.8.4.	Semi-quantitative analysis via modified Ashcroft Scoring.....	141
4.4.	Results	143
4.4.1.	BLM induces acute lung injury.....	143
4.4.2.	Therapeutic blockade of IL-33 signalling fails to reduce established BLM-induced fibrosis	144
4.4.3.	Both the α ST2 antibody and the ST2-Fc fusion protein have biological effects in the BLM mouse model	147
4.4.3.1.	The α ST2 antibody and ST2-Fc fusion protein increase <i>IL33</i> gene expression in the lungs of BLM-treated mice	147
4.4.3.2.	ST2-Fc administration increased ST2 levels in the BALF and lung tissue	149
4.4.3.3.	The ST2-Fc fusion protein modulates BLM-induced inflammation	150
4.5.	Discussion	153
Chapter 5: Investigating the pro-fibrotic activity of IL-33 using human precision cut lung slices (PCLS)		158
5.	Investigating the pro-fibrotic activity of IL-33 using human precision cut lung slices (PCLS).....	159
5.1.	Introduction	159
5.2.	Aims	160
5.3.	Methods.....	160
5.3.1.	Human lung tissue.....	160
5.3.2.	Generation and maintenance of human PCLS	161
5.3.3.	Processing of PCLS for biochemical analysis	163
5.3.3.1.	Processing of PCLS for RNA	163
5.3.3.2.	Processing of PCLS for protein.....	163
5.3.4.	Lactate dehydrogenase (LDH) assay	163
5.3.4.1.	Principle	163
5.3.4.2.	Method	164
5.4.	Results	165
5.4.1.	PCLS remain viable for at least 7 days	165

5.4.2.	Tissue slicing induces pro-inflammatory cytokine release from PCLS	166
5.4.3.	Slicing induces IL-33 release from PCLS.....	169
5.4.4.	RNA can be extracted from cultured PCLS	170
5.4.5.	Protein can be extracted from cultured PCLS	171
5.4.6.	Cultured PCLS secrete cytokines in response to exogenous pro-inflammatory stimuli	173
5.4.7.	Cultured PCLS secrete cytokines in response to recombinant IL-33	174
5.4.8.	IL-33 appears to have no effect on fibrotic gene and protein expression by PCLS	176
5.5.	Discussion	178
Chapter 6: The effects of oxidised IL-33 (oxIL-33) on fibroblasts		182
6.	The effects of oxidised IL-33 (oxIL-33) on fibroblasts	183
6.1.	Introduction	183
6.2.	Aims	185
6.3.	Methods	185
6.3.1.	NF- κ B translocation assay	186
6.3.1.1.	Principle	186
6.3.1.2.	Method	186
6.3.2.	Phosphorylated EGFR (p-EGFR) homogenous time-resolved fluorescence (HTRF) assay	187
6.3.2.1.	Principle	187
6.3.2.2.	Method	188
6.3.3.	Phospho-kinase arrays.....	188
6.3.3.1.	Principle	188
6.3.3.2.	Method	189
6.3.4.	SDS-PAGE and in-gel protein detection.....	190
6.3.4.1.	Principle	190
6.3.4.2.	Method	190
6.4.	Results	191
6.4.1.	IL-33 oxidises in IMDM media	191
6.4.2.	IL-33 loses its ST2-dependent activity following oxidation.....	193
6.4.3.	IL-33 gains ST2-independent activity in epithelial cells following oxidation	195
6.4.4.	oxIL-33 induces EGFR phosphorylation in fibroblasts	196

6.4.5.	oxIL-33 is unlikely to have direct effects on the pro-fibrotic activity of fibroblasts.....	198
6.4.6.	Identifying oxIL-33-induced signalling pathways in fibroblasts	199
6.4.7.	oxIL-33 signalling in fibroblasts is EGFR-dependent	203
6.4.8.	Using AKT phosphorylation as a readout of TGF β -induced IL-33 release from fibroblasts	204
6.5.	Discussion	208
Chapter 7: Summary, conclusions and future directions.....		217
7.	Summary, conclusions and future directions	218
7.1.	Summary and conclusions.....	218
7.2.	Future directions.....	221
7.3.	Concluding remarks	224
References		225
8.	References	226
Appendix.....		279
9.	Appendix.....	280
9.1.	Materials.....	280
9.1.1.	Reagents and general lab supplies.....	280
9.1.2.	qPCR Primers.....	285
9.1.2.1.	SYBR® Green qPCR primers.....	285
9.1.2.2.	TaqMan™ assays	286
9.1.3.	Antibodies	286
9.1.4.	Kits	287
9.1.5.	Lab equipment.....	288
9.1.6.	Software	289
9.2.	Media, buffer, western blot gels and histology stain recipes	289
9.2.1.	Media recipes	289
9.2.2.	Buffer recipes	290
9.2.3.	Western blot gel recipes	291
9.2.4.	Histology stain recipes	291

Abbreviations

4E-BP1	Eukaryotic translation initiation factor 4E-binding
ABC	Avidin-biotin complex
AE	Acute exacerbation
AEC	Alveolar epithelial cell
AEC1	Type 1 alveolar epithelial cell
AEC2	Type 2 alveolar epithelial cell
AF488	Alexa Fluor™ 488
AGEs	Advanced glycation end products
AKAP13	A-kinase anchor protein 13
ALI	Air liquid interface culture
AM	Alveolar macrophage
ANOVA	Analysis of variance
ATP	Adenosine triphosphate
BAL	Bronchoalveolar lavage
BALF	Bronchoalveolar lavage fluid
BCA	Bicinchoninic acid
BLM	Bleomycin
BSA	Bovine serum albumin
CA	Cancer antigen
CBM	Chromatin binding motif
CCL	C-C motif chemokine ligand
cDNA	Complementary deoxyribonucleic acid
CKD	Chronic kidney disease
Co-IP	Co-immunoprecipitation
COPD	Chronic obstructive pulmonary disease
Ct	Cycle threshold
CTGF	Connective tissue growth factor
CXCL	C-X-C motif ligand
DAB	3,3'-Diaminobenzidine
DC	Dendritic cell
DF	Dilution factor
DMEM	Dulbecco's Modified Eagle medium

DNA	Deoxyribonucleic acid
DNAse	Deoxyribonuclease
dNTP	Deoxyribonucleotide triphosphate
EBC	Exhaled breath condensate
EBV	Epstein-Barr virus
ECL	Enhanced chemiluminescence
ECM	Extracellular matrix
EGF	Epidermal growth factor
EGFR	Epidermal growth factor receptor
EGFR-TKI	Epidermal growth factor receptor-tyrosine kinase inhibitor
ELISA	Enzyme-linked immunosorbent assay
ELK-1	ETS Like-1
EMT	Epithelial-to-mesenchymal transition
ER	Endoplasmic reticulum
ERK	Extracellular signal-regulated kinases
ET	Endothelin receptor
FCS	Fetal calf serum
FGF	Fibroblast growth factor
FITC	Fluorescein isothiocyanate
flIL-33	Full length interleukin-33
FOP	Fibrosing organising pneumonia
FRET	Fluorescence resonance energy transfer
FVC	Forced vital capacity
GAPDH	Glyceraldehyde 3-phosphate dehydrogenase
GDP	Guanosine diphosphate
GEF	Guanine nucleotide exchange factor
GER	Gastroesophageal reflux
GTP	Guanosine triphosphate
GWAS	Genome-wide association study
H&E	Haematoxylin and eosin
HEVs	High endothelial venules
HFL-1	Human fetal lung fibroblast 1
HHV	Human Herpesvirus
HLF	Human lung fibroblast

HMGB1	High mobility group box 1
HPAEC	Human Pulmonary Artery Endothelial Cell
HRCT	High-resolution computed tomography
HRP	Horse radish peroxidase
HSC	Hepatic stellate cell
HTRF	Homogeneous time resolved fluorescence
HUVECs	Human umbilical vein endothelial cells
Hyp	Hydroxyproline
i.p.	Intraperitoneal
i.t.	Intratracheal
IBD	Inflammatory bowel disease
IFN	Interferon
IHC	Immunohistochemistry
IIP	Idiopathic interstitial pneumonia
IL	Interleukin
IL-1RAcP	Interleukin-1 receptor accessory protein
ILC	Innate lymphoid cell
ILD	Interstitial lung disease
IMDM	Iscoe's Modified Dulbecco's medium
IPF	Idiopathic pulmonary fibrosis
IRAK	Interleukin-1 receptor-associated kinase
IRI	Ischemia-reperfusion injury
JNK	c-Jun N-terminal kinase
LDH	Lactate dehydrogenase
LLOD	Lower limit of detection
LPA	Lysophosphatidic acid
LPS	Lipopolysaccharide
MAPK	Mitogen-activated protein kinase
MDM	Monocyte derived macrophage
mIL-33	Mature interleukin-33
M-MLV	Moloney murine leukemia virus
MMP	Matrix metalloproteinase
mRNA	Messenger ribonucleic acid
mTOR	Mammalian target of rapamycin

mTORC1	Mammalian target of rapamycin complex 1
mTORC2	Mammalian target of rapamycin complex 2
MUC5B	Mucin 5B
MyD88	Myeloid differentiation primary response 88
NAC	N-acetyl-cysteine
NAD	Nicotinamide adenine dinucleotide
NADH	Nicotinamide adenine dinucleotide hydrogen
NAT	Normal adjacent tissue
NF-HEV	Nuclear factor in high endothelial venules
NFQ	Nonfluorescent quencher
NF- κ B	Nuclear factor- κ B
NK cell	Natural killer cell
NLS	Nuclear localisation sequence
NMR	Nuclear magnetic resonance
NTC	No template control
NTfC	Non transfected control
NTs	Non-targeting siRNA
o.p.	Oropharyngeal
OD	Optical Density
oxIL-33	Oxidised interleukin-33
p70S6K	p70 S6 Kinase
PAGE	Polyacrylamide gel electrophoresis
PBS	Phosphate-buffered saline
PCLS	Precision cut lung slices
PCR	Polymerase chain reaction
PDE	Phosphodiesterase
PDGF	Platelet-derived growth factor
PK1	Phosphoinositide-dependent kinase-1
p-EGFR	Phosphorylated epidermal growth factor receptor
PI3K	Phosphoinositide 3-kinase
PIP2	Phosphatidylinositol biphosphate
PIP3	Phosphatidylinositol triphosphate
PRAS40	Proline-rich Akt substrate of 40 kDa
PVDF	Polyvinylidene difluoride

qPCR	Quantitative real-time PCR
RAGE	Receptor for advanced glycation endproducts
rDNase	Recombinant deoxyribonuclease
redIL-33	Reduced interleukin-33
RISC	RNA-induced silencing complex
RNA	Ribonucleic acid
RNA-seq	RNA sequencing
ROS	Reactive oxygen species
RPMI	Roswell Park Memorial Institute (medium)
RT	Reverse transcription
RTK	Receptor tyrosine kinase
S	Serine
SAP	Serum amyloid P
SASP	Senescence-associated secretory phenotype
SD	Standard deviation
SDS	Sodium dodecyl sulfate
SDS-PAGE	Sodium dodecyl sulfate-polyacrylamide gel electrophoresis
SIGIRR	Single Ig IL-1-related receptor
siRNA	Small interfering ribonucleic acid
SLB	Surgical lung biopsy
SNP	Single nucleotide polymorphism
SP	Surfactant protein
SSc	Systemic sclerosis
sST2	Soluble ST2
STAT3	Signal transducer and activator of transcription 3
T	Threonine
TERC	Telomerase RNA component
TERT	Telomerase reverse transcriptase
TGF	Transforming growth factor
T _H 1	T-helper 1 CD4 ⁺ T cells
T _H 2	T-helper 2 CD4 ⁺ T cells
TMB	3,3',5,5'-Tetramethylbenzidine
TNF	Tumor necrosis factor
TOLLIP	Toll interacting protein

TRAF	Tumor necrosis factor receptor–associated factor
Tregs	Regulatory T cell
TSC2	Tuberous sclerosis complex 2
TSLP	Thymic stromal lymphopoietin
UIP	Usual interstitial pneumonia
ULK1	Unc-51 like autophagy activating kinase 1
UPR	Unfolded protein response
UUO	Unilateral ureteral obstruction
VEGF	Vascular endothelial growth factor
WNK-1	Lysine deficient protein kinase 1
Y	Tyrosine
α SMA	α -smooth muscle actin
β ME	β -mercaptoethanol
$\Delta\Delta$ CT	Delta-delta Ct

List of Tables

Table 1.1: Pharmacological therapies trialled for use in IPF.	50
Table 1.2: IL-33 release following cell damage and/or death.	59
Table 1.3: ST2 expressing cells.	64
Table 1.4: The pathophysiological role of IL-33 in the development of inflammatory diseases.	73
Table 4.1: Scoring criteria for quantifying BLM-Induced fibrosis.	142
Table 9.1: SYBR Green qPCR primer sequences	285
Table 9.2: TaqMan assay IDs.	286
Table 9.3: Details of antibodies used	286

List of Figures

Figure 1.1: The classification of IPF.	26
Figure 1.2: Pattern of UIP observed via HRCT and SLB.	29
Figure 1.3: The progression of IPF.	30
Figure 1.4: The 5 year survival rate of IPF versus different types of cancer.	31
Figure 1.5: The pathogenesis of IPF.	33
Figure 1.6: PI3K/AKT/mTOR signalling.	49
Figure 1.7: The primary structure of human IL-33 protein.	55
Figure 1.8: ST2-dependent IL-33 signalling.	65
Figure 1.9: The role of IL-33 in inflammation and wound healing.	67
Figure 2.1: Cell counting using a haemocytometer.	82
Figure 3.1: IL-33 expression in IPF lung tissue.	109
Figure 3.2: IL-33 gene and protein expression by primary HLFs <i>in vitro</i>	110
Figure 3.3: The effect of TGF β stimulation on <i>IL33</i> gene expression by HLFs over time.	111
Figure 3.4: The effect of TGF β on IL-33 protein expression by HLFs over time. ..	112
Figure 3.5: IL-33 and ST2 release from TGF β -stimulated HLFs over time.	113
Figure 3.6: The effect of IL-33 on pro-fibrotic gene expression by HLFs over time.	114
Figure 3.7: The effect of IL-33 on <i>IL6</i> and <i>CXCL8</i> gene expression by HLFs over time.	115

Figure 3.8: The effect of increasing concentrations of recombinant IL-33 on gene expression by non-IPF HLFs.	116
Figure 3.9: The ability of recombinant IL-33 to induce ST2-dependent signalling in HUVECs.	117
Figure 3.10: Basal ST2 gene expression by HLFs.	118
Figure 3.11: ST2 protein expression by HLFs.	119
Figure 3.12: Optimisation of IL-33 siRNA knockdown in HLFs.	121
Figure 3.13: IL-33 expression by HLFs following IL-33 knockdown and TGF β stimulation.	122
Figure 4.1: The BLM mouse model of pulmonary fibrosis.	132
Figure 4.2: BLM study overview.	135
Figure 4.3: The effect of BLM treatment on mouse body weight.	143
Figure 4.4: The effect of BLM, the α ST2 antibody and the ST2-Fc fusion protein on lung weight and hydroxyproline content.	144
Figure 4.5: The effect of BLM, the α ST2 antibody and the ST2-Fc fusion protein on lung morphology and modified Ashcroft score.	145
Figure 4.6: Retrospective analysis of body weight data and modified Ashcroft scoring.	146
Figure 4.7: The effect of the α ST2 antibody and ST2-Fc fusion protein on IL-33 levels.	148
Figure 4.8: The effect of the α ST2 antibody and ST2-Fc fusion protein on ST2 expression.	149
Figure 4.9: The effects of the α ST2 antibody and ST2-Fc fusion protein on type 2 cytokine release.	151
Figure 4.10: The effect of the α ST2 antibody and ST2-Fc fusion protein on BLM-induced pulmonary inflammation.	152
Figure 5.1: Generation of human PCLS.	162
Figure 5.2: Principle of the LDH assay.	164
Figure 5.3: Overview of PCLS optimisation experiments.	165
Figure 5.4: LDH release by cultured PCLS over time.	166
Figure 5.5: Pro-inflammatory cytokine release by PCLS over time.	167
Figure 5.6: Comparing pro-inflammatory cytokine release from PCLS at days 1 and 4.	168
Figure 5.7: IL-33 release by non-diseased and diseased PCLS over time.	169
Figure 5.8: RNA extraction from cultured PCLS.	171
Figure 5.9: Protein extraction from cultured PCLS.	172
Figure 5.10: The response of PCLS to LPS.	174
Figure 5.11: The response of PCLS to recombinant IL-33.	175

Figure 5.12: Assessing the effect of IL-33 on fibrotic gene and protein expression by human PCLS.	176
Figure 5.13: The effect of recombinant IL-33 on fibrotic gene expression by PCLS.	177
Figure 5.14: The effect of recombinant IL-33 on fibronectin expression by PCLS.	177
Figure 6.1: Principle of the p-EGFR HTRF assay.	187
Figure 6.2: The generation of crude oxIL-33 from recombinant IL-33.	191
Figure 6.3: Determining the oxidation statuses of redIL-33 and oxIL-33 crude preps.	193
Figure 6.4: Assessing the ST2-dependent activity of crude oxIL-33.	194
Figure 6.5: Determining the ST2-independent activity of crude oxIL-33.	196
Figure 6.6: The effect of crude oxIL-33 on EGFR phosphorylation in HLFs.	197
Figure 6.7: The effect of oxIL-33 on the pro-fibrotic activity of HLFs.	198
Figure 6.8: Identification of oxIL-33 signalling pathways in HLFs using phospho-kinase arrays.	200
Figure 6.9: Validation of phospho-kinase array hits via western blot.	201
Figure 6.10: The effect of oxIL-33 on AKT phosphorylation in HLFs.	202
Figure 6.11: The effect of inhibiting EGFR on oxIL-33 signalling in HLFs.	203
Figure 6.12: The effect of TGF β on AKT phosphorylation and IL-33 expression by HLFs over time.	205
Figure 6.13: The effect of the α IL-33 antibody on the oxidation of IL-33.	206
Figure 6.14: The effect of an α IL-33 antibody on TGF β -induced AKT phosphorylation at 12 hours.	207

Chapter 1: Introduction

1. Introduction

1.1. Idiopathic pulmonary fibrosis (IPF)

1.1.1. Introduction to IPF

Idiopathic pulmonary fibrosis (IPF) is a chronic, progressive interstitial lung disease (ILD) characterised by the excessive deposition of extracellular matrix (ECM) in the lung interstitium (Martinez et al., 2017, Lederer and Martinez, 2018, Yanagihara et al., 2019). Resulting in the disruption of normal lung architecture and an irreversible loss of lung function, IPF is a debilitating ILD with a median survival time of 3-5 years from diagnosis (Hambly et al., 2017, Fernández Fabrellas et al., 2018). Despite its incidence continuing to increase, the aetiology and underlying pathophysiology of IPF are poorly understood. Furthermore, despite ongoing efforts, treatment options for IPF patients remain limited with only two therapies, pirfenidone and nintedanib, currently available (Hewitt and Maher, 2019).

1.1.2. The classification and epidemiology of IPF

IPF is the most common form of ILD accounting for 50-60% of diseases affecting the lung interstitium (Navaratnam et al., 2011, Valeyre, 2011). Belonging to the idiopathic interstitial pneumonia (IIP) subgroup of ILDs (Fig. 1.1), IPF is classified as a major chronic fibrosing interstitial pneumonia (Travis et al., 2013).

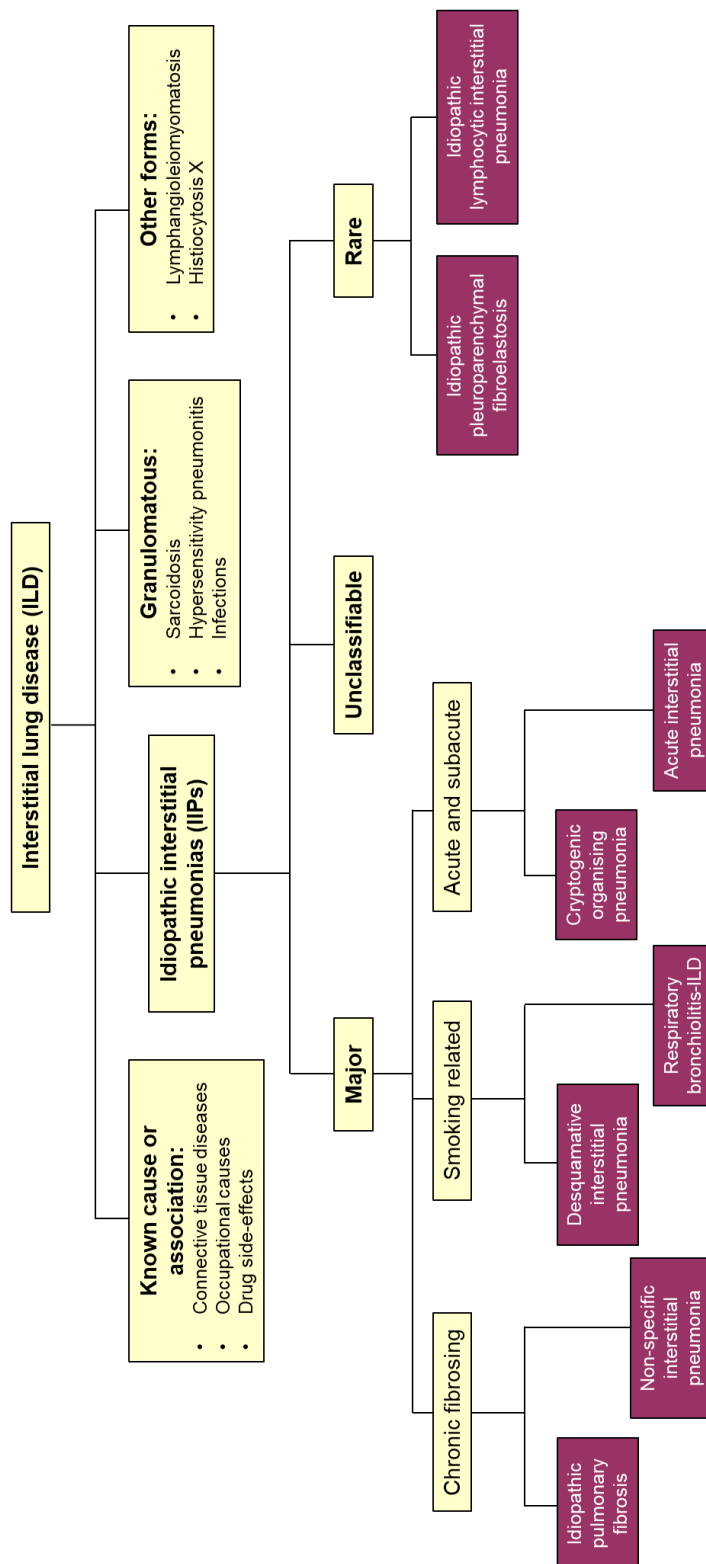


Figure 1.1: The classification of IPF.

ILDs are categorised into several subgroups. IPF is classified as a chronic fibrosing major IIP. Figure adapted from D Zibrak and Price (2014).

Due to differences in the clinical definitions used, the incidence and prevalence of IPF varies between studies with precise figures unknown (Esposito et al., 2015, Ley et al., 2017, Olson et al., 2018). Currently however, the annual incidence of IPF in North America and Europe is estimated to range between 2.8 and 19 cases per 100,000 people (Hutchinson et al., 2015, Olson et al., 2018). Although lower figures have been reported in South America and East Asia (less than 4 cases per 100,000 people), the global incidence of IPF continues to rise (Hutchinson et al., 2015, Lederer and Martinez, 2018). Indeed, over a 10 year period, a 78% increase in cases was reported in the UK alone with approximately 6000 people diagnosed annually (British Lung Foundation, 2016, Strongman et al., 2018). Globally, the incidence of IPF is higher in older individuals with a mean age of ≥ 65 years at diagnosis (Collard, 2010, Raghu et al., 2016a, Lederer and Martinez, 2018). Moreover, although no difference in median survival has been observed between genders (King et al., 2001), significantly more men than women develop IPF (Hutchinson et al., 2015, European Respiratory Society, 2019).

In the United States the prevalence of IPF ranges from 10 to 60 cases per 100,000 people (Esposito et al., 2015, Raghu et al., 2016a) with similar figures reported in the UK and Europe (British Lung Foundation, 2016, Olson et al., 2018). Consequently, approximately 32,500 individuals in the UK suffer from IPF accounting for 9000 hospital admissions and 5300 deaths annually (British Lung Foundation, 2016, Snell et al., 2016).

1.1.3. The clinical presentation of IPF

Currently, a multidisciplinary approach involving experienced clinicians, radiologists and pathologists is required to diagnose IPF (Martinez et al., 2017, Lederer and Martinez, 2018). Typically presenting with chronic exertional dyspnoea, dry cough, inspiratory bibasilar crackles and digital clubbing, patients with IPF are identified by the presence of subpleural honeycombing, peripheral alveolar septal thickening and traction bronchiectasis (Lederer and Martinez, 2018). Known collectively as usual interstitial pneumonia (UIP), this pattern, observed via high-resolution computed tomography (HRCT) scan and confirmed (if necessary) by surgical lung biopsy (SLB) (Fig. 1.2), is key for a diagnosis of IPF (Daccord and Maher, 2016). However, as this pattern and the clinical symptoms of IPF are shared with other ILDs (Leslie, 2009, Spagnolo et al., 2012), UIP in the absence of a detailed medical history is insufficient for a reliable diagnosis of IPF to be made. Moreover, as SLBs cannot be performed on patients with advanced lung disease and/or substantial comorbidities (Han et al., 2015, Hutchinson et al., 2016), accurate diagnosis can pose a significant challenge (Lederer and Martinez, 2018).

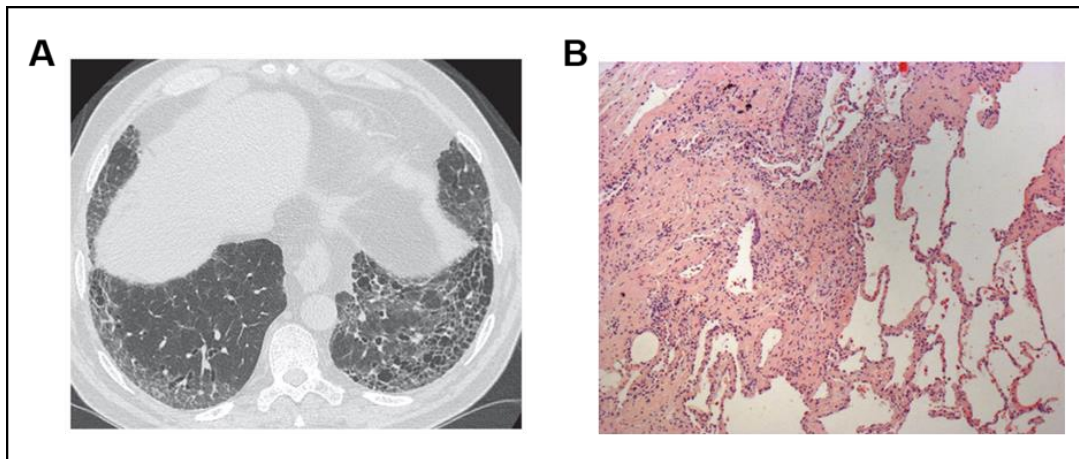


Figure 1.2: Pattern of UIP observed via HRCT and SLB.

Representative HRCT scan showing subpleural and basal honeycombing in the lungs of an IPF patient. **(A)**. Representative photomicrograph of a SLB from an IPF patient showing areas of active fibrosis (consisting of fibroblastic foci, inflammatory cell infiltrate and ECM) interspersed with histologically normal areas of lung tissue **(B)**. Images from Daccord and Maher (2016).

1.1.4. The progression of IPF and acute exacerbations

Despite most IPF patients experiencing a slow and gradual decline in lung function, some patients progress rapidly with death from respiratory failure (or complicating co-morbidities) occurring less than a year from diagnosis (Fig. 1.3). As some patients remain stable for a number of years (Fig. 1.3), it is widely accepted that the natural progression of IPF varies between individuals (Raghu et al., 2011).

Disappointingly, HRCT (with or without SLB) cannot predict the natural progression of IPF. Consequently, decisions regarding the most appropriate course of treatment can be difficult to make at diagnosis (Daccord and Maher, 2016). Although surfactant protein-D (SP-D), matrix metalloproteinase 7 (MMP7), cancer antigen (CA) 125 and CA19-9 have been identified as potential prognostic biomarkers (Takahashi et al., 2000, Rosas et al., 2008, Morais et al., 2015, Maher et al., 2017), further work is needed to successfully stratify IPF patients.

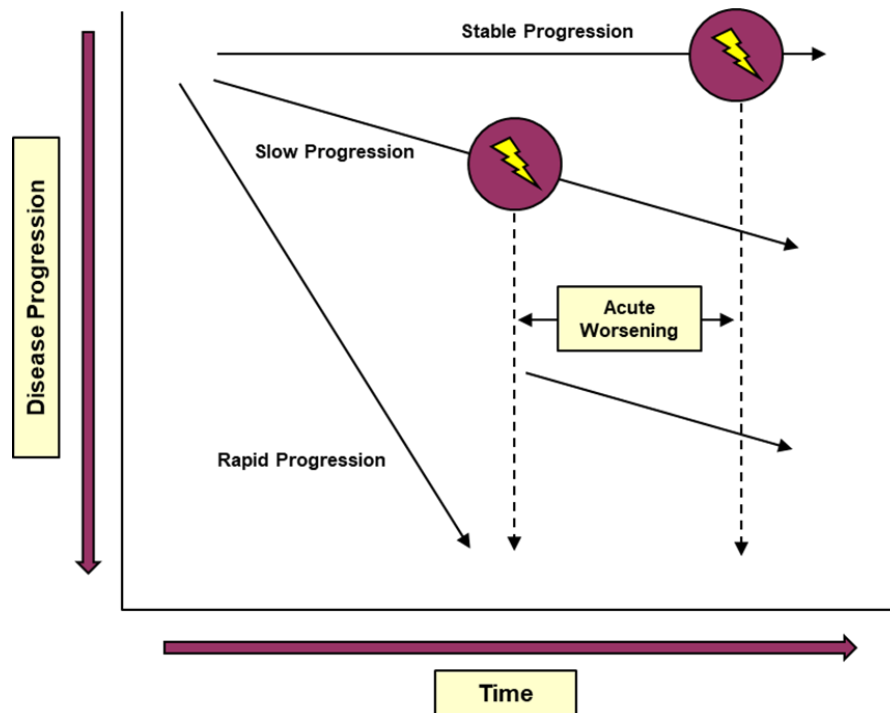


Figure 1.3: The progression of IPF.

The natural progression of IPF varies between patients. Although some individuals remain stable for a number of years, the majority of patients experience a slow, progressive decline in lung function over time. In contrast, a small subset of patients progress rapidly. Additionally, some patients experience acute exacerbations (AEs) (indicated by lightning bolts) which can alter natural disease progression. Figure adapted from Raghu et al. (2011).

Per year, approximately 4-20% of IPF patients experience acute respiratory worsening (Collard et al., 2016). Referred to as acute exacerbations (AEs), these events alter the natural progression of IPF (Fig. 1.3), shortening life expectancy and frequently resulting in death (Raghu et al., 2011, Ryerson et al., 2015, Hambly et al., 2017). Characterised by an unexplained worsening of dyspnoea, severely impaired gas exchange, alveolar infiltration and diffuse alveolar damage, AEs can occur at any point in IPF including at diagnosis (Parambil et al., 2005, Kim et al., 2006, Collard et al., 2007, Sakamoto et al., 2009). Although the cause of AEs in IPF is currently unknown, procedures such as bronchoalveolar lavage (BAL) and thoracic surgery (including SLB) have been identified as risk factors (Hiwatari et al., 1994, Utz et al., 2001,

Kondoh et al., 2006, Yüksel et al., 2006). Moreover, despite conflicting evidence, infection with bacteria or viruses may lead to AEs (Vannella and Moore, 2008, Wootton et al., 2011, Molyneaux et al., 2017, Weng et al., 2019, Sheng et al., 2020).

1.1.5. The prognosis for IPF patients

Although disease progression varies, the prognosis for IPF patients is poor with a median survival time of 3-5 years from diagnosis (Hambly et al., 2017, Fernández Fabrellas et al., 2018). As a result, the 5 year survival rate for IPF is worse than all but two types of cancer (Fig. 1.4) (Vancheri et al., 2010). Due to this, much research has focused on the pathogenesis of IPF in the hope that a better understanding of the fibrotic response will lead to the identification of novel therapeutic targets and the development of more effective treatments.

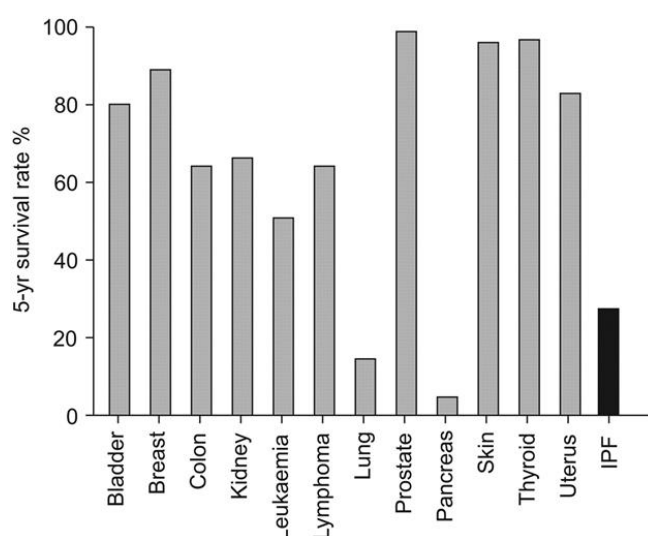


Figure 1.4: The 5 year survival rate of IPF versus different types of cancer.

The 5 year survival rate for IPF is worse than all but two types of cancer. Figure taken from Vancheri et al. (2010).

1.1.6. The pathogenesis of IPF

Historically, chronic inflammation was proposed to cause IPF (Crystal et al., 1976, Keogh and Crystal, 1982). Consequently, multiple anti-inflammatory therapies have been trialled for use in IPF (see section 1.1.7 for more details). However, as these therapies failed to improve clinical outcomes and increased the risk of hospitalisation and death (Raghu et al., 2012), the inflammatory hypothesis of IPF has since been rejected (Xu et al., 2016b, Martinez et al., 2017, Yanagihara et al., 2019).

Although not fully understood, it is currently believed that repeated alveolar epithelial cell (AEC) injury in genetically susceptible individuals initiates a series of events which cumulate in the excessive deposition of ECM by fibroblasts/myofibroblasts in the lungs (Martinez et al., 2017, Lederer and Martinez, 2018, Yanagihara et al., 2019). The key risk factors, cell types, mediators and signalling pathways implicated in the pathogenesis of IPF are described in detail in the following sections and are summarised in Figure 1.5.

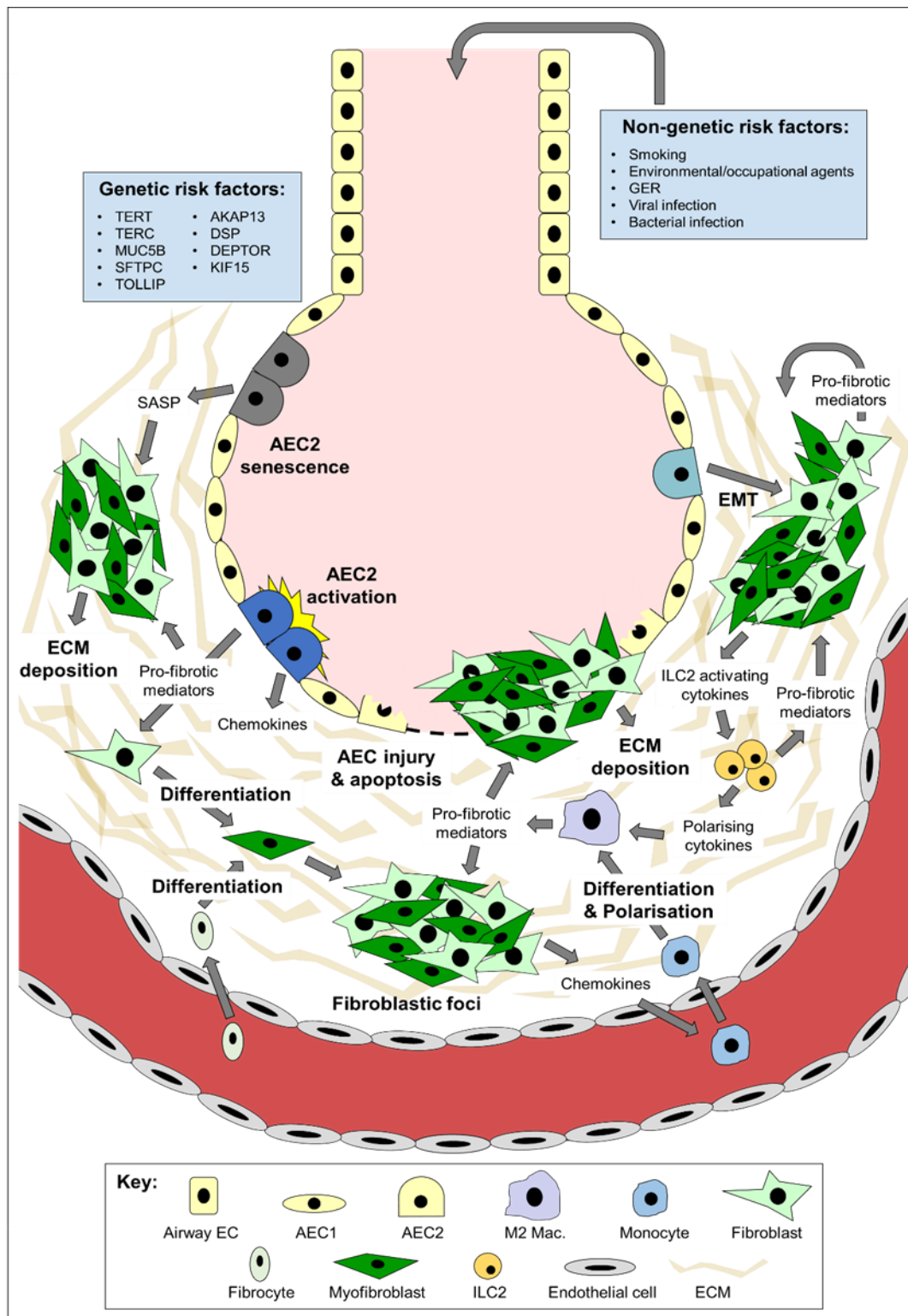


Figure 1.5: The pathogenesis of IPF.

In genetically susceptible individuals, repeated exposures to non-genetic risk factors results in AEC injury, senescence, activation and death (apoptosis). The release of profibrotic mediators by (activated/senescent) AEC2s, fibroblasts/myofibroblasts, monocyte-derived M2 macrophages and activated ILC2s results in the formation of fibroblastic foci (via increased fibroblast proliferation and survival, fibroblast/fibrocyte differentiation and EMT) and the excessive deposition of ECM by fibroblasts/myofibroblasts. Figure adapted from Lederer and Martinez (2018).

1.1.6.1. Risk factors for the development of IPF

As indicated by its name, the cause of IPF remains unknown. Despite this, several genetic and non-genetic risk factors have been identified. Strikingly, the majority of these risk factors are associated with epithelial cell injury, dysfunction and death (Fig. 1.5).

1.1.6.1.1. Non-genetic risk factors

A number of non-genetic risk factors are associated with the development of IPF. Believed to result in epithelial cell injury, activation, senescence and death (Fig. 1.5), non-genetic risk factors for IPF include smoking, environmental/occupational exposures, gastroesophageal reflux and infection with viruses or bacteria.

1.1.6.1.1.1. Smoking

In comparison to life-long non-smokers, current and former smokers are 60% more likely to develop IPF (Baumgartner et al., 1997). Indeed, 41-83% of IPF patients have a history of smoking (Carrington et al., 1978, Turner-Warwick et al., 1980, Coultas et al., 1994, Baumgartner et al., 1997, Daniil et al., 1999). Interestingly, former smokers appear to be at higher risk of developing IPF with those who have quit most recently at greatest risk (Baumgartner et al., 1997).

1.1.6.1.1.2. Environmental/occupational exposures

Exposure to environmental/occupational agents is associated with the development of IPF (Iwai et al., 1994, Hubbard et al., 1996, Hubbard et al., 2000, Miyake et al., 2005, Gustafson et al., 2007) with meta-analysis of previous studies revealing that exposure to metal dust, wood dust, livestock, sand/stone and agriculture/farming increases the risk of developing IPF (Taskar and Coultas, 2006). Additionally, chronic exposure to

air pollutants may increase the risk of developing IPF with high levels of nitrogen dioxide associated with an increased incidence of IPF in the Lombardy region of Italy (Conti et al., 2018).

1.1.6.1.1.3. Gastroesophageal reflux

Characterised by the reflux/regurgitation of stomach acid into the oesophagus, gastroesophageal reflux (GER) is common in patients with IPF with the microaspiration of stomach acid believed to cause epithelial cell injury (Tobin et al., 1998, Patti et al., 2005, Raghu et al., 2006a). Interestingly, anti-acid treatment stabilised lung function in a small cohort of IPF patients (Raghu et al., 2006b). As similar observations were made when 124 IPF patients received anti-acid therapy (Lee et al., 2013), these results suggest that GER may play a role in the development of IPF.

1.1.6.1.1.4. Viral Infection

DNA and protein from Epstein-Barr virus (EBV) has been detected in IPF lung tissue (Egan et al., 1995, Stewart et al., 1999, Tsukamoto et al., 2000, Tang et al., 2003). Present predominately in alveolar epithelial cells (AECs), EBV has been shown to replicate in the lungs of IPF patients (Kelly et al., 2002). In addition to EBV, other herpesviruses including human herpesvirus (HHV)-8, cytomegalovirus and Kaposi sarcoma-associated herpesvirus have been detected in IPF lung tissue (Tang et al., 2003). In type 2 AECs (AEC2s), these viruses co-localise with markers of the unfolded protein response (UPR) and endoplasmic reticulum (ER) stress (Lawson et al., 2008). As these processes can lead to cell death via apoptosis (see section 1.1.6.2.1), repeated viral infection is regarded as a risk factor for IPF (Burman et al., 2018, Sheng et al., 2020).

1.1.6.1.1.5. Bacterial Infection

BAL samples from IPF patients have higher bacterial loads than those taken from healthy controls with increases in the percentages of *Haemophilus* spp., *Staphylococcus* spp., *Streptococcus* spp., *Neisseria* spp., *Veillonella* spp. and *Prevotella* spp. reported (Richter et al., 2009, Han et al., 2014, Molyneaux et al., 2014, Takahashi et al., 2018). As these changes in the lung microbiome are associated with more rapid disease progression and death (Han et al., 2014, Molyneaux et al., 2014, Takahashi et al., 2018), bacterial infection is proposed to play a pathophysiological role in IPF.

1.1.6.1.2. Genetic risk factors

Due to the ubiquitous nature of cigarette smoke, occupational irritants and pathogens in the environment, an important question arises as to why only a relatively small percentage of the population suffers from IPF. Recently, large genome wide association studies (GWAS) have started to provide answers to this intriguing paradox with polymorphisms in several genes associated with the development of IPF. The genes in which polymorphisms have been identified include *TERT*, *TERC*, *MUC5B*, *SFTPC*, *TOLLIP*, *AKAP13*, *DSP*, *KIF15* and *DEPTOR*. As detailed in the following sections, mutations in these genes likely predispose individuals to epithelial injury, dysfunction and death (Fig. 1.5).

1.1.6.1.2.1. *TERT* and *TERC*

Found at the ends of all chromosomes, telomeres are repetitive nucleotide sequences that prevent chromosomal deterioration and fusion (Shay and Wright, 2019). Although telomere attrition (shortening) occurs with age, mutations in the genes responsible for the maintenance of telomeres results in premature shortening, cell death and

senescence (Armanios, 2013). In IPF, mutations in the genes encoding telomerase reverse transcriptase (*TERT*) and telomerase RNA component (*TERC*) have been identified (Armanios et al., 2007, Tsakiri et al., 2007, Alder et al., 2008). As these mutations lead to premature telomere attrition in AEC2s, AEC2 death and AEC2 senescence (Alder et al., 2015, Snetselaar et al., 2017), variants of *TERT* and *TERC* are associated with the development of IPF (Armanios et al., 2007, Tsakiri et al., 2007, Alder et al., 2008).

1.1.6.1.2.2. *MUC5B*

Secreted by epithelial cells, mucin 5B (*MUC5B*) is an important component of mucus (Ridley and Thornton, 2018). Recently, a single nucleotide polymorphism (SNP) in the promoter of the *MUC5B* gene (rs35705950) has been identified in 34-38% of IPF patients and is associated with an increased risk of developing IPF (Seibold et al., 2011, Borie et al., 2013, Fingerlin et al., 2013, Noth et al., 2013, Stock et al., 2013). Leading to *MUC5B* overexpression in small airway epithelial cells and AEC2s (Nakano et al., 2016), rs35705950 is proposed to promote fibrosis by impairing mucociliary clearance (Hancock et al., 2018).

1.1.6.1.2.3. *SFTPC*

Specifically expressed by AEC2s, surfactant protein C (SP-C) is encoded by the *SFTPC* gene (Keller et al., 1991, Beers and Moodley, 2017). Interestingly, heterozygous mutations in the *SFTPC* gene have been reported in IPF (Nogee et al., 2001, Thomas et al., 2002, Cameron et al., 2005, van Moorsel et al., 2010, Ono et al., 2011). As these lead to protein misfolding, ER stress and AEC2 apoptosis, mutations in *SFTPC* are associated with the development of IPF (Mulugeta et al., 2005, Mulugeta et al., 2007, Zhou and Wang, 2016, Selman and Pardo, 2020). Indeed, transgenic mice

expressing *SFTPC* mutants develop spontaneous fibrosis and exhibit exaggerated fibrotic responses to bleomycin (BLM) (Lawson et al., 2011, Nureki et al., 2018).

1.1.6.1.2.4. *TOLLIP*

Encoded by the *TOLLIP* gene on chromosome 11, Toll interacting protein (TOLLIP) inhibits transforming growth factor- β (TGF β) and toll-like receptor (TLR) signalling (Zhang and Ghosh, 2002, Zhu et al., 2012). In 2013, 3 SNPs (rs111521887, rs5743894 and rs5743890) in the *TOLLIP* gene were identified (Noth et al., 2013). Reducing TOLLIP expression by 40 and 50% respectively, rs111521887 and rs5743894 are associated with increased susceptibility to IPF (Noth et al., 2013). Although the precise mechanism is unclear, it is possible that these polymorphisms promote lung fibrosis by increasing TGF β signalling (see section 1.1.6.3 for the importance of TGF β in IPF).

1.1.6.1.2.5. *AKAP13*

A-kinase anchoring protein 13 (AKAP13), is a guanine nucleotide exchange factor (GEF) expressed by $\alpha\text{v}\beta 6$ integrin positive AECs and encoded by the *AKAP13* gene. Catalysing the release of GDP and the binding of GTP, AKAP13 activates RhoA (Allen et al., 2017, Organ et al., 2019). Recently, a SNP (rs62025270) associated with increased AKAP13 expression has been identified. Since GTP-bound RhoA activates TGF β via $\alpha\text{v}\beta 6$ (which in turn increases the pro-fibrotic activity of fibroblasts and other cell types -see section 1.1.6.2.2), it is not surprising that rs62025270 is associated with increased susceptibility to IPF (Allen et al., 2017, Organ et al., 2019, Allen et al., 2020).

1.1.6.1.2.6. *DSP*, *KIF15* and *DEPTOR*

Mutations in genes promoting cell adhesion (*DSP*), mitotic spindle assembly (*KIF15*) and PI3K/AKT/mTOR signalling (*DEPTOR*) are associated with the development of IPF (Mathai et al., 2016, Allen et al., 2020). Biologically, these mutations likely predispose individuals to AEC apoptosis, senescence and activation following repeated AEC injury.

1.1.6.2. Key cell types in the pathogenesis of IPF

As detailed in the following sections, epithelial cells, fibroblasts and immune cells appear to play key pathophysiological roles during the development of IPF.

1.1.6.2.1. Epithelial cells

The majority of IPF susceptibility genes are associated with epithelial cell survival and function. Consequently, epithelial cells are proposed to play an important role during the development of IPF (Martinez et al., 2017, Lederer and Martinez, 2018, Yanagihara et al., 2019). Indeed, AEC apoptosis, senescence and activation appear key for the initiation of pulmonary fibrosis.

In IPF lung tissue, apoptotic AECs are found in areas of active fibrosis (Uhal et al., 1998, Barbas-Filho et al., 2001, Maeyama et al., 2001, Jablonski et al., 2017). Furthermore, mutations in *SFTPC* result in AEC2-specific apoptosis and the initiation/worsening of fibrotic responses *in vivo* (Nureki et al., 2018, Lawson et al., 2011). As the selective depletion of AEC2s (via diphtheria toxin induced apoptosis) causes spontaneous pulmonary fibrosis in mice (Sisson et al., 2010), these results collectively suggest that AEC apoptosis plays an important role in the development of IPF. Indeed, it is currently believed that injury induced-AEC apoptosis leads to the

destruction of the alveolar-capillary basement membrane, initiation of the coagulation cascade and the recruitment of fibrogenic cells into the alveolar interstitium (King et al., 2011b, Martinez et al., 2017, Lederer and Martinez, 2018) (Fig. 1.5).

In addition to hyperplastic epithelial cells, senescent AECs have been reported in IPF lung tissue (Qunn et al., 2002, Alder et al., 2008, Minagawa et al., 2011). Primarily induced by age and/or TERT/TERC related telomere attrition, ER stress and mitochondrial dysfunction, senescence is characterised by cell-cycle arrest and the acquisition of a senescence-associated secretory phenotype (SASP) (Yanagihara et al., 2019, Parimon et al., 2020). Interestingly, AEC2-specific deletion of the telomere shelterin protein TRF1 results in telomere attrition, AEC2 senescence and age-induced fibrosis in mice (Naikawadi et al., 2016). Similarly, AEC2-specific silencing of the chromatin regulator Sin3a, drives AEC2 senescence and the development of spontaneous pulmonary fibrosis *in vivo* (Yao et al., 2019). As senolytic drugs (drugs that selectively induce the death of senescent cells via apoptosis) reduce AEC2 senescence and fibrotic marker expression by fibrotic mouse precision cut lung slices (PCLS) (Lehmann et al., 2017), these *in vivo* and *ex vivo* results suggest that AEC senescence contributes to the development of IPF. Although the mechanisms underpinning this potential involvement remain unclear, it is possible that the secretion of SASP factors (including fibroblast growth factor (FGF), vascular endothelial growth factor (VEGF) and C-C motif chemokine ligand 2 (CCL2)) by senescent AECs increases the pro-fibrotic activity of fibroblasts and immune cells (Schafer et al., 2017, Liu and Liu, 2020). Moreover, since AEC2s act as progenitor cells for AEC1s (Olajuyin et al., 2019, Parimon et al., 2020), it is feasible that AEC2 senescence prevents alveolar re-epithelialisation following injury (Fig. 1.5).

Activated AECs release multiple mediators including TGF β , connective tissue growth factor (CTGF), platelet-derived growth factor (PDGF), FGF, VEGF, CXC chemokine ligand 12 (CXCL12) and CCL2 (Martinez et al., 2017, Yanagihara et al., 2019). Since these promote the recruitment, differentiation and activation of fibrogenic cells (such as monocytes, fibrocytes and fibroblasts), AEC activation is believed to play a crucial role in the pathogenesis of IPF (Martinez et al., 2017, Yanagihara et al., 2019) (Fig. 1.5).

1.1.6.2.2. Fibroblasts

Derived from embryonic mesoderm, fibroblasts are tissue resident mesenchymal cells that can secrete ECM and differentiate into myofibroblasts (Kendall and Feghali-Bostwick, 2014). Despite being essential for maintaining normal lung architecture, fibroblasts are regarded as the central effector cell type in IPF (Moore and Herzog, 2013, Daccord and Maher, 2016, Martinez et al., 2017, Yanagihara et al., 2019).

As detailed in the previous section, activated AECs release mediators such as TGF β , PDGF, CTGF and FGF. Resulting in mesenchymal activation, these factors drive the proliferation of lung resident fibroblasts and their differentiation into myofibroblasts (Klingberg et al., 2013, Yanagihara et al., 2019) (Fig. 1.5). Due to their ability to secrete ECM and exert traction forces, α -smooth muscle actin (α SMA) expressing myofibroblasts play a key role in normal wound healing (Pakshir et al., 2020). In IPF however, a combination of uncontrolled fibroblast proliferation, increased myofibroblast differentiation, decreased fibroblast/myofibroblast apoptosis and defective autophagy (a regulated homeostatic mechanism where autophagosome formation results in the degradation and recycling of cytosolic components) results in the formation of fibroblastic foci (Rahimi et al., 2009, Maher et al., 2010, Goc et al.,

2011, Nho et al., 2011, Patel et al., 2012, Nho and Hergert, 2014, Im et al., 2015, Mercer et al., 2016, Romero et al., 2016, Hinz and Lagares, 2020). Synonymous with the pathogenesis of IPF, these foci accumulate in the lung interstitium with their component fibroblasts and myofibroblasts secreting excessive amounts of collagen rich-ECM (Klingberg et al., 2013, Moore and Herzog, 2013) (Fig. 1.5). Although initially resulting in areas of patchy subpleural fibrosis, the sustained activation of fibroblasts and myofibroblasts leads to the widespread disruption of normal lung architecture and an irreversible loss of lung function (Martinez et al., 2017, Lederer and Martinez, 2018, Yanagihara et al., 2019). Interestingly, fibroblast specific deletion of the type II TGF β receptor attenuates bleomycin induced pulmonary fibrosis (Hoyles et al., 2011). In addition to highlighting the central importance of TGF β during the fibrotic response, this result suggests that fibroblasts play a crucial role during the development of IPF. Indeed, many emerging and current therapies for IPF (including pirfenidone, nintedanib, GLPG1690 and FG-3019) have been shown to exert at least some of their anti-fibrotic effects by decreasing the recruitment, proliferation and pro-fibrotic activity (i.e. the ability of fibroblasts to secrete ECM and differentiate into myofibroblasts) of fibroblasts (Conte et al., 2014, Hostettler et al., 2014, Raghu et al., 2016b, Maher et al., 2018).

In addition to responding to such factors, fibroblasts and myofibroblasts can secrete multiple pro-fibrotic mediators including TGF β and CTGF (Kendall and Feghali-Bostwick, 2014) (Fig. 1.5). Although other cell types such as AECs (see section 1.1.6.2.1) and macrophages (see section 1.1.6.2.3) release TGF β , fibroblasts and myofibroblasts may act as an important autocrine/paracrine source of TGF β in IPF (Kendall and Feghali-Bostwick, 2014). Indeed, after using microRNA to inhibit the

expression and release of TGF β by human fetal lung fibroblasts (HFL-1 cells), Horie et al. (2014) reported significant reductions in HFL-1 proliferation and collagen production. Interestingly, fibroblast-specific overexpression of CTGF results in spontaneous pulmonary fibrosis in mice (Sonnylal et al., 2010). Due to this, and the aforementioned effects of CTGF on fibroblasts and myofibroblasts, fibroblast-derived CTGF is proposed to play an important role in IPF (Sonnylal et al., 2010, Kendall and Feghali-Bostwick, 2014).

Activated fibroblasts secrete a number of cytokines and chemokines such as CCL2 and thymic stromal lymphopoietin (TSLP) (Hogaboam et al., 1998, Datta et al., 2013, Van Linthout et al., 2014). Due to their respective abilities to drive monocyte recruitment and activate group 2 innate lymphoid cells (ILC2s) (and the apparent importance of these immune cells in the pathogenesis of IPF (see section 1.1.6.2.3)), CCL2 and TSLP are proposed to promote pulmonary fibrosis (Matsushima et al., 1989, Yoshimura et al., 1989, Moore et al., 2006, Baran et al., 2007, Datta et al., 2013, Lee et al., 2017). Although other cell types release these mediators, their production by activated fibroblasts suggests that, in addition to secreting ECM, fibroblasts play an important immunoregulatory role during the development of IPF (Moore and Herzog, 2013, Kendall and Feghali-Bostwick, 2014, Van Linthout et al., 2014) (Fig. 1.5).

Fibrocytes are bone-marrow derived circulating mesenchymal progenitor cells that express haemopoietic (CD34), leukocyte (CD11b, CD13 and CD45) and fibroblast markers (collagen 1 and fibronectin) (Yang et al., 2002, Reilkoff et al., 2011). *In vitro*, TGF β -treated fibrocytes differentiate into myofibroblasts (Lama and Phan, 2006). Since AEC2s undergo TGF β -induced epithelial-to-mesenchymal transition (EMT), fibrocytes and AEC2s have been proposed to act as myofibroblast precursors in IPF

(Willis et al., 2005) (Fig. 1.5). Interestingly however, fibrocytes transferred into the lungs of transforming growth factor- α (TGF α)-overexpressing mice fail to express α SMA (Madala et al., 2014). As AEC2 lineage tracing experiments suggest that EMT does not occur in the bleomycin mouse model of pulmonary fibrosis (Rock et al., 2011), these results question the contribution of fibrocytes and AECs to the expanded myofibroblast population observed in IPF (Moore and Herzog, 2013). Although further experiments are required to confirm the relative contributions of fibroblasts, fibrocytes and AECs to the myofibroblast pool, it is tempting to speculate that tissue resident fibroblasts are the predominant source of myofibroblasts in IPF.

1.1.6.2.3. Immune cells

In addition to epithelial cells and fibroblasts, multiple immune cells including neutrophils, T cells, B cells, macrophages and innate lymphoid cells (ILCs) have been implicated in the development of IPF (Desai et al., 2018, Heukels et al., 2019, Huang et al., 2020a). Of these, macrophages and ILCs are of particular interest for this thesis.

1.1.6.2.3.1. Macrophages

In addition to phagocytosing invading pathogens, alveolar and interstitial macrophages are implicated in the development of pulmonary fibrosis (Larson-Casey et al., 2016, Peng et al., 2016). Although a spectrum of macrophage polarisation exists, cells are broadly classified as either classically activated M1 or alternatively activated M2 macrophages (Mosser and Edwards, 2008, Xue et al., 2014). Polarised by IL-4, IL-13 and IL-33 (Pechkovsky et al., 2010, Kurowska-Stolarska et al., 2009, Li et al., 2014b, Murray et al., 2014), M2 macrophages release several factors (including TGF β , PDGF, VEGF and MMPs) that increase the pro-fibrotic activity of fibroblasts, promote ECM-remodelling and drive angiogenesis (Stockmann et al., 2010, Rohani et al., 2015,

Young et al., 2016, Li et al., 2014b, Wynn and Vannella, 2016). Given the importance of these processes during the fibrotic response, M2 macrophages are proposed to play an important pathophysiological role in IPF (Wynn and Vannella, 2016, Desai et al., 2018). Indeed, increased numbers of these cells have been reported in both animal models and IPF patients (Misson et al., 2004, Pechkovsky et al., 2010, Murray et al., 2011, Peng et al., 2011, Ji et al., 2014, Misharin et al., 2017) (Fig. 1.5).

Predominantly arising from fetal progenitors, alveolar macrophages (AMs) are long-lived self-renewing cells. Due to this, the contribution of circulating monocytes to the population of AMs in healthy lungs is minimal (Guilliams et al., 2013, Hashimoto et al., 2013, Yona et al., 2013, van de Laar et al., 2016). In disease however, macrophage depletion results in CCL2-dependent monocyte recruitment and the differentiation of monocytes into AMs (Matsushima et al., 1989, Yoshimura et al., 1989, Landsman and Jung, 2007, Lavin et al., 2014, Gibbings et al., 2015). Interestingly, depletion of circulating monocytes reduces collagen deposition in multiple murine models of pulmonary fibrosis (Moore et al., 2001, Gibbons et al., 2011). Since monocyte derived rather than resident AMs drive bleomycin-induced fibrosis, these results collectively suggest that monocyte derived AMs may play an important role in the development of IPF (Misharin et al., 2017) (Fig. 1.5).

1.1.6.2.3.2. Innate lymphoid cells (ILCs)

Originating from the common lymphoid progenitor, ILCs are innate immune cells that secrete cytokines in the absence of antigen specific receptors (Mikami et al., 2018, Vivier et al., 2018). Predominantly found at epithelial barriers such as those in the lungs, ILCs are classified into 3 subgroups (ILC1, ILC2 and ILC3) based on their expression of lineage determining transcription factors and release of signature

cytokines (Mikami et al., 2018, Vivier et al., 2018). Compared to healthy controls, significantly more ILC2s were identified in the BAL of IPF patients (Hams et al., 2014). As the depletion or adoptive transfer of ILC2s attenuates and exacerbates bleomycin-induced pulmonary fibrosis respectively (Li et al., 2014b, Zhao et al., 2018), these findings implicate ILC2s in the pathogenesis of IPF. Activated by IL-33, TSLP and IL-25, ILC2s secrete type 2 cytokines such as IL-4 and IL-13 (Mikami et al., 2018, Vivier et al., 2018). Based on *in vitro* experiments with IL-4 and IL-13 (Saito et al., 2003, Fichtner-Feigl et al., 2006, Murray et al., 2014), activated ILC2s are proposed to promote pulmonary fibrosis by inducing M2 macrophage polarisation and increasing the proliferation and pro-fibrotic activity of fibroblasts (Fig. 1.5).

1.1.6.3. Pro-fibrotic mediators in IPF

Multiple extracellular mediators have been implicated in the development of IPF. Although several reports suggest important fibrogenic roles for CTGF, PDGF, VEGF and FGF (Chen et al., 2003, Bonner, 2004, Sonnylal et al., 2010, Wang et al., 2011, Hostettler et al., 2014, Richeldi et al., 2014, Wollin et al., 2014, Wollin et al., 2015, Raghu et al., 2016b, Kim et al., 2017, Lehmann et al., 2018, Kishi et al., 2019), the pleiotropic cytokine TGF β is of particular interest for this thesis.

Secreted by multiple cell types (see sections 1.1.6.2.1, 1.1.6.2.2 and 1.1.6.2.3) as part of a biologically inactive complex, TGF β is sequestered in the ECM and can be activated by several molecules including reactive oxygen species, proteases, thrombospondin and integrins (Annes et al., 2003, Jenkins, 2008, Tatler and Jenkins, 2012). Despite playing an important homeostatic role in the lungs, TGF β is increased in the lungs of IPF patients with a significant positive correlation reported between TGF β levels and disease progression (Khalil et al., 1991, Limper et al., 1991, Bergeron

et al., 2003, Bartram and Speer, 2004). As inhibitors of TGF β activation and signalling attenuate pulmonary fibrosis in multiple animal models (Higashiyama et al., 2007, Horan et al., 2008, Kim et al., 2009, Li et al., 2011, John et al., 2020), these results suggest that TGF β acts as a key pro-fibrotic mediator during the development of IPF. Indeed, due to its ability to induce AEC2 apoptosis (Lee et al., 2006), increase the pro-fibrotic activity of fibroblasts (Eickelberg et al., 1999, Conte et al., 2014, Woodcock et al., 2019), drive fibrocyte to myofibroblast differentiation (Lama and Phan, 2006) and promote EMT (Willis et al., 2005), TGF β is widely regarded as the prototypic pro-fibrotic cytokine in IPF (Yue et al., 2010, Fernandez and Eickelberg, 2012, Saito et al., 2018). Consequently, TGF β is often used as a stimulus to model the pro-fibrotic environment *in vitro* and induce fibrotic gene and protein expression by human lung fibroblasts (HLFs) and PCLS (Eickelberg et al., 1999, Conte et al., 2014, Roach et al., 2018, Woodcock et al., 2019).

1.1.6.4. Signalling pathways in IPF

Several signalling pathways have been implicated in the pathogenesis of IPF. Although multiple studies suggest important pro-fibrotic roles for TGF β /SMAD, MAPK/ERK, WNT/ β -catenin, Rho-kinase and Notch signalling (Fernandez and Eickelberg, 2012, Madala et al., 2012, Knipe et al., 2015, Hu and Phan, 2016, Baarsma and Königshoff, 2017), the PI3K/AKT/mTOR pathway is of particular interest for this thesis.

The serine-threonine protein kinase mammalian target of rapamycin (mTOR) associates with multiple proteins to form two distinct complexes. Referred to as mTOR complex 1 (mTORC1) and mTOR complex 2 (mTORC2), the activity of mTOR within these complexes is tightly regulated (Saxton and Sabatini, 2017, Liu and Sabatini, 2020). Although the mechanism of mTORC2 activation remains poorly understood,

receptor tyrosine kinase (RTK) transautophosphorylation, phosphoinositide 3-kinase (PI3K) activation and AKT-mediated phosphorylation of tuberous sclerosis complex 2 (TSC2) and proline-rich AKT substrate of 40 kDa (PRAS40) activates mTORC1 (Kovacina et al., 2003, Sancak et al., 2007, Vander Haar et al., 2007, Wiza et al., 2012, Menon et al., 2014)(Fig. 1.6). Referred to from this point onwards as mTOR, mTORC1 drives cell growth/proliferation by phosphorylating p70S6 kinase (p70S6K) and eukaryotic initiation factor 4E-binding protein 1 (4E-BP1) and inhibits autophagy and apoptosis by phosphorylating unc-51 like autophagy activating kinase 1 (ULK1) (Kim et al., 2011, Egan et al., 2011) (Fig. 1.6).

Despite playing an important homeostatic role in the lungs, dysregulated PI3K/AKT/mTOR signalling is implicated in the pathogenesis of IPF with pharmacological inhibition of this pathway shown to reduce bleomycin- and TGF α -induced pulmonary fibrosis *in vivo* (Korfhagen et al., 2009, Patel et al., 2012). *In vitro*, inhibition of PI3K/AKT/mTOR signalling via small molecules or genetic manipulation, reduces growth factor-induced fibroblast proliferation, differentiation and ECM deposition (Rahimi et al., 2009, Goc et al., 2011, Mercer et al., 2016). Furthermore, activation of the PI3K/AKT/mTOR pathway in IPF fibroblasts prevents starvation- and collagen- induced apoptosis by suppressing autophagy (Nho and Hergert, 2014, Im et al., 2015, Romero et al., 2016). Taken together, these results suggest that the PI3K/AKT/mTOR signalling pathway promotes pulmonary fibrosis by increasing the proliferation, differentiation and survival of ECM-secreting fibroblasts.

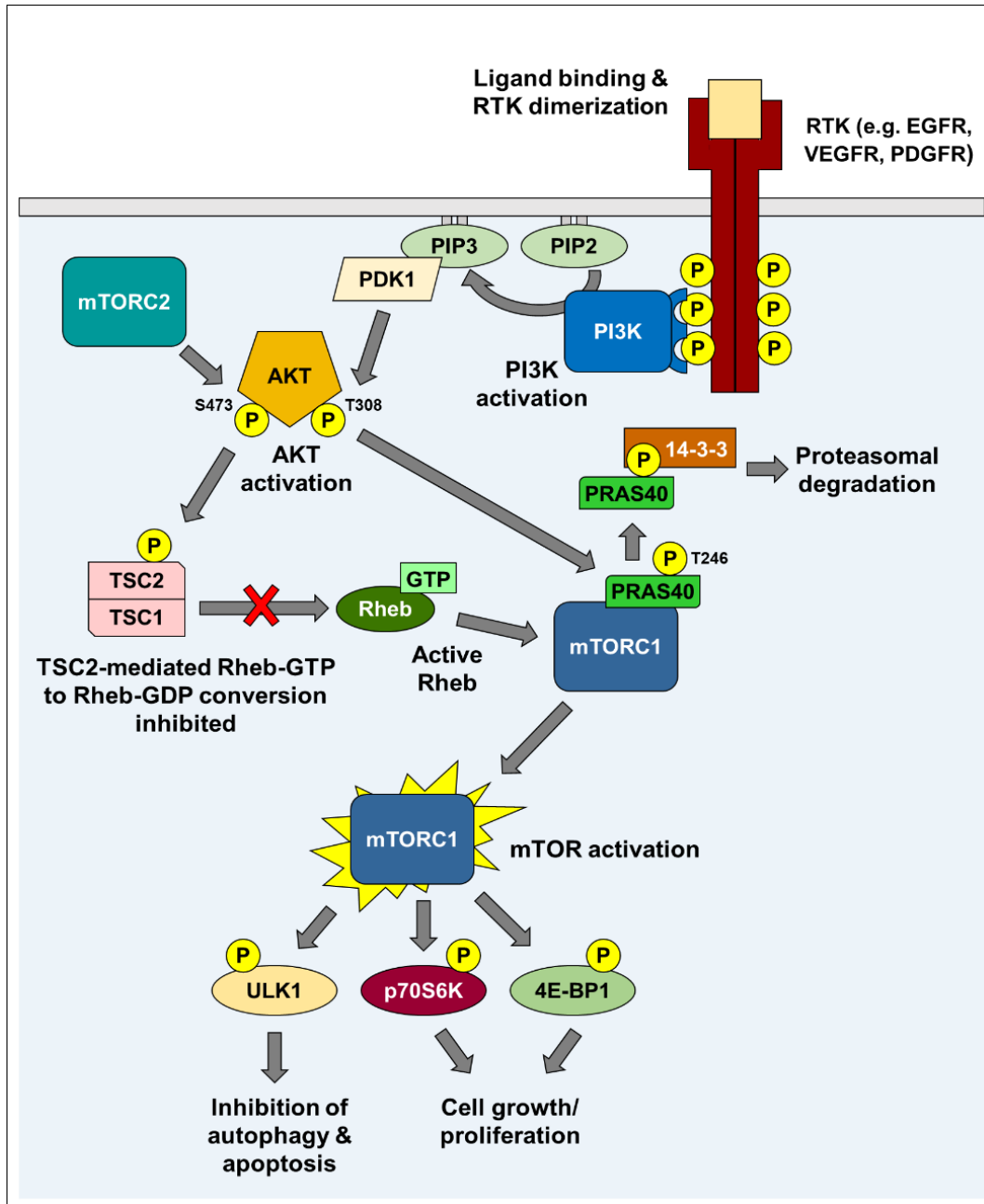


Figure 1.6: PI3K/AKT/mTOR signalling.

Following ligand binding, the dimerization and transautophosphorylation of RTKs (such as EGFR, PDGFR and VEGFR) results in the recruitment and activation of PI3K. Phosphorylating the 3' hydroxyl group of the plasma membrane component PIP2, PI3K converts PIP2 to PIP3. Resulting in the recruitment of AKT and PDK1, PIP3 facilitates the PDK1-dependent phosphorylation of AKT at threonine 308. Following mTORC2-mediated phosphorylation at serine 473, activated AKT phosphorylates a number of protein substrates including TSC2 and PRAS40. This results in the activation of mTORC1 and the mTOR-mediated phosphorylation of multiple proteins including p70S6K, 4E-BP1 and ULK1. As these proteins regulate cell growth/proliferation, autophagy and apoptosis, PI3K/AKT/mTOR signalling plays an important role in both health and disease. Figure adapted from Wiza et al. (2012).

1.1.7. The treatment of IPF

Although shown in multiple studies to improve patient quality of life, pulmonary rehabilitation and oxygen therapy have no effect on disease progression and life expectancy (Nishiyama et al., 2008, Nishiyama et al., 2013, Dowman et al., 2017a, Dowman et al., 2017b). Moreover, despite increasing life expectancy (Titman et al., 2009, Singer et al., 2017), common contraindications (such as advanced non-pulmonary organ failure, cancer and chronic viral/bacterial infection) and limited tissue availability mean that lung transplantation is not an option for many IPF patients (Weill et al., 2015, Lederer and Martinez, 2018). Consequently, much research has focused on the development of new drugs to treat IPF. As summarised in Table 1.1, numerous therapies with varying mechanisms of action have entered or are in clinical trials. Currently however, only two drugs, pirfenidone and nintedanib, are licenced to treat IPF (Hewitt and Maher, 2019).

Table 1.1: Pharmacological therapies trialled for use in IPF.

Ineffective therapies		
Drug	Mechanism of action	Clinical efficacy
Prednisone	Immunosuppressant	• Little to no efficacy reported when prednisone, azathioprine or NAC used alone (Raghu et al., 1991, Martinez et al., 2014) • When combined as triple therapy, prednisone, azathioprine and NAC demonstrated to slow decline in FVC (Raghu et al., 2011) • Triple therapy no longer used as standard clinical intervention due to recent trial showing no effect on FVC and increased hospitalisation and mortality vs. placebo (Raghu et al., 2012, Jones, 2013)
Azathioprine	Immunosuppressant	
NAC	Antioxidant	
Etanercept	anti-TNF α mAb	• No effect on FVC vs. placebo (Raghu et al., 2008)
Carlumab	anti-CCL2 mAb	• Trial halted due to increased rate of FVC decline vs. placebo (Raghu et al., 2015)
Imatinib	RTK inhibitor	• No effect on FVC or survival time vs. placebo (Daniels et al., 2010)
Warfarin	Anti-coagulant	• Improved the one year survival rate for patients hospitalised due to exacerbation vs. placebo (Kubo et al., 2005)

		Clinical trials halted due to increased mortality (Noth et al., 2012)
IFNγ	Immunomodulation	No effect on rate of decline in FVC, quality of life and survival vs. placebo (Raghu et al., 2004)
Sildenafil	PDE-5 inhibitor	No significant improvement in 6 minute walk test distance or quality of life vs. placebo (Zisman et al., 2010)
Bosentan	ET antagonist	No effect on rate of decline in FVC, quality of life and survival vs. placebo (King et al., 2011a)
Licensed therapies		
Drug	Mechanism of action	Clinical efficacy
Pirfenidone	Exact mechanism unclear but effects on pro-fibrotic cytokines/signalling such as (TGF β , PDGF, TNF α etc.)	- Slowed the rate of decline in FVC vs placebo and increased 6 minute walk distance (Taniguchi et al., 2010, Noble et al., 2011, King et al., 2014, Lederer et al., 2015, Noble et al., 2016) - Adverse effects include rash, nausea, diarrhoea, fatigue, elevated liver enzymes, photosensitivity, dyspepsia and weight loss (Valeyre et al., 2014)
Nintedanib	RTK inhibitor	- Slowed the rate of decline in FVC vs placebo, increased the time to first exacerbation after withdrawal and improved quality of life (Richeldi et al., 2011, Richeldi et al., 2014) - Non-compliance common due to diarrhoea (Richeldi et al., 2014) - Other adverse effects include nausea, vomiting and elevated liver enzymes (Ogura et al., 2015).
Potential therapies (currently in clinical trials)		
Drug	Mechanism of action	Clinical efficacy
GLPG1690	Autotaxin inhibitor	- Inhibits LPA production - Well tolerated with limited adverse effects - Mean FVC remained stable in phase IIa however not sufficiently powered to attain significance vs placebo. Currently in Phase III trials (Maher and Strek, 2019)
BMS-986020	LPA receptor inhibitor	- Slowed the rate of decline in FVC vs placebo in phase II - Increases liver enzymes and has off-target effects on hepatobiliary transporters (Palmer et al., 2018)
PRM-151	Pentraxin-2 (SAP)	- Pattern recognition receptor - Slowed the rate of decline in FVC vs placebo in phase II - Cough observed as adverse event (Raghu et al., 2018)
FG-3019 (Pamrevlumab)	anti-CTGF mAb	Slowed the rate of decline in FVC and some improvements in FVC vs placebo. Reduced fibrosis via HRCT (Raghu et al., 2016b, Goldin et al., 2018)
TD139	Galectin-3 inhibitor	- Target engagement with no adverse events (Mackinnon et al., 2017) - Currently in phase IIb
BG00011 & GSK3008348	$\alpha_v\beta_6$ mAbs	BG00011 - Target engagement with no adverse events. Currently in phase IIb (Mouded et al., 2018)

		• GSK3008348 - No adverse events reported (Maden et al., 2018)
Lebrikizumab	Anti-IL-13 mAb	• No effect on rate of decline in FVC and survival vs. placebo (Kondoh et al., 2018, Ogura et al., 2018) • Likely to be ineffective
GSK2126458 (Omipalisib)	PI3K/mTOR inhibitor	• Target engagement in phase Ib trial. Diarrhoea as adverse event in some patients (Lukey et al., 2019)

Forced vital capacity (FVC), a measurement of lung function, is frequently used as the primary endpoint during clinical trials. The distance walked in a 6 minute period (the 6 minute walk test (6MWT) is often used as a secondary endpoint. **Abbreviations:** ET: endothelin receptor, IFN: interferon, mAb: monoclonal antibody, NAC: N-acetyl-cysteine, PDE: phosphodiesterase, SAP: serum amyloid P and TNF: tumour necrosis factor.

Pirfenidone is an orally bioavailable pyridone compound demonstrated to improve lung function and slow disease progression (Taniguchi et al., 2010, Noble et al., 2011, King et al., 2014, Lederer et al., 2015, Noble et al., 2016). Although its exact mechanism of action remains unclear, pirfenidone inhibits TGF β -induced collagen synthesis by fibroblasts and decreases fibronectin expression by TGF β , PDGF, tumour necrosis factor- α (TNF α) and LPA treated PCLS (Conte et al., 2014, Lehmann et al., 2018). As it also reduces TGF β , IL-6, TNF α and hydroxyl radical levels in animal models of pulmonary fibrosis (Gurujeyalakshmi et al., 1999, Iyer et al., 1999, Iyer et al., 2000, Misra and Rabideau, 2000, Oku et al., 2008), these *in vitro* and *in vivo* findings suggest that pirfenidone exerts its anti-fibrotic effects by inhibiting fibrotic mediators, blocking inflammatory cytokines and scavenging reactive oxygen species (ROS).

Nintedanib is an orally bioavailable RTK inhibitor that binds to VEGF, FGF and PDGF receptors and prevents downstream signalling (Hilberg et al., 2008, Hostettler et al., 2014). Shown to decrease the pro-fibrotic activity of fibroblasts, reduce fibrotic changes in PCLS and attenuate pulmonary fibrosis in animal models (Hostettler et al., 2014, Wollin et al., 2014, Wollin et al., 2015, Lehmann et al., 2018), nintedanib

improves lung function and slows disease progression in IPF patients (Richeldi et al., 2011, Richeldi et al., 2014).

Despite improving lung function and increasing life expectancy, neither pirfenidone nor nintedanib halt disease progression (Lederer and Martinez, 2018, Maher and Streck, 2019, Yanagihara et al., 2019). Given this and the fact that both drugs have potentially use limiting side effects (including diarrhoea, nausea, vomiting, weight loss, photosensitivity and altered liver enzymes/function)(Richeldi et al., 2014, Valeyre et al., 2014, Ogura et al., 2015), further research to identify novel therapeutic targets is required. Recently, emerging evidence suggests that the cytokine, interleukin-33 (IL-33), may play an important role in the development of IPF. In the following sections I will discuss the biology of IL-33 and outline what is currently known about its role in IPF.

1.2. Interleukin-33 (IL-33)

1.2.1. The discovery of IL-33

Following research to identify novel genes expressed in high endothelial venules (HEVs), a nuclear factor specific to HEV endothelial cells (HEVECs) was discovered (Baekkevold et al., 2003). Designated nuclear factor from HEVs (NF-HEV), high levels of NF-HEV mRNA and protein were observed in lymph nodes, human tonsils and Peyer's patches *in situ* (Baekkevold et al., 2003). Encoded by a gene on the short arm of chromosome 9 (at 9p24.1) in humans, NF-HEV was subsequently discovered to be a member of the IL-1 family of cytokines (Schmitz et al., 2005, Carriere et al., 2007). Renamed as IL-33, canine and murine orthologues were identified (Onda et al.,

1999, Baekkevold et al., 2003, Schmitz et al., 2005) with IL-33 found to induce type 2 inflammation via the orphan receptor ST2 (Schmitz et al., 2005).

1.2.2. The molecular biology of IL-33

1.2.2.1. The IL-33 gene

Despite being located on different chromosomes (9p24.1 in humans and 19qC1 in mice), the IL-33 gene consists of 8 exons in both humans and mice (Schmitz et al., 2005). Spanning approximately 42 kb in humans, the IL-33 gene is regulated by a single promoter (Cayrol and Girard, 2018). In mice, two alternative promoters regulate IL-33 gene expression with *IL33a* and *IL33b* transcribed. Although the 5'-untranslated regions of these transcripts differ, *IL33a* and *IL33b* encode the same protein (Talabot-Ayer et al., 2009, Polumuri et al., 2012).

1.2.2.2. IL-33 protein

Comprising of 270 amino acids in humans (266 in mice), the IL-33 protein consists of an N-terminal nuclear domain (residues 1-65), a central linker domain (residues 66-111) and a C-terminal IL-1-like cytokine domain (residues 112-270) (Baekkevold et al., 2003, Schmitz et al., 2005) (Fig 1.7). Given the highly conserved nature of the N- and C- terminal domains, 52% sequence homology has been reported for human and mouse IL-33 (Baekkevold et al., 2003).

Spanning the first 65 amino acids and encoded by exons 2-3, the N-terminal nuclear domain of IL-33 contains a chromatin binding motif (CBM) and a nuclear localisation sequence (Carriere et al., 2007, Roussel et al., 2008) (Fig. 1.7). As suggested by its name, the N-terminal nuclear domain is necessary and sufficient for the nuclear localisation of IL-33 *in vitro* and *in vivo* (Carriere et al., 2007, Bessa et al., 2014, Xi et

al., 2016). Although initially thought to interact with DNA directly via a homeodomain-like helix-turn-helix DNA-binding motif (Baekkevold et al., 2003), elegant point mutation experiments have shown that IL-33 uses its CBM to bind to the histone H2A-H2B heterodimer at the surface of chromatin (Roussel et al., 2008).

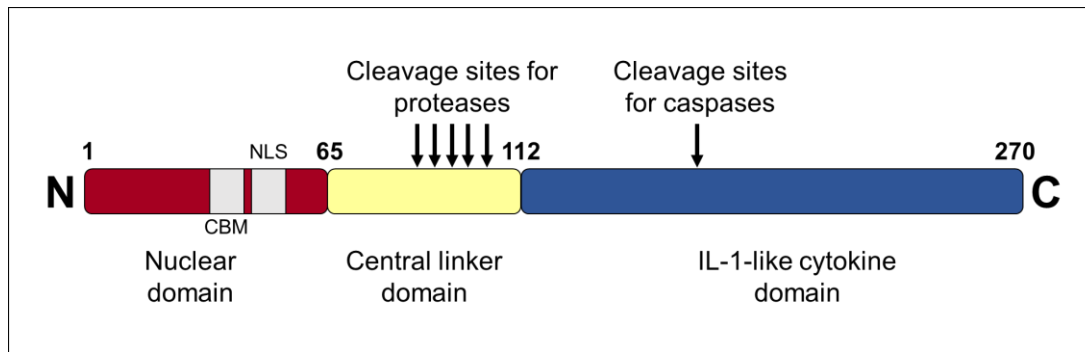


Figure 1.7: The primary structure of human IL-33 protein.

The different domains of human IL-33 protein are shown. Protease and caspase cleavage sites are also indicated. Figure adapted from Cayrol and Girard (2018).

Encoded by exon 4, the amino acid sequence of the central linker domain varies across species (Baekkevold et al., 2003, Schmitz et al., 2005). In humans, the linker domain of IL-33 contains multiple cleavage sites sensitive to processing by cell, allergen and microbial derived proteases (Hayakawa et al., 2009, Lefrançois et al., 2012, Lefrançois et al., 2014, Cayrol et al., 2018, Scott et al., 2018a) (Fig. 1.7).

Following analysis by X-ray crystallography and nuclear magnetic resonance (NMR) spectroscopy, the C-terminal domain of IL-33 was shown to adopt the tertiary β -trefoil structure characteristic of the IL-1 cytokine family (Lingel et al., 2009, Liu et al., 2013). Encoded by exons 5-8, the C-terminal IL-1-like cytokine domain can bind to ST2 or be cleaved by caspases or proteases (Cayrol and Girard, 2009, Lingel et al., 2009, Liu et al., 2013, Lüthi et al., 2009, Ali et al., 2010, Bae et al., 2012, Waern et al., 2013) (Fig. 1.7).

1.2.3. The expression of IL-33

IL-33 is constitutively expressed by multiple cell types in human and mouse tissues. Indeed, under homeostatic conditions, IL-33 is found in the nuclei of epithelial cells in barrier tissues, endothelial cells in vascular beds and fibroblast-like cells in lymphoid organs (Carriere et al., 2007, K  chler et al., 2008, Moussion et al., 2008, Pichery et al., 2012). Despite these cell types acting as major sources of IL-33 in humans and mice, species-specific differences in constitutive IL-33 expression have been reported. For instance, in contrast to humans where it is constitutively expressed along the vascular tree, IL-33 is present in very few vascular beds in mice (Liew et al., 2016). Moreover, under homeostatic conditions, IL-33 is expressed by airway epithelial cells in humans but not mice (Prefontaine et al., 2009, Byers et al., 2013, Kearley et al., 2015). Since the opposite is true for AEC2s (i.e. IL-33 is expressed by AEC2s in mice but not humans) (Hardman et al., 2013, Kearley et al., 2015), care is required when extrapolating IL-33-specific findings from murine models to human disease.

Compared with non-diseased control tissues, higher levels of IL-33 are present in the airway epithelium of asthma and COPD patients, the intestinal epithelium of graft versus host disease patients and the endothelium and skin keratinocytes of atopic dermatitis patients (Savinko et al., 2012, Byers et al., 2013, Kearley et al., 2015, Reichenbach et al., 2015). As the same is true for AEC2s in the lungs of mice following allergen challenge, nematode infection and cigarette smoke exposure (Yasuda et al., 2012, Barlow et al., 2013, Hardman et al., 2013, Kearley et al., 2015), these reports demonstrate that basal IL-33 expression can be increased during inflammation.

In addition to upregulating basal expression by epithelial, endothelial and fibroblast-like cells, inflammatory stimuli can induce IL-33 expression in numerous cell types *in vivo*. Indeed, although IL-33 is not generally expressed by fibroblasts/myofibroblasts under homeostatic conditions (Moussion et al., 2008), these cells express high levels of this cytokine during inflammation and/or fibrosis (Palmer et al., 2009, Kobori et al., 2010, Marvie et al., 2010, Masamune et al., 2010, Nishida et al., 2010, Sponheim et al., 2010, Luzina et al., 2012, Luzina et al., 2013, Chen et al., 2015, Millar et al., 2015, Sundnes et al., 2015). Similarly, hepatocytes, smooth muscle cells and CD45⁺ immune cells (including macrophages, ILCs and mast cells) have all been shown to express IL-33 under inflammatory conditions (Prefontaine et al., 2009, Hsu et al., 2010, Arshad et al., 2011, Chang et al., 2011, Arshad et al., 2012, Kim et al., 2012, Fock et al., 2013, Shimokawa et al., 2017).

In vitro, multiple cytokines modulate IL-33 expression. For instance, IL-1 β , IL-4 and TNF α upregulate IL-33 expression by fibroblasts whilst TGF β downregulates IL-33 expression by smooth muscle cells (Xu et al., 2008, Prefontaine et al., 2009, Nomura et al., 2012, Zhao and Hu, 2012). Interestingly, despite increasing IL-33 levels in human skin keratinocytes, IFN γ has been shown to decrease IL-33 expression by human lung fibroblasts *in vitro* (Meephansan et al., 2012, Kopach et al., 2014, Sundnes et al., 2015). Due to this, it is plausible that cytokine-induced IL-33 expression is cell type and/or organ specific.

1.2.4. The intracellular role of IL-33

Although cytoplasmic staining has been reported (Kakkar et al., 2012, Luzina et al., 2019), IL-33 is predominantly observed in the nucleus of cells (Moussion et al., 2008, Louten et al., 2011, Haenuki et al., 2012, Luzina et al., 2012, Pichery et al., 2012,

Luzina et al., 2013). Combined with its ability to bind chromatin and interact with the transcription factor NF- κ B, IL-33 has been proposed to regulate gene expression via an intracellular mechanism (Schmitz et al., 2005, Carriere et al., 2007, Ali et al., 2011). However, despite being reported to alter p53, ICAM-1, IL-6 and IL-13 mRNA levels (Ali et al., 2011, Choi et al., 2012, Shao et al., 2014, Ni et al., 2015), knockdown of IL-33 had no detectable effect on protein expression by endothelial cells (Gautier et al., 2016). Moreover, although shown to increase *COX-2* and *IDO* mRNA expression by mesenchymal stem cells (MSCs) *in vitro* (Chen et al., 2019), nuclear IL-33 was found to have no effect on MSC gene expression *in vivo* (Rana et al., 2019). Given that IL-33 overexpression failed to alter global gene expression by transduced epithelial cells (Travers et al., 2018), it remains unclear whether intracellular IL-33 regulates gene expression. Likewise, as only endothelial cells, MSCs and epithelial cells have been studied, it is currently unknown whether IL-33 plays an active intranuclear role in fibroblasts.

1.2.5. The release of IL-33

Unlike other cytokines, IL-33 lacks the signal sequence required for release via the classical ER-Golgi secretory pathway (Liew et al., 2016). Due to this and the high expression of IL-33 by barrier forming cells (see section 1.2.3), IL-33 was proposed by Moussion et al. (2008) to act as an alarmin alerting the immune system to cell damage and death.

Following several *in vitro* and *in vivo* experiments, IL-33 has been shown to function as an alarmin with mechanical injury (cell damage) and necrotic cell death resulting in IL-33 release in multiple model systems (Table 1.2). Despite suggesting that cell damage and death are required for IL-33 release, it is important to note that cyclical

stretch induced IL-33 release from fibroblasts in the absence of cell death (Kakkar et al., 2012). Since ATP (Kouzaki et al., 2011) and uric acid (Hara et al., 2014) drove IL-33 release from viable epithelial cells, these findings suggest that IL-33 release can occur independently of cell damage and death.

Table 1.2: IL-33 release following cell damage and/or death.

Model system	Key observations	Release mechanism	Reference(s)
Cell scraping (<i>In vitro</i>)	IL-33 present in supernatants collected from scraped endothelial cells. No IL-33 detected in supernatants from unscraped controls	Cell damage via mechanical injury	Cayrol and Girard (2009)
Repeated freeze-thaw cycles (<i>In vitro</i>)	- IL-33 detected in supernatants following repeated freeze-thaw cycles. No IL-33 detected in supernatants prior to freeze-thaw cycles - IL-33 levels increased with the number of freeze-thaw cycles	Necrotic cell death	
Hydrogen peroxide or Streptolysin O treatment (<i>In vitro</i>)	- Hydrogen peroxide and Streptolysin O induced THP-1 cell necrosis - Treatment with either Hydrogen peroxide or Streptolysin O (pore forming bacterial toxin) induced IL-33 release from THP-1 cells pre-treated with LPS and PMA. No IL-33 detected following LPS and PMA pre-treatment alone	Necrotic cell death	Lüthi et al. (2009)
Oleic acid (OA) induced lung injury (<i>In vivo</i>)	- OA induced alveolar epithelial cell damage in mice (indicated by increases in lung weight and protein leakage into the BALF versus PBS control) - IL-33 detected in BALF of OA treated mice after 2 hours. No IL-33 present in BALF of PBS treated controls	Cell damage	Lefrançois et al. (2012)
Rhinovirus, Influenza A or RSV infection (<i>In vitro</i>)	- Infection with Rhinovirus, Influenza A or RSV induced IL-33 release from primary human bronchial epithelial cells. No IL-33 detected in supernatants collected from uninfected cells - IL-33 release correlated with LDH release	Necrotic cell death	Kearley et al. (2015)
Influenza A infection (<i>In vivo</i>)	- Loss of intracellular IL-33 staining in areas of lung tissue where Influenza A present. Intracellular IL-33 present in lungs of uninfected mice - Loss of IL-33 staining correlated with LDH levels in the BALF of infected mice	Necrotic cell death	

Tape stripping (<i>Ex vivo</i> & <i>In vivo</i>)	- Compared to control, more IL-33 detected in supernatants collected from cultured tape-stripped skin - Tape stripping induced systemic IL-33 release. No IL-33 detected in serum of control mice	Cell damage via mechanical injury	Galand et al. (2016)
Phospholipase A ₂ treatment (<i>In vivo</i>)	Compared to untreated control, higher concentrations of IL-33 detected in peritoneal fluid following intraperitoneal injection of Phospholipase A₂ (major allergen in bee venom)	Cell damage	Palm et al. (2013)
Alum (<i>In vivo</i>)	- Alum treatment resulted in necrotic cell death - Compared to PBS control, higher concentrations of IL-33 detected in peritoneal fluid following intraperitoneal injection of Alum (frequently used vaccine adjuvant)	Necrotic cell death	Rose Ii et al. (2015)
<i>Alternaria alternata</i> (<i>In vivo</i>)	- Rapid IL-33 release into the BALF 15-60 minutes after <i>Alternaria alternata</i> challenge. No IL-33 detected in BALF of control animals - Dose of <i>Alternaria</i> used induces airway epithelial cell damage and necrotic cell death*	Cell damage and necrotic cell death	Cohen et al. (2015) Hristova et al. (2016) Snelgrove et al. (2014) *Murai et al. (2012)

1.2.6. The forms of IL-33

Multiple forms of IL-33 exist with full length IL-33, mature IL-33, IL-33 splice variants and oxidised IL-33 reported.

1.2.6.1. Full length IL-33 (flIL-33)

Since other IL-1 family members (i.e. IL-1 β and IL-18) are synthesised as inactive precursor proteins (Fields et al., 2019), full length IL-33 (flIL-33) was initially thought to have no biological activity (Schmitz et al., 2005, Kakkar and Lee, 2008). Indeed, it was proposed that caspase-1 mediated cleavage of flIL-33 was required for the activation of IL-33 (Schmitz et al., 2005). Despite being widely supported, this hypothesis was challenged by the finding that flIL-33 could bind to ST2 and activate NF- κ B (Cayrol and Girard, 2009, Lüthi et al., 2009). As full length protein induces IL-

6 release from P815 mastocytoma cells (Talabot-Ayer et al., 2009) and MC/9 mast cells (Lefrançais et al., 2012), it is now widely accepted that fIL-33 is biologically active.

1.2.6.2. Mature IL-33 (mIL-33)

In addition to full length protein (31 kDa), shorter forms (18-21 kDa) of IL-33 elicit ST2-dependent responses *in vitro* and *in vivo*. Collectively known as mature IL-33 (mIL-33), these n-terminal truncated isoforms are generated following the release and subsequent cleavage of fIL-33 by extracellular proteases.

Serine proteases from mast cells and neutrophils cleave fIL-33 within its central linker domain (Lefrançais et al., 2012, Lefrançais et al., 2014). Since serine and cysteine proteases from allergens, bacteria and necrotic epithelial cells also generate mIL-33 (Hayakawa et al., 2009, Cayrol et al., 2018, Scott et al., 2018a), it is not surprising that mIL-33 is the predominant form of IL-33 found in the BALF of chitin (Mohapatra et al., 2016), *Alternaria alternata* (Cohen et al., 2015, Osbourn et al., 2017, Cayrol et al., 2018) and oleic acid challenged mice (Lefrançais et al., 2012).

Compared to mIL-33, 3-60 fold higher concentrations of fIL-33 were needed to activate mast cells, endothelial cells and ILC2s *in vitro* (Lefrançais et al., 2012, Lefrançais et al., 2014, Scott et al., 2018a). Although direct side by side comparisons are required to assess the relative importance of fIL-33 and mIL-33 *in vivo*, these *in vitro* observations suggest that proteolytic cleavage of fIL-33 is crucial for the induction and/or amplification of IL-33 mediated responses especially when fIL-33 is present at low levels.

1.2.6.3. IL-33 splice variants

Analogous to other cytokines (Alms et al., 1996, Tsytsikov et al., 1996, Korte et al., 1999, Bihl et al., 2002, Wu et al., 2005, Rahman et al., 2007, Whitaker et al., 2011), multiple splice variants of IL-33 have been identified in immortalised and primary human cells (Hong et al., 2011, Tsuda et al., 2012, Gordon et al., 2016b). Characterised by the deletion (Δ) of some or all of exons 3, 4 or 5, the proteins encoded by these alternatively spliced transcripts differ from each other in terms of their ST2-dependent activity. For instance, splice variants lacking exon 3 (Δ exon 3) or exons 3 and 4 (Δ exon 3, 4) induce IL-8 secretion from HMC-1 cells whilst those missing exon 5 do not (Gordon et al., 2016b). Interestingly, expression of the Δ exon 3, 4 splice variant positively correlates with *periostin*, *serpinB2* and *CLCA1* mRNA levels in asthmatic airway epithelial cells (Gordon et al., 2016b). Since *periostin*, *serpinB2* and *CLCA1* are readouts of IL-13 mediated epithelial cell activation (Woodruff et al., 2007, Bhakta et al., 2013), this splice variant has been proposed to drive type 2 inflammation in mild to moderate asthma (Gordon et al., 2016b). Despite this however, further work is required to validate this finding *in vivo* and determine the pathophysiological importance of alternative IL-33 splicing in other diseases.

1.2.6.4. Oxidised IL-33 (oxIL-33)

Following its release from cells, IL-33 readily oxidises. Occurring at cysteine 208, 227, 232 and 259 in humans, oxidation leads to the formation of two intrachain disulphide bonds (Cohen et al., 2015). Resulting in a substantial conformational change in its tertiary structure, oxidised IL-33 cannot bind to ST2 (Cohen et al., 2015). Since this prevents NF- κ B activation and the release of pro-inflammatory cytokines *in*

vitro, IL-33 oxidation is proposed to limit/control ST2-dependent inflammation *in vivo* (Cohen et al., 2015).

1.2.7. ST2-dependent IL-33 signalling

Once in the extracellular space, IL-33 exerts its biological effects by binding to the IL-33 receptor. Comprising of IL-1 receptor accessory protein (IL-1RAcP) and ST2 (also known as ST2L, IL1RL1, FIT-1, T1 and DER4), it is the latter transmembrane protein that is specific to the IL-33 receptor (Ali et al., 2007, Chackerian et al., 2007, Palmer et al., 2008, Liew et al., 2016).

First identified in murine fibroblasts, ST2 is a member of the IL-1 receptor-like subfamily of the Toll-like/IL-1-receptor superfamily (Yanagisawa et al., 1993). Encoded by the *IL1RL1* gene on chromosome 2 (at 2q12.1) in humans and chromosome 1 (at 1 B|1 19.19 cM) in mice, ST2 consists of an intracellular Toll/Interleukin-1 receptor (TIR) domain and an extracellular domain comprising of three linked immunoglobulin motifs (Kakkar and Lee, 2008). Present on the surface of a wide range of cell types (Table 1.3), ST2 can be induced and/or increased by pro-inflammatory stimuli (including IFN γ , TNF α , IL-1 β and IL-4) and serum derived factors (Kumar et al., 1997, Yanagisawa et al., 1993, Nishida et al., 2010).

Table 1.3: ST2 expressing cells.

Cell type	Cell	Reference(s)
Immune cells	ILC2s	Moro et al. (2010), Neill et al. (2010), Price et al. (2010), Mjösberg et al. (2011)
	Mast cells	Xu et al. (2008), Enoksson et al. (2011), Morita et al. (2015)
	T _H 1 CD4 ⁺ T cells	Kearley et al. (2015), Reichenbach et al. (2015)
	T _H 2 CD4 ⁺ T cells	Löhning et al. (1998), Coyle et al. (1999), Smithgall et al. (2008),
	CD8 ⁺ T cells	Bonilla et al. (2012), Peine et al. (2016)
	Macrophages	Kurowska-Stolarska et al. (2009), Li et al. (2014b),
	DCs	Turnquist et al. (2008), Rank et al. (2009), Turnquist et al. (2010), Matta et al. (2014),
	Tregs	Turnquist et al. (2011), Schiering et al. (2014), Arpaia et al. (2015), Kolodin et al. (2015), Molofsky et al. (2015a), Vasanthakumar et al. (2015), Matta et al. (2016)
	Eosinophils	Cherry et al. (2008), Johnston and Bryce (2017)
	Basophils	Smithgall et al. (2008), Suzukawa et al. (2008)
	NK cells	Smithgall et al. (2008), Bourgeois et al. (2009), Kearley et al. (2015)
Structural cells	Endothelial cells	Cayrol and Girard (2009), Gautier et al. (2016)
	Epithelial cells	Mahapatro et al. (2016)
	Fibroblasts	Yanagisawa et al. (1993), Kumar et al. (1997), Kurokawa et al. (2011), Zhu and Carver (2012)

Abbreviations: DCs: dendritic cells, NK: natural killer, T_H1:T-helper 1, T_H2:T-helper 2.

As illustrated in Figure 1.8, surface charge complementarity enables the binding of IL-33 to ST2 (Liu et al., 2013). The resulting conformational change in ST2 drives the recruitment of IL-1RACp and the formation of a ternary complex (Ali et al., 2007, Chackerian et al., 2007, Palmer et al., 2008). Colocalization of ST2 and IL-1RACp's TIR domains leads to the binding of the adaptor protein myeloid differentiation primary response protein 88 (MYD88) and the recruitment of IL-1 associated kinase (IRAK) 1, IRAK4 and TNF receptor associated factor 6 (TRAF6). Subsequent TRAF6-mediated activation of NF-κB and mitogen activated protein kinases (JNK, ERK and p38) alters the expression of several genes (Schmitz et al., 2005, Liew et al., 2016). As these genes promote cell survival, proliferation and cytokine secretion, ST2-dependent IL-33 signalling plays a crucial role in inflammatory responses and wound healing (Liew et al., 2016, Martin and Martin, 2016, Cayrol and Girard, 2018).

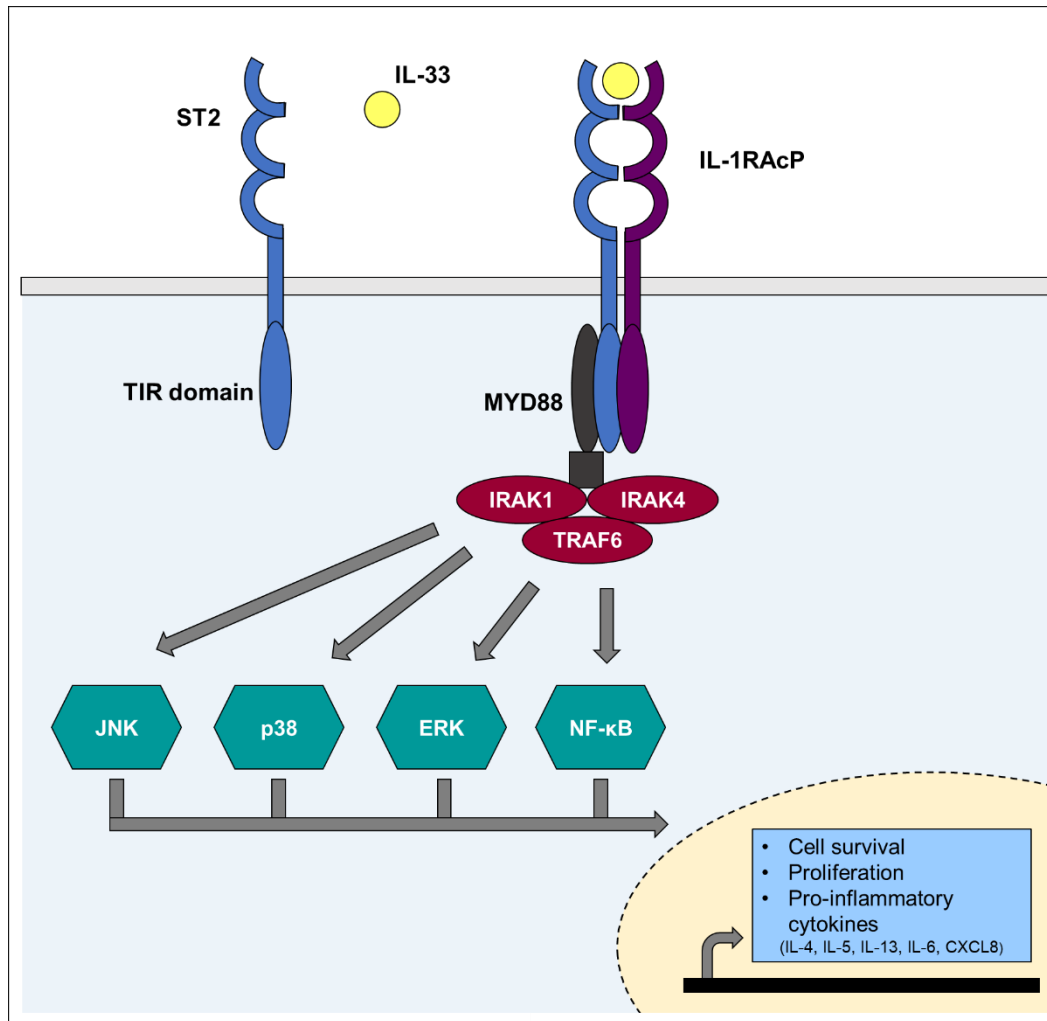


Figure 1.8: ST2-dependent IL-33 signalling.

Surface charge complementarity results in the binding of IL-33 to ST2 inducing a conformational change. IL-1RAcP then associates with ST2 before the adaptor protein MYD88 is recruited via heterotypic TIR domain interactions. MYD88 in turn facilitates the binding of IRAK1, IRAK4 and TRAF6 resulting in the activation of MAPK pathway components (including JNK, p38 and ERK) and NF-κB. Alterations in gene expression subsequently promote cell survival, proliferation and cytokine secretion. Figure adapted from Liew et al. (2016).

1.2.8. The biological effects of IL-33

As illustrated in Table 1.3, a large number of structural and immune cells express ST2.

Due to this, IL-33 plays an important and diverse role in inflammation and wound healing.

Characterised by IL-4, IL-5 and IL-13 release from ILC2s and T-helper 2 (T_H2) CD4⁺ T cells, type 2 inflammation can occur following exposure to allergens and parasites

(Humphreys et al., 2008, Hepworth et al., 2012, Le et al., 2012, Lambrecht and Hammad, 2015, Maizels et al., 2018). Due to its ability to drive T_H2 cell polarisation (Rank et al., 2009, Besnard et al., 2011), generate ILC2s (Vivier et al., 2018, Frisbee et al., 2019), activate mast cells (Lunderius Andersson et al., 2012, Saluja et al., 2015) and stimulate type 2 cytokine release by ILC2 and T_H2 cells (Lefrançois et al., 2014, Peine et al., 2016, Alvarez et al., 2019, Frisbee et al., 2019), IL-33 plays a key role in the initiation of type 2 responses (Fig. 1.9).

In addition to initiating type 2 responses, IL-33 supports $IFN\gamma$ and $TNF\alpha$ dominated type 1 responses (Fig. 1.9). Indeed, following bacterial and viral infection, IL-33 augments $IFN\gamma$ and $TNF\alpha$ release by T-helper 1 (T_H1) $CD4^+$ T cells, $CD8^+$ T cells and natural killer (NK) cells (Smithgall et al., 2008, Bourgeois et al., 2009, Bonilla et al., 2012, Kearley et al., 2015, Reichenbach et al., 2015).

Recently, $ST2^+$ regulatory T cells (Tregs) were identified in primary and secondary lymphoid organs (Turnquist et al., 2011). Subsequently found in gut, adipose and lung tissue, these cells proliferate in response to IL-33 and suppress inflammatory responses (Schiering et al., 2014, Arpaia et al., 2015, Kolodin et al., 2015, Molofsky et al., 2015b, Vasanthakumar et al., 2015, Matta et al., 2016, Siede et al., 2016). Consequently, IL-33 is proposed to play an important role in both the initiation and resolution of inflammation (Fig. 1.9) (Molofsky et al., 2015a, Cayrol and Girard, 2018).

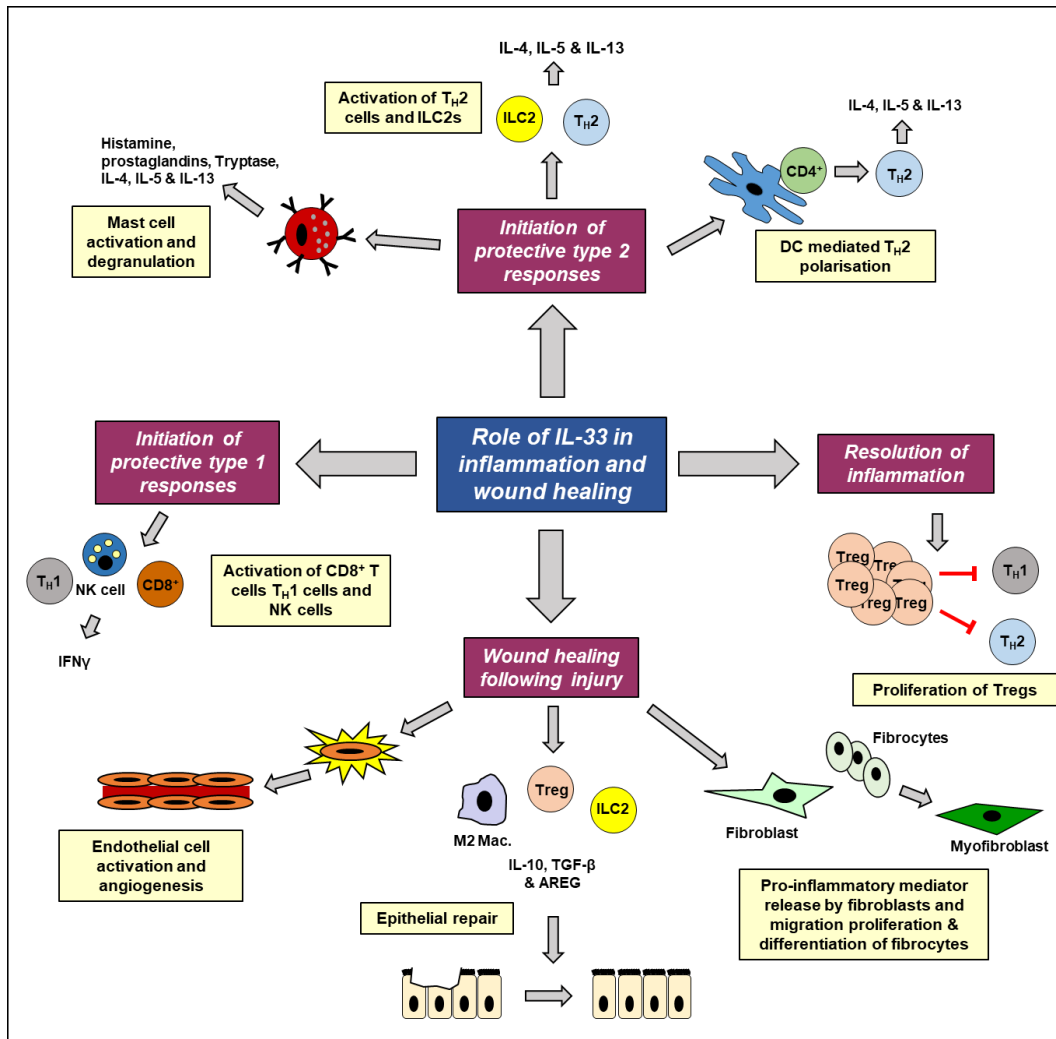


Figure 1.9: The role of IL-33 in inflammation and wound healing.

IL-33 has multiple roles during inflammation and is involved in the initiation of type 2 responses to allergens and parasites by promoting dendritic cell (DC)-mediated T_H2 cell polarisation, driving ILC2 generation, activating mast cells and stimulating the secretion of type 2 cytokines (IL-4, IL-5 and IL-13) by T_H2 cell and ILC2s. Additionally, following bacterial and viral infection, IL-33 stimulates $IFN\gamma$ and $TNF\alpha$ release from T_H1 cells, NK cells and $CD8^+$ T cells, augmenting type 1 inflammation. Since IL-33 drives the proliferation of immunosuppressive $ST2^+$ Tregs, IL-33 plays an important role in regulating the immune responses it helps to initiate. Following injury IL-33 activates endothelial cells, drives M2 macrophage polarisation, activates fibroblasts/fibrocytes and stimulates the secretion of IL-10, $TGF\beta$ and amphiregulin (AREG) by M2 macrophages, Tregs and ILC2s. As these events promote epithelial cell repair, ECM deposition and the formation of new blood vessels, IL-33 is considered to play a key role in wound healing. Figure adapted from (Liew et al., 2016).

The delay in wound healing observed in IL-33 and $ST2$ knockout mice infers that IL-33 is required for the rapid restoration of tissue homeostasis following injury (Lee et al., 2016, Rak et al., 2016, Dagher et al., 2020). Indeed, by promoting M2 macrophage polarisation (Kurowska-Stolarska et al., 2009, Li et al., 2014b, Fanny et al., 2018,

Dagher et al., 2020), increasing amphiregulin production by Tregs and ILC2s (Arpaia et al., 2015, Monticelli et al., 2015) and activating endothelial cells (Pollheimer et al., 2013), IL-33 has been shown to stimulate the repair of epithelial cells and blood vessels. Additionally, IL-33 increases the release of pro-inflammatory mediators by fibroblasts (Kurokawa et al., 2011, Zhu and Carver, 2012) and promotes the migration, proliferation and differentiation of fibrocytes into ECM-secreting myofibroblasts (Bianchetti et al., 2012, Hayashi et al., 2014). Taken together, these observations suggest that IL-33 plays an important role in wound healing (Fig. 1.9).

1.2.9. The regulation of IL-33 activity

Given the importance of IL-33 in many processes, multiple mechanisms controlling the expression, biological activity and signalling of this cytokine are required to maintain tissue homeostasis.

1.2.9.1. The regulation of IL-33 gene, mRNA and protein expression

At the gene level, several SNPs have been identified in the first intron and promoter region of IL-33 (Sakashita et al., 2008, Gudbjartsson et al., 2009, Moffatt et al., 2010, Torgerson et al., 2011, Ramasamy et al., 2012, Bønnelykke et al., 2014, Li et al., 2014a). Given their close proximity to the transcription start site, it is likely that these SNPs regulate IL-33 gene expression. Indeed, although one rare SNP is associated with decreased susceptibility to asthma (Smith et al., 2017), individuals with SNPs in their IL-33 gene are at greater risk of developing diseases where IL-33 levels are raised (Sakashita et al., 2008, Gudbjartsson et al., 2009, Prefontaine et al., 2009, Moffatt et al., 2010, Torgerson et al., 2011, Ramasamy et al., 2012, Tang et al., 2013, Bønnelykke et al., 2014, Li et al., 2014a).

At the mRNA level, alternative splicing regulates the biological activity of IL-33 (see section 1.2.6.3). Moreover, multiple studies have demonstrated that IL-33 mRNA is degraded shortly after synthesis decreasing the expression of this cytokine (Meephansan et al., 2012, Polumuri et al., 2012, Meephansan et al., 2013, Seltsmann et al., 2013).

Finally, at the protein level, IL-33 expression is altered by multiple cytokines (see section 1.2.3). Furthermore, ubiquitination and proteasomal degradation has been shown to decrease IL-33 protein expression (Tao et al., 2014, Clerman et al., 2017).

1.2.9.2. Mechanisms regulating the biological activity of IL-33

As detailed in the following sections, several intracellular and extracellular mechanisms have been shown to regulate the biological activity of IL-33.

1.2.9.2.1 Intracellular mechanisms

Intracellularly, nuclear sequestration/localisation appears to play a key role in regulating the biological activity of IL-33, with chromatin association shown to reduce necrosis-induced IL-33 release *in vitro* (Travers et al., 2018). In mice, deletion of the N-terminal nuclear domain results in constitutive IL-33 release, ST2-dependent inflammation and death 3 months after birth (Bessa et al., 2014). Moreover, mIL-33 rather than fIL-33 drives cutaneous ST2-dependent inflammation *in vivo* (Kurow et al., 2017). Taken together with the highly conserved nature of the N-terminal domain (Carriere et al., 2007), these *in vitro* and *in vivo* observations demonstrate the importance of nuclear sequestration/localisation in regulating the biological activity of IL-33.

In addition to nuclear sequestration/localisation, caspase mediated cleavage regulates the biological activity of IL-33. Indeed, following treatment with apoptotic stimuli, caspase-1, -3 and -7 cleave intracellular IL-33 at Asp178 rendering it inactive (Cayrol and Girard, 2009, Lüthi et al., 2009, Ali et al., 2010). Interestingly, IL-33-dependent inflammation was increased in the lungs of caspase-1 and caspase-7 deficient mice following allergen challenge (Kim et al., 2015, Madouri et al., 2015). Considered with earlier *in vitro* observations, this result suggests that caspases play a crucial role in regulating the biological activity of IL-33 during apoptosis.

1.2.9.2.2 Extracellular mechanisms

Following its release, the biological activity of extracellular IL-33 is regulated. Despite some proteases increasing the ST2-dependent activity of IL-33 *in vitro* (see section 1.2.6.2), certain neutrophil and mast cell derived proteases can cleave IL-33 rendering it inactive. For instance, mast cell chymase degrades IL-33 *in vivo* (Waern et al., 2013) whilst proteinase 3 prevents IL-33-induced CXCL8 and TNF α release from mast cells and monocytes respectively (Bae et al., 2012).

In addition to cleavage by inflammatory proteases, cysteine oxidation prevents IL-33 from inducing ST2-dependent responses *in vitro* and is proposed to limit IL-33 driven inflammation *in vivo* (see section 1.2.6.4 for further details).

1.2.9.3. The regulation of IL-33 signalling

The biological activity of IL-33 can be modulated by regulating ST2-dependent signalling. This regulation has been shown to occur at the level of both the receptor and ligand.

At the receptor level, SNPs in the *IL1RL1* gene have been shown to increase ST2 expression *in vitro* and *in vivo* and enhance ST2-dependent signalling (Ho et al., 2013, Gordon et al., 2016a). Moreover, inflammatory cytokines (including IFN γ , TNF α , IL-1 β and IL-4) and serum derived factors have been shown to upregulate ST2 expression *in vitro* (Kumar et al., 1997, Yanagisawa et al., 1993, Nishida et al., 2010). In contrast, IL-33 decreases the expression of its own receptor, inducing the phosphorylation, polyubiquitination and proteasomal degradation of ST2 *in vitro* (Zhao et al., 2012). Additionally, the expression of single immunoglobulin domain IL-1 receptor-related molecule (SIGIRR) by cells limits the ST2-dependent activity of IL-33 with SIGIRR-ST2 dimerization demonstrated to prevent IL-1RAcP recruitment by ST2 (Bulek et al., 2009). Finally, exosomes from the helminth *Heligmosomoides polygyrus* have been shown to decrease ST2 expression by epithelial cells and ILCs (Buck et al., 2014). Since these exosomes were found to contain several microRNAs, small non-coding RNAs have been proposed to regulate ST2-dependent signalling (Buck et al., 2014).

At the ligand level, extracellular IL-33 binds to a soluble isoform of ST2. With its expression and release increased following stimulation with inflammatory stimuli (Mildner et al., 2010), soluble ST2 (sST2) acts as a decoy receptor sequestering extracellular IL-33 and preventing it from interacting with its transmembrane receptor (Sanada et al., 2007, Mildner et al., 2010, Chen et al., 2015, Scott et al., 2018b). Although encoded by the same gene as ST2, alternative splicing results in the generation of sST2 (Yanagisawa et al., 1993, Bergers et al., 1994). Lacking the transmembrane domain possessed by the ST2 receptor (ST2L), sST2 is the predominant ST2 isoform expressed by cells (Kumar et al., 1997). Acting as a surrogate readout of IL-33 levels (Azazi et al., 2014), high concentrations of sST2 have

been observed in several inflammatory diseases with positive correlations reported between serum sST2 levels and disease severity (Hayakawa et al., 2007, Bandara et al., 2015). Indeed, in addition to being used to monitor responses to various therapies (Díaz-Jiménez et al., 2016), sST2 acts as a predictive biomarker in heart failure, chronic kidney disease (CKD) and asthma exacerbations (Pascual-Figal et al., 2009, de Boer et al., 2011, Ky et al., 2011, Bayes-Genis et al., 2012, Broch et al., 2012, Bayes-Genis et al., 2014, Plawecki et al., 2018, Watanabe et al., 2018, Alam et al., 2019). Following exogenous instillation, sST2 significantly reduces IL-33 driven inflammation *in vivo* (Löhning et al., 1998, Oshikawa et al., 2002a, Oshikawa et al., 2002b, Hayakawa et al., 2007, Sanada et al., 2007, Teufelberger et al., 2018). As a result, sST2 is proposed to act as a major regulator of IL-33 signalling (Felix and Savvides, 2017, Scott et al., 2018b).

Given the numerous and diverse range of regulatory strategies employed to harness the activity of IL-33, it is unsurprising that this cytokine has been shown to play a pathophysiological role in several diseases.

1.2.10. IL-33 in inflammatory diseases, infectious diseases and cancer

Since its discovery, IL-33 has been implicated in the pathogenesis of a number of diseases. Indeed, due to its stimulatory effects on multiple immune cells (see section 1.2.8), IL-33 has been linked to the development of several inflammatory conditions (Liew et al., 2010, De la Fuente et al., 2015). Although the cellular and molecular mechanisms underlying this involvement are beyond the scope of this thesis, key observations regarding the pathophysiological role of IL-33 in inflammatory bowel

disease (IBD), chronic obstructive pulmonary disease (COPD), cardiovascular disease, musculoskeletal disorders and allergic conditions are summarised in Table 1.4.

Table 1.4: The pathophysiological role of IL-33 in the development of inflammatory diseases.

Disease type	Condition	Evidence IL-33 is involved and its role in pathogenesis
Allergic	Asthma	• Positive correlation between IL-33 levels and asthma severity (Prefontaine et al., 2009) • Individuals with SNPs in IL-33 and/or <i>IL1RL1</i> (ST2) gene are at higher risk of developing asthma (Moffatt et al., 2010, Torgerson et al., 2011) • IL-33 shown to act as an alarmin in murine models of airway inflammation activating ILC2s and promoting DC mediated T_H2 cell polarisation. T_H2 cells and ILC2s produce Th2 cytokines (IL-4, IL-5 and IL-13) in response to IL-33 driving M2 macrophage polarisation and airway eosinophilia (Kurowska-Stolarska et al., 2009, Stolarski et al., 2010, Bartemes et al., 2012)
	Atopic Dermatitis (AD)	• IL-33 elevated in skin biopsies from AD patients (Savinko et al., 2012) • Overexpression of <i>IL33</i> by keratinocytes results in dermatitis (Imai et al., 2013) • Similar cellular mechanism to asthma proposed (Salimi et al., 2013)
	Allergic rhinitis	• SNPs in IL-33 gene associated with increased susceptibility to allergic rhinitis (Sakashita et al., 2008) • IL-33 knockout mice protected from allergic rhinitis (Haenuki et al., 2012) • Similar cellular mechanism to asthma and AD proposed (Durham et al., 1992)
Cardiovascular	Myocardial infarction (MI)	• sST2 (surrogate biomarker of IL-33 levels) increased after MI with a negative correlation between sST2 levels, left ventricular function and prognosis (Shimpo et al., 2004) • Cellular mechanism(s) not yet clear
Musculoskeletal	Rheumatoid arthritis (RA)	• IL-33 and sST2 significantly elevated in synovium of RA patients with levels indicative of outcome (Tang et al., 2013) • rs7044343 SNP in IL-33 gene associated with decreased levels of IL-33 in the serum and reduced risk of RA (Li et al., 2014a) • sST2 administration, ST2 gene deletion or the use of an anti-IL-33 antibody decreased RA severity <i>in vivo</i>. In contrast, IL-33 exacerbates RA in murine models (Leung et al., 2004, Xu et al., 2008, Palmer et al., 2009) • IL-33-induced type 2 cytokine secretion by T_H2 cells may activate a subset of innate B cells (B1 cells) capable of producing autoantibodies synonymous with the pathogenesis of RA • IL-33 may also increase the activation of mast cells, the recruitment of neutrophils into the joints and the release of pro-inflammatory cytokines (Komai-Koma et al., 2011)

	Tendinopathy	IL-33 reduces miRNA29a expression in stressed tendon cells increasing their production of type 3 collagen. This results in tendon remodelling and early tendon failure (Millar et al., 2015)
Other	COPD	- IL-33 expression is increased in the lungs of COPD patients (Byers et al., 2013) - IL-33 is released from airway epithelial cells following viral or bacterial infection. Released IL-33 synergises with IL-12 increasing IFNγ release by NK cells exacerbating COPD (Kearley et al., 2015)
	IBD (Ulcerative colitis and Crohn's disease)	- IL-33 increased in ulcerative colitis and Crohn's disease with levels correlated with disease severity (Pastorelli et al., 2010, Sedhom et al., 2013) - IL-33 and ST2 knockout animals protected from dextran sodium sulphate induced colitis (Oboki et al., 2010, Sedhom et al., 2013) - IL-33 shown to act as an alarmin in the gut resulting in type 2 inflammation in a similar manner to that seen in allergic asthma (Seo et al., 2017)

In addition to its role in inflammatory diseases, IL-33 expression is increased in patients with myeloproliferative neoplasms and colorectal cancer (Mager et al., 2015, Maywald et al., 2015). As IL-33 promotes tumorigenesis in multiple animal models (Maywald et al., 2015), these observations suggest that IL-33 contributes to the development of certain cancers. Furthermore, despite IL-33 signalling playing a largely protective role during fungal and protozoa infections (Jones et al., 2010, Nelson et al., 2011, Le et al., 2012, Besnard et al., 2015), ST2 knockout mice were found to have lower fungal and parasitic loads (compared to wild-type control animals) following infection with *Cryptococcus neoformans* and *Leishmania donovani* (Flaczyk et al., 2013, Piehler et al., 2013, Rostan et al., 2013). Due to this, IL-33 is considered to play a complex role in infectious disease. Finally, despite its initial positive contribution to wound healing (see section 1.2.8), excessive and uncontrolled activation of the IL-33:ST2 axis may lead to fibrosis (Kotsiou et al., 2018).

1.2.11. IL-33 in organ fibrosis

Despite being linked to pancreatic (Dib et al., 2002, Masamune et al., 2010, Nishida et al., 2010) and intestinal fibrosis (Masterson et al., 2015, Eissmann et al., 2018) the strongest evidence implicating IL-33 in organ fibrosis comes from the liver, kidneys and skin.

1.2.11.1 IL-33 in liver fibrosis

During liver fibrosis, hepatic stellate cells (HSCs) transdifferentiate into α SMA⁺ fibroblasts/myofibroblasts and secrete ECM (Friedman, 2010, Tan et al., 2018). Interestingly, IL-33 treatment increases α SMA expression and collagen secretion by HSCs *in vitro* (Tan et al., 2018). *In vivo*, IL-33 overexpression results in the spontaneous development of hepatic fibrosis. Moreover, IL-33 knockout mice are protected from carbon tetrachloride-induced liver fibrosis (McHedlidze et al., 2013). As IL-33 mRNA and protein expression is increased in fibrotic livers and positively correlates with disease severity (Marvie et al., 2010, Li et al., 2014c, Sun et al., 2014, Sun et al., 2017), these *in vitro* and *in vivo* results collectively suggest that IL-33 plays a key pathophysiological role in liver fibrosis.

1.2.11.2 IL-33 in renal fibrosis

IL-33 levels are increased in the unilateral urinary obstruction (UUO) and ischemia-reperfusion injury (IRI) models of renal (kidney) fibrosis (Chen et al., 2016, Li et al., 2019). Furthermore, IL-33 and ST2 knockout mice are protected from UUO- and IRI-induced fibrosis respectively (Chen et al., 2016, Ferhat et al., 2018, Liu et al., 2019b). Importantly, IL-33 drives tubular epithelial cell EMT in a dose dependent fashion (Xu et al., 2018). Since this process is key in the pathogenesis of renal fibrosis (Chen et al.,

2016), these *in vitro* and *in vivo* observations demonstrate the importance of IL-33 during the development of renal fibrosis.

1.2.11.3 IL-33 in skin fibrosis

In addition to being increased in fibrotic skin biopsies from systemic sclerosis (SSc) patients (Manetti et al., 2010), IL-33 is elevated in the serum of SSc patients with a significant positive correlation observed between serum IL-33 levels and fibrotic skin score (Yanaba et al., 2011). As ST2 knockout mice are protected from IL-33-induced skin fibrosis (Rankin et al., 2010), these results collectively suggest that IL-33 plays an important pro-fibrotic role in the skin. Combined with evidence from the liver and kidneys, these findings infer that IL-33 may promote fibrosis in other organs such as the lungs. Indeed, IL-33 has been implicated in the development of IPF.

1.2.12. IL-33 in IPF

IL-33 levels are significantly increased in the exhaled breath condensate (EBC), BALF and lung tissue homogenates of IPF patients (Luzina et al., 2012, Lee et al., 2017, Majewski et al., 2019b) with more IL-33 positive cells visible in IPF lung sections versus non-IPF control tissue (Luzina et al., 2012, Luzina et al., 2013). Since IPF patients have higher serum concentrations of sST2 (a surrogate biomarker of IL-33 levels) during AEs (Tajima et al., 2003), IL-33 has been proposed to play an important pathophysiological role during the development of IPF.

In addition to data from other organs (see section 1.2.11), evidence to support this hypothesis comes primarily from the bleomycin (BLM) mouse model of pulmonary fibrosis. Indeed, following BLM exposure, IL-33 mRNA and protein expression is increased in lung homogenates with more IL-33 positive cells observed in tissue

sections from BLM-treated mice (Luzina et al., 2013, Li et al., 2014b, Xu et al., 2016a). Importantly, BLM-induced fibrosis is reduced in ST2 knockout mice (Li et al., 2014b, Fanny et al., 2018, Zhao et al., 2018). Moreover, prophylactic dosing with IL-33 blocking reagents (such as lentivirus expressed sST2 and IL-33 neutralising antibodies) attenuates BLM-driven collagen deposition in wildtype animals (Li et al., 2014b, Gao et al., 2016). As IL-33 overexpression and treatment potentiates BLM-induced fibrosis (Luzina et al., 2013, Li et al., 2014b), these *in vivo* observations suggest that IL-33 may act as an important pro-fibrotic mediator during the development of IPF.

Despite being implicated in the pathogenesis of IPF, the cellular and molecular mechanisms by which IL-33 exerts its potential pro-fibrotic effects remain poorly understood. Although IL-33 increases pro-inflammatory gene and protein expression by rat cardiac and murine lung fibroblasts (Kurokawa et al., 2011, Zhu and Carver, 2012), it is unknown whether IL-33 has direct effects on the pro-fibrotic activity of human lung fibroblasts (HLFs). Furthermore, as all previous *in vivo* studies (Li et al., 2014b, Gao et al., 2016, Fanny et al., 2018, Zhao et al., 2018) block IL-33 signalling during the inflammatory rather than the fibrotic phase of the BLM mouse model, the true pro-fibrotic activity of IL-33 in this model is yet to be determined.

1.3. Aims and Hypothesis

The overall aim of this thesis was to investigate the role of IL-33 in the development of IPF and test the hypothesis that IL-33 acts as a key pro-fibrotic mediator in the lungs. To do this, the initial aims of this piece of work were to:

- Investigate the effects of IL-33 on the pro-fibrotic activity of primary HLFs
- Determine the true pro-fibrotic activity of IL-33 in the BLM mouse model of pulmonary fibrosis

Chapter 2: Materials and Methods

2. Materials and Methods

The general methods used throughout this thesis are outlined below with chapter-specific methods detailed in relevant chapters. All of the materials, kits, antibodies, primers, media and buffer recipes used in this thesis are listed in the appendix.

2.1. Cell culture

2.1.1. Primary human lung fibroblasts (HLFs)

Cryopreserved non-IPF and IPF HLFs were obtained from the Nottingham Respiratory Research Unit. Upon receipt, cells were thawed and seeded into T150 flasks at a density of 5×10^5 cells/flask. Cells were cultured in serum supplemented Dulbecco's Modified Eagle's Medium (DMEM) (see appendix) and maintained at $37^\circ\text{C} + 5\% \text{CO}_2$. For the majority of experiments using HLFs, cells were seeded at a density of 4×10^4 cells/well into 6 well plates and cultured at $37^\circ\text{C} + 5\% \text{CO}_2$ (see chapter-specific methods for any assay-specific changes in seeding densities and plate formats). Once 100% confluent, cells were serum starved for 24 hours prior to stimulation in serum free DMEM (see appendix). For all experiments, HLFs were used at passage 6 and confirmed as mycoplasma negative.

2.1.2. Human umbilical vein endothelial cells (HUVECs)

Cryopreserved HUVECs were purchased from PromoCell® and seeded into T75 flasks at a density of 5×10^5 cells/flask. Cells were cultured in supplemented Endothelial Cell Growth Medium (see appendix) and maintained at $37^\circ\text{C} + 5\% \text{CO}_2$. To assess changes

in gene expression, HUVECs were seeded at a density of 4×10^4 cells/well into 6 well plates and cultured at $37^\circ\text{C} + 5\% \text{CO}_2$ until 90% confluent. To measure changes in cytokine release, HUVECs were seeded at a density of 1×10^4 cells/well into 96 well plates and cultured at $37^\circ\text{C} + 5\% \text{CO}_2$ until 90% confluent. For all experiments, HUVECs were used at passage 3 and confirmed as mycoplasma negative.

2.1.3. A549 cells

Cryopreserved A549 cells were purchased from ATCC and expanded by the Core Tissue Culture Group at AstraZeneca Cambridge. Upon request, mycoplasma negative cells were received in serum supplemented RPMI (see appendix) and cultured (at $37^\circ\text{C} + 5\% \text{CO}_2$) in T175 flasks until confluent. Unless stated otherwise, cells were seeded at a density of 1×10^6 cells/well into 6 well plates and cultured at $37^\circ\text{C} + 5\% \text{CO}_2$. Once 100% confluent, cells were serum starved for 24 hours prior to stimulation in serum free RPMI (see appendix). For all experiments, cells were used at passage 4-7.

2.2. Cell counting using a haemocytometer

For *in vitro* experiments and total bronchoalveolar lavage (BAL) cell counts, cells were counted using an Improved Neubauer haemocytometer and a light microscope. Specifically, a small sample of cell suspension/BAL was diluted 1:1 in trypan blue and 10 μl of this mixture added to the haemocytometer. Cells were visualised at x20 magnification and the number of viable (trypan blue-negative) cells in all 4 corner squares counted (Fig. 2.1A). Using this number, the number of cells per ml of cell suspension/BAL was calculated using the equation in Figure 2.1B. Finally, total

cell/BAL cell count was determined by multiplying the number of cells per ml by the total volume of the cell suspension/BAL (Fig. 2.1C).

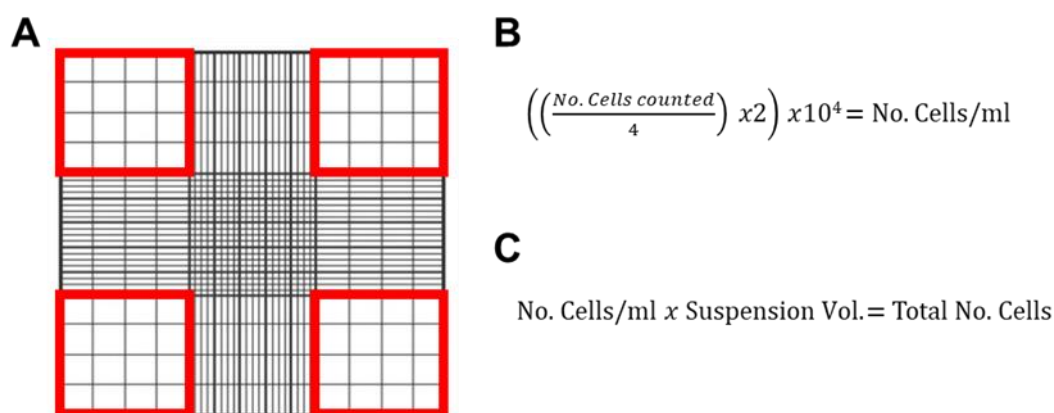


Figure 2.1: Cell counting using a haemocytometer.

Grid layout of Improved Neubauer haemocytometer with all 4 corner squares highlighted in red (**A**) Image taken from Abcam (2020). Equations used to calculate the number of cells per ml (**B**) and the total number of cells (**C**).

2.3. RNA extraction

Depending on sample type, three different methods of RNA extraction were used. The principles and methods of each are detailed below.

2.3.1. NucleoSpin® RNA extraction

2.3.1.1. Principle

The NucleoSpin® RNA extraction kit uses silica membrane technology to isolate RNA from cell lysates (Macherey-Nagel, 2019).

2.3.1.2. Method

For *in vitro* experiments conducted with HLFs and HUVECs, RNA was isolated using the NucleoSpin® RNA extraction kit according to the manufacturer's instructions. Briefly, cells were washed with sterile PBS and lysed in 350 µl of RA1 lysis buffer

supplemented with 10 µl/ml of β-mercaptoethanol (βME). Following storage at -80°C, thawed samples were transferred into homogenisation columns and centrifuged at 15,000 x g for 1 minute. Upon completion, the resulting flow through was mixed with 350 µl of 70% ethanol and all samples transferred into NucleoSpin® filter columns. Following centrifugation at 15,000 x g for 30 seconds, 350 µl of membrane desalting buffer was added and all filter columns centrifuged at 15,000 x g for 1 minute. After combining 10 µl of rDNase with 90 µl of rDNase reaction buffer, 95 µl of diluted rDNase was added to each sample. Following 15 minutes at room temperature, 200 µl of RAW2 buffer was added and all samples centrifuged at 15,000 x g for 30 seconds. 600 µl of RA3 buffer was subsequently added and each filter column centrifuged at 15,000 x g for 30 seconds. After discarding all flow through, a further 250 µl of RA3 buffer was added and all samples centrifuged at 15,000 x g for 2 minutes. Upon completion, filter columns were transferred into 1.5 ml RNase free Eppendorf tubes and 40 µl of nuclease free water added. Finally, RNA was eluted by centrifuging all samples at 15,000 x g for 1 minute and stored at -80°C for future use.

2.3.2. TRIzol RNA extraction

2.3.2.1. Principle

Tissue/cells are lysed in TRIzol and chloroform added. Centrifugation results in the phase separation of proteins, DNA and RNA. Following collection of the top aqueous phase, RNA is precipitated using isopropyl alcohol and resuspended (after centrifugation) in nuclease free water (Chomczynski, 1993).

2.3.2.2. Method

Ground lung tissue was transferred into 1.5 ml Eppendorf tubes and lysed in 1 ml of TRIzol. After vortexing for approximately 20 seconds, samples were incubated at room temperature for 10 minutes. Phase separation was achieved by the addition of 200 µl of chloroform and centrifugation at 13,000 x g for 20 minutes at 4°C. Following separation, the aqueous phase was transferred into a new 1.5 ml Eppendorf tube and 500 µl of 100% isopropyl alcohol added. RNA was precipitated at room temperature for 10 minutes and pelleted by centrifugation at 13,000 x g for 10 minutes at 4°C. After discarding all supernatant, the resulting RNA pellet was resuspended in 1ml of 75% ethanol and incubated at -80°C for 1 hour. Following centrifugation at 13,000 x g for 5 minutes at 4°C, all supernatant was removed and the RNA pellet air dried at room temperature for 30 minutes. Once dry, 25 µl of nuclease free water was added and the resulting RNA stored at -80°C for future use.

2.3.3. Direct-Zol RNA extraction

2.3.3.1. Principle

The Direct-Zol™ RNA extraction kit uses silica membrane technology to isolate RNA from TRI Reagent® treated samples. This method is quicker and easier than the TRIzol RNA extraction method and is better suited to extracting RNA from tissue than the NucleoSpin® RNA extraction kit (Zymo-Research, 2017).

2.3.3.2. Method

For *ex vivo* experiments conducted with human precision cut lung slices (PCLS), RNA was extracted using the Direct-zol™ RNA Miniprep kit according to the manufacturer's instructions. Briefly, RNAlater®-treated PCLS samples were thawed

on ice before being washed with 1 ml of PBS to remove any residual RNAlater[®]. Samples were subsequently transferred into 1.2 ml collection tubes containing a single 5 mm stainless steel bead before 600µl of TRI Reagent[®] was added to each tube. Using a Tissue Lyser II, all slices were homogenised at 20Hz for a total of 6 minutes. After transferring into 1.5 ml Eppendorf tubes, all samples were centrifuged at 16,000 x g for 30 seconds and the resulting supernatants transferred into fresh 1.5 ml Eppendorf tubes. 600 µl of 100% ethanol was subsequently added and all samples thoroughly vortexed. The resulting mixtures were transferred into Zymo-Spin[™] IICR columns (spin columns) prior to centrifugation at 16,000 x g. After spinning for 30 seconds, spin columns were transferred into fresh 2 ml collection tubes and 400 µl of RNA wash buffer added. Following centrifugation at 16,000 x g for 30 seconds, DNA was digested by adding 80 µl of diluted DNase I (30 U of DNase I diluted in DNA Digestion buffer) to each spin column matrix. After 15 minutes at room temperature, 400 µl of Direct-zol[™] RNA PreWash was added to each spin column and samples centrifuged at 16,000 x g for 30 seconds. After discarding all flow through, samples were washed again with 400 µl of Direct-zol[™] RNA PreWash and all spin columns transferred into fresh 2 ml collection tubes. 700 µl of RNA Wash Buffer was then added and all samples centrifuged at 16,000 x g for 2 minutes. Upon completion, spin columns were transferred into 1.5 ml RNase free Eppendorf tubes and 25 µl of nuclease free water added. Finally, RNA was eluted by centrifuging all samples at 16,000 x g for 30 seconds and stored at -80°C for future use.

2.4. Determination of RNA concentration

Prior to reverse transcription, the concentration and purity of extracted RNA was determined using a NanoDrop™ spectrophotometer. To quantify RNA samples at the University of Nottingham a NanoDrop™ 2000 spectrophotometer was used. To quantify RNA samples at AstraZeneca a NanoDrop™ 1000 spectrophotometer was used.

Irrespective of the NanoDrop™ spectrophotometer used, 2 µl of nuclease free water was used to calibrate the machine. 2 µl of neat RNA sample was then loaded onto the spectrophotometer and absorbance at 260 and 280 nm measured. RNA concentration was calculated using the following equation:

$$\text{RNA } (\mu\text{g/ml}) = \text{OD at 260 nm} \times 38 \text{ (extinction coefficient of RNA)} \times \text{DF}$$

OD = optical density, DF = dilution factor

RNA with a 260/280 ratio of approximately 1.8-2.1 and a 230/260 of approximately 2-2.2 was deemed to be of suitable purity for analysis via quantitative real-time PCR (qPCR).

2.5. Reverse transcription

2.5.1. Principle

Reverse transcription is the process by which complementary DNA (cDNA) is synthesised from messenger RNA (mRNA) using an RNA-dependent DNA polymerase. The resulting cDNA can be analysed via qPCR (Martín-Alonso et al.).

2.5.2. Methods

To convert RNA into cDNA, three different methods were employed. Unless stated otherwise, M-MLV reverse transcriptase was used to reverse transcribe RNA derived from *in vitro* cell culture experiments. For RNA isolated from murine lung tissue, SuperScript® IV Reverse Transcriptase was used. Finally, for RNA derived from human PCLS and certain *in vitro* cell culture experiments, a High-Capacity RNA-to-cDNA™ kit was used.

2.5.2.1. M-MLV Reverse transcriptase

500 ng of RNA was reverse transcribed into cDNA using M-MLV reverse transcriptase according to the manufacturer's instructions. Briefly, 15 µl of mastermix containing RT buffer, 200 U of M-MLV reverse transcriptase, 25 U of RNAsin, 0.5 µg of oligo (DT) primers and 2 mM of each deoxyribonucleotide triphosphate (dNTP) was added to 10 µl of diluted RNA. After (brief) vortexing to mix, the 25 µl reaction mixtures were loaded into a ProFlex™ PCR system and heated to 42°C for 90 minutes. After 10 minutes at 95°C the resulting cDNA samples were stored at -20°C for future use.

2.5.2.2. SuperScript® IV Reverse Transcriptase

500 ng of RNA was reverse transcribed into cDNA using SuperScript® IV Reverse Transcriptase according to the manufacturer's instructions. Briefly, 50 µM of oligo (DT) primers, 10 mM of dNTP mix and 500 ng of RNA were combined with nuclease free water in a 13 µl reaction volume. After (brief) vortexing to mix, samples were loaded into a ProFlex™ PCR system, heated at 65°C for 5 minutes and cooled to 4°C for 1 minute. A master mix containing SuperScript® IV buffer, 25 U of RNAsin, 100

nM of DTT and 200 U of SuperScript® IV Reverse Transcriptase was then made and 7 µl (of mastermix) added to each sample to give a final reaction volume of 20 µl. Following 10 minutes at 55°C and a further 10 minutes at 80°C, the resulting cDNA samples were stored at -20°C for future use.

2.5.2.3. High-Capacity RNA-to-cDNA™ kit

200 ng of RNA was reverse transcribed into cDNA using a High-Capacity RNA-to-cDNA™ kit according to the manufacturer's instructions. Briefly, 11 µl of mastermix containing RT buffer mix and RT enzyme was added to 9 µl of diluted RNA. After (brief) vortexing to mix, the resulting 20 µl reaction mixtures were loaded into a GeneAmp® PCR System 9700 and incubated at 37°C for 60 minutes. Following 5 minutes at 95°C, the resulting cDNA samples were stored at -20°C for future use.

2.6. Quantitative real-time PCR (qPCR)

In quantitative real-time PCR (qPCR), amplification of a specific (c)DNA molecule is measured in real-time (i.e. amplification is measured during the PCR reaction rather than at the end). Due to differences in the equipment available at the University of Nottingham and AstraZeneca, two qPCR methods were used to measure changes in gene expression. At the University of Nottingham qPCR SYBR® Green dye was used. At AstraZeneca TaqMan™ assays were used.

2.6.1. qPCR using SYBR[®] Green

2.6.1.1. Principle

During SYBR[®] Green qPCR, SYBR[®] Green dye binds to double stranded PCR products. At the end of each amplification cycle, fluorescence is measured meaning that DNA amplification is measured in real-time. In general, the intensity of the fluorescent signal recorded is proportional to the amount of PCR product present in the sample. However, as SYBR[®] Green dye binds to all double stranded DNA, melting curve analysis is required at the end of SYBR[®] Green qPCR to confirm that the fluorescent signal recorded is due to the amplification of a single DNA product rather than non-specific primer-DNA interactions and/or primer dimer formation (Tajadini et al., 2014).

2.6.1.2. Method

For each reaction, 200 nM of forward and reverse primer (see Table 9.1 in the appendix for all sequences), 10 µl of KAPA SYBR[®] FAST qPCR MasterMix and 8.2 µl of nuclease free water was added to 1 µl of cDNA. Samples were thoroughly mixed before being loaded into a Mx3000P[®] qPCR machine. After initial denaturation at 95°C for 30 seconds, 40 cycles of 95°C for 5 seconds, 60°C for 30 seconds and 72°C for 15 seconds were performed. The integration of SYBR[®] green into amplified DNA was measured after each 60°C annealing step with a dissociation curve (produced by heating all amplified samples for 1 minute at 95°C, 30 seconds at 60°C and 30 seconds at 95°C) generated at the end of the 40 cycles. All samples were run in duplicate with a no template control (NTC) used to assess contamination and primer-dimer formation. Amplification of a single DNA product was confirmed by melting curve analysis and

cycle threshold (Ct) values for the target and housekeeping gene determined using MxPro qPCR software.

2.6.2. qPCR using TaqMan™ probes

2.6.2.1. Principle

TaqMan™ assays consist of a pair of unlabelled primers and a TaqMan® MGB probe. The 5' end of each probe is labelled with a fluorogenic dye (e.g. FAM™ or VIC™) whilst the 3' end is labelled with a nonfluorescent quencher (NFQ). Prior to amplification, the close proximity of the dye and the NFQ means that the signal from the 5' dye is quenched. Following heat-induced cDNA denaturation, a reduction in temperature enables the primers and probes to anneal to their target sequences. Using the unlabelled primers and cDNA template, *Taq* DNA polymerase synthesises new polynucleotide strands. Upon reaching the DNA-bound TaqMan® probe, the polymerase uses its endogenous nuclease activity to cleave the 5' end of the probe. This separates the 5' dye from the 3' quencher resulting in a fluorescent signal. The intensity of the fluorescent signal recorded at the end of each amplification cycle is proportional to the amount of DNA present in the sample (Tajadini et al., 2014).

2.6.2.2. Method

For each reaction, 1 µl of TaqMan™ assay (consisting of 900 nM of unlabelled forward and reverse primer and 250 nM of 6-FAM™ dye-labelled TaqMan® MGB probe), 10 µl of TaqMan™ Fast Advanced Master Mix and 8 µl of nuclease free water was added to 1 µl of cDNA (see Table 9.2 in appendix for details of TaqMan™ assays used). Samples were thoroughly mixed before being loaded into a 7900HT Fast Real-Time PCR machine. After initial denaturation at 95°C for 20 seconds, 40 cycles of

95°C for 1 second and 60°C for 20 seconds were performed. Fluorescence due to DNA synthesis and 6-FAM™ dye release was measured after each 60°C annealing step. All samples were run in duplicate with a no template control (NTC) used to assess contamination. Finally, Ct values for the target and housekeeping gene were calculated using SDS 2.4 software.

2.6.3. Analysis of qPCR data

Irrespective of whether SYBR® Green or TaqMan™ assays were used, all qPCR data was analysed using the Delta-Delta Ct ($\Delta\Delta Ct$) method. Specifically, the Ct value for the housekeeping gene was subtracted from that of the gene of interest. The resulting ΔCt value for the untreated control (media alone/saline), was then subtracted from all other ΔCt values to give $\Delta\Delta Ct$ values for each treatment condition. The following formula was subsequently used to calculate the expression of the target gene relative to the untreated control:

$$\text{Fold Change (relative expression)} = 2^{-\Delta\Delta Ct}$$

2.7. Protein extraction

Depending on sample type, different protein extraction methods were used.

2.7.1. Protein extraction from cells

Following the collection of all supernatant, cells were washed once with ice-cold PBS. For assays performed using 6 well plates, 150 μl /well of cell lysis buffer (see appendix) was added. For assays performed using 15 cm dishes, 1 ml/dish of cell lysis buffer was added. To ensure complete lysis, cells were scraped on ice and the resulting lysates collected into 1.5 ml Eppendorf tubes. Following centrifugation at 14,000 x g for 10

minutes at 4°C, lysates were transferred into fresh 1.5 ml Eppendorf tubes and quantified via BCA assay (see section 2.8.2).

2.7.2. Protein extraction from murine lung tissue

Ground lung tissue was transferred into 7 ml Bijou tubes and 500 µl of ice-cold tissue lysis buffer (see appendix) added to each sample. After vortexing to mix, samples were incubated on ice for 30 minutes prior to storage overnight at -80°C. The next day, samples were thawed on ice before being transferred into 1.5 ml Eppendorf tubes and centrifuged at 14,000 x g for 10 minutes at 4°C. The resulting protein lysates were collected into fresh 1.5 ml Eppendorf tubes and quantified via BCA assay (see section 2.8.2).

2.7.3. Protein extraction from human lung tissue

Approximately 400 mg of human lung tissue (see chapter 5 section 5.3.1 for tissue details) was transferred into 7 ml Bijou tubes and 1 ml of ice-cold tissue lysis buffer (see appendix) added to each sample. Following overnight storage at -80°C, samples were thawed on ice and homogenised using an OMNI Tissue Homogenizer. After transferring into 1.5 ml Eppendorf tubes, all samples were centrifuged at 14,000 x g for 10 minutes at 4°C. The resulting lung lysates were collected into fresh 1.5 ml Eppendorf tubes and quantified via BCA assay (see section 2.8.2).

2.7.4. Protein extraction from human PCLS

Stored protein samples were thawed on ice prior to homogenisation using an OMNI Tissue Homogenizer. After transferring into 1.5 ml Eppendorf tubes, all samples were centrifuged at 14,000 x g for 10 minutes at 4°C and the resulting protein lysates collected into fresh 1.5 ml Eppendorf tubes. All PCLS protein lysates were quantified by BCA assay (see section 2.8.2).

2.8. Protein quantification

2.8.1. Principle

The Pierce™ BCA Protein Assay is a colorimetric assay where protein concentrations are extrapolated from a BSA standard curve (Smith et al., 1985).

2.8.2. Method

Protein concentration was determined using the Pierce™ BCA Protein Assay Kit according to the manufacturer's instructions. Briefly, a 7-point BSA standard curve was prepared and 10 µl/well of standard added to a 96 well plate in duplicate. After adding 10 µl/well of sample to the same plate in duplicate, reagent B was diluted 1:50 in reagent A. 190 µl/well of the resulting solution was added to each standard/sample and the plate incubated at 37°C + 5% CO₂ for 30 minutes. Absorbance at 562 nm was measured using a FLUOstar® Omega microplate reader or a Gen5 Cytation plate reader. The concentration of each sample was calculated from the BSA standard curve using MARS Data Analysis software or GraphPad Prism 8.

2.9. Western blotting

2.9.1. Principle

Western blotting is a semiquantitative technique used to detect and characterise specific proteins in cell/tissue homogenates. In this technique samples are reduced and denatured (by boiling in sodium dodecyl sulphate (SDS) containing, reducing agent (i.e. β ME) supplemented sample buffer) prior to separation by SDS polyacrylamide gel electrophoresis (SDS-PAGE). Under reduced and denatured conditions proteins are separated based on their molecular weight (not their native charge or confirmation). Once separated, proteins are transferred onto a polyvinylidene fluoride (PVDF) or nitrocellulose membrane using an electric current. After blocking with milk to prevent non-specific antibody binding, the membrane is probed with a primary antibody specific to the protein of interest. Using a horse radish peroxidase (HRP)-conjugated secondary antibody and enhanced chemiluminescence (ECL) substrate, primary antibody bound protein is visualised and expression relative to an appropriate loading control calculated (Mahmood and Yang, 2012).

2.9.2. Methods

Due to differences in the equipment available at the University of Nottingham and AstraZeneca, two western blot methods were used to assess protein expression.

2.9.2.1. Western blotting at the University of Nottingham

2.9.2.1.1. Sample preparation

To standardise the concentrations of all lysates, the appropriate volume of lysis buffer (based on results from the BCA assay and the concentration of total protein stated in

the relevant western blot figure legends) was added to each sample. After diluting 3:1 in laemmli buffer (see appendix) + 6% β ME, all samples were boiled at 100°C for 5 minutes and analysed by western blot.

2.9.2.1.2. SDS-PAGE

Gel casting apparatus was assembled according to the manufacturer's instructions and used to cast a 10% polyacrylamide resolving gel (see appendix). Once set, stacking gel (see appendix) was poured on top of the resolving gel and a 10 or 15 well comb inserted. After allowing the stacking gel to set at room temperature, the comb was removed and the resulting gel attached to the electrophoresis module of a Mini-PROTEAN® Tetra Electrophoresis Cell tank. After filling the electrophoresis module with running buffer (see appendix), 8 μ l of Rainbow™ Molecular Weight Marker was added to the first lane. Boiled protein samples were loaded into subsequent lanes and the tank filled with running buffer. Finally, the tank was connected to a Bio-Rad PowerPac™ and gels run at 150 volts for 70 minutes.

2.9.2.1.3. Protein transfer

Two pieces of filter paper and four sponges were soaked in transfer buffer (see appendix). Following SDS-PAGE, gels were removed from the electrophoresis module and submerged in transfer buffer. After 20 seconds in 100% methanol, activated PVDF membrane was washed with tap water and incubated in transfer buffer for 4 minutes. To transfer proteins, two pieces of sponge and one piece of filter paper were placed on the open transfer cassette. After laying the activated membrane on the filter paper, the gel was placed on top of the membrane and all bubbles removed. Once closed, the transfer cassette was inserted into a Criterion™ Blotter and samples transferred at 110 volts for 40 minutes on ice.

2.9.2.1.4. Protein detection

After the successful transfer of proteins, the resulting membrane was washed twice with TBST (see appendix) for 5 minutes. To prevent non-specific binding, the membrane was submerged in 5% blotto (freeze dried skimmed milk) in TBST and incubated at room temperature for 90 minutes. Following two 5-minute washes with TBST, primary antibody (see appendix for antibody clones and concentrations) diluted in 5% blotto in TBST was added to the membrane. After overnight incubation at 4°C, three 5-minute washes with TBST were performed and HRP-conjugated secondary antibody (see appendix for antibody clones and concentrations) applied in 5% blotto in TBST. Following 60 minutes at room temperature, five 5-minute washes with TBST were performed. To visualise the protein of interest, ECL™ detection reagent was prepared according to the manufacturer's instructions and added to the membrane for 1 minute. After removing excess ECL, the membrane was enclosed in saran wrap and developed in the dark using Hyperfilm ECL™. Using the Rainbow™ Marker, the molecular weights of the resulting bands were determined. To confirm equal protein loading, monoclonal antibodies against GAPDH or β -tubulin were used (see appendix for antibody clones and concentrations). Finally, densitometric analysis was performed using ImageJ and protein expression relative to loading control calculated.

2.9.2.2. Western blotting at AstraZeneca

2.9.2.2.1. Sample preparation

To standardise the concentrations of all lysates, the appropriate volume of lysis buffer (based on results from the BCA assay and the concentration of total protein stated in the relevant western blot figure legends) was added to each sample. After diluting 3:1

in Bolt™ LDS Sample Buffer + 10% βME, all samples were boiled at 95°C for 5 minutes and analysed by western blot.

2.9.2.2.2. SDS-PAGE

10%, 12% and 4-12% Bolt™ Bis-Tris Plus gels were purchased from Invitrogen and placed into the chamber of a Mini Gel Electrophoresis Tank. After securing the gels using cassette clamps, the electrophoresis tank was filled with Bolt™ MOPS SDS Running Buffer and 8 µl of Rainbow™ Molecular Weight Marker added to the first lane of each gel. Finally, boiled protein samples were loaded into subsequent lanes and gels run at 180 volts for 45 minutes using a Bio-Rad PowerPac™.

2.9.2.2.3. Protein transfer

Trans-Blot® Turbo™ Midi 0.2 µm Nitrocellulose Transfer Packs were purchased from Bio-Rad. To transfer proteins, the anode stack and nitrocellulose membrane were placed into the cassette of a Trans-Blot® Turbo™ Transfer system. Once run, gels were removed from their cassettes and laid on top of the nitrocellulose membrane. After placing the cathode stack on top of the gel, all bubbles were removed and the transfer cassette lid attached. Finally, the transfer cassette was inserted into the Trans-Blot® Turbo™ Transfer system and proteins transferred at 25 volts for 7 or 15 minutes depending on the molecular weight of the protein of interest (7 minutes for low molecular weight proteins and 15 minutes for high molecular weight proteins).

2.9.2.2.4. Protein detection

After the successful transfer of proteins, the resulting membrane was washed twice with PBS-T (see appendix) for 5 minutes. To prevent non-specific binding, the membrane was submerged in 5% freeze dried skimmed milk in PBS-T and incubated

at room temperature for 90 minutes. After two 5-minute washes with PBS-T, primary antibody (see appendix for antibody clones and concentrations) was applied in 5% bovine serum albumin (BSA) in PBS-T. Following overnight incubation at room temperature, three 5-minute washes with PBS-T were performed and HRP-conjugated secondary antibody (see appendix for antibody clones and concentrations) applied in 5% BSA in PBS-T. After 60 minutes at room temperature, five 5-minute washes with PBS-T were performed. To visualise the protein of interest, Clarity™ or Clarity Max™ ECL substrate was prepared according to the manufacturer's instructions and added to the membrane for 3 minutes. Upon the removal of excess ECL substrate, the membrane was placed face down on the imaging surface of a LI-COR C®-DiGit Blot Scanner and the blot imaged for 6 or 12 minutes. Using the Rainbow™ Marker and a WesternSure Pen, the molecular weights of the resulting bands were determined. To confirm equal loading for unphosphorylated proteins, monoclonal antibodies against GAPDH and β -tubulin were used. To confirm equal loading for phosphorylated proteins, monoclonal antibodies specific for the total protein of interest were used (for example for p-EGFR, an antibody specific to total EGFR was used). Finally, densitometric analysis was performed using Image Studio™ software and protein expression relative to loading control calculated.

2.10. Enzyme-linked immunosorbent assays (ELISAs)

2.10.1. Principle

Enzyme-linked immunosorbent assays (ELISAs) are microplate-based immunoassays used to measure the concentration of specific proteins in biological samples (i.e. supernatants, BAL fluid (BALF) and protein lysates). Commercially available DuoSet[®] ELISAs were used throughout this thesis. In these colorimetric sandwich ELISAs, samples are added to capture antibody-coated microplates and the protein of interest allowed to bind. The immobilised protein is then recognised by a biotinylated detection antibody and streptavidin-HRP added. The addition of 3,3',5,5'-Tetramethylbenzidine (TMB) substrate solution results in a blue coloured solution which intensifies over time. After reaching the desired colour intensity, stop solution is added and absorbance at 450 nm measured. Absorbance is proportional to the amount of analyte in the sample with protein concentrations extrapolated from a standard curve (R&D Systems, 2020).

2.10.2. Method

To measure the concentrations of IL-33, ST2, IL-6 and CXCL8 in supernatants, protein lysates and BALF, species-specific DuoSet[®] ELISA kits were used according to the manufacturer's instructions. Briefly, high binding 96 well plates were coated with 100 µl/well of diluted capture antibody. After overnight incubation at room temperature, each well was washed three times with 400 µl/well of ELISA wash buffer (see appendix). Whilst plates were being blocked at room temperature with 300 µl/well

of reagent diluent (see appendix), a 7-point, 1:2 standard curve was prepared from the supplied protein standard. After blocking for 2 hours at room temperature, all plates were washed three times with 400 µl/well of ELISA wash buffer and 100 µl/well of standard or sample added in duplicate. Following 2 hours at room temperature, all plates were washed three times with 400 µl/well of ELISA wash buffer and 100 µl/well of diluted detection antibody added. After 2 hours at room temperature and three 400 µl/well washes with ELISA wash buffer, 100 µl/well of diluted Streptavidin-HRP was added and all plates incubated at room temperature for 20 minutes. All plates were subsequently washed three times with 400 µl/well of ELISA wash buffer and 100 µl/well of TMB substrate solution added. After 20 minutes at room temperature, 50 µl/well of stop solution (see appendix) was added and absorbance at 450 nm (with wavelength correction at 540 nm) measured using a FLUOstar® Omega microplate reader or a Gen5 Cytation plate reader. Finally, the concentration of the protein of interest was calculated from the standard curve using MARS Data Analysis software or GraphPad Prism 8.

2.11. MSD assays

2.11.1. Principle

MSD assays are plate-based sandwich immunoassays used to measure the concentration of specific proteins in biological samples (i.e. supernatants, BALF and protein lysates). In these assays electrode-bound capture antibody binds to the protein of interest. The immobilised protein is then recognised by sulfo-tagged detection antibody and MSD ECL substrate (read buffer) added. After loading into a specialised MSD plate reader, voltage is applied to the plate electrodes causing the detection

antibody-bound sulfo-tags to emit light. The intensity of the light emitted is proportional to the amount of analyte in the sample with protein concentrations extrapolated from a standard curve. Commercially available multiplex and ‘in-house’ singleplex MSD assays were used in this thesis. Compared to singleplex assays where a single protein analyte is measured, multiplex assays enable the simultaneous measurement of up to 10 proteins in a single small volume sample. Compared to standard sandwich ELISAs, MSD assays are quicker to run and require less sample. They are also more sensitive than ELISAs and have a broader dynamic range (Meso Scale Discovery, 2013).

2.11.2. Methods

2.11.2.1. Commercial MSD assays

IL-4 and IL-5 in mouse BALF samples were quantified using the mouse V-PLEX Proinflammatory Panel 1 MSD assay kit. IL-6, CXCL8, IL-1 β , IL-10, IL-2, IL-4, IFN γ , TNF α , IL-12p70 and IL-13 in PCLS supernatants were quantified using the human V-PLEX Proinflammatory Panel 1 MSD assay kit. Both kits were used according to the manufacturer’s instructions. Briefly, all plates were washed three times with 150 μ l/well of MSD wash buffer (see appendix). For the mouse V-PLEX assay, lyophilised calibrator was reconstituted in 1 ml of diluent 41 and a 7-point, 1:4 serial dilution performed. For the human V-PLEX assay, lyophilised calibrator was reconstituted in 1 ml of diluent 2 and a 7-point, 1:4 serial dilution performed. 50 μ l/well of sample or calibrator was then added in duplicate to the washed MSD assay plates. After shaking (400 rpm) at room temperature for 2 hours, plates were washed three times with 150 μ l/well of MSD wash buffer and 25 μ l/well of detection antibody solution (made by adding 60 μ l of each sulfo-tagged detection antibody to 2.4 ml of

either diluent 45 (mouse) or diluent 3 (human)) added. After shaking (400 rpm) at room temperature for 2 hours, plates were washed three times with 150 µl/well of MSD wash buffer and 150 µl/well of Read buffer T® (see appendix) added. All plates were subsequently read using a SECTOR® Imager 6000 and all data analysed using MSD DISCOVERY WORKBENCH® software.

2.11.2.2. ‘In-house’ reduced and oxidised IL-33 specific MSD assays

Capture antibodies specific to either reduced or oxidised IL-33 (in-house proprietary antibodies) were diluted in PBS to give a final concentration of 5 µg/ml and 25 µl/well of diluted capture antibody added to MSD standard multi-array plates. Following overnight incubation at 4°C, plates were washed three times with 300 µl/well of MSD wash buffer and blocked with 200 µl/well of reagent diluent (see appendix). Samples were diluted in reagent diluent to give a top concentration of 100 ng/ml and a 9-point, 1:3 dilution series performed. After blocking for 1 hour at room temperature, plates were washed three times with 300 µl/well of MSD wash buffer and 25 µl/well of sample added in duplicate. For the reduced IL-33 assay, an in-house sulfo-tagged detection antibody was diluted in reagent diluent to give a final concentration of 1 µg/ml. For the oxidised IL-33 assay a commercially available anti-IL-33 antibody (AF3625) was sulfo-tagged and diluted in reagent diluent to give a final concentration of 1 µg/ml. Following 2 hours at room temperature, plates were washed three times with 300 µl/well of MSD wash buffer and 50 µl/well of diluted detection antibody added. After 2 hours at room temperature, plates were washed three times with 300 µl/well of MSD wash buffer and 150 µl/well of Read buffer T ® added. Once Read

buffer T® had been added to all plates, plates were read immediately using a SECTOR® Imager 6000.

2.12. Statistical analysis

The distribution of all data was determined by Shapiro-Wilk normality test. Data are reported as either mean (parametric) or median (non-parametric) with all individual data points shown where appropriate. For parametric data, unpaired and paired t-tests were used to determine statistical significance between two unmatched and matched groups whilst One- and Two-way (ordinary and repeated measures) ANOVAs were used to compare multiple (unpaired and paired) groups and variables. For non-parametric unpaired data, statistical significance between two or more groups was assessed by Mann-Whitney U test and Kruskal-Wallis test respectively. For non-parametric paired data, statistical significance between two or more groups was assessed by Wilcoxon signed-rank test and Friedman test respectively. All statistical analysis was performed using GraphPad Prism 8 with the particular statistical test(s) used stated in each figure legend. P values less than or equal to 0.05 were considered statistically significant.

Chapter 3: The effects of IL-33 on the pro-fibrotic activity of fibroblasts

3. The effects of IL-33 on the pro-fibrotic activity of fibroblasts

3.1. Introduction

Despite being implicated in the pathogenesis of IPF (Tajima et al., 2003, Luzina et al., 2012, Luzina et al., 2013, Li et al., 2014b, Gao et al., 2016, Lee et al., 2017, Fanny et al., 2018, Zhao et al., 2018), the cellular and molecular mechanisms by which IL-33 exerts its potential pro-fibrotic effects remain unclear. Given the central role of fibroblasts during the development of IPF (see chapter 1 section 1.1.6.2.2), it is plausible that IL-33 exerts some of its reported pro-fibrotic effects (Luzina et al., 2013, Li et al., 2014b, Gao et al., 2016, Fanny et al., 2018) via this cell type. Although IL-33 has been shown to induce IL-6 and CCL2 expression by rat cardiac fibroblasts (Zhu and Carver, 2012) and increase eotaxin production by murine lung fibroblasts (Kurokawa et al., 2011), the response of human lung fibroblasts (HLFs) to IL-33 stimulation is not well characterised. Indeed, it is unknown whether IL-33 has direct effects on the pro-fibrotic activity of HLFs.

3.2. Aims

To test the hypothesis that IL-33 directly increases the pro-fibrotic activity of fibroblasts during the development of IPF, the aims of this chapter were to:

- Determine if IPF fibroblasts express IL-33
- Establish the effect of the pro-fibrotic environment on IL-33 expression by fibroblasts

- Investigate whether IL-33 directly increases the pro-fibrotic activity of fibroblasts

3.3. Methods

Methods specific to this chapter are detailed below. For general methods including HLF and HUVEC culture, RNA extraction, reverse transcription, qPCR, protein extraction, western blotting and ELISAs, please refer to the relevant sections in chapter 2. For details of all the materials, kits, antibodies, qPCR primers, media and buffer recipes used in this chapter, please refer to the appendix.

3.3.1. IL-33 Immunohistochemistry (IHC)

3.3.1.1. Principle

IHC is a form of immunostaining where a protein of interest is identified in deparaffinised, rehydrated tissue sections. During this process, antigens are retrieved and tissue sections probed with an antigen-specific primary antibody. Following the addition of a biotinylated secondary antibody (which binds to the primary antibody), the protein of interest can be visualised using avidin-biotinylated HRP and the HRP substrate 3,3'-Diaminobenzidine (DAB). Using HRP and DAB, the protein of interest is stained brown (Ramos-Vara, 2005).

3.3.1.2. Method

Following informed consent and local ethics approval, formalin fixed, paraffin embedded lung tissue from 3 non-IPF and 43 IPF patients was received from W. Wallace (University of Edinburgh, Edinburgh, UK) and 5 µm-thick sections cut using a microtome. Tissue was deparaffinised and rehydrated by two 5-minute washes in

xylene, two 5-minute washes in 100% ethanol, two 5-minute washes in 70% ethanol and two 5-minute washes in distilled water. Following rehydration, antigen retrieval was performed by heating all sections in 10 mM sodium citrate buffer (pH 6.0) for 20 minutes. Endogenous peroxidase activity was then blocked by incubating all slides in 3% hydrogen peroxide diluted in methanol. Following 30 minutes at room temperature, two 1-minute washes with TBST were performed before 5% horse serum in TBST was added to all sections to prevent non-specific antibody binding. After blocking overnight at 4°C, 3 µg/ml of mouse anti-IL-33 antibody (Nessy-1) was added and all slides incubated at room temperature for 90 minutes. Following two 5-minute washes with TBST, 75 µg/ml of biotinylated horse anti-mouse secondary antibody was added to all slides. After 60 minutes at room temperature and two 5-minute washes with TBST, 400 µl of avidin/biotinylated enzyme complex (ABC) solution was added to each tissue section and all slides incubated at room temperature for 30 minutes. 400 µl of DAB solution was subsequently added to each slide and all tissue sections incubated at room temperature for a further 10 minutes. After 5 minutes in distilled water, all tissue sections were counterstained with Mayer's haematoxylin for 1 minute before being washed with distilled water. To 'blue' the haematoxylin, all slides were incubated in 10 mM PBS (pH 8.0) for 1 minute prior to washing with distilled water. To dehydrate and clear all stained tissue sections, two 3-minute washes in 95% ethanol, three 3-minute washes in 100% ethanol and two 3-minute washes in xylene were performed. After mounting in DPX Mountant and applying coverslips, all tissue was visualised using a Nikon 90i light microscope.

3.3.2. Transfection of HLFs with small interfering RNA (siRNA)

3.3.2.1. Principle

Genes/proteins of interest can be selectively knocked down using small interfering RNA (siRNA). Following Lipofectamine™ mediated transfection, cytoplasmic siRNA is processed by the RNA silencing complex (RISC). The resulting RISC-bound single stranded siRNA molecule recognises/binds mRNA transcribed from the gene of interest leading to RISC-mediated mRNA degradation. As mRNA is translated into protein, successful siRNA transfection results in targeted reductions in mRNA and protein expression (i.e. only the gene/protein of interest is knocked down). Non-targeting negative control siRNAs are used during siRNA knockdown experiments to confirm that any biological effects are due to silencing of the gene/protein of interest and not non-specific effects of siRNA transfection (Kim and Rossi, 2008).

3.3.2.2. Method

HLFs were seeded at a density of 1.8×10^5 cells/well into 6 well plates and incubated overnight at 37°C + 5% CO₂. The following day, Lipofectamine™ RNAiMAX Transfection Reagent (Lipofectamine™), ON-TARGETplus Human *IL33* siRNA (IL-33-targeting siRNA) and ON-TARGETplus Non-targeting Control Pool siRNA (non-targeting control siRNA) were diluted in L-glutamine supplemented DMEM (see appendix) and equal volumes of diluted Lipofectamine™ and diluted siRNA (either IL-33-targeting or non-targeting control siRNA) combined. After 15 minutes at room temperature, 400 µl/well of the appropriate Lipofectamine™-siRNA solution (or L-glutamine supplemented DMEM alone for the non-transfected control (NTfC) condition) was added to cells. 1.6 ml/well of transfection media (see appendix) was

subsequently added and cells incubated at 37°C + 5% CO₂ for 24 hours. For all siRNA knockdown experiments, HLFs were transfected with 5 nM siRNA using 7.5 µl/well of Lipofectamine™.

3.4. Results

3.4.1. IL-33 is expressed by fibroblasts in IPF

To determine the pattern of IL-33 expression in IPF lung tissue, lung tissue from 43 IPF patients was analysed via IHC.

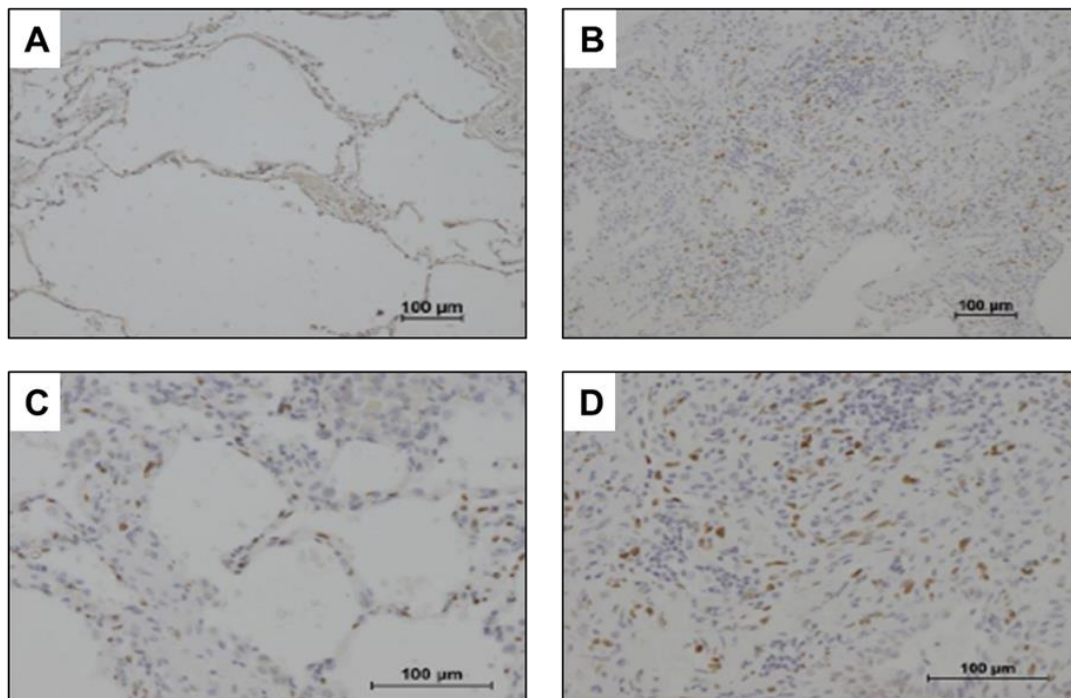


Figure 3.1: IL-33 expression in IPF lung tissue.

IL-33 expressing cells in the lungs of 43 IPF patients and 3 non-IPF controls were identified via IHC. Representative images of IL-33 staining in the lungs of non-IPF controls at x10 magnification (A) and IPF patients at x10 magnification (B) and x20 magnification (C & D) are shown.

Compared with non-IPF controls (Fig. 3.1A), a greater number of IL-33 positive cells were present in IPF lung sections (Fig. 3.1B) with staining predominately localised to the nucleus (Fig. 3.1C & 3.1D). Morphological analysis of IPF tissue sections

suggested that a number of IL-33 positive cells were fibroblasts. (Fig. 3.1D). However, as no fibroblast specific markers were used to confirm the identity of these cells, the expression of IL-33 by IPF fibroblasts was validated *in vitro* by qPCR and western blotting.

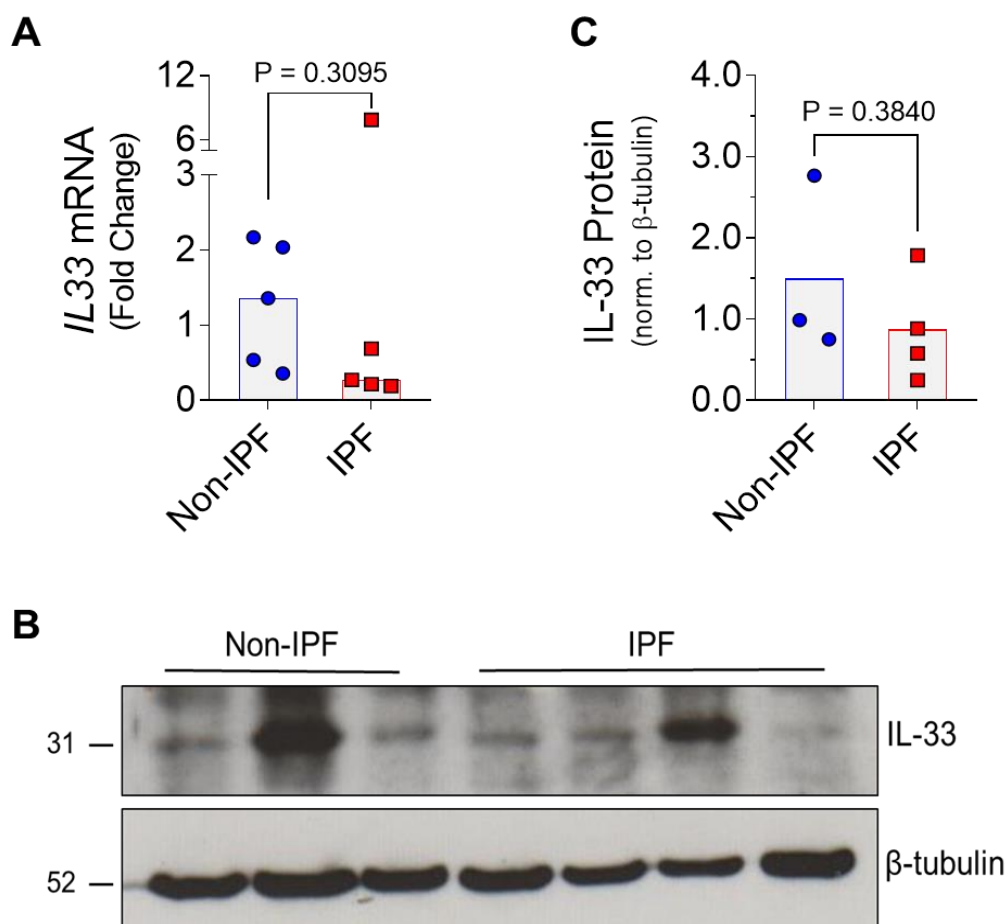


Figure 3.2: IL-33 gene and protein expression by primary HLFs *in vitro*.

Basal *IL33* gene expression by non-IPF (n=5) and IPF (n=5) HLFs was assessed by qPCR and normalised to the average fold change for non-IPF cells. Non-IPF and IPF donors are shown in blue and red respectively. Bars indicate median values and each data point represents a single donor. Mann-Whitney U test (**A**). 20 μ g/lane of total protein was separated by SDS-PAGE and basal IL-33 protein expression by non-IPF (n=3) and IPF (n=4) HLFs assessed by western blot (**B**) and quantified relative to β -tubulin via densitometry (**C**). A representative western blot is shown. Non-IPF and IPF donors are shown in blue and red respectively. Bars indicate mean values and each data point represents a single donor. Unpaired t-test.

Both non-IPF and IPF HLFs expressed IL-33 mRNA (Fig. 3.2A) and protein (Fig. 3.2B & 3.2C) *in vitro*. Although IPF HLFs appeared to express lower levels of IL-33 than non-IPF controls, substantial donor to donor variability (combined with relatively

small sample sizes) meant that no significant difference was recorded between control and IPF derived cells (Fig. 3.2A & 3.2C).

3.4.2. TGF β increases IL-33 expression by fibroblasts

After establishing that IL-33 is expressed by IPF fibroblasts, the effect of the pro-fibrotic environment on the expression of this cytokine was assessed. Specifically, non-IPF and IPF HLFs were stimulated with TGF β and *IL33* gene expression measured at 4, 8 and 24 hours by qPCR.

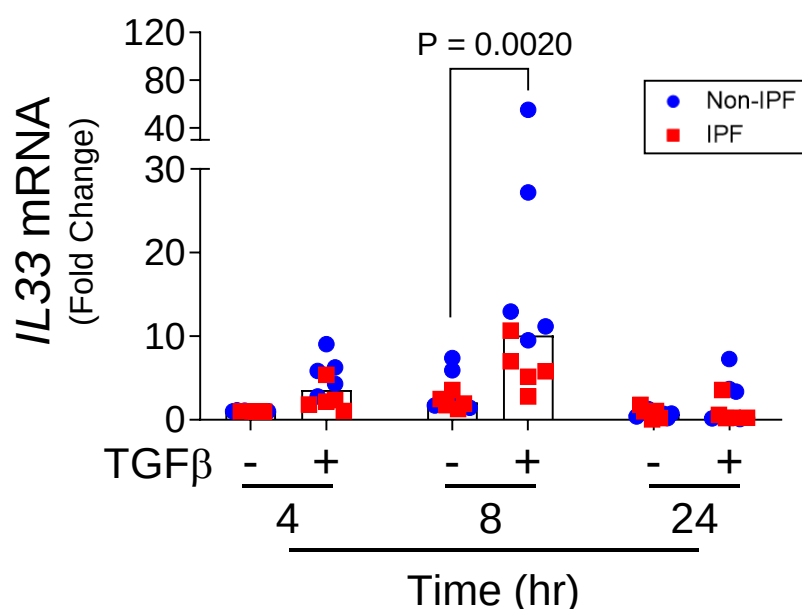


Figure 3.3: The effect of TGF β stimulation on *IL33* gene expression by HLFs over time.

Non-IPF (n=5) and IPF (n=5) HLFs were stimulated with 2 ng/ml of TGF β . Cells were lysed at 4, 8 and 24 hours for RNA and *IL33* gene expression measured by qPCR. For each donor, all data was normalised to the unstimulated media alone control at 4 hours. Non-IPF and IPF donors are shown in blue and red respectively. Bars indicate median values and each data point represents a single donor. Wilcoxon signed-rank test.

Following TGF β stimulation, an initial increase in *IL33* gene expression was observed for non-IPF and IPF HLFs at 4 hours (Fig. 3.3). mRNA levels were subsequently found to peak 8 hours post TGF β stimulation, with a significant increase relative to 8-hour

unstimulated control cells recorded (Fig. 3.3). Despite *IL33* mRNA levels returning to baseline after 24 hours (Fig. 3.3), these results suggest that TGF β increases *IL33* gene expression by non-IPF and IPF HLFs.

To establish whether TGF β had a comparable effect on IL-33 protein expression, non-IPF and IPF HLFs were stimulated with TGF β and IL-33 expression at 8 and 24 hours measured by western blot.

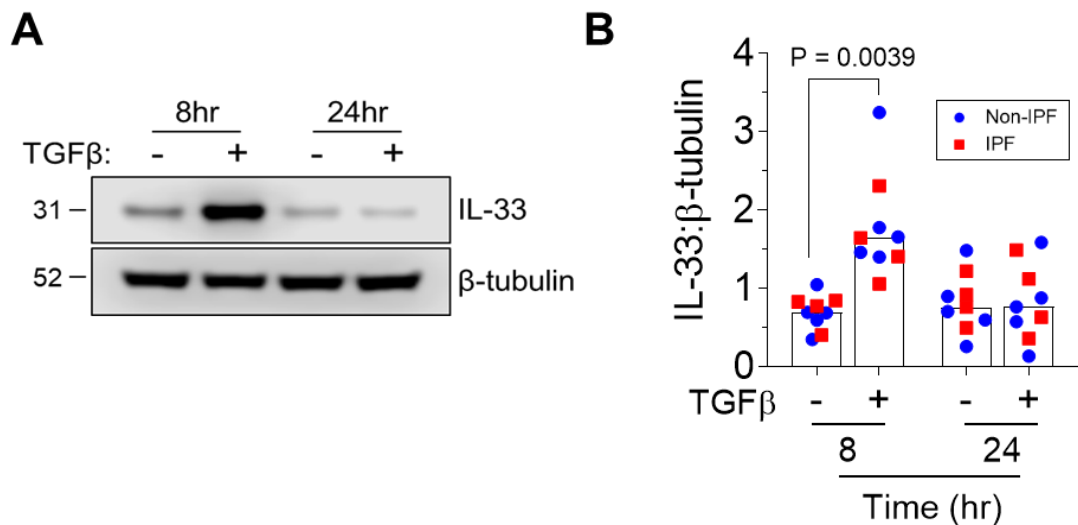


Figure 3.4: The effect of TGF β on IL-33 protein expression by HLFs over time.

Non-IPF (n=5) and IPF (n=4) HLFs were stimulated with 2 ng/ml of TGF β . Whole cell lysates were generated at 8 and 24 hours and 20 μ g/lane of total protein separated by SDS-PAGE. IL-33 protein expression was assessed by western blot (**A**) and quantified relative to β -tubulin via densitometry (**B**). A representative western blot from a single non-IPF donor is shown. Non-IPF and IPF donors are shown in blue and red respectively. Bars indicate median values and each data point represents a single donor. Wilcoxon signed-rank test.

Relative to unstimulated control, 8-hour TGF β stimulation significantly increased IL-33 protein expression by non-IPF and IPF HLFs (Fig. 3.4A & 3.4B). Since levels returned to baseline after 24 hours (Fig. 3.4A & 3.4B), these results demonstrate that TGF β transiently upregulates IL-33 protein expression by fibroblasts.

3.4.3. IL-33 cannot be detected in supernatants collected from TGF β -treated fibroblasts

To investigate whether the reduction in TGF β -induced IL-33 expression at 24 hours was due to the release of IL-33 from fibroblasts, supernatants from TGF β -stimulated HLFs were collected and the concentrations of IL-33 at 4, 8 and 24 hours measured by ELISA.

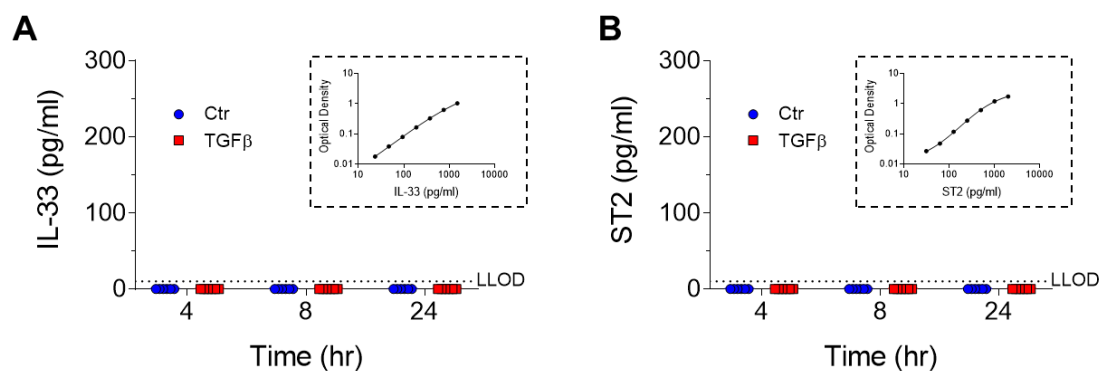


Figure 3.5: IL-33 and ST2 release from TGF β -stimulated HLFs over time.

Non-IPF (n=3) and IPF (n=3) HLFs were stimulated with 2 ng/ml of TGF β . Supernatants were collected at 4, 8 and 24 hours and the concentrations of IL-33 (**A**) and ST2 (**B**) measured by ELISA. Unstimulated control (Ctr) and TGF β treated samples are shown in blue and red respectively. IL-33 (**A**) and ST2 (**B**) standard curves are shown as positive controls.

Irrespective of time and TGF β stimulation, IL-33 could not be detected in supernatants collected from non-IPF and IPF HLFs (Fig. 3.5A). Since IL-33 can complex with soluble ST2 (sST2), I hypothesised that high concentrations of this decoy receptor could explain my findings. To address this possibility, ST2 levels in the same samples were measured by ELISA. Surprisingly, ST2 could not be detected in supernatants collected from non-IPF and IPF cells stimulated with TGF β (Fig. 3.5B). Taken together, these results suggest that TGF β does not induce IL-33 release from fibroblasts. However, given the previously documented issues with the ELISA kits used (Scott et al., 2018b), further experiments are required to confirm this finding.

3.4.4. Extracellular IL-33 fails to increase the pro-fibrotic activity of fibroblasts

To determine whether IL-33 has direct pro-fibrotic effects independent of TGF β , *in vitro* experiments with recombinant IL-33 were performed. Specifically, HLFs were stimulated with either TGF β or IL-33 and the levels of *ACTA2* and *COL1A1* mRNA measured at 4, 8 and 24 hours by qPCR.

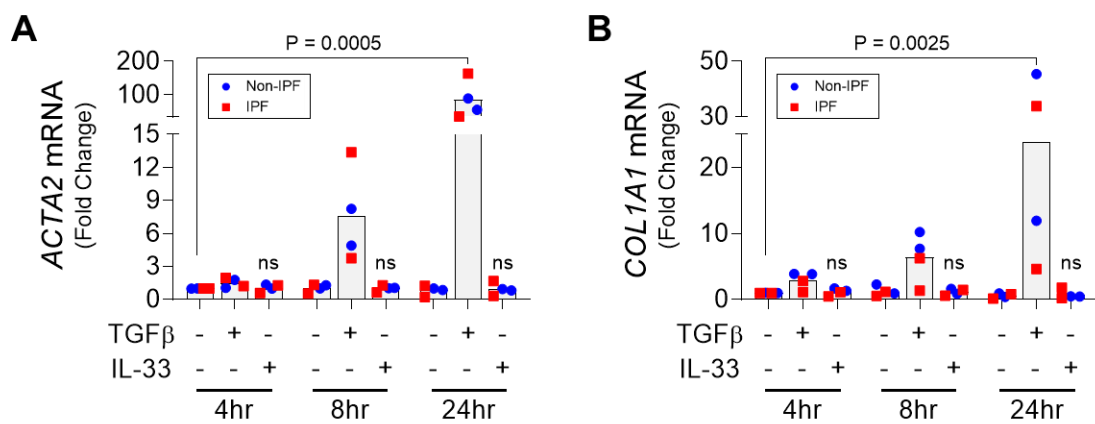


Figure 3.6: The effect of IL-33 on pro-fibrotic gene expression by HLFs over time.

Non-IPF (n=2) and IPF (n=2) HLFs were stimulated with either 2 ng/ml of TGF β or 10 ng/ml of IL-33. Cells were lysed at 4, 8 and 24 hours for RNA and *ACTA2* (**A**) and *COL1A1* (**B**) gene expression measured by qPCR. For each donor, all data was normalised to the unstimulated media alone control at 4 hours. Non-IPF and IPF donors are shown in blue and red respectively. Bars indicate mean values and each data point represents a single donor. ns = P > 0.9999 vs. 4 hour unstimulated control. Two-Way repeated measures ANOVA with Tukey's multiple comparisons test.

As expected, TGF β increased *ACTA2* (Fig. 3.6A) and *COL1A1* (Fig. 3.6B) expression by non-IPF and IPF HLFs over time, with significant increases for both genes observed at 24 hours (Fig 3.6A & 3.6B). In contrast, IL-33 had no significant effect on *ACTA2* and *COL1A1* mRNA levels at any time point (Fig 3.6A & 3.6B). To determine if this lack of response to IL-33 was a gene specific effect, genes known to be induced by IL-33 in other cell types, namely *IL6* and *CXCL8*, were assessed.

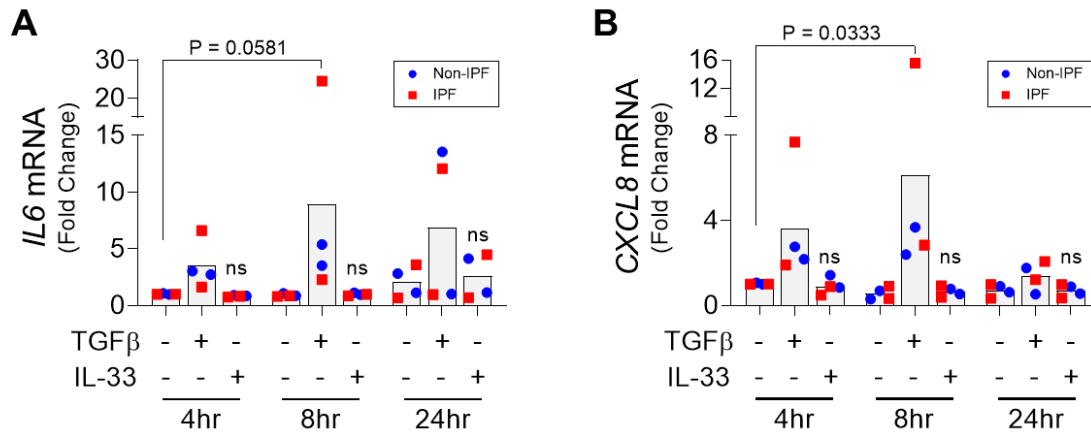


Figure 3.7: The effect of IL-33 on *IL6* and *CXCL8* gene expression by HLFs over time.

Non-IPF (n=2) and IPF (n=2) HLFs were stimulated with either 2 ng/ml of TGFβ or 10 ng/ml of IL-33. Cells were lysed at 4, 8 and 24 hours for RNA and *IL6* (A) and *CXCL8* (B) gene expression measured by qPCR. For each donor, all data was normalised to the unstimulated media alone control at 4 hours. Non-IPF and IPF donors are shown in blue and red respectively. Bars indicate mean values and each data point represents a single donor. ns = P > 0.9999 vs. 4 hour unstimulated control. Two-Way repeated measures ANOVA with Tukey's multiple comparisons test.

Unlike TGFβ, 8-hour stimulation with IL-33 failed to increase *IL6* (Fig. 3.7A) and *CXCL8* (Fig. 3.7B) gene expression by non-IPF and IPF HLFs. As IL-33 had no effect on the expression of these genes at either 4 or 24 hours (Fig 3.7A & 3.7B), it was hypothesised that 10 ng/ml of recombinant IL-33 may be insufficient to activate fibroblasts and increase their pro-fibrotic activity.

To explore this possibility, non-IPF HLFs were stimulated with increasing concentrations of IL-33 and the levels of *ACTA2*, *COL1A1*, *IL6* and *CXCL8* mRNA measured after 8 hours by qPCR.

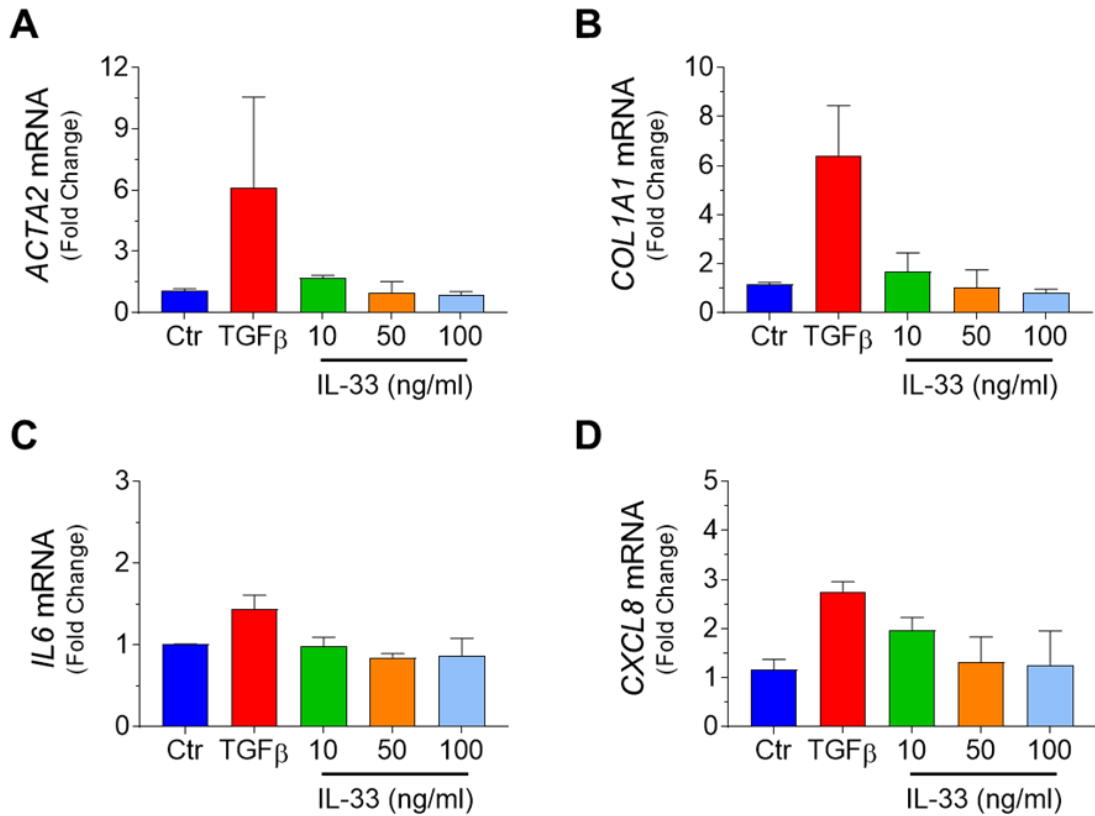


Figure 3.8: The effect of increasing concentrations of recombinant IL-33 on gene expression by non-IPF HLFs.

Non-IPF HLFs were stimulated with either 2 ng/ml of TGF β or 10, 50 or 100 ng/ml of IL-33. After 8 hours the levels of *ACTA2* (A), *COL1A1* (B), *IL6* (C) and *CXCL8* (D) mRNA were measured by qPCR. For each gene, data was normalised to the media alone control (Ctr) and is presented as mean $\pm$ SD, n=2.

Consistent with my previous findings, 8-hour stimulation with 10 ng/ml of recombinant IL-33 had no effect on *ACTA2* (Fig. 3.8A), *COL1A1* (Fig. 3.8B), *IL6* (Fig. 3.8C), and *CXCL8* (Fig. 3.8D) expression by HLFs. Moreover, unlike TGF β , stimulation with 50 or 100 ng/ml of IL-33 failed to increase the expression of these genes (Fig. 3.8A – 3.8D). Taken together, these results imply that the concentration of IL-33 used in previous experiments is unlikely to explain the failure of IL-33 to activate fibroblasts and increase their pro-fibrotic activity.

Recent reports suggest that the majority (>90%) of recombinant IL-33 oxidises in tissue culture media within 4 hours. Since oxIL-33 is unable to induce ST2-dependent

responses (Cohen et al., 2015), it is feasible that the inability of fibroblasts to respond to exogenous IL-33 stems from the oxidation of this cytokine in culture. To address this possibility, ST2-expressing HUVECs were stimulated with recombinant IL-33 and the expression and release of IL-6 and CXCL8 used as readouts of ST2-dependent activity.

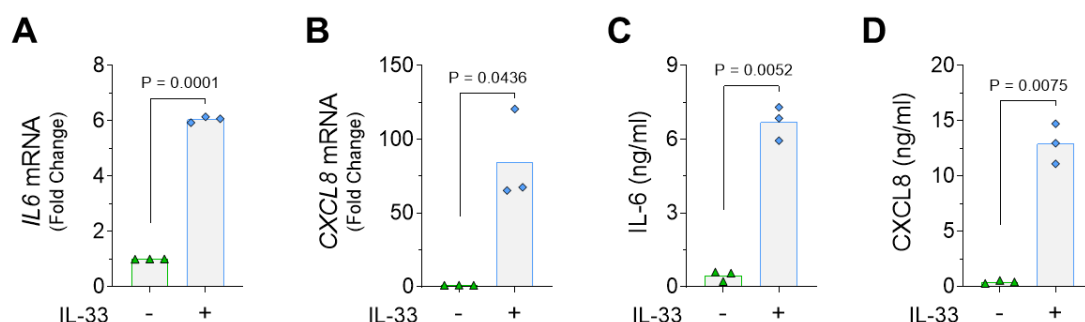


Figure 3.9: The ability of recombinant IL-33 to induce ST2-dependent signalling in HUVECs.

HUVECs were stimulated with 10 ng/ml of recombinant human IL-33. After 8 hours, cells were lysed for RNA with *IL6* (A) and *CXCL8* (B) mRNA levels measured by qPCR and normalised to unstimulated control. After 24 hours, supernatants were collected and the concentrations of IL-6 (C) and CXCL8 (D) measured by ELISA. For all graphs, bars indicate mean values and each data point represents an independent experiment. n=3. Paired t-test.

Following 8-hour stimulation with IL-33, significant increases in *IL6* (Fig. 3.9A) and *CXCL8* (Fig. 3.9B) gene expression were recorded versus unstimulated control. As 24-hour stimulation resulted in IL-6 (Fig. 3.9C) and CXCL8 (Fig. 3.9D) release from HUVECs, these results suggest that the recombinant IL-33 used in all previous experiments can elicit ST2-dependent responses.

Considering all previous data, it was hypothesised that the inability of HLFs to respond to IL-33 could be due to the failure of these cells to express ST2. To investigate this theory, cDNA derived from HLFs and HUVECs was analysed using a qPCR primer set specific for the transmembrane form of ST2.

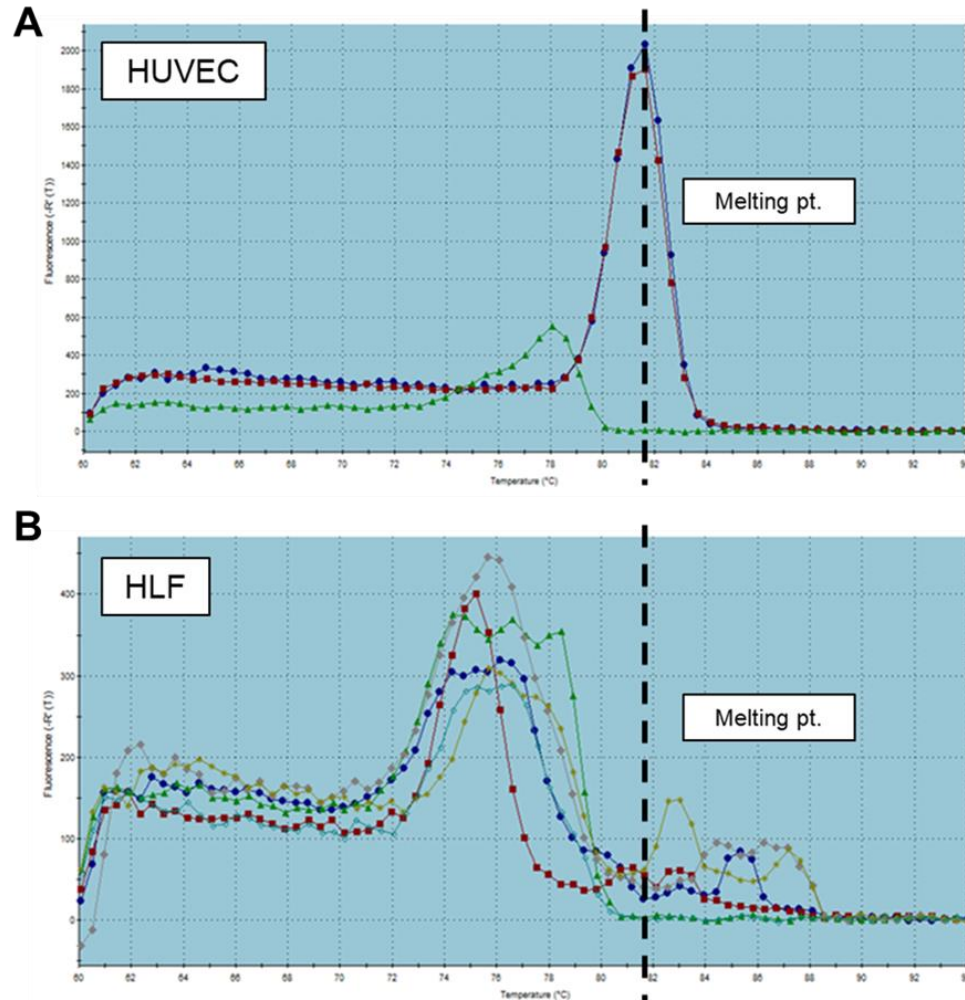


Figure 3.10: Basal ST2 gene expression by HLFs.

The dissociation curves obtained by qPCR when cDNA from HUVECs (A), non-IPF (n=3) and IPF (n=3) HLFs (B) was amplified using a primer set specific for the transmembrane form of ST2. For all images the no template control is shown in dark green.

Compared to HUVECs (Fig. 3.10A), a dissociation curve with a single melting temperature could not be detected when cDNA from HLFs was analysed by qPCR (Fig. 3.10B). Despite suggesting that HLFs do not express ST2, this result required validation at the protein level. Consequently, whole cell lysates from monocyte derived macrophages (MDMs), human pulmonary artery endothelial cells (HPAECs), HUVECs and HLFs were generated and ST2 protein expression assessed by western blot.

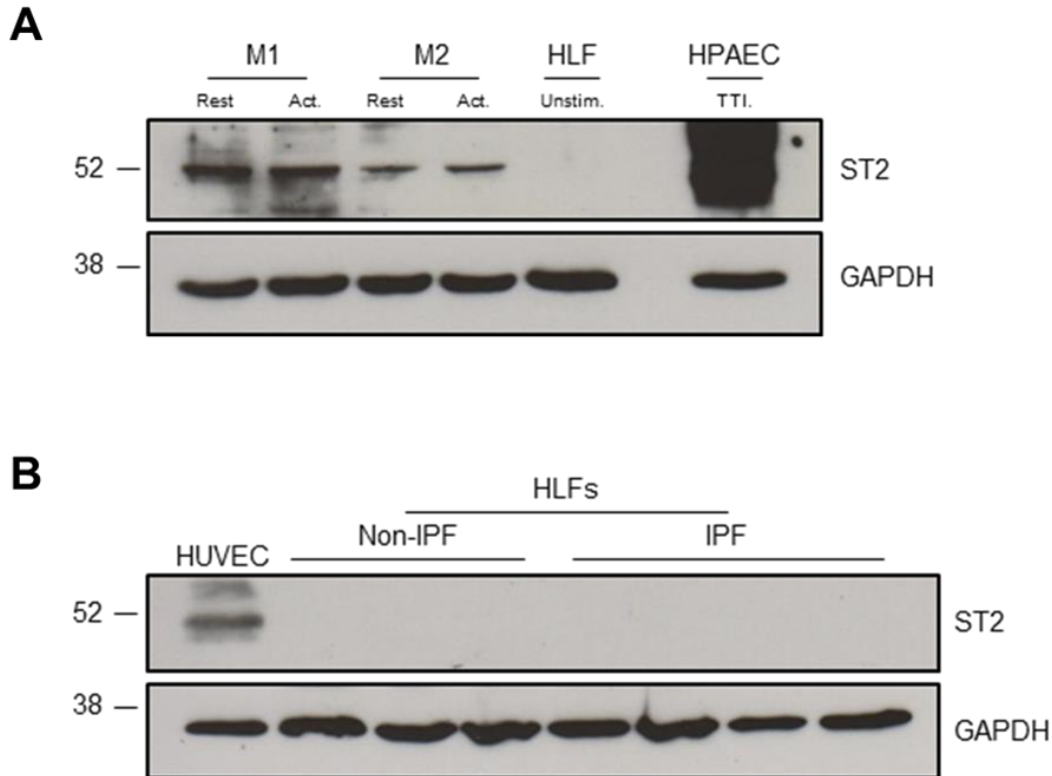


Figure 3.11: ST2 protein expression by HLFs.

Whole cell lysates from resting (rest.) M1 and M2 MDMs, activated (act.) M1 (IFN γ stimulated) and M2 (IL-4 stimulated) MDMs, HPAECs stimulated with 5 ng/ml of TNF α , 5 ng/ml of TGF β and 0.1 ng/ml of IL-1 β (TTI) and a single non-IPF HLF donor were generated. 20 μ g/lane of protein was separated by SDS-PAGE and the expression of ST2 and GAPDH measured by western blot (**A**). Whole cell lysates from HUVECs, non-IPF (n=3) and IPF (n=4) HLFs were subsequently generated. 20 μ g/lane of protein was separated by SDS-PAGE and the expression of ST2 and GAPDH determined by western blot (**B**). Representative western blots are shown. MDM and HPAEC protein lysates were kind gifts from Dr Andrew Jackson and Alice Pasini.

In contrast to HPAECs, MDMs (Fig. 3.11A) and HUVECs (Fig. 3.11B), ST2 could not be detected when protein lysates from non-IPF and IPF HLFs were analysed by western blot (Fig. 3.11A & 3.11B). Combined with my qPCR data, this result suggests that HLFs fail to express the receptor required for IL-33 responsiveness. Considering this, it is unlikely that extracellular IL-33 has direct, ST2-dependent pro-fibrotic effects on fibroblasts during the development of IPF.

3.4.5. Exploring the intracellular role of TGF β -induced IL-33

Intranuclear IL-33 has previously been shown to regulate gene expression by endothelial cells and mesenchymal stem cells (Ali et al., 2011, Choi et al., 2012, Shao et al., 2014, Chen et al., 2019). As I was unable to measure IL-33 release from TGF β treated HLFs, it was theorised that TGF β -induced IL-33 may play an intracellular rather than extracellular role in IPF. Specifically, it was hypothesised that intranuclear TGF β -induced IL-33 increases the expression of pro-fibrotic genes by fibroblasts. To test this hypothesis, a protocol to knockdown IL-33 using siRNA was optimised.

HLFs were transfected with IL-33-targeting- or non-targeting control- siRNA and IL-33 expression at 24 hours assessed by qPCR and western blotting (Fig. 3.12A). Compared with non-transfected control (NTfC), non-targeting control siRNA appeared to increase IL-33 expression by non-IPF and IPF HLFs (Fig. 3.12B – 12D). However, as cells treated with transfection reagent alone (Lipofectamine) had an identical effect on both IL-33 mRNA (Fig. 3.12B) and protein (Fig. 3.12C & 3.12D) levels, it was concluded that this result was due to the transfection reagent used rather than an off-target effect of the control siRNA. Importantly, transfection with IL-33-targeting siRNA led to a significant ~90% reduction in *IL33* gene expression relative to non-targeting control (Fig. 3.12B). As an identical trend was observed at the protein level (Fig. 3.12C & 3.12D), this optimised transfection protocol was used to knockdown IL-33 in all future experiments with HLFs.

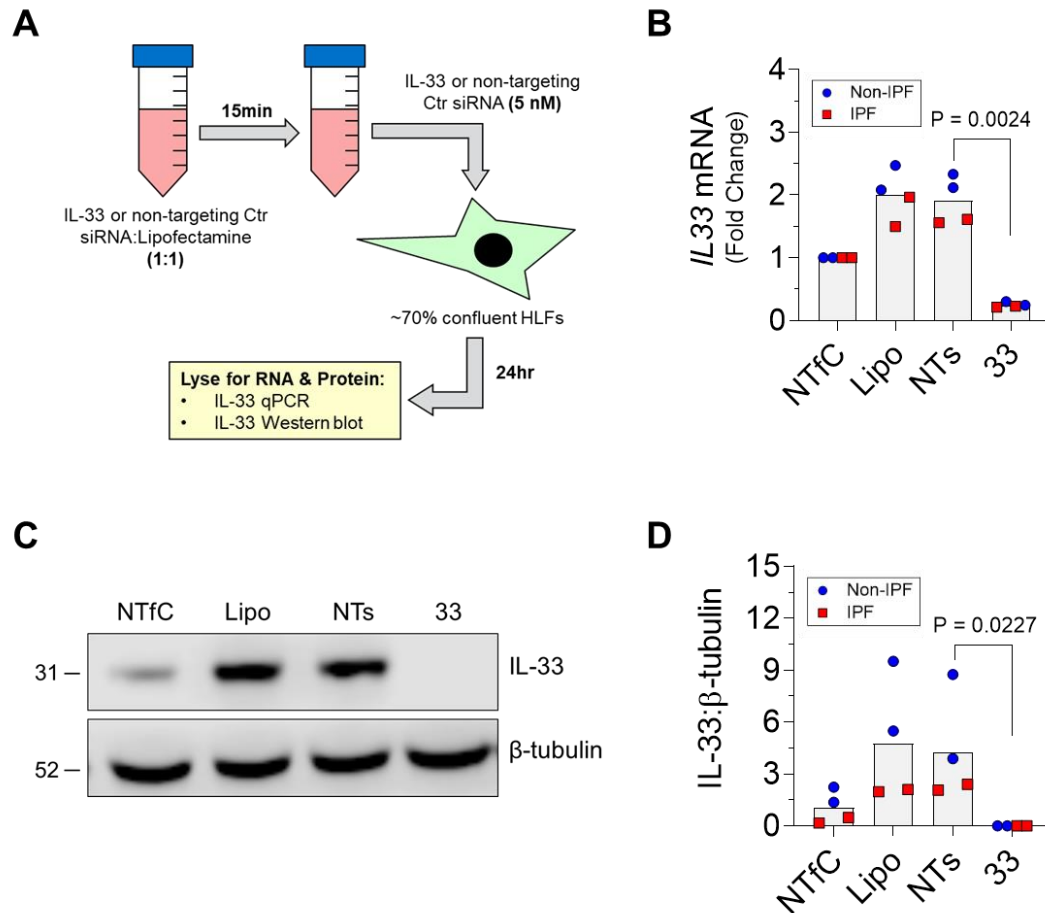


Figure 3.12: Optimisation of IL-33 siRNA knockdown in HLFs.

Equal volumes of diluted Lipofectamine and siRNA were combined and incubated at room temperature for 15 minutes. When 70% confluent, non-IPF (n=2) and IPF (n=2) HLFs were treated with either media (non-transfected control (NTfC)) or Lipofectamine alone (Lipo.). Alternatively, cells were transfected with 5 nM of either non-targeting control siRNA (NTs) or IL-33-targeting (33) siRNA. After 24 hours, cells were lysed for RNA and protein and IL-33 gene and protein expression assessed (**A**). *IL33* gene expression was assessed by qPCR and normalised to the NTfC for each donor (**B**). IL-33 protein expression was assessed by western blot (**C**) and quantified relative to β-tubulin via densitometry (**D**). A representative western blot from a single non-IPF donor is shown. Non-IPF and IPF donors are shown in blue and red respectively. Bars indicate mean values and each data point represents a single donor. Paired t-test.

To determine the potential intracellular effects of TGFβ-induced IL-33 on pro-fibrotic gene expression, IL-33 was knocked down in HLFs. Following 24-hour serum starvation, cells were stimulated with TGFβ and lysed at 4, 8 and 24 hours for RNA and protein (Fig. 3.13A). Before using TaqMan™ gene expression array cards to analyse the resulting RNA samples, it was important to confirm (given that TGFβ upregulates IL-33 expression by fibroblasts (Fig. 3.3 & 3.4)) that IL-33 knockdown

was sustained in TGF β treated HLFs. To achieve this, IL-33 western blots were performed.

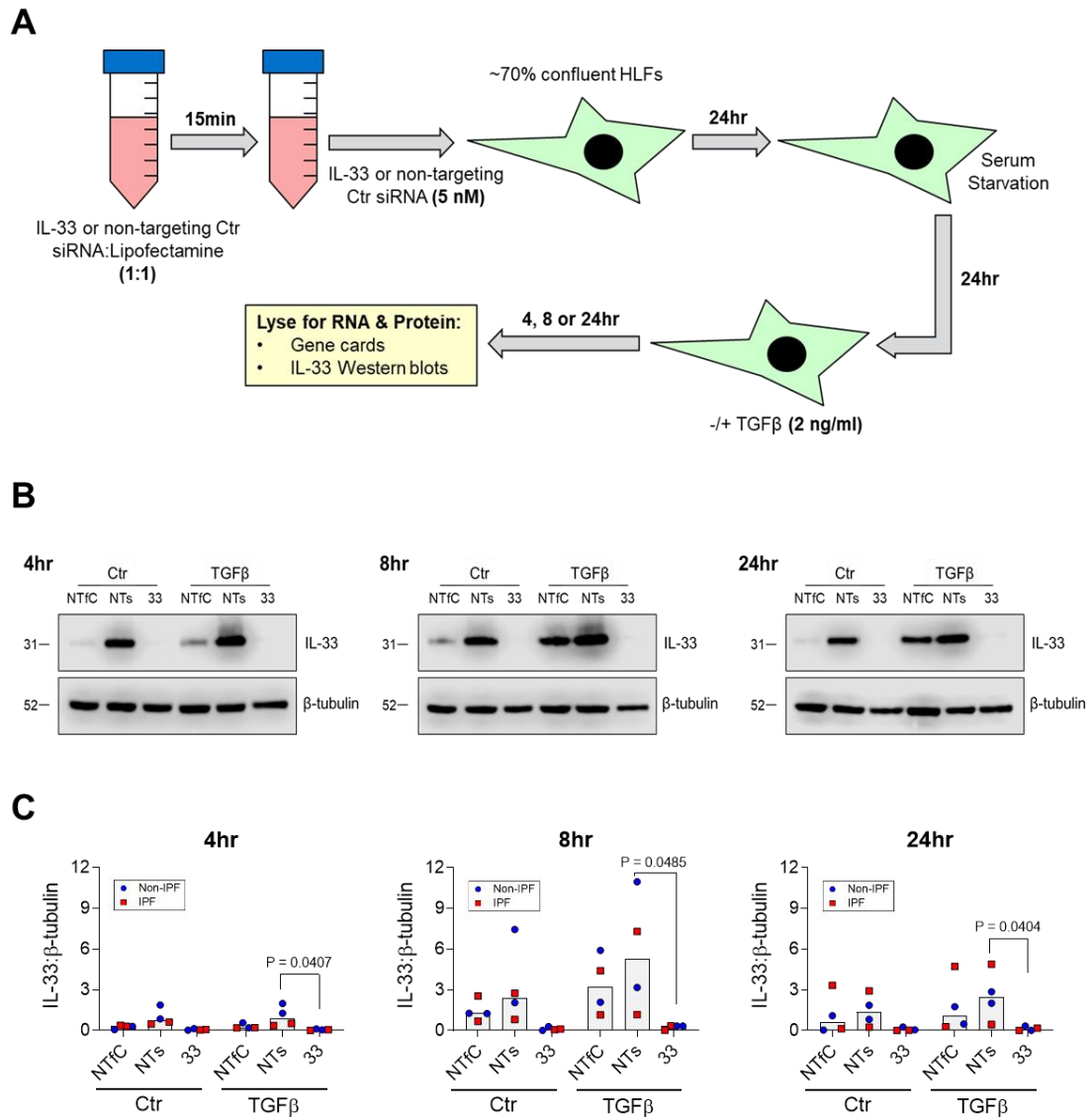


Figure 3.13: IL-33 expression by HLFs following IL-33 knockdown and TGF β stimulation.

Equal volumes of diluted Lipofectamine and siRNA were combined and incubated at room temperature for 15 minutes. When 70% confluent, non-IPF (n=2) and IPF (n=2) HLFs were treated with media (non-transfected control (NTfC)) or transfected with 5 nM of either non-targeting siRNA (NTs) or IL-33-targeting (33) siRNA. After 24 hour transfection, cells were washed with sterile PBS prior to 24-hour serum starvation. Following serum starvation, cells were stimulated with 2 ng/ml of TGF β and lysed for RNA and protein at 4, 8 and 24 hours (A). IL-33 protein expression was assessed by western blot (B) and quantified relative to β -tubulin via densitometry (C). Representative western blots from a single non-IPF donor are shown. Non-IPF and IPF donors are shown in blue and red respectively. Bars indicate median values and each data point represents a single donor. Paired t-test.

Following TGF β stimulation, IL-33 knockdown was sustained at all time points, with significant reductions in IL-33 protein expression observed versus non-targeting control (Fig. 3.13B & 3.13C). Consequently, all RNA samples were reverse transcribed ready for analysis by TaqMan™ gene expression array cards. Unfortunately, due to the COVID-19 pandemic, the resulting cDNA was not analysed. It is therefore unknown what effects intracellular TGF β -induced IL-33 has on the expression of pro-fibrotic genes by fibroblasts.

3.5. Discussion

Recent evidence suggests that IL-33 plays an important pathophysiological role during the development of IPF. Despite this, it is currently unclear how IL-33 promotes pulmonary fibrosis. In particular, it is unknown whether IL-33 directly increases the pro-fibrotic activity of fibroblasts. Here I show that despite being expressed by fibroblasts and upregulated by TGF β , IL-33 has no direct effect on the pro-fibrotic activity of these cells. As fibroblasts were subsequently found not to express ST2, these results imply that, if IL-33 acts as a pro-fibrotic mediator in IPF, alternative cell types likely mediate the effects of this cytokine during the fibrotic response.

IHC was used to assess the pattern of IL-33 expression in IPF lung tissue. Consistent with previous reports (Luzina et al., 2012, Luzina et al., 2013), more IL-33 positive cells were present in IPF tissue, with a number of these cells appearing to be fibroblasts based on their morphology. Although the expression of IL-33 by cultured IPF HLFs suggests that these spindle-shaped cells are likely to be fibroblasts, markers such as vimentin, α SMA and fibroblast-specific protein-1 (FSP-1) could be used to confirm my morphological observations. However, despite being widely used to identify

fibroblasts/myofibroblasts (Strutz et al., 1995, Lawson et al., 2005, Chanda et al., 2016, Surolia et al., 2019), it is important to realise that all of these markers are expressed at varying levels by other cell types. To overcome this issue, serial sections could be probed (either individually or with appropriate combinations of antibodies where possible) with antibodies specific to IL-33, vimentin, α SMA, FSP-1, α v β 6 and CD31. Since α v β 6 and CD31 are expressed by epithelial and endothelial cells respectively (Breuss et al., 1995, Righi et al., 2003), this staining strategy (in addition to morphological analysis) would allow me to definitively determine whether IPF fibroblasts express IL-33 *in situ*.

In line with my IHC data, HLFs expressed IL-33 under basal conditions. Interestingly, no significant difference in IL-33 expression was observed between non-IPF and IPF cells. Despite contradicting Luzina et al.'s (2013) findings, this result should be interpreted cautiously given the relatively small number of donors used in my study and the considerable donor to donor variation observed. Future studies with more non-IPF and IPF HLFs are required to determine if basal IL-33 expression differs significantly between non-IPF and IPF fibroblasts.

Multiple studies have demonstrated the pathophysiological importance of TGF β in IPF (Higashiyama et al., 2007, Horan et al., 2008, Kim et al., 2009, Li et al., 2011, Conte et al., 2014, Roach et al., 2018). Because of this, TGF β was used to establish the effect of the pro-fibrotic environment on IL-33 expression by fibroblasts. In contrast to Sponheim et al.'s (2010) observations with gut-derived fibroblasts, TGF β increased IL-33 expression by non-IPF and IPF HLFs. Since TGF β decreases IL-33 expression by alveolar epithelial (John et al., 2016) and smooth muscle cells (Prefontaine et al.,

2009), my findings imply that TGF β regulates IL-33 expression in a cell type and organ specific fashion.

Following stimulation with TGF β , IL-33 gene (mRNA) and protein expression peaked at the same time point in HLFs. Given that mRNA is translated into protein (Clancy and Brown, 2008), this result is somewhat surprising. However, as I only measured protein expression at 8 and 24 hours, the 16 hour window between these time points means that the precise peak in IL-33 protein expression may have been missed. Consequently, more extensive time course experiments (including timepoints between 8 and 24 hours) are required to confirm the kinetics of IL-33 protein expression following TGF β stimulation.

Compared with samples from non-IPF patients, TGF β levels in the BALF of IPF patients are elevated (Hiwatari et al., 1997, Khalil et al., 2001). Likewise, TGF β expression (both at the mRNA and protein level) is upregulated in IPF lung tissue (Khalil and Greenberg, 1991, Bergeron et al., 2003). Since fibroblast numbers increase during the development of IPF (see chapter 1 section 1.1.6.2.2), the increased levels of IL-33 in the BALF (Lee et al., 2017) and lung tissue (Luzina et al., 2012, Luzina et al., 2013) of IPF patients may be explained by the ability of TGF β to upregulate IL-33 expression by fibroblasts. However, as TGF β has been shown to decrease IL-33 expression by other cell types in the lungs (Prefontaine et al., 2009, John et al., 2016), further work is required to test this hypothesis.

Reductions in IL-33 protein expression have been shown to correlate with IL-33 release (Cayrol and Girard, 2009). Since IL-33 protein expression returned to baseline 24 hours post TGF β stimulation, it was suspected that IL-33 release had occurred from

my cells. Surprisingly however, IL-33 was not detected in supernatants collected from TGF β stimulated HLFs. Given that cell damage and death is necessary for IL-33 release from other cell types (Cayrol and Girard, 2009, Lüthi et al., 2009, Le Goffic et al., 2011, Rickard et al., 2014), it is possible that TGF β stimulation is not sufficient to induce IL-33 release from fibroblasts. However, since IL-33 release in the absence of cell death has been reported by Kouzaki et al. (2011), Kakkar et al. (2012) and Hara et al. (2014), it is feasible that IL-33 was released from my cells but could not be measured. Indeed, it is important to realise that the IL-33 ELISA kit used in my experiments is known to have several limitations including poor sensitivity (Scott et al., 2018b). As all other commercially available ELISA kits suffer from similar issues (Scott et al., 2018b), biological readouts of IL-33 release (from fibroblasts) are required.

IL-33 can be degraded by the proteasome (Tao et al., 2014, Clerman et al., 2017). Consequently, the kinetics of IL-33 expression by HLFs may be indicative of this rather than TGF β -induced release. Since MG132 and Bortezomib prevent proteasomal degradation (Mujtaba and Dou, 2011, Goldberg, 2012), experiments with these inhibitors could be conducted to explore this hypothesis.

In contrast to previous studies using rat cardiac (Zhu and Carver, 2012) and murine lung fibroblasts (Kurokawa et al., 2011), IL-33 had no effect on pro-inflammatory gene expression by HLFs. After thorough investigation (Fig. 3.8, 3.9, 3.10 and 3.11), the most likely explanation for this result is the failure of both non-IPF and IPF HLFs to express ST2. As this component of the IL-33 receptor was first identified in 3T3 fibroblasts (Yanagisawa et al., 1993), the lack of ST2 expression by HLFs is somewhat surprising. However, given that 3T3 cells are derived from mouse embryos (Todaro

and Green, 1963), it is possible that differences in species and/or developmental stage may explain my findings with adult HLFs. Indeed, it is important to note that my results regarding ST2 expression and IL-33 responsiveness are consistent with previous reports using commercially available normal HLFs (NHLFs) (Yagami et al., 2010).

Cytokines elevated in the lungs of IPF patients (IL-1 β , TNF α , IL-1 α & IL-4) have been shown to upregulate ST2 expression by fibroblasts (Kumar et al., 1997, Ziegenhagen et al., 1998, Ogushi et al., 2001, Park et al., 2009, Nishida et al., 2010, Wilson et al., 2010). Additionally, repeated passaging on tissue culture treated plastic can alter the phenotype of IPF HLFs *in vitro* (Xia et al., 2008, Lawrence and Nho, 2018). Considering these reports, it is possible that HLFs express ST2 *in situ* but not *in vitro*. To test this hypothesis, ST2 expression by fibroblasts in IPF lung tissue could be assessed by *in situ* hybridisation and IHC. Although this work is required to validate my *in vitro* findings, it is important to note that *IL1RL1* (the gene encoding ST2) was not expressed by fibroblasts when IPF lung tissue was analysed by single-cell RNA-seq (Reyfman et al., 2019). Because of this it is tempting to speculate that, during the development of IPF, IL-33 is unlikely to have direct, ST2-dependent effects on the pro-fibrotic activity of fibroblasts.

Despite conflicting reports, intranuclear IL-33 has been shown to regulate gene expression by endothelial cells and mesenchymal stem cells (Ali et al., 2011, Choi et al., 2012, Shao et al., 2014, Gautier et al., 2016, Chen et al., 2019, Rana et al., 2019). Due to this and; 1) the predominantly nuclear pattern of IL-33 expression in IPF lung tissue and 2) the apparent failure of TGF β to induce IL-33 release from HLFs; it was hypothesised that TGF β -induced IL-33 increases the pro-fibrotic activity of HLFs via

an intracellular mechanism. To test this theory, HLFs were transfected with IL-33-targeting siRNA and treated with TGF β . Disappointingly, cDNA from this experiment was not analysed due to the COVID-19 pandemic. Until these samples are assessed using TaqMan™ gene expression array cards (and any results validated), no conclusions can be made regarding the intracellular effects of TGF β -induced IL-33. Nevertheless, the western blot data presented in Figures 3.12 and 3.13 validates the primary antibody used to measure (human) IL-33 protein expression throughout this thesis.

Due to their ability to deposit extracellular matrix, fibroblasts play a central pathophysiological role during the development of IPF (see chapter 1, section 1.1.6.2.2). Although further work is required to confirm its intracellular effects, the failure of extracellular IL-33 to directly increase the pro-fibrotic activity of HLFs suggests that this cytokine may not act as a major fibrotic mediator in IPF. However, since macrophages and ILC2s appear to mediate the pro-fibrotic effects of IL-33 in the bleomycin mouse model of pulmonary fibrosis (Li et al., 2014b, Zhao et al., 2018), it is possible that IL-33 has indirect effects on fibroblasts during the fibrotic response.

In summary, despite being expressed by HLFs and upregulated by TGF β , IL-33 had no direct effects on the pro-fibrotic activity of these cells *in vitro*. Since fibroblasts fail to express ST2, I conclude that alternative cell types likely mediate the reported pro-fibrotic effects of IL-33 *in vivo*.

Chapter 4: The effects of blocking IL-33 signalling in the bleomycin (BLM) mouse model of pulmonary fibrosis

4. The effects of blocking IL-33 signalling in the BLM mouse model of pulmonary fibrosis

4.1. Introduction

Although IL-33 had no ST2-dependent effects on HLFs, it is possible that alternative cell types (such as macrophages and ILC2s) mediate the reported pro-fibrotic effects of this cytokine (Luzina et al., 2013, Li et al., 2014b, Gao et al., 2016, Fanny et al., 2018, Zhao et al., 2018). Whilst *in vitro* co-cultures could be used to test this hypothesis, it would be difficult to fully recapitulate the complex cellular cross-talk that occurs in IPF (Lederer and Martinez, 2018, Yanagihara et al., 2019). To overcome this issue and study the pathogenesis of IPF in a metabolically active, immune responsive system, animal models of pulmonary fibrosis have been developed (B Moore et al., 2013, Tashiro et al., 2017).

Spontaneous pulmonary fibrosis occurs in larger animals such as cats, dogs and horses (Webb and Armstrong, 2002, Cohn et al., 2004, Williams et al., 2007). Although more clinically relevant to IPF in terms of disease onset, histological features and response to treatment (Webb and Armstrong, 2002, Cohn et al., 2004, Williams et al., 2007), the impracticality and increased costs associated with using larger animals means that rodent models of pulmonary fibrosis tend to be favoured for IPF research (Moeller et al., 2008, Tashiro et al., 2017). In general these rodent models can be divided into two categories: environmental models where exposure to agents such as bleomycin (BLM) (Moeller et al., 2008), silica (Davis et al., 1998), FITC (Roberts et al., 1995) and ionising radiation (Karvonen et al., 1987) induces a fibrotic response in the lungs and

transgenic models where deletion (e.g. RAGE, ELK1, and relaxin) or overexpression (e.g. TGF β , TGF α and IL-13) of certain genes results in the development of pulmonary fibrosis (Korfhagen et al., 1994, Lee et al., 2001, Samuel et al., 2003, Lee et al., 2004, Hardie et al., 2007, Englert et al., 2008, Cairns et al., 2020). Out of these *in vivo* systems, the BLM-mouse model of pulmonary fibrosis is the most widely used for pre-clinical assessment of anti-fibrotic compounds (Moeller et al., 2008, Jenkins et al., 2017, Tashiro et al., 2017).

Bleomycin (BLM) is a bacterial derived chemotherapeutic antibiotic (Froudarakis et al., 2013). Although important for the treatment of certain cancers, low levels of bleomycin hydrolase (the enzyme responsible for inactivating BLM) in the lungs means that BLM toxicity occurs in approximately 10% of patients (Adamson, 1976, Froudarakis et al., 2013). Since this leads to the development of pulmonary fibrosis, BLM is commonly used to study the fibrotic response *in vivo* (Moeller et al., 2008).

In the BLM mouse model, intratracheal (i.t.) or oropharyngeal (o.p) administration of BLM induces DNA damage, oxidative stress and epithelial cell death (Tashiro et al., 2017). This in turn results in an inflammatory phase characterised by high levels of inflammatory cytokines, alveolitis and pulmonary oedema (Moeller et al., 2008). Subsiding approximately 10 days post BLM treatment, the inflammatory phase gives way to a fibrotic phase that peaks 21-28 days post BLM exposure (Moeller et al., 2008, Moore and Hogaboam, 2008, Tashiro et al., 2017) (Fig. 4.1).

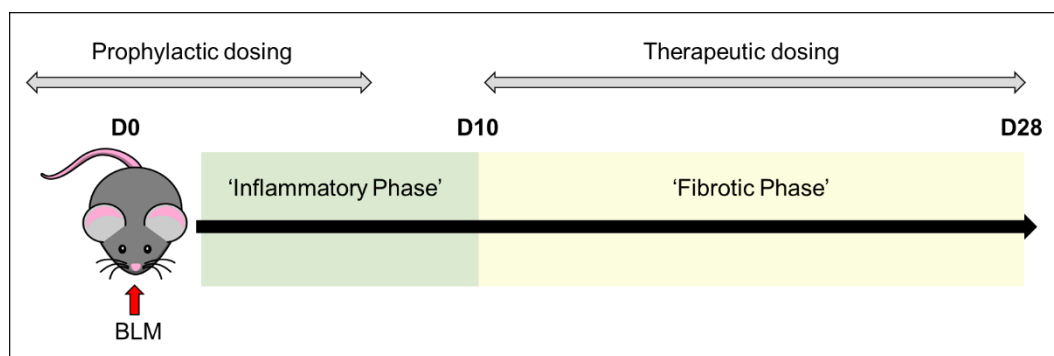


Figure 4.1: The BLM mouse model of pulmonary fibrosis.

The BLM mouse model of pulmonary fibrosis can be divided into 2 phases: an inflammatory phase from day 0-10 and a fibrotic phase from day 10-28. Drug treatment prior to or during the first 7 days of the BLM model is considered as prophylactic dosing whilst drug treatment from day 10 onwards is regarded as therapeutic dosing. Figure adapted from Moeller et al. (2008)

Similar to all animal models, the BLM mouse model has some limitations. For instance, although several of the histological hallmarks of IPF are recapitulated in the BLM mouse model (i.e. collagen deposition, intra-alveolar buds and alveolar destruction), subpleural honeycombing does not occur (Usuki and Fukuda, 1995, Moeller et al., 2008). Moreover, unlike IPF, BLM-induced fibrosis is dependent on inflammation and has been reported to spontaneously resolve over time (Izbicki et al., 2002, Moeller et al., 2008) (although the latter is controversial with Scotton et al. (2013) observing fibrotic lesions in the lungs ≥ 6 months post BLM-exposure). Despite these potential weaknesses the BLM mouse model is regarded as the gold standard *in vivo* model for pre-clinical testing (Scotton and Chambers, 2010). Furthermore, the relative ease, accessibility and speed of this model mean that it is frequently used to study the cellular and molecular mechanisms of IPF (Moeller et al., 2008, B Moore et al., 2013, Tashiro et al., 2017). Indeed, the BLM mouse model has been used to investigate the importance of IL-33 during the development of IPF.

As detailed in chapter 1 section 1.2.12, experiments with ST2 knockout mice (Li et al., 2014b, Fanny et al., 2018, Zhao et al., 2018), IL-33 neutralising antibodies (Li et al., 2014b) and exogenous sST2 (Gao et al., 2016) suggest that IL-33 plays an important pro-fibrotic role during the development of IPF. However, it is important to note that all of these studies block IL-33 signalling prior to or during the inflammatory phase of the BLM model (i.e. use prophylactic rather than therapeutic dosing – see Figure 4.1). Given the pro-inflammatory nature of the IL-33:ST2 axis (Liew et al., 2016, Cayrol and Girard, 2018) it is possible that the reductions in fibrosis reported by Li (2014b), Gao (2016), Fanny (2018) and Zhao et al. (2018) represent a reduction in the inflammation required for the development of BLM-induced fibrosis rather than a true anti-fibrotic effect of inhibiting IL-33 signalling. Due to this, it is unclear whether IL-33 acts as a pro-fibrotic mediator during the BLM mouse model and the development of IPF.

4.2. Aims

To test the hypothesis that IL-33 acts as a fibrotic molecule *in vivo*, the aims of this chapter were to:

- Use BLM to induce pulmonary fibrosis in C57BL/6 mice
- Block IL-33 signalling during the fibrotic phase of the BLM mouse model of pulmonary fibrosis to establish the true pro-fibrotic activity of IL-33 *in vivo*

4.3. Methods

Methods specific to this chapter are detailed below. For general methods including RNA extraction, reverse transcription, qPCR, protein extraction, western blotting, ELISAs and MSD assays, please refer to the relevant sections in chapter 2. For details of all the materials, kits, antibodies, qPCR primers and buffer recipes used in this chapter, please refer to the appendix.

4.3.1. Mice

6-8-week-old male C57BL/6 mice were purchased from Charles River laboratories and housed in cages of up to 4 mice at the Biomedical Services Unit at the University of Nottingham. All animals had free access to food (Tekland Global 18% protein rodent diet) and water and were held in pathogen free conditions under 12:12 light/dark cycles. Mice were allowed to acclimatise to these conditions for at least 7 days post-delivery.

4.3.2. BLM-induced pulmonary fibrosis

Experiments were planned and reported in compliance with the ARRIVE (Animal Research: Reporting of *In vivo* Experiments) guidelines. Specifically, a power calculation was performed to establish the minimum number of mice required per treatment group to achieve statistical significance. Moreover, mice were randomly assigned into treatment groups and all data analysed blind to avoid bias (i.e. data was analysed using the experimental ID numbers given to animals prior to dosing with no knowledge of treatment condition until all analysis was completed). All animal care and procedures were approved by the University of Nottingham Ethical Review

Committee and performed under Home Office Project and Personal License Authority within the Animal (Scientific Procedures) Act 1986.

To induce pulmonary fibrosis, male C57BL/6 mice were anaesthetised using 2.5% isoflurane (2 L/min flow of oxygen) and 60 IU of bleomycin (BLM) sulphate (in 50 μ l sterile 0.9% saline) or 50 μ l of sterile 0.9% saline administered via oropharyngeal (o.p) aspiration. 14 days post BLM exposure, 10 mg/kg of isotype control antibody, anti-ST2 (α ST2) antibody or ST2-Fc fusion protein (all supplied by AstraZeneca, Cambridge, UK) was delivered via intraperitoneal (i.p.) injection. Dosing was repeated every 48 hours with mice euthanised 28 days post BLM treatment via i.p. overdose of Euthatal (Fig. 4.2).

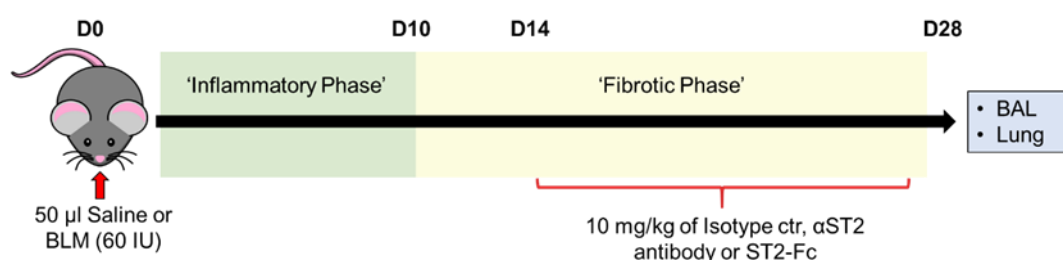


Figure 4.2: BLM study overview.

Pulmonary fibrosis was induced using BLM and IL-33/ST2 blocking reagents (α ST2 antibody and ST2-Fc fusion protein) administered during the fibrotic phase of the model. 28 days post bleomycin treatment, bronchoalveolar lavage (BAL) was collected and the lungs of all mice processed for either biochemical or histological analysis.

The health of all mice was monitored over the 28 day study period with animals weighed on a daily basis and any clinical abnormalities (including abnormal activity, hunching, increased respiration, laboured respiration, piloerection, dehydration and diarrhoea) recorded on a scale of 0-3 (where 0 = not present, 1 = mild, 2 = moderate and 3 = severe). Mice were euthanised early if weight loss exceeded 25% of their starting body weight or severe clinical abnormalities (i.e a score of 3) were observed.

4.3.3. Bronchoalveolar lavage (BAL)

Following euthanasia, bronchoalveolar lavage (BAL) was collected via cannulation of the trachea (Fig. 4.2). Specifically, 1 ml of ice-cold sterile PBS was slowly introduced into the lungs and lavage fluid collected into a sterile syringe. After transferring the sample into a 1.5 ml Eppendorf tube, total and differential cell counts were performed (see section 4.3.4). The cellular and fluid components of the sample were then separated by centrifugation at 500 x g for 5 minutes at 4°C. All cell pellets and BAL fluid (BALF) were stored at -80°C for future use.

4.3.4. Total and differential BAL cell counts

Following gentle inversion to mix, a representative, 200 µl sample of BAL was taken. 10 µl was diluted 1:1 in trypan blue and the total number of cells in the BAL quantified using a haemocytometer (see chapter 2, section 2.2). The remainder was cytospun onto glass slides and stained using a Diff-Quick staining kit according to the manufacturer's instructions. Once dry, all slides were viewed at x20 magnification and differential cell counts performed. Specifically, the number of macrophages, lymphocytes and neutrophils per 100 cells was counted and the resulting percentages combined with total BAL cell counts to give the total number of each cell type.

4.3.5. Processing of lung tissue for biochemical analysis

Following BAL collection, lungs were removed *en bloc*. After separating into lobes, all lung tissue was snap frozen and stored at -80°C for future use. For biochemical analysis, snap frozen lung tissue was immersed in liquid nitrogen and ground into a

powder using a pestle and mortar. After weighing, 50% of the resulting powder was analysed via hydroxyproline assay (see section 4.3.6). The remaining powder was split into two further aliquots – one for RNA extraction (see chapter 2, section 2.3.2) and one for protein extraction (see chapter 2, section 2.7.2).

4.3.6. Hydroxyproline assay

4.3.6.1. Principle

Resulting from the hydroxylation of the amino acid proline, hydroxyproline is a major component of collagen (Campa et al., 1990). Due to this, lung hydroxyproline content is used as a surrogate readout of collagen content. In contrast to commercially available Sircol assays (which measure newly formed pepsin- and acid-soluble collagens only), the assay detailed below measures both newly formed soluble collagen and mature insoluble collagen (Jenkins et al., 2017).

4.3.6.2. Method

To remove any residual liquid following BAL fluid collection, ground lung tissue was dried at 60°C for 90 minutes. Once weighed, the dried lung tissue was transferred into glass Pyrex® test tubes and resuspended in 1 ml of distilled water. Each sample was subsequently mixed with 125 µl of 50% Trichloroacetic acid and all tubes incubated on ice for 20 minutes. Samples were then centrifuged at 500 x g for 10 minutes at 4°C and the resulting supernatants discarded. Next, 1 ml of 12 N HCl was added to each tissue pellet and all samples incubated at 110°C overnight. Once all liquid had evaporated, samples were resuspended in 2 ml of distilled water before being shaken vigorously at 750 rpm on an orbital shaker. After 3 hours with intermittent vortexing, 200 µl of each sample was transferred into 1.5 ml Eppendorf tubes. To calculate the

concentration of hydroxyproline in each sample, 50 mg of trans-4-hydro-1-proline was diluted in 100 ml of distilled water and a 7-point, 1:2 serial dilution (ranging from 500-7.82 µg/ml) performed. Next, 200 µl of standard or sample was transferred into 1.5 ml Eppendorf tubes and 500 µl of chloramine T added. Following 20 minutes at room temperature, 500 µl Ehrlich's solution was added and all samples and standards incubated at 65°C for 15 minutes. After cooling at room temperature for 2 hours, 100 µl/well of sample or standard was added in duplicate to a 96 well plate and absorbance at 550 nm measured using a FLUOstar® Omega microplate reader. Using MARS Data Analysis software the concentration of hydroxyproline in each sample was extrapolated from the standard curve and hydroxyproline content/lung calculated using wet and dry lung weight.

4.3.7. Processing of lung tissue for histological analysis

For lungs requiring histological analysis, the trachea was cannulated and the pulmonary vasculature perfused with 10 ml of heparinised PBS (40 U/ml) via the right ventricle. Lungs were insufflated with formalin via the tracheal cannula under constant gravitational pressure (20 cm H₂O) and the trachea tied off prior to removal of the lungs *en bloc* (Fig 4.2). All lungs were then fixed in 15 ml of formalin for 24 hours prior to paraffin embedding.

4.3.8. Histological analysis of tissue sections

Formalin fixed lungs were dissected into lobes, placed into cassettes and sent to the Nottingham Health Sciences Biobank (NHSB) for paraffin embedding and sectioning.

H&E, Sirius red and Masson's trichrome staining were performed with the resulting 5 µm-thick tissue sections.

4.3.8.1. H&E staining

4.3.8.1.1. Principle

H&E (Haematoxylin and Eosin) staining is used to assess tissue structure. Haematoxylin stains nuclei blue whilst Eosin stains cytoplasm and extracellular matrix pink (Chan, 2014).

4.3.8.1.2. Method

Tissue was deparaffinised and rehydrated by two 3-minute washes in xylene, two 3-minute washes in 100% ethanol, two 3-minute washes in 70% ethanol and one 3-minute wash in distilled water. Following rehydration, slides were submerged in Mayer's haematoxylin (see appendix) for 1 minute before being washed under running water for a further minute. To remove excess haematoxylin and define nuclei, slides were placed in acid-alcohol (see appendix) for 1 minute before being rinsed in water. Slides were then incubated in Eosin (see appendix) for 3 minutes, washed under running water (until the water ran clear) and submerged in 75% ethanol for 30 seconds. Tissue was then dehydrated and cleared by two 3-minute washes in 100% ethanol and two 3-minute washes in xylene. Finally, tissue was mounted in DPX Mountant and coverslips applied to all slides.

4.3.8.2. Sirius red staining

4.3.8.2.1. Principle

Sirius red stains collagen fibres in formalin-fixed paraffin-embedded tissue sections. Although able to differentiate type I and type III collagen fibres under polarised light (Junqueira et al., 1979), Sirius red staining was used in this thesis to visualise general collagen deposition with all collagen fibres appearing red via bright-field microscopy.

4.3.8.2.2. Method

Tissue was deparaffinised and rehydrated by two 3-minute washes in xylene, two 3-minute washes in 100% ethanol, two 3-minute washes in 75% ethanol and two 3-minute washes in distilled water. Following rehydration, tissue sections were placed in Weigert's haematoxylin (see appendix) for 8 minutes. After washing under running water for 10 minutes, all slides were submerged in Picro-Sirius red (see appendix) and collagen fibres stained for 1 hour. Following two 2-minute washes in acidified water (see appendix), slides were dehydrated and cleared by three 2-minute washes in 100% ethanol and two 3-minute washes in xylene. Tissue was then mounted in DPX Mountant and coverslips applied.

4.3.8.3. Masson's trichrome staining

4.3.8.3.1. Principle

Masson's trichrome staining is used to visualise collagen fibres in formalin-fixed paraffin-embedded tissue sections. As suggested by its name, the stain consists of three dyes; Weigert's haematoxylin that stains nuclei black, Biebrich scarlet-acid fuchsin that stains cytoplasm and muscle fibres red and Aniline blue that stains collagen fibres blue (Szunyogova and Parson, 2016).

4.3.8.3.2. Method

Tissue was deparaffinised and rehydrated by two 3-minute washes in xylene, two 3-minute washes in 100% ethanol, two 3-minute washes in 70% ethanol and two 3-minute washes in distilled water. Following rehydration, slides were incubated in Bouin's solution (see appendix) overnight. The following morning all tissue sections were rinsed under running water (until the water ran clear) and stained with Weigert's haematoxylin for 5 minutes. After washing with water for 5 minutes, slides were briefly submerged in distilled water and incubated in Biebrich scarlet-acid fuchsin solution (see appendix) for a further 5 minutes. Sections were then rinsed with distilled water and incubated in Phosphotungstic Phosphomolybdic acid (see appendix) for 7 minutes. Slides were subsequently transferred into Aniline blue solution (see appendix) for 5 minutes, rinsed with distilled water for 2 minutes and incubated in 1% acetic acid for 4 minutes. Tissue was then dehydrated and cleared by two 3-minute washes in 70% ethanol, two 3-minute washes in 100% ethanol and two 3-minute washes in xylene. Finally, tissue was mounted in DPX Mountant and coverslips applied to all slides.

4.3.8.4. Semi-quantitative analysis via modified Ashcroft Scoring

The severity of BLM-induced fibrosis was quantified via modified Ashcroft Scoring. Specifically, H&E, Sirius red and Masson's trichrome stained tissue sections were scanned at the Nottingham Health Science Biobank and visualised using CaseViewer software. For each stain, 25 images per slide (5 images/lobe) were taken at random. Each image was scored using the criteria outlined in Table 4.1 and the mean score for each animal calculated. In the event that two descriptions of the scoring system were

evident in one image, a score between the two descriptions was given. For instance, moderate alveolar thickening with patches of damage and fibrosis was given a score of 4. All scoring was performed blind.

Table 4.1: Scoring criteria for quantifying BLM-Induced fibrosis.

Adapted from Ashcroft et al. (1988).

Grade of Fibrosis	Histological features
0	Normal lung
1	Minimal thickening of alveolar or bronchiolar walls
2	
3	Moderate thickening of alveolar or bronchiolar walls but no damage to underlying lung architecture
4	
5	Increased fibrosis with small fibrotic masses and definite damage to underlying lung architecture
6	
7	Severe distortion of lung architecture and large fibrotic regions. Honeycombing of the lung is included in this category.
8	Total fibrous obliteration of the field of view

4.4. Results

4.4.1. BLM induces acute lung injury

Although the BLM model utilised in this thesis is well established (John et al., 2013, Habgood et al., 2016, Tatler et al., 2016b), the extent of lung injury and resulting fibrosis can be variable. To confirm that BLM treatment had induced acute lung injury in this cohort of animals, lung injury-induced weight loss was recorded over time.

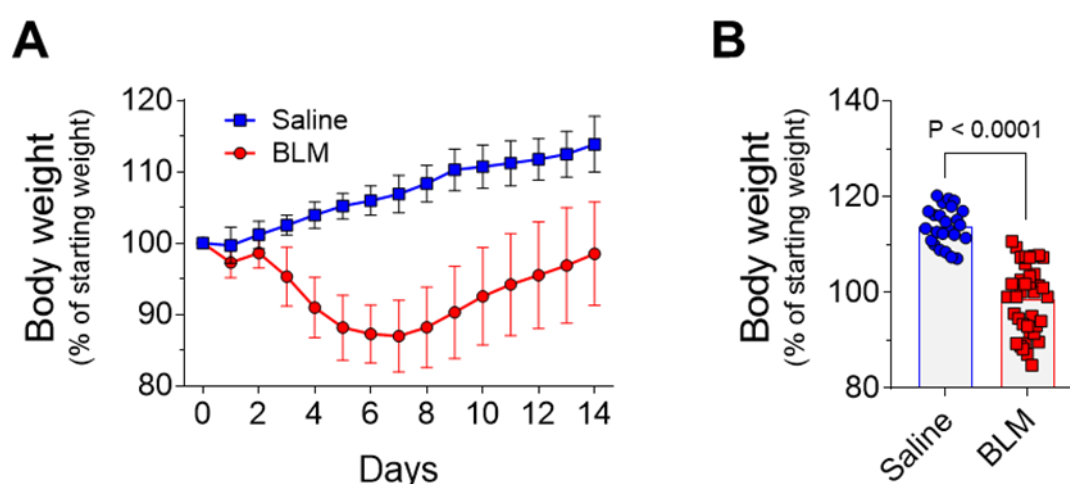


Figure 4.3: The effect of BLM treatment on mouse body weight.

Mice were weighed on a daily basis following BLM treatment. Body weight over time (A) and at day 14 prior to dosing with either isotype control antibody, α ST2 antibody or ST2-Fc fusion protein (B). All data is expressed as a percentage of starting body weight. For Figure A, data is presented as mean $\pm$ SD. For Figure B, bars indicate mean values and each data point represents a single mouse. $n = 63$. Unpaired t-test.

In contrast with saline mice that gained weight, BLM treated animals progressively lost weight from days 3 to 7 of my model (Fig. 4.3A). Maximal weight loss was observed at day 7 with BLM treated mice losing an average of 13% of their starting body weight (Fig. 4.3A). Despite gaining weight from day 7 onwards, BLM treated animals weighed significantly less than saline controls prior to dosing with antibody or fusion protein at day 14 (Fig. 4.3B). Taken together, these results indicate that bleomycin induced acute lung injury in my model.

4.4.2. Therapeutic blockade of IL-33 signalling fails to reduce established BLM-induced fibrosis

To confirm that BLM had induced pulmonary fibrosis and determine the importance of IL-33 during the fibrotic response, changes in lung weight, hydroxyproline content, lung morphology and modified Ashcroft score were assessed following BLM, α ST2 antibody and ST2-Fc fusion protein treatment.

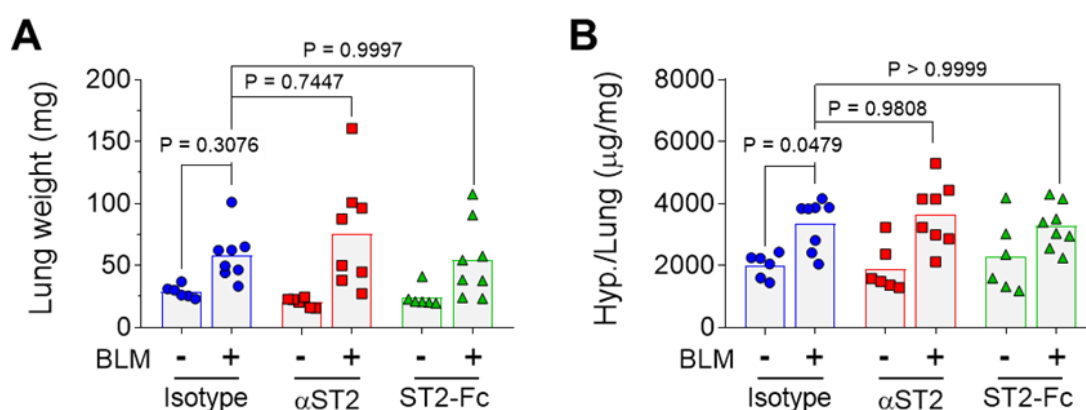


Figure 4.4: The effect of BLM, the α ST2 antibody and the ST2-Fc fusion protein on lung weight and hydroxyproline content.

After drying all lung tissue was weighed (**A**) and lung hydroxyproline (Hyp.) content measured (**B**). For all data sets, bars indicate mean values and each data point represents a single mouse. n=6-8 per treatment group. Two-way ANOVA with Tukey's multiple comparisons test.

BLM exposure increased dry lung weight (Fig. 4.4A) and significantly increased hydroxyproline levels (Fig. 4.4B) relative to saline control with immune cell infiltration and collagen deposition evident in lung sections stained with H&E, Sirius red and Masson's trichrome (Fig. 4.5A). Combined with corresponding increases in modified Ashcroft score (versus saline control) (Fig 4.5B) these results confirm that BLM successfully induced pulmonary fibrosis in my model.

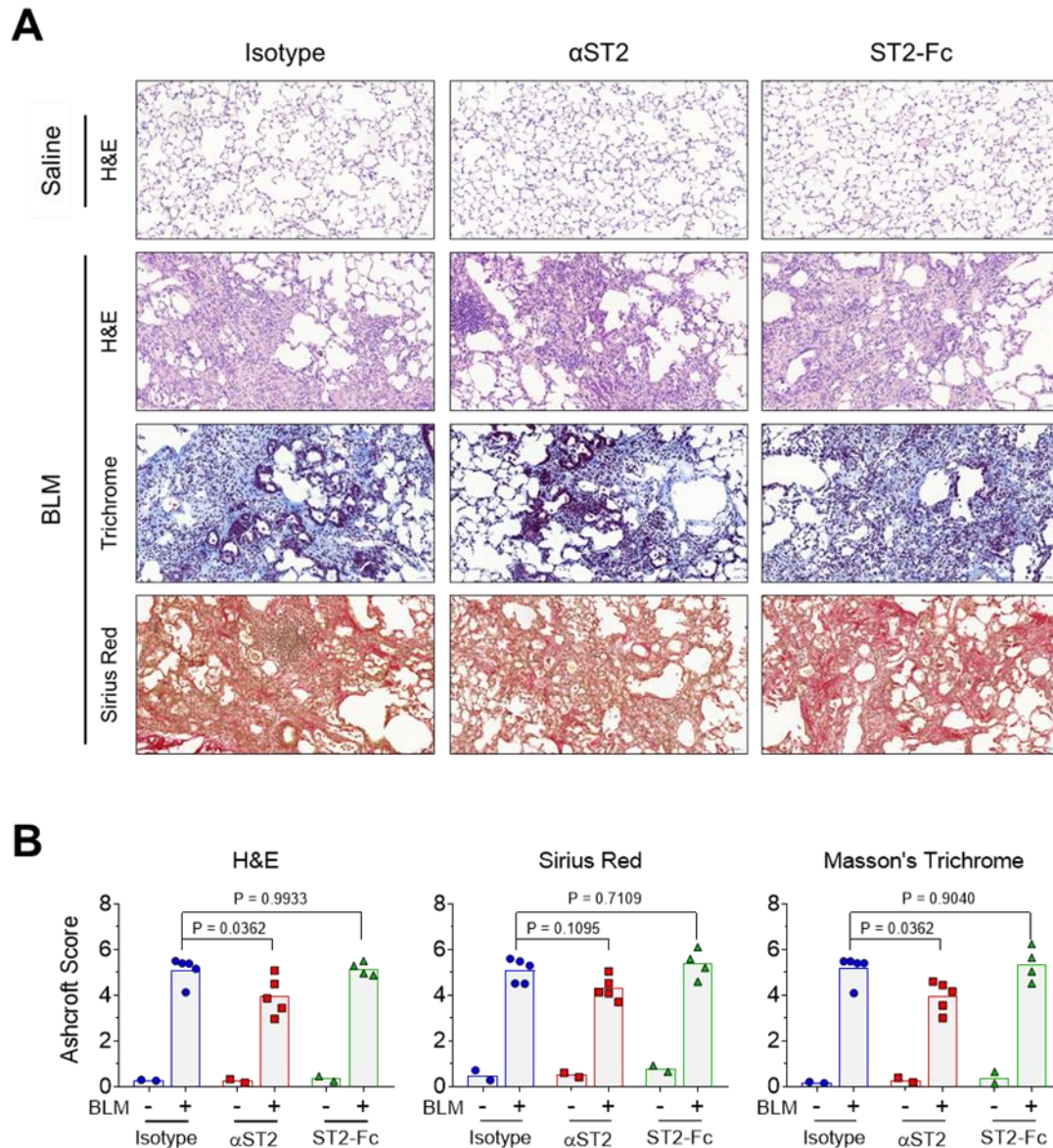


Figure 4.5: The effect of BLM, the α ST2 antibody and the ST2-Fc fusion protein on lung morphology and modified Ashcroft score.

Representative, low magnification (x10) images of H&E, Sirius red and Masson's trichrome stained lung tissue sections (A). For all 3 stains, the severity of BLM-induced fibrosis was quantified by modified Ashcroft scoring (B). For all graphs, bars indicate mean values and each data point represents a single mouse. n=2-5 per treatment group. One-way ANOVA with Tukey's multiple comparisons test.

Interestingly, therapeutic dosing with either the α ST2 antibody or the ST2-Fc fusion protein failed to reduce BLM-dependent increases in lung weight (Fig. 4.4A) and hydroxyproline content (Fig. 4.4B). Moreover, compared to isotype control, no obvious differences in lung morphology and collagen deposition were evident in the

lungs of BLM exposed α ST2 or ST2-Fc treated mice (Fig. 4.5A). Surprisingly, following semi-quantitative analysis of H&E and Masson's Trichrome stained tissue sections, significant reductions in modified Ashcroft score were apparent for α ST2 antibody treated mice versus isotype control (Fig. 4.5B). To assess the validity of this result, retrospective analysis of body weight data was performed.

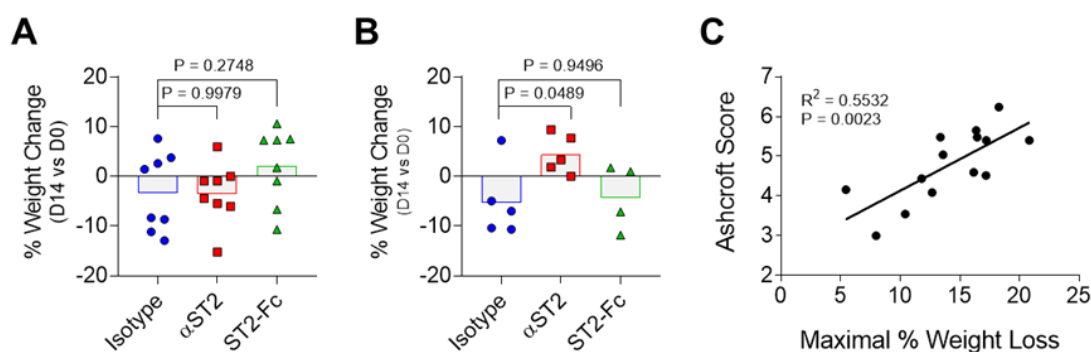


Figure 4.6: Retrospective analysis of body weight data and modified Ashcroft scoring.

Percentage weight change at day 14 (versus day 0) for BLM-treated mice taken for biochemical (A) and histological (B) analysis. Bars indicate mean values and each data point represents a single mouse. $n=4-8$ per treatment group. One-way ANOVA with Tukey's multiple comparisons test. The relationship between maximal percentage weight loss and Masson's trichrome Ashcroft score at day 28 (C). $n=14$. Linear regression with line of best fit.

In contrast to BLM-treated mice taken for biochemical analysis (Fig. 4.6A), BLM-treated animals randomly allocated to the α ST2 histology group were found to have lost significantly less weight prior to dosing than those assigned to either the isotype control or ST2-Fc histology groups (Fig. 4.6B). Given that a significant positive correlation between maximal percentage weight loss and modified Ashcroft score was observed (Fig. 4.6C) and that no reductions in dry lung weight or hydroxyproline content were recorded following α ST2 treatment (Fig. 4.4), the reduction in modified Ashcroft score with the α ST2 antibody is highly likely to be an artefact of a lower level of initial lung injury in this treatment group rather than a true biological effect.

Consequently, these data suggest that IL-33 does not act as a key pro-fibrotic mediator in the BLM mouse model of pulmonary fibrosis.

4.4.3. Both the α ST2 antibody and the ST2-Fc fusion protein have biological effects in the BLM mouse model

To conclude that IL-33 does not play a substantial pro-fibrotic role in the BLM model, it was important to confirm that my results with the α ST2 antibody and the ST2-Fc fusion protein could not be explained by a lack of target engagement. Since no assays were available to directly assess target engagement (i.e. the binding of α ST2 and ST2-Fc to ST2 and IL-33 respectively), the biological effects of both drugs were used as surrogate readouts of target engagement. Specifically, the effects of the α ST2 antibody and the ST2-Fc fusion protein on IL-33 expression, ST2 levels and BLM-induced inflammation were assessed.

4.4.3.1. The α ST2 antibody and ST2-Fc fusion protein increase *IL33* gene expression in the lungs of BLM-treated mice

Since both biologics target the IL-33:ST2 axis, it was hypothesised that they may alter IL-33 levels in the lungs following BLM exposure. To explore this possibility, IL-33 expression in the lungs was assessed by western blot, ELISA and qPCR and IL-33 levels in the BALF measured by ELISA.

IL-33 was detectable in the lung tissue homogenates of all mice by western blot (Fig. 4.7A & 4.7B) and ELISA (Fig. 4.7C). In contrast, IL-33 levels in the BALF were below the lower limit of detection of the ELISA kit used (Fig. 4.7D). In the lungs of

mice receiving isotype control, both saline and BLM treated animals showed similar IL-33 gene (Fig. 4.7E) and protein expression (Fig. 4.7A - 4.7C). Interestingly, repeated exposure to either the α ST2 antibody or the ST2-Fc fusion protein increased *IL33* gene expression in the lungs of BLM treated mice relative to isotype control (Fig. 4.7E). Although not evident at the protein level (Fig. 4.7A - 4.7C), this result suggests that both biologics had some effect on IL-33 expression during the fibrotic phase of the BLM model.

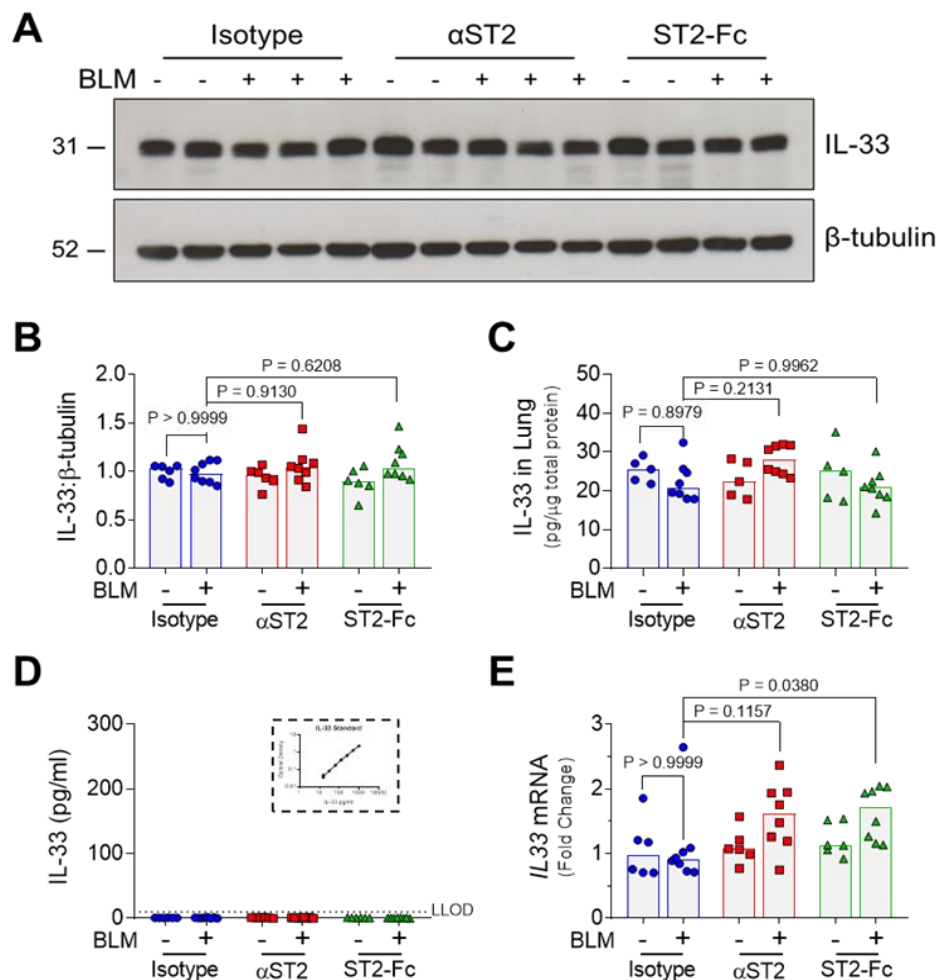


Figure 4.7: The effect of the α ST2 antibody and ST2-Fc fusion protein on IL-33 levels.

Lung homogenates were generated and 15 μ g/lane of total protein separated by SDS PAGE. IL-33 protein expression in the lungs of all mice was determined by western blot (A), quantified relative to β -tubulin via densitometry (B) and validated by ELISA (C). The concentration of IL-33 in the BALF was measured by ELISA (D). *IL33* mRNA levels in the lungs were assessed via qPCR with all values normalised to saline isotype control (E). A representative IL-33 western blot is shown. For all data sets, bars indicate mean values and each data point represents a single mouse. n=6-8 per treatment group. Two-way ANOVA with Tukey's multiple comparisons test.

4.4.3.2. ST2-Fc administration increased ST2 levels in the BALF and lung tissue

IL-33 can complex with the decoy receptor sST2 (Sanada et al., 2007, Mildner et al., 2010). If present at high concentrations, sST2 may prevent detection of bound IL-33 by ELISA. To address this possibility, ST2 levels were measured in the BALF and lung tissue.

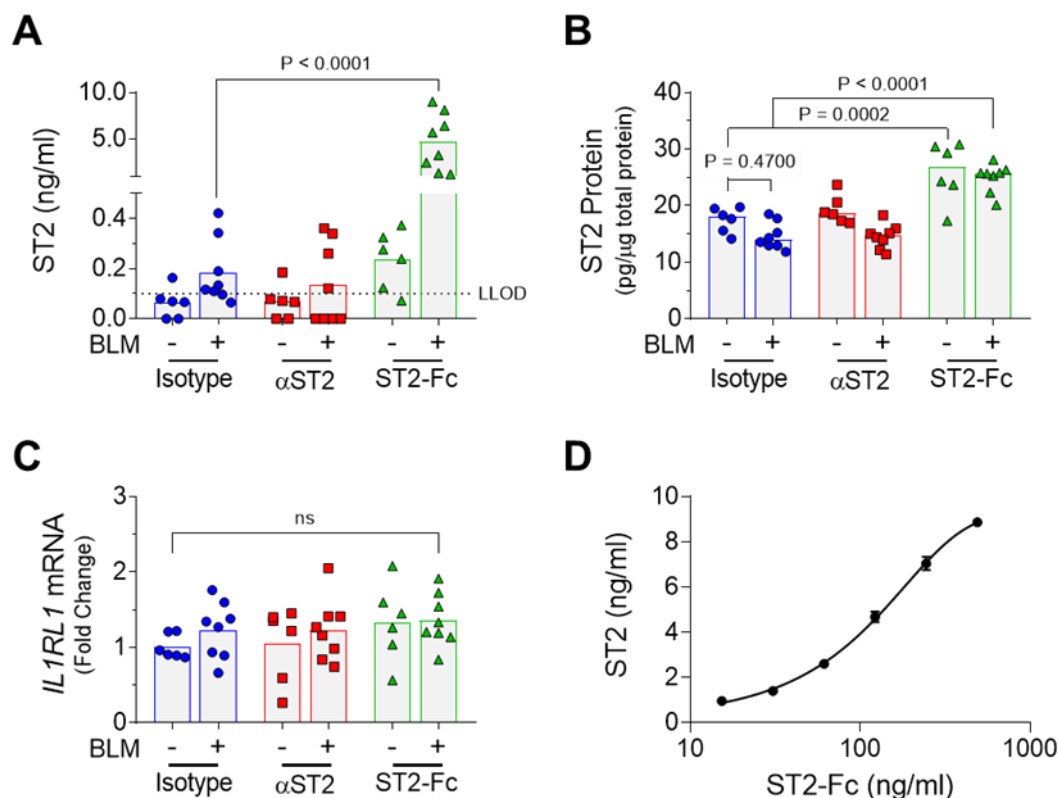


Figure 4.8: The effect of the α ST2 antibody and ST2-Fc fusion protein on ST2 expression.

The concentration of ST2 in the BALF (**A**) and lung tissue (**B**) of mice was measured by ELISA. *IL1RL1* (ST2) gene expression in the lungs was assessed by qPCR with all values normalised to saline isotype control (**C**). Cross-reactivity between the ST2-Fc fusion protein and the ST2 ELISA kit was determined (**D**). For Figures A-C, bars indicate mean values and each data point represents a single mouse. $n=6-8$ per treatment group. Two-way ANOVA with Tukey's multiple comparisons test. For Figure D, data is presented as mean $\pm$ SD.

28 days post BLM exposure, ST2 levels in the BALF of the majority of isotype control and α ST2 antibody treated mice were below the lower limit of detection of the ELISA kit used (Fig. 4.8A). Although ST2 was detectable in the lung homogenates of these

animals, BLM-induced lung injury and fibrosis had no effect on ST2 protein expression relative to saline control animals (Fig. 4.8B). In contrast, ST2 levels in the BALF and lung tissue of ST2-Fc treated mice were elevated post BLM treatment with significant increases evident versus isotype control (Fig. 4.8A & 4.8B). Interestingly, the same trend was observed in the BALF and lung tissue of saline ST2-Fc treated mice (Fig. 4.8A & 4.8B).

Since ST2 (*IL1RL1*) gene expression was unchanged across treatment groups (Fig. 4.8C), I hypothesised that cross-reactivity between the fusion protein and the ST2 ELISA kit capture antibody may explain the high levels of ST2 detected in the BALF and lung tissue of ST2-Fc treated mice. To address this possibility, the ST2-Fc fusion protein was serially diluted in PBS and analysed by ST2 ELISA. Following titration of the fusion protein, a concentration-dependent increase in ST2 was detected by ELISA (Fig. 4.8D). Due to this cross-reactivity, my results appear to be indicative of successful fusion protein delivery to the lungs rather than a biological effect of blocking IL-33 signalling.

4.4.3.3. The ST2-Fc fusion protein modulates BLM-induced inflammation

After establishing the effect of both the α ST2 antibody and ST2-Fc fusion protein on IL-33 and ST2 levels in the BALF and lung tissue, the effect of these biologics on BLM-induced inflammation was assessed. Since IL-33 stimulates type 2 cytokine release *in vivo* (Luzina et al., 2012, Fanny et al., 2018), the levels of IL-4 and IL-5 in the BALF were measured and the effects of the α ST2 antibody and ST2-Fc fusion protein determined.

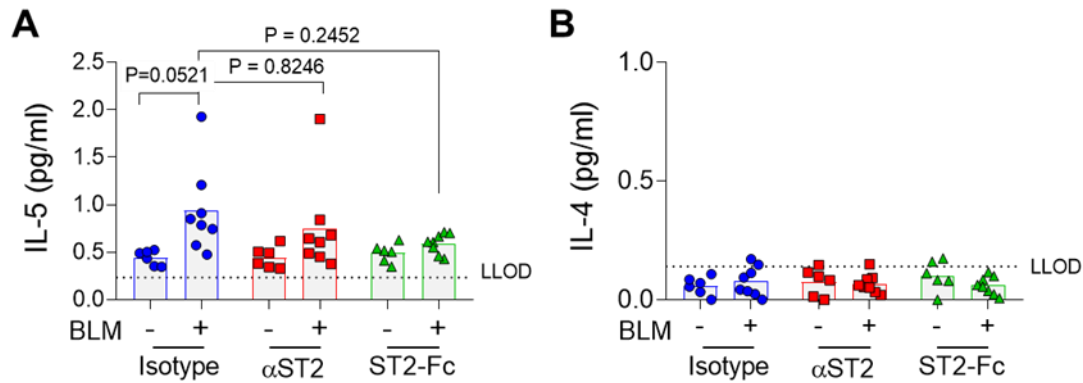


Figure 4.9: The effects of the α ST2 antibody and ST2-Fc fusion protein on type 2 cytokine release. The concentrations of IL-5 (**A**) and IL-4 (**B**) in the BALF were measured by MSD assay. For both cytokines, bars indicate mean values and each data point represents a single mouse. n=6-8 per treatment group. Two-way ANOVA with Tukey's multiple comparisons test.

Compared with saline control animals, exposure to BLM increased IL-5 levels in the BALF of mice treated with isotype control (Fig. 4.9A). Although treatment with the α ST2 antibody had no effect on IL-5 levels, a slight trend towards a reduction in IL-5 was observed for ST2-Fc treated mice relative to isotype control (Fig. 4.9A). IL-4 levels were below the lower limit of detection in all treatment groups (Fig. 4.9B).

In addition to inducing type 2 cytokine release *in vivo*, IL-33 promotes immune cell recruitment into the lungs following BLM treatment (Luzina et al., 2013, Li et al., 2014b, Fanny et al., 2018). To assess whether either biologic had blocked IL-33 signalling in my model, total and differential BAL cell counts were performed and the effects of the α ST2 antibody and ST2-Fc fusion protein on BLM-induced pulmonary inflammation determined.

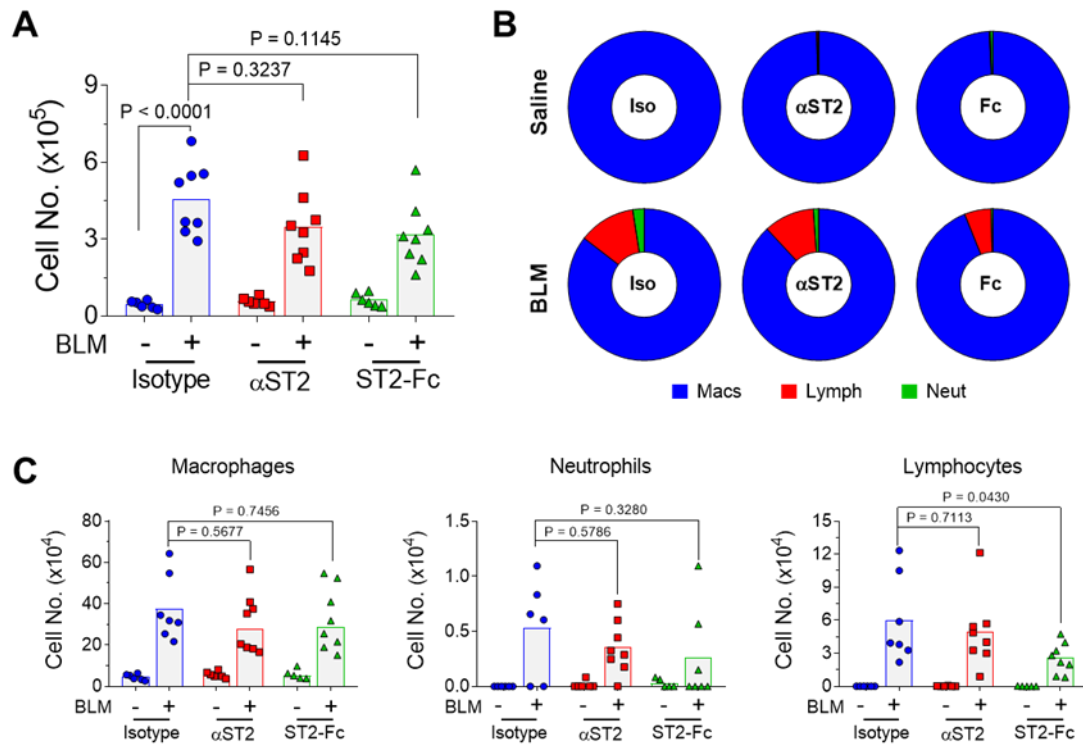


Figure 4.10: The effect of the α ST2 antibody and ST2-Fc fusion protein on BLM-induced pulmonary inflammation.

Total BAL cell counts were performed using a haemocytometer. Two-way ANOVA with Tukey's multiple comparisons test (**A**). Following Diff-quick staining, the percentage (**B**) and number (**C**) of macrophages, neutrophils and lymphocytes was calculated. One-way ANOVA with Tukey's multiple comparisons test. For all data sets, bars indicate mean values and each data point represents a single mouse. $n=6-8$ per treatment group.

Inflammation was apparent in the lungs of isotype control mice with a significant increase in total BAL cell number observed for BLM mice versus saline control (Fig. 4.10A). Compared with the isotype control, neither the α ST2 antibody nor the ST2-Fc fusion protein significantly reduced the total number of cells in the BAL post BLM exposure (Fig. 4.10A). Despite this however, therapeutic dosing with ST2-Fc led to significant reductions in both the percentage (Fig. 4.10B) and number of lymphocytes (Fig. 4.10C) relative to isotype control with a trend towards a reduction in the number of neutrophils (Fig. 4.10C). As the α ST2 antibody had no effect on the percentage (Fig. 4.10B) and number (Fig. 4.10C) of these cells post BLM treatment, these results

suggest that only the ST2-Fc fusion protein reduced BLM-induced pulmonary inflammation in my model.

4.5. Discussion

Using the BLM mouse model of pulmonary fibrosis, IL-33 has been proposed to act as a pro-fibrotic mediator during the development of IPF and has subsequently been suggested as a target for anti-fibrotic therapies. However, as all previous studies have blocked IL-33 signalling during the inflammatory, rather than the fibrotic phase of the BLM model, the true pro-fibrotic activity of IL-33 remains unclear. Here I show that blocking IL-33/ST2 during the fibrotic phase of the BLM mouse model increases *IL33* gene expression and reduces pulmonary inflammation. Interestingly, neither the α ST2 antibody nor the ST2-Fc fusion protein reduced established BLM-induced fibrosis. Taken together, these results suggest that, when signalling via ST2, IL-33 does not act as a substantial pro-fibrotic mediator in the BLM mouse model of pulmonary fibrosis.

Consistent with previous reports, BLM treatment resulted in reductions in body weight, increases in lung weight and increases in collagen deposition in the lungs versus saline control (Egger et al., 2013, Gilhodes et al., 2017, Barbayianni et al., 2018, Cowley et al., 2019). Importantly, a significant, ~2-fold increase in lung hydroxyproline content was also observed. Since hydroxyproline (arising from the post-translational modification of proline) is a major component of collagen (Campa et al., 1990, Cundy et al., 2014), these results indicate the successful induction of pulmonary fibrosis and demonstrate the suitability of my model to assess the anti-fibrotic activity of the α ST2 antibody and the ST2-Fc fusion protein.

Both the α ST2 antibody and the ST2-Fc fusion protein increased *IL33* gene expression in the lungs of BLM-treated mice. Although no such increase was observed at the protein level, potentially due to the time point when samples were taken and/or the inability to measure IL-33 protein levels in the BALF, this result suggests that both biologics had an effect on the IL-33:ST2 axis.

Consistent with reports by Saito et al. (2008) and Fanny et al. (2018), BLM induced pulmonary inflammation with increases in IL-5 and total BAL cell number evident versus saline control. Moreover, similar to previous studies using ST2 knockout mice (Li et al., 2014b, Fanny et al., 2018, Zhao et al., 2018), IL-33 neutralising antibodies (Li et al., 2014b) and soluble ST2 (Gao et al., 2016), treatment with the ST2-Fc fusion protein reduced the number of lymphocytes and neutrophils in the BAL of BLM treated mice. As adenoviral overexpression and intranasal delivery of IL-33 increases the numbers of these cells (Luzina et al., 2012, Luzina et al., 2013, Li et al., 2014b), my results suggest that IL-33 promoted BLM-induced inflammation in my model and imply that the fusion protein blocked IL-33 signalling *in vivo*.

In contrast with the fusion protein, treatment with the α ST2 antibody had no effect on BLM-induced inflammation. Given that the EC_{50} of the α ST2 antibody is approximately 5 fold greater than that of the fusion protein *in vitro* (personal communication from Dr Suzanne Cohen regarding historical AstraZeneca data), it is possible that 10 mg/kg of the α ST2 antibody was an insufficient dose to block all IL-33 signalling in my model. Combined with potential differences in absorption, distribution and/or excretion, this hypothesis could explain the failure of the α ST2 antibody to modulate pulmonary inflammation *in vivo*.

IL-33 promotes the M2 polarisation of macrophages and the generation and activation of ILC2s (Kurowska-Stolarska et al., 2009, Li et al., 2014b, Fanny et al., 2018, Vivier et al., 2018). Since inhibition of IL-33 signalling decreases the numbers of M2 macrophages and ILC2s in the lungs of BLM treated mice (Li et al., 2014b, Fanny et al., 2018, Zhao et al., 2018), reductions in the numbers of these cells could have been used as additional surrogate readouts of drug target engagement. Indeed, if I were to repeat the same study again, flow cytometric analysis similar to that used by Li et al. (2014b) and Fanny et al. (2018) would be performed on lung homogenates and the effects of the α ST2 antibody and ST2-Fc fusion protein on the numbers of M2 macrophages and ILC2s quantified.

In contrast to previous reports using ST2 knockout mice (Li et al., 2014b, Fanny et al., 2018, Zhao et al., 2018), IL-33 neutralising antibodies (Li et al., 2014b) and soluble ST2 (Gao et al., 2016), both the α ST2 antibody and the ST2-Fc fusion protein failed to reduce BLM-induced fibrosis. Due to my results regarding *IL33* gene expression and BLM-induced inflammation, I am confident that the α ST2 antibody and the ST2-Fc fusion protein had biological effects in my model and believe that the failure of both reagents to reduce established fibrosis cannot be explained by a lack of target engagement.

All previous studies have blocked the IL-33:ST2 axis prior to or during the inflammatory phase of the BLM mouse model (Li et al., 2014b, Gao et al., 2016, Fanny et al., 2018, Zhao et al., 2018). My use of IL-33/ST2 blocking reagents during the fibrotic phase of the BLM model (to assess the role of IL-33 signalling during established fibrosis rather than acute lung injury and inflammation) suggests that IL-33 does not act as a major pro-fibrotic mediator in this *in vivo* system. Indeed,

considering the pro-inflammatory nature of IL-33 (Liew et al., 2016, Cayrol and Girard, 2018, Chan et al., 2019), I strongly believe that the improvements in fibrosis reported by Li (2014b), Gao (2016), Fanny (2018) and Zhao et al. (2018) simply reflect a reduction in the inflammation required for the development of BLM-induced fibrosis rather than a true anti-fibrotic effect of targeting the IL-33:ST2 axis.

Despite making important contributions to IPF research, mouse models of pulmonary fibrosis (including the BLM model) have some limitations. Firstly, none of the mouse models currently used for IPF research and pre-clinical testing fully recapitulate the fibrotic response believed to occur in the lungs of IPF patients (Tashiro et al., 2017, Yanagihara et al., 2020). For example, in contrast to IPF where repeated AEC injury is thought to result in progressive fibrosis, many murine models of pulmonary fibrosis are driven by acute lung injury and inflammation (B Moore et al., 2013, Tashiro et al., 2017, Yanagihara et al., 2020). Moreover, the pattern of lung fibrosis observed in most mouse models is not representative of the subpleural fibrosis observed in IPF patients and has been reported to spontaneously resolve over time (Moeller et al., 2008, Degryse and Lawson, 2011, B Moore et al., 2013). Secondly, some of the mouse models used for IPF research (including silica, ionising radiation and transgenic models) are expensive, time consuming and technically challenging to perform (Moore and Hogaboam, 2008, Degryse and Lawson, 2011). Furthermore, the severity, persistence and pattern of fibrosis induced in such models can vary depending on the strain of mice used, the batch and dose of fibrosis-inducing agent and the route of administration (Degryse and Lawson, 2011, Tashiro et al., 2017). Thirdly, the results from some murine models of IPF can be heavily influenced by the study design used leading to potentially misleading conclusions (Moeller et al., 2008, Tashiro et al.,

2017, Yanagihara et al., 2020). Indeed, the continued use of prophylactic rather than therapeutic dosing in murine models of IPF has contributed (along with species-specific differences between humans and mice) to the high attrition rates of candidate anti-fibrotic therapies in patient clinical trials (Moeller et al., 2008, Yanagihara et al., 2020). Finally, species-specific differences between humans and mice mean that the findings from murine models of IPF may not translate fully to human disease (Degryse and Lawson, 2011, Tashiro et al., 2017, Yanagihara et al., 2020). For instance, differences in the pattern of IL-33 expression in human and mouse lungs (Liew et al., 2016, Cayrol and Girard, 2018) may lead to the importance of this cytokine in IPF being over or underestimated. To overcome this and some of the other limitations of IPF mouse models, complex, species-relevant culture systems such as human precision cut lung slices (PCLS) are increasingly being used for basic IPF research and pre-clinical testing (Liu et al., 2019a, Alsafadi et al., 2020).

In summary, despite having effects on *IL33* gene expression and pulmonary inflammation, blocking IL-33 signalling during the fibrotic phase of the BLM mouse model had no effect on established fibrosis. I therefore conclude that, when signalling via ST2, IL-33 does not act as a key pro-fibrotic mediator in the BLM mouse model of pulmonary fibrosis and speculate that IL-33 may not play a pathophysiological role during the development of IPF. However, given the known differences in IL-33 expression between humans and mice, further work with human precision cut lung slices (PCLS) is required to support this hypothesis and confirm whether targeting the IL-33:ST2 axis is likely to be of therapeutic benefit in IPF.

Chapter 5: Investigating the pro-fibrotic activity of IL-33 using human precision cut lung slices (PCLS)

5. Investigating the pro-fibrotic activity of IL-33 using human precision cut lung slices (PCLS)

5.1. Introduction

Despite a number of similarities, species-specific differences in the pattern of IL-33 expression exist between humans and mice (Liew et al., 2016, Cayrol and Girard, 2018). Consequently, my findings regarding IL-33 in the BLM-mouse model of pulmonary fibrosis may not translate fully to human disease. To address similar species-specific issues, methods to generate human precision cut lung slices (PCLS) have been developed (Alsafadi et al., 2017, Roach et al., 2018, Gerckens et al., 2019).

First described by Fisher et al. (1994), human PCLS are thin (150-500 μm), uniform tissue slices cut from agarose-inflated human lung tissue (Liu et al., 2019a). Despite having some limitations, such as lack of a circulatory system and recruited immune cells, human PCLS have several advantages compared with 2D cell monolayers and 3D co-culture, air liquid interface (ALI) and organ-on-a-chip systems. For example, preservation of native lung architecture allows natural interactions between cells, mediators and extracellular matrix to be studied *ex vivo*. Moreover, analysis of PCLS from patient lung tissue provides direct insight into disease relevant mechanisms (Liu et al., 2019a, Alsafadi et al., 2020). Consequently, human lung slices have emerged as valuable tools to study the pathogenesis of IPF and screen novel therapies. Indeed, using human PCLS, the importance of PI3K/AKT/mTOR signalling in the development of IPF has been demonstrated and the anti-fibrotic effects of Omipalisib

(a PI3K/mTOR inhibitor – see chapter 1 section 1.1.7) validated (Mercer et al., 2016). Furthermore, the pro-fibrotic effects of TGF β , LPA, PDGF-AB and TNF α have been highlighted (Alsafadi et al., 2017, Roach et al., 2018) and mechanistic differences between pirfenidone and nintedanib identified (Lehmann et al., 2018). As of yet however, the ability of IL-33 to induce fibrotic changes in human PCLS has not been explored.

5.2. Aims

To validate my *in vivo* findings in a complex species-relevant system the aims of this chapter were to:

- Develop a method to generate PCLS from non-diseased (normal adjacent) and diseased human lung tissue
- Determine the ability of IL-33 to induce fibrotic changes in human PCLS

5.3. Methods

Methods specific to this chapter are detailed below. For general methods including MSD assays, ELISAs, RNA extraction, reverse transcription, qPCR, protein extraction and western blotting, please refer to the relevant sections in chapter 2. For details of all the materials, kits, antibodies, qPCR primers, media and buffer recipes used in this chapter, please refer to the appendix.

5.3.1. Human lung tissue

Normal adjacent lung tissue (NAT) from cancer resection surgeries and IPF, COPD and fibrosing organising pneumonia (FOP) lung tissue from transplant surgeries was

received from the Royal Papworth Hospital Research Tissue Bank. Written consent was obtained from all patients with studies approved by the NRES Committee of East England. Post-surgery, tissue was stored in AQIX[®] medium at 4°C with all samples received and processed (i.e. sliced or homogenised for protein) within 24 hours.

5.3.2. Generation and maintenance of human PCLS

Using a 21g needle, lung tissue was inflated with 20-40 ml (depending on size) of 3% low melting point agarose. Once firm to the touch, samples were incubated on ice and all agarose allowed to set at 4°C for 60 minutes. Inflated tissue was then cut into 1 cm thick sections and 8 mm ‘cores’ generated using disposable skin biopsy punches. Using a stainless steel histology base mould, the resulting cores were set into blocks of 3% low melting point agarose (maximum of 6 cores/block) before being attached to the mounting stage of a Leica VT1200 S vibratome using 3M Vetbond Tissue Adhesive. Once adhered, the agarose-tissue block was submerged in ice-cold PBS and 450 µm-thick PCLS cut at a speed of 0.3 mm/s and an amplitude of 2 mm. Following the removal of excess agarose, slices were transferred into 24 well plates at a density of 1 PCLS/well (Fig. 5.1). Slices were cultured in 500 µl/well of supplemented Small Airway Epithelial Cell Media (see appendix) and maintained at 37°C + 5% CO₂. For all PCLS experiments, all media was collected and replaced every 24 hours with the resulting supernatants centrifuged at 13,000 x g for 5 minutes and stored at -80°C for future analysis. Following stimulation, PCLS were collected at the desired time points and processed for biochemical analysis.

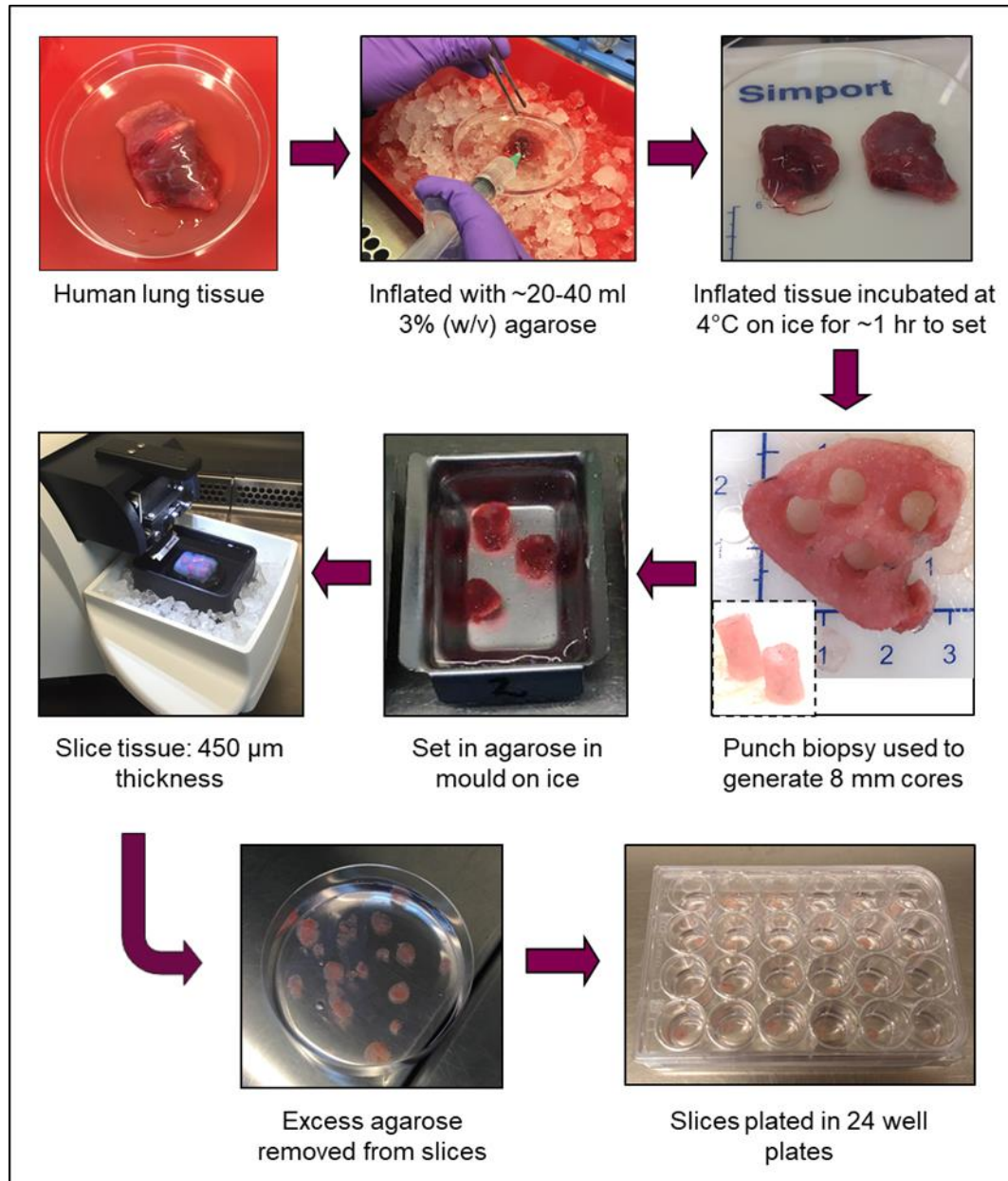


Figure 5.1: Generation of human PCLS.

Human lung tissue was inflated with approximately 20-40 ml of 3% low melting point agarose. After incubating at 4°C on ice for 1 hour, inflated tissue was cut into 1 cm thick sections and 8 mm cores generated. Using a stainless steel histology base mould, the resulting cores were set into blocks of 3% low melting point agarose. After attaching these to the mounting stage of a Leica VT1200 S vibratome, 450 µm thick PCLS were generated. Following the removal of excess agarose, the resulting slices were plated into 24 well plates and cultured at 37°C + 5% CO₂ in supplemented small airway epithelial cell media.

5.3.3. Processing of PCLS for biochemical analysis

5.3.3.1. Processing of PCLS for RNA

Each PCLS was washed once with 500 µl of sterile PBS. For each treatment condition, 3 slices were pooled into a single 1.5 ml Eppendorf tube containing 1 ml of RNAlater[®]. Following 24 hours at 4°C, all samples were removed from RNAlater[®], snap frozen using dry ice and stored at -80°C for future use.

5.3.3.2. Processing of PCLS for protein

Each PCLS was washed once with 500 µl of sterile PBS. Unless stated otherwise, 3 slices per treatment condition were pooled into a single 7 ml Bijou tube and 150 µl of ice-cold tissue lysis buffer (see appendix) added. All samples were snap frozen using dry ice and stored at -80°C for future use.

5.3.4. Lactate dehydrogenase (LDH) assay

5.3.4.1. Principle

The cytoplasmic enzyme lactate dehydrogenase (LDH) catalyses the oxidation of lactate and the reduction of NAD⁺. Resulting in pyruvate and NADH respectively, LDH is released from cells following death and is used as a readout of cell/tissue viability (Cummings and Schnellmann, 2004).

In the LDH assay, LDH in cell/tissue supernatants reacts with LDH detection reagent (consisting of lactate, NAD⁺, reductase, reductase substrate and Ultra-Glo[™] rLuciferase) resulting in a luminescent signal (Fig. 5.2). Luminescence is proportional

to the amount of LDH in each sample with LDH concentrations extrapolated from a LDH standard curve (Promega, 2018).

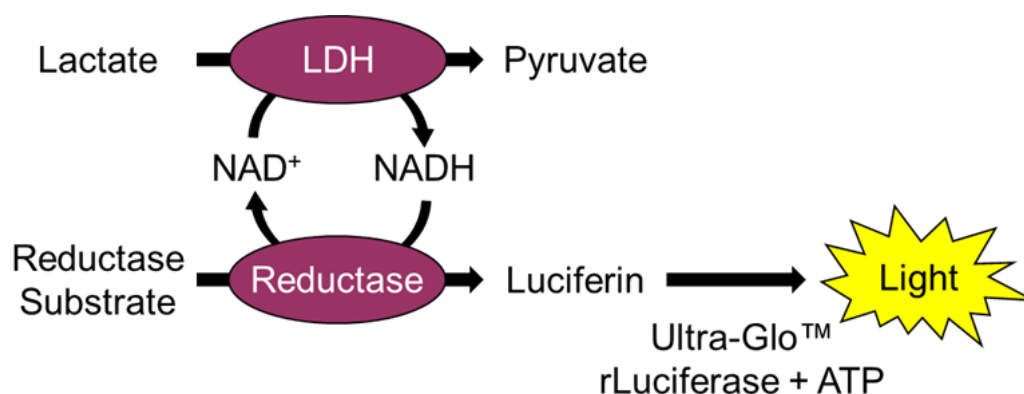


Figure 5.2: Principle of the LDH assay.

LDH catalyses the conversion of lactate and NAD^+ to pyruvate and NADH respectively. Using NADH, reductase generates luciferin from reductase substrate. Luciferin is converted into a bioluminescent (light) signal by Ultra-Glo™ rLuciferase and ATP. Figure adapted from Promega (2018).

5.3.4.2. Method

LDH release from cultured PCLS was measured using the LDH-Glo™ Cytotoxicity Assay according to the manufacturer's instructions. Briefly, supernatants were thawed at room temperature and diluted 1:10 in LDH assay buffer (see appendix). To calculate the concentration of LDH in each sample, purified LDH was reconstituted in 275 μl of LDH assay buffer (to give a stock concentration of 1000 U/ml) and a 7-point, 1:2 serial dilution (ranging from 32-0.5 mU/ml) performed. 50 μl /well of sample or standard was transferred into white 96 well clear-bottomed plates in duplicate before 50 μl /well of LDH detection reagent was added. After 60 minutes at room temperature, luminescence was measured using a Gen5 Cytation plate reader and the concentration of LDH in each sample extrapolated from the standard curve using GraphPad Prism 8.

5.4. Results

A method to generate human PCLS was developed based on previous literature (Gerckens et al., 2019) and advice from collaborators (see section 5.3.2). To optimise the developed technique, tissue slices were prepared from non-diseased (normal adjacent (NAT)) and diseased lung tissue and cultured for 7 days. Supernatants were collected every 24 hours and the effects of slicing and prolonged culture on PCLS viability and cytokine release assessed over time (Fig. 5.3). Methods to extract RNA and protein from cultured slices were tested after 7 days (Fig. 5.3).

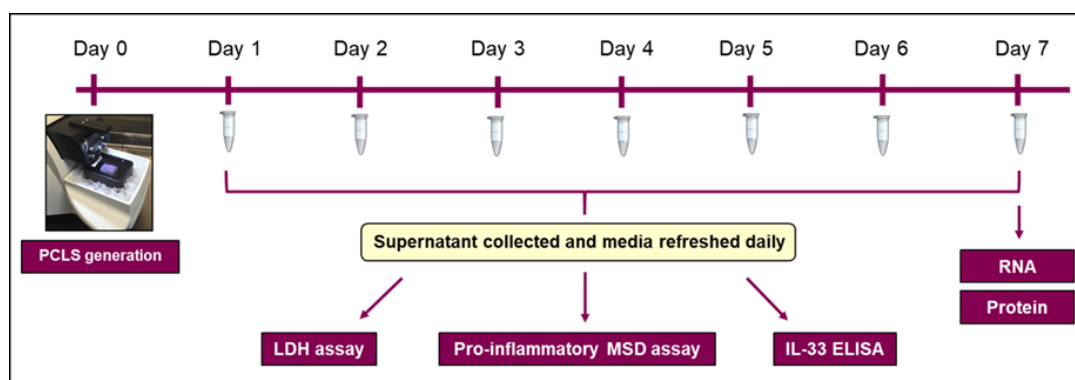


Figure 5.3: Overview of PCLS optimisation experiments.

PCLS were generated using the method detailed in section 5.3.2 and cultured for 7 days at 37°C + 5% CO₂. All media was collected and replaced every 24 hours and the release of LDH (section 5.4.1), pro-inflammatory cytokines (section 5.4.2) and IL-33 (section 5.4.3) measured. 7 days post slicing, slices were pooled for either RNA (section 5.4.4) or protein (section 5.4.5).

5.4.1. PCLS remain viable for at least 7 days

LDH release occurs following cell death (Cummings and Schnellmann, 2004). To determine the effect of prolonged culture on PCLS viability, LDH release was measured over time (Fig 5.3).

LDH levels peaked at day 1 indicating that tissue slicing resulted in a degree of damage-induced cell death (Fig. 5.4A). Although variability was evident between slices and donors, LDH release from non-diseased (NAT) and diseased (IPF, FOP and COPD) PCLS decreased over time (Fig. 5.4A). Indeed, compared to levels at day 1, a significant reduction in LDH was observed at day 7 for all donors (Fig. 5.4B). Taken together, these results suggest that slices can be cultured for up to 7 days with no major negative effects on tissue viability.

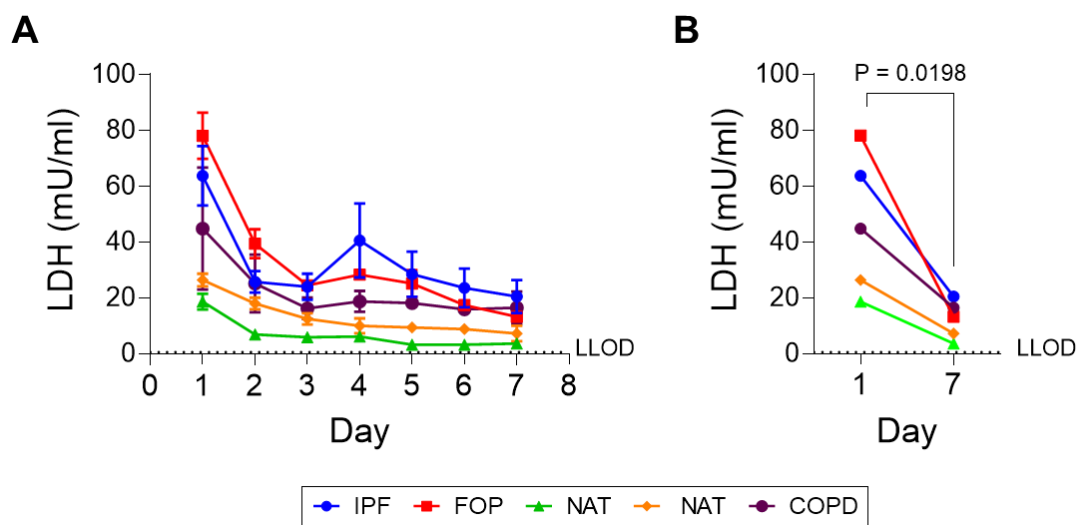


Figure 5.4: LDH release by cultured PCLS over time.

LDH release from 3 slices per donor was assessed over time (A). Mean $\pm$ SD. n=5 individual donors. LDH release at day 1 versus day 7 (B). Each data point represents mean LDH release from 3 PCLS per donor. n=5 independent donors Paired t-test.

5.4.2. Tissue slicing induces pro-inflammatory cytokine release from PCLS

Tissue slicing induces the release of multiple pro-inflammatory cytokines from human PCLS (Alsafadi et al., 2020). Since this initial ‘cytokine storm’ can alter the responses of slices to exogenous pro-inflammatory stimuli, PCLS are often ‘rested’ prior to treatment with agents such as lipopolysaccharide (LPS) (Temann et al., 2017, Alsafadi

et al., 2020). To establish whether this was necessary in my model post-slicing, pro-inflammatory cytokine release from non-diseased and diseased PCLS was assessed over time (Fig. 5.3).

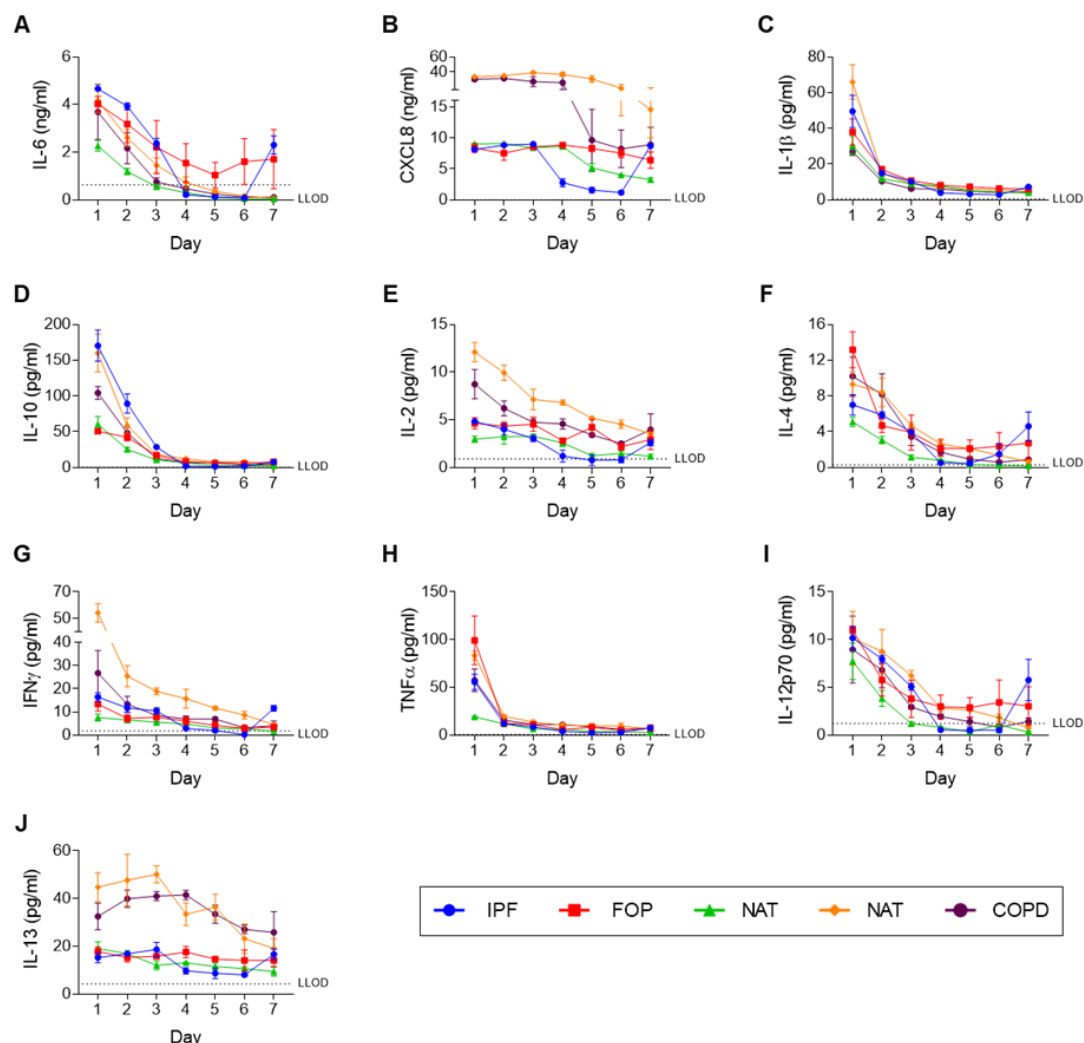


Figure 5.5: Pro-inflammatory cytokine release by PCLS over time.

IL-6 (A), CXCL8 (B), IL-1 β (C), IL-10 (D), IL-2 (E), IL-4 (F), IFN γ (G), TNF α (H), IL-12p70 (I) and IL-13 (J) release from 3 slices per donor was assessed over time. Mean $\pm$ SD. n=5 independent donors.

Although variability was evident between donors, slicing induced pro-inflammatory cytokine release from all PCLS (Fig. 5.5). With the exception of CXCL8 (Fig. 5.5B) and IL-13 (Fig. 5.5J), cytokine levels decreased over the 7 day time course (Fig. 5.5). Indeed, compared to day 1, significant reductions in the concentrations of IL-6, IL-1 β ,

IL-10, IL-2, IL-4, TNF α and IL-12p70 (Fig. 5.6A, 5.6C, 5.6D, 5.6E, 5.6F, 5.6H & 5.6I) were observed at day 4. As a similar trend was recorded for IFN γ (Fig. 5.6G), these results suggest that (once generated) slices should be ‘rested’ for 96 hours prior to treatment with pro-inflammatory stimuli.

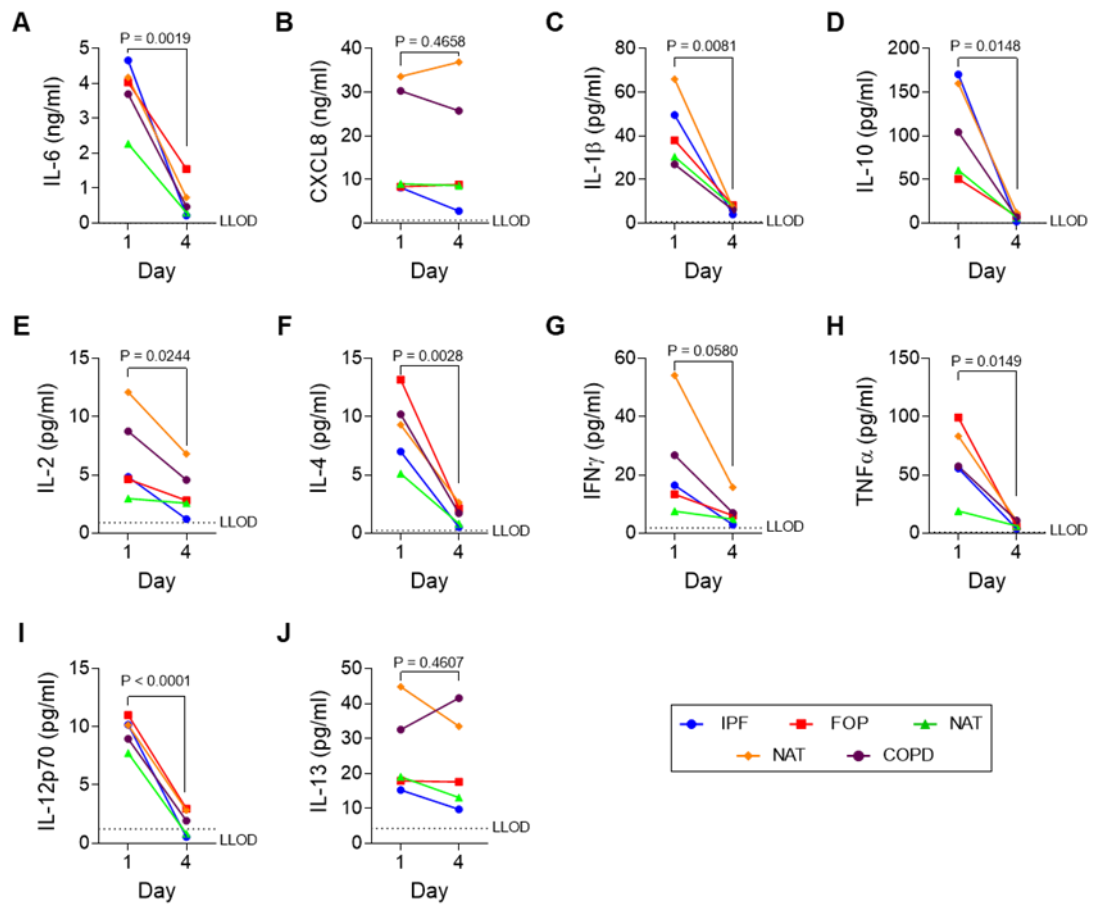


Figure 5.6: Comparing pro-inflammatory cytokine release from PCLS at days 1 and 4.

IL-6 (A), CXCL8 (B), IL-1 β (C), IL-10 (D), IL-2 (E), IL-4 (F), IFN γ (G), TNF α (H), IL-12p70 (I) and IL-13 (J) release at day 1 versus day 4. Each data point represents mean cytokine release from 3 PCLS per donor. n=5 independent donors Paired t-test.

5.4.3. Slicing induces IL-33 release from PCLS

IL-33 is expressed in human lung tissue and is released following cell damage/death (Lüthi et al., 2009, Le Goffic et al., 2011, Rickard et al., 2014). Due to this, it may be difficult to differentiate the effects of recombinant and endogenous IL-33 on PCLS post slicing. To avoid this possibility, IL-33 release was measured over time (Fig. 5.3) and the optimal window to stimulate slices with recombinant IL-33 determined.

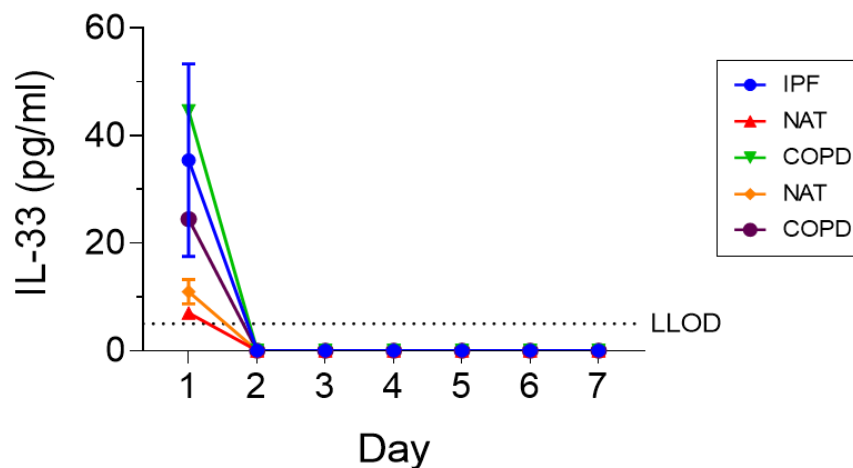


Figure 5.7: IL-33 release by non-diseased and diseased PCLS over time.

IL-33 release from 2 PCLS per donor was assessed over time. Mean $\pm$ SD. n=5 independent donors.

For both non-diseased and diseased slices, low levels of IL-33 were detected in supernatants collected at day 1 (Fig. 5.7) with COPD and IPF slices releasing more IL-33 than NAT ones (Fig. 5.7). Taken together, these results imply that slicing induces IL-33 release from both non-diseased and diseased PCLS.

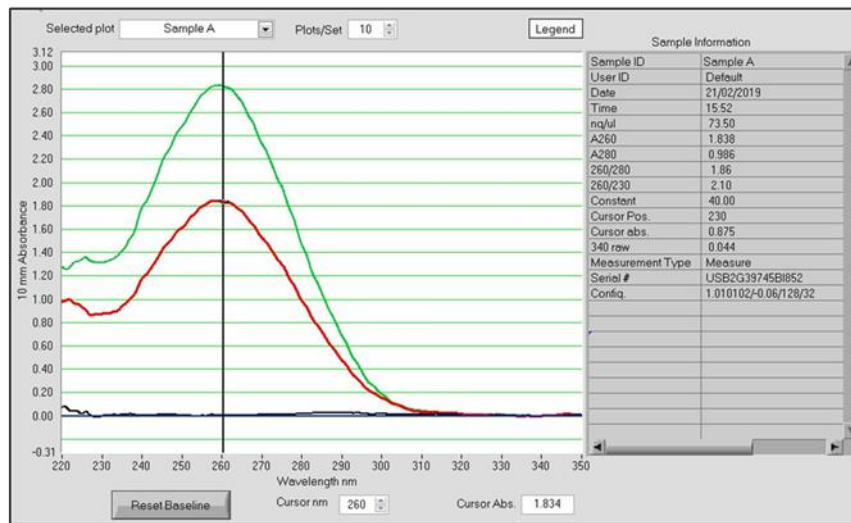
From day 2 onwards, IL-33 could not be detected in supernatants collected from either non-diseased or diseased lung slices (Fig. 5.7). Although these data suggest that slices can be stimulated with recombinant IL-33 from day 2, it is important to note that the IL-33 ELISA kit used in these experiments is not particularly sensitive (Scott et al.,

2018b). Since other pro-inflammatory cytokines were released 2-3 days post slicing (Fig. 5.5), it was possible that the same was true for IL-33. Consequently, it was concluded that the optimal window to stimulate PCLS with recombinant IL-33 was 4-6 days post slicing.

5.4.4. RNA can be extracted from cultured PCLS

Since I wanted to determine the effects of IL-33 on pro-fibrotic gene expression by PCLS, it was important to establish if RNA could be extracted from cultured slices. Moreover, given the relatively small size of the slices generated, it was necessary to assess whether pooling 3 PCLS would result in a sufficient concentration of RNA to measure the expression of multiple genes by qPCR. After culturing for 7 days (Fig. 5.3), 3 lung slices per sample were pooled and RNA extracted using the protocol detailed in chapter 2 section 2.3.3.2. To assess RNA integrity and yield, 2 μ l of each sample was analysed using a Nanodrop spectrophotometer.

UV absorbance spectra indicative of nucleic acid were obtained (Fig. 5.8A). Importantly, the 260/280 ratios calculated for NAT and IPF samples fell between 1.85 and 2 (Fig. 5.8B). Moreover, all 260/230 ratios were greater than 2 (Fig 5.8B). Taken together, these ratios suggest that pure nucleic acid can be extracted from cultured PCLS. Although further analysis (by agarose gel or bioanalyzer) is required to confirm the type of nucleic acid extracted, it is important to note that DNase I was used during the extraction process. As 15 minute incubation with this enzyme has been shown to digest all contaminating DNA (Zymo-Research, 2017), it is highly likely that the nucleic acid extracted from my PCLS is RNA.

A**B**

Tissue type	Sample	Conc. (ng/μl)	Total RNA (ng)	260/280	260/230
NAT	A	73.50	1690.50	1.86	2.10
	B	113.14	2602.22	1.92	2.15
IPF	A	90.27	2076.21	1.97	2.21
	B	62.75	1443.25	1.97	2.13
NAT	A	41.91	963.93	1.91	2.07
	B	24.88	572.24	1.92	2.07

Figure 5.8: RNA extraction from cultured PCLS.

Representative UV absorbance spectra of 2 RNA samples from a single non-diseased (NAT) donor (**A**). The quantity and purity of RNA extracted from cultured non-diseased (NAT) and IPF PCLS. 2 RNA samples from each donor are shown (**B**). n=3 independent donors.

Pooling 3 slices resulted in approximately 550-2600 ng of RNA (Fig. 5.8B). Since future qPCR experiments required a minimum of 200 ng per sample, it was concluded that 3 slices per treatment condition should be combined for a single RNA sample.

5.4.5. Protein can be extracted from cultured PCLS

To determine if protein could be extracted from cultured PCLS, slices from normal adjacent lung tissue were generated. After culturing for 7 days (Fig. 5.3), 2 or 3 slices

were pooled and lysates generated according to chapter 2 section 2.7.4. Since the resulting samples were detected by BCA assay (Fig. 5.9A & 5.9B), it demonstrates that protein can be extracted from cultured PCLS.

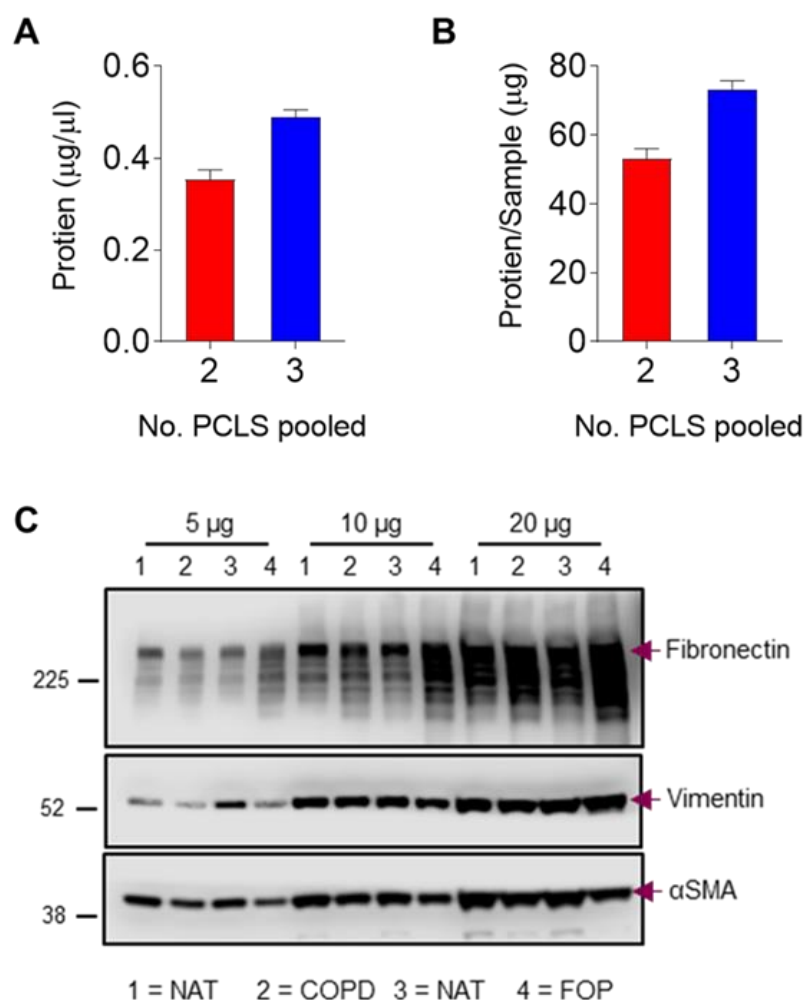


Figure 5.9: Protein extraction from cultured PCLS.

Following 7 day culture, 2 or 3 PCLS from a single normal adjacent donor were pooled. The concentration of protein per µl (**A**) and the amount of protein per sample (**B**) was determined by BCA assay. Protein lysates were generated from normal adjacent (NAT), fibrosing organising pneumonia (FOP) and COPD lung tissue. Increasing amounts of total protein were separated by SDS-PAGE with fibronectin, vimentin and αSMA detected by western blot (**C**).

To establish the minimum amount of protein needed to assess the effects of IL-33 on fibrotic protein expression by PCLS, lysates from non-diseased and diseased lung tissue were generated (see chapter 2 section 2.7.3). After separating increasing

amounts of protein by SDS-PAGE, fibronectin, vimentin and α SMA were detected by western blot (Fig. 5.9C).

Although fibronectin was detected when 5 and 20 μ g of protein was loaded, optimal signal resolution was observed at 10 μ g (Fig. 5.9C). As the same was true for both vimentin and α SMA (Fig. 5.9C), it was concluded that 30 μ g of protein per treatment condition was required for future PCLS experiments. Despite approximately 50 μ g of protein being extracted from 2 PCLS (Fig. 5.9B), it was decided that 3 slices should be pooled per treatment condition to allow for donor to donor variability and the assessment of additional fibrotic proteins if required.

5.4.6. Cultured PCLS secrete cytokines in response to exogenous pro-inflammatory stimuli

After establishing that RNA and protein could be extracted from cultured PCLS, the ability of cultured PCLS to respond to exogenous pro-inflammatory stimuli was assessed. Since LPS has previously been shown to induce TNF α and IL-6 release from human lung slices (Temann et al., 2017), ‘rested’ PCLS were stimulated with 1 μ g/ml of LPS and the release of TNF α and IL-6 measured by MSD assay (Fig. 5.10A).

Although not significant due to variability between donors, LPS treatment increased TNF α (Fig. 5.10B) and IL-6 (Fig. 5.10C) release relative to unstimulated control. Considered together, these results demonstrate that non-diseased and diseased slices can respond to exogenous pro-inflammatory stimuli.

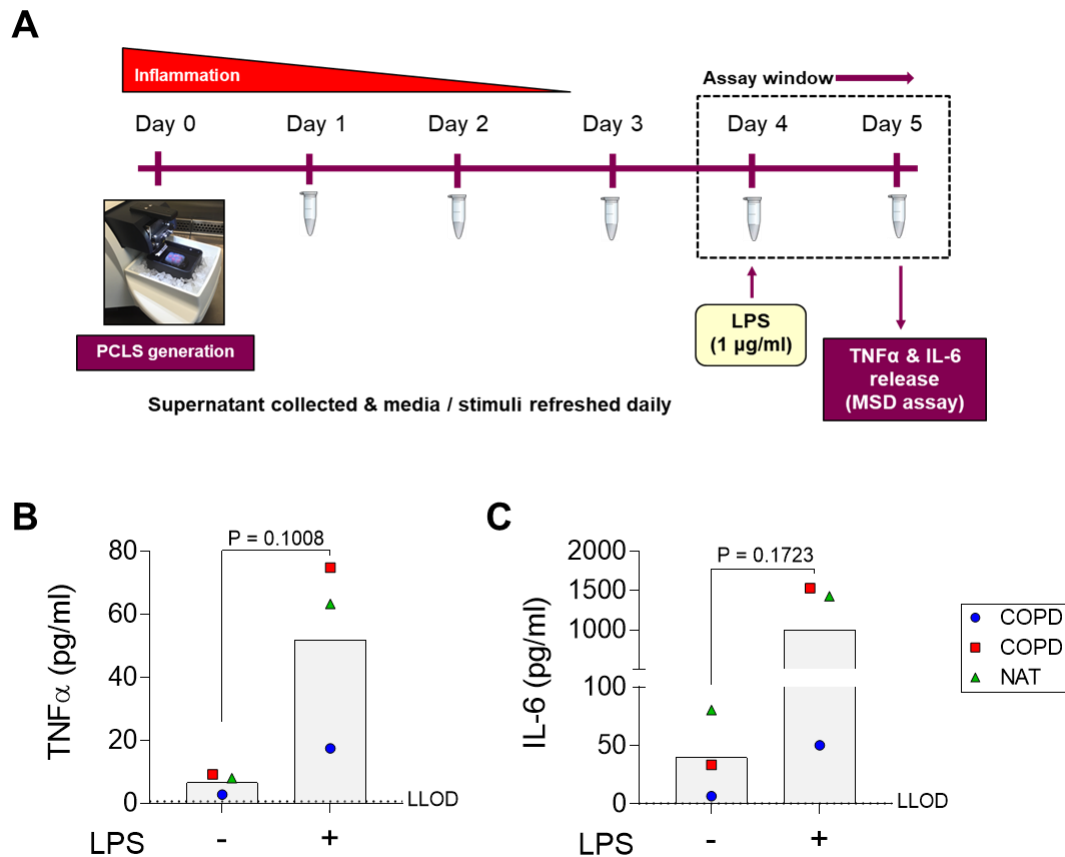


Figure 5.10: The response of PCLS to LPS.

PCLS were generated from normal adjacent (NAT) and COPD lung tissue. After resting for 96 hours, slices were stimulated with 1 µg/ml of LPS. 24 hours post stimulation, supernatants were collected and TNFα and IL-6 release measured by MSD assay (A). TNFα (B) and IL-6 (C) release from unstimulated and LPS treated PCLS. Bars indicate mean cytokine release from unstimulated and LPS treated PCLS. Each data point represents mean cytokine release from 2 PCLS per donor. n=3 independent donors. Paired t-test.

5.4.7. Cultured PCLS secrete cytokines in response to recombinant IL-33

Before establishing whether IL-33 induces fibrotic changes in human PCLS, the ability of slices to respond to this cytokine was assessed. Specifically, ‘rested’ PCLS were stimulated with 30 ng/ml of recombinant IL-33 and the release of IL-6 and CXCL8 after 24 hours measured by MSD assay (Fig. 5.11A).

IL-33 consistently induced IL-6 (Fig. 5.11B) and CXCL8 (Fig. 5.11C) release from non-diseased and diseased PCLS. Indeed, compared to unstimulated control,

significant increases in the concentrations of IL-6 (Fig. 5.11B) and CXCL8 (Fig. 5.11C) were observed following IL-33 treatment. Taken together, these results demonstrate that both non-diseased and diseased PCLS can respond to 30 ng/ml of recombinant IL-33.

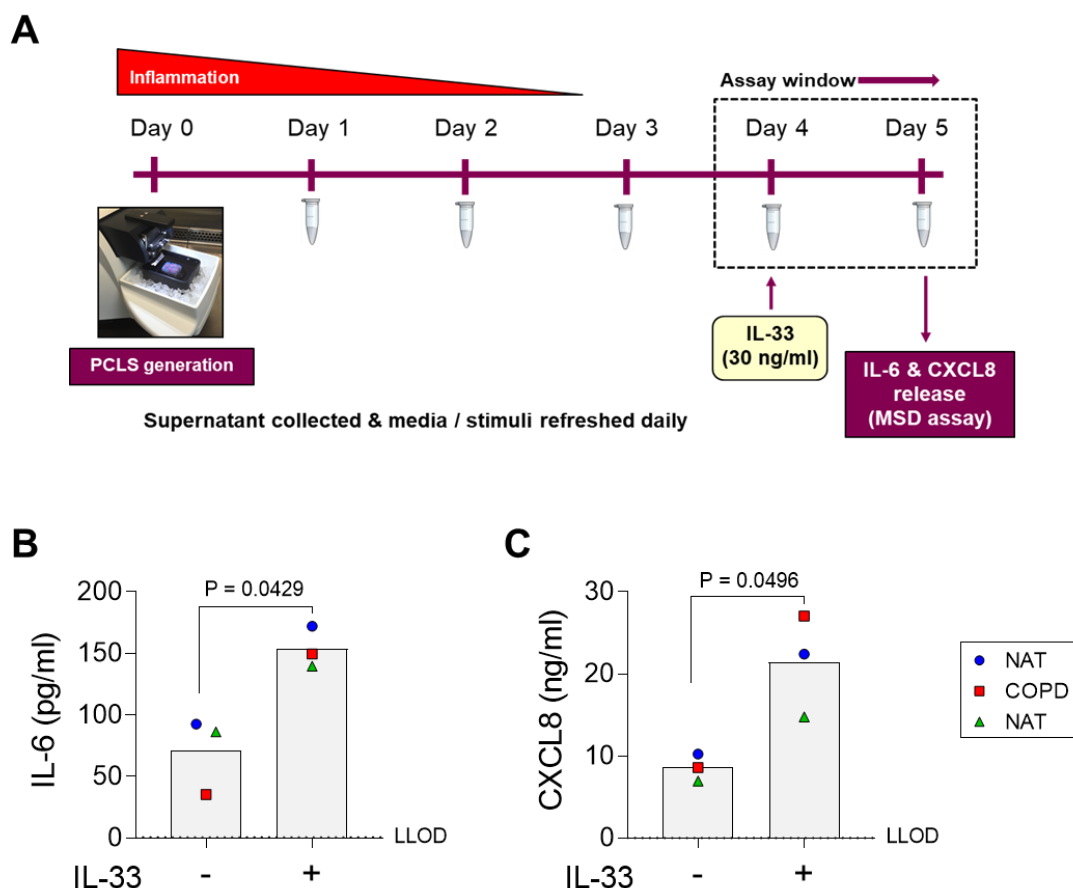


Figure 5.11: The response of PCLS to recombinant IL-33.

PCLS were generated from normal adjacent (NAT) and COPD lung tissue. After resting for 96 hours, slices were stimulated with 30 ng/ml of recombinant IL-33. 24 hours post stimulation, supernatants were collected and IL-6 and CXCL8 release measured by MSD assay (A). IL-6 (B) and CXCL8 (C) release from unstimulated and IL-33 treated PCLS. Bars indicate mean cytokine release from unstimulated and IL-33 treated PCLS. Each data point represents mean cytokine release from 2 PCLS per donor. n=3 independent donors. Paired t-test.

5.4.8. IL-33 appears to have no effect on fibrotic gene and protein expression by PCLS

Based on the results from previous experiments (Fig. 5.4 – 5.11), it was concluded that viable and responsive PCLS could be generated from non-diseased and diseased human lung tissue. To assess the pro-fibrotic activity of IL-33 in this model system, PCLS were generated from normal adjacent (NAT) and IPF lung tissue. After resting for 96 hours, slices were stimulated daily with 2 ng/ml of TGF β or 30 ng/ml of IL-33. 7 days post-slicing, RNA and protein were extracted and fibrotic changes assessed by qPCR and western blotting (Fig. 5.12).

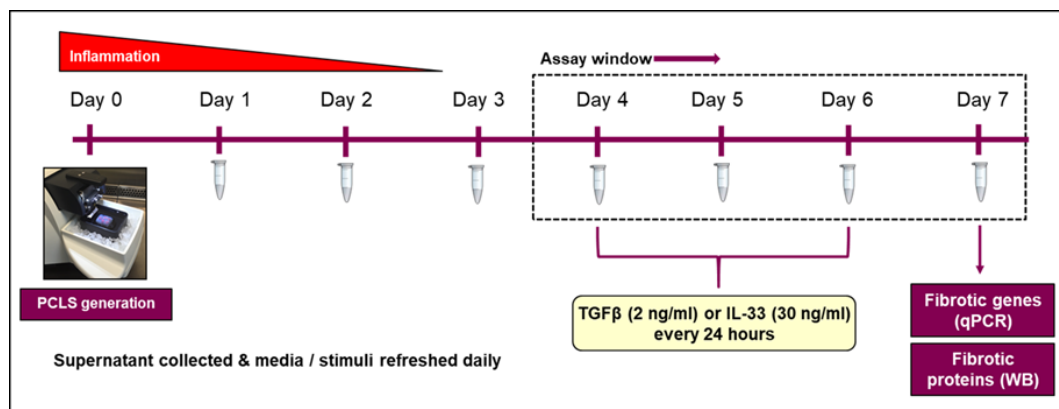


Figure 5.12: Assessing the effect of IL-33 on fibrotic gene and protein expression by human PCLS. PCLS were generated from normal adjacent (NAT) and IPF lung tissue. After resting for 96 hours, slices were stimulated with 2 ng/ml of TGF β or 30 ng/ml of IL-33 every 24 hours. 7 days post slicing, RNA and protein were extracted and the expression of fibrotic genes and proteins assessed by qPCR and western blotting respectively.

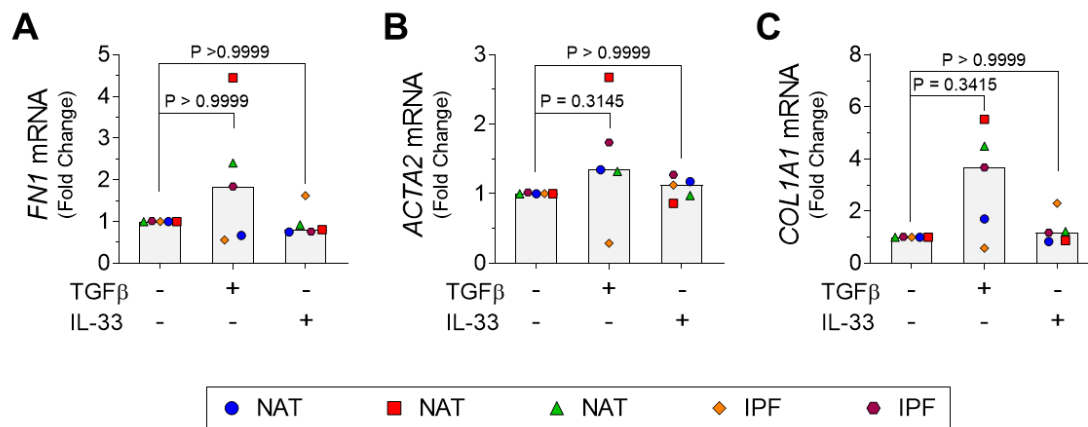


Figure 5.13: The effect of recombinant IL-33 on fibrotic gene expression by PCLS.

FN1 (A), *ACTA2* (B) and *COL1A1* (C) mRNA expression by NAT and IPF PCLS was measured by qPCR and normalised relative to unstimulated control. Bars represent median values and each data point represents an independent donor. n=3. Friedman test with Dunn's multiple comparisons test.

For the majority of donors, TGFβ increased *FN1* (Fig. 5.13A), *ACTA2* (Fig. 5.13B) and *COL1A1* (Fig. 5.13C) levels relative to unstimulated control. In contrast, IL-33 had no effect on the expression of these genes by NAT and IPF PCLS. Since an identical trend was observed for fibronectin at the protein level (Fig. 5.14A & 5.14B), these results suggest that IL-33 does not induce fibrotic changes in human PCLS.

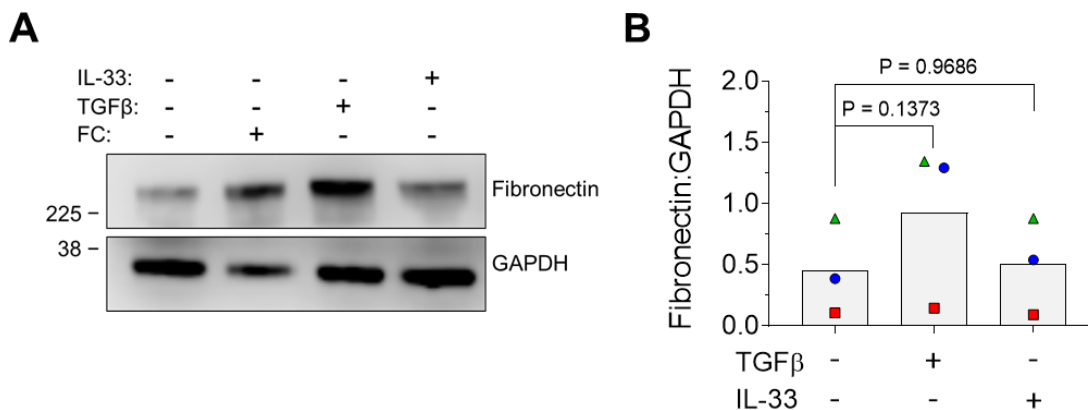


Figure 5.14: The effect of recombinant IL-33 on fibronectin expression by PCLS.

10 µg/lane of total protein was separated by SDS-PAGE. Fibronectin expression by TGFβ and IL-33 treated PCLS was assessed by western blot (A) and quantified relative to GAPDH via densitometry (B). In initial experiments assessing fibrotic changes in human PCLS, a fibrotic cocktail (FC) consisting of 5 ng/ml of TGFβ, 10 ng/ml of TNFα, 5 µM of PDGF-AB and 5 µM LPA was used as an additional positive control alongside TGFβ. As TGFβ treatment alone was sufficient to increase fibronectin expression by NAT PCLS, the FC was not used in further PCLS experiments. Consequently, the FC condition was excluded from densitometric analysis and is only shown in Fig. 5.14A to avoid excessive cropping of the western blot image. A representative western blot from a single donor is shown. Bars represent median values and each data point represents an independent donor. n=3. One-way repeated measures ANOVA with Tukey's multiple comparisons test.

5.5. Discussion

Due to species-specific differences in the pattern of IL-33 expression, my findings from the BLM mouse model of pulmonary fibrosis may not translate fully to human IPF. To investigate this possibility, a method to generate human PCLS was developed and the pro-fibrotic activity of IL-33 assessed. Here I show that, in contrast to TGF β , IL-33 treatment had no apparent effect on fibrotic gene and protein expression by human PCLS. Despite some limitations, the data presented in this chapter implies that IL-33 is unable to induce fibrotic changes in human PCLS. Taken together with my previous *in vitro* (chapter 3) and *in vivo* (chapter 4) findings, this result suggests that the IL-33:ST2 axis is unlikely to play a key pathophysiological role in IPF.

Following advice from collaborators, I cultured my PCLS in small airway epithelial cell media. As DMEM and DMEM-F12 are more commonly used (Alsafadi et al., 2017, Temann et al., 2017, Roach et al., 2018), it was necessary to assess the viability of my cultured slices. After initial slicing induced cell damage/death, the slices seemed to recover and LDH release from my PCLS decreased over time. Despite differences in the media and viability readouts used, my results regarding PCLS viability are highly consistent with those previously published (Alsafadi et al., 2017, Temann et al., 2017, Roach et al., 2018). Although possible that some cell types may persist for longer in my cultured slices than others (due to potential, cell-type specific differences in viability over time), overall my LDH data suggests that PCLS can be cultured in small airway epithelial cell media without significant negative effects on tissue viability over time.

Consistent with unpublished data from Borthwick et al. (personal communication), pro-inflammatory cytokine release from PCLS decreased over time. In addition to demonstrating the importance of resting my slices prior to stimulation with exogenous pro-inflammatory stimuli, these results imply that necrotic cell death does not occur following prolonged culture.

Since IL-33 release occurs following cell damage and death (Lüthi et al., 2009, Le Goffic et al., 2011, Rickard et al., 2014), it was not surprising that IL-33 was detected in supernatants 1 day post tissue slicing. Although further work with more sensitive IL-33 assays is required to confirm my results, the fact that no IL-33 could be detected from day 2 onwards suggests that (in agreement with my LDH data) significant cell death does not occur in my model over time.

In line with previous reports (Temann et al., 2017), LPS treatment induced TNF α and IL-6 release from non-diseased and diseased PCLS. Since IL-33 stimulation drove IL-6 and CXCL8 release, these results demonstrate the ability of my slices to respond to exogenous stimuli. Due to this (and all previous optimisation data), it was concluded that my lung slice model could be used to assess whether IL-33 induces fibrotic changes in human PCLS.

Consistent with Roach et al.'s (2018) findings, TGF β increased fibrotic gene and protein expression by human PCLS. Despite failing to achieve statistical significance (most likely due to donor variability and small sample size), these results demonstrate the ability of my slices to respond to pro-fibrotic stimuli.

In contrast with TGF β , IL-33 stimulation had no effect on *ACTA2*, *COL1A1*, *FN1* and fibronectin expression by human PCLS. Despite implying that this cytokine is unlikely

to promote pulmonary fibrosis, it is important to note that IL-33 was used at a single concentration in all experiments and that the treatment period used was relatively short (only 3 days). Although 30 ng/ml resulted in IL-6 and CXCL8 release, it is possible that a higher concentration of IL-33 and/or more chronic treatment is needed to induce fibrotic changes in human PCLS. To test this hypothesis and confirm my results in additional donors, dose response and time course experiments are desirable.

Due to COVID-19 related time constraints, the effects of IL-33 on α SMA and vimentin expression were not assessed. Moreover, difficulties blotting for collagen 1 meant that I was unable to validate my *COL1A1* qPCR data. Because of these issues, only *FN1*, *ACTA2*, *COL1A1* and fibronectin were measured. Given the relatively small number of fibrotic readouts used, it is possible that the pro-fibrotic effects of IL-33 have been missed. Although PCR arrays similar to those used by Roach et al. (2018) could be used to assess this possibility, more comprehensive analysis via bulk and single cell RNA sequencing (RNA-seq) is preferred. Additionally, immunofluorescence staining (similar to that used by Alsafadi et al. (2017) and Lehmann et al. (2018)) could be performed to validate my western blot data and determine the effects of IL-33 on α SMA and collagen 1 expression. Finally, given their success in quantifying the anti-fibrotic effects of caffeine (Tatler et al., 2016a) and CZ415 (Woodcock et al., 2019) on PCLS, hydroxyproline and procollagen type 1 amino-terminal propeptide could be measured to assess the effects of IL-33 on collagen deposition.

Although some experiments with IPF PCLS were performed, I did not receive many IPF lung samples. Due to this, I was unable to assess whether treatment with an IL-33 neutralising antibody reduces established fibrosis *ex vivo*. To overcome issues with IPF tissue supply and determine the pro-fibrotic activity of endogenous IL-33, the

fibrotic cocktail developed by Alsafadi et al. (2017) could be used to induce fibrosis in more readily available NAT. However, given that recombinant IL-33 failed to induce fibrotic changes in NAT PCLS, it is possible that blocking endogenous IL-33 will have no effect on fibrotic cocktail-induced fibrosis.

In summary, a method to generate human PCLS was developed and used to assess the pro-fibrotic activity of IL-33 *ex vivo*. Unlike TGF β , IL-33 treatment had no effect on *ACTA2*, *COL1A1*, *FN1* and fibronectin expression by human PCLS. Although dose response and time course experiments are desirable to confirm this finding in additional donors, this result suggests that IL-33 is unable to induce fibrotic changes in human PCLS. Considered together with the data presented in chapters 3 and 4, the apparent failure of IL-33 to promote fibrosis in a complex species-relevant system suggests that the IL-33:ST2 axis is unlikely to play a central pro-fibrotic role in IPF.

Chapter 6: The effects of oxidised IL-33 (oxIL-33) on fibroblasts

6. The effects of oxidised IL-33 (oxIL-33) on fibroblasts

6.1. Introduction

IL-33 is a dynamic molecule and a number of different forms of IL-33 have been described (see chapter 1, section 1.2.6). Oxidation of IL-33 at cysteine residues results in the formation of two intrachain disulphide bonds (Cohen et al., 2015). Due to the considerable distance between the cysteines involved (Lingel et al., 2009), oxidation induces a substantial conformational change in the tertiary structure of IL-33 (Cohen et al., 2015). As this prevents the formation of the β -barrel core and hairpin loop required for ST2 binding (Liu et al., 2013), once oxidised, IL-33 can no longer interact with its receptor (Cohen et al., 2015). Although IL-33 oxidation was originally proposed to act as a mechanism to limit/resolve ST2-dependent inflammation (Cohen et al., 2015), unpublished data from the Cohen group suggests that oxidised IL-33 (oxIL-33) can signal via the epidermal growth factor receptor (EGFR).

EGFR is a receptor tyrosine kinase (RTK) expressed on the surface of multiple cell types including epithelial cells, fibroblasts and macrophages (Epstein Shochet et al., 2019, Reyfman et al., 2019, Schiller et al., 2019, Vieira Braga et al., 2019). Following the binding of ligands such as epidermal growth factor (EGF), transforming growth factor- α (TGF α) and amphiregulin (AREG), EGFR dimerization and transautophosphorylation results in the activation of several signalling pathways including the MAPK/ERK pathway and the PI3K/AKT/mTOR pathway (Oda et al., 2005, Wee and Wang, 2017). Since these pathways promote the growth, proliferation and survival of EGFR expressing cells, dysregulated EGFR signalling has been linked

to the development of several cancers (Wee and Wang, 2017, Sigismund et al., 2018). Moreover, given its reported effects on inflammatory gene expression (Rayego-Mateos et al., 2018) and TGF β -induced fibroblast to myofibroblast differentiation (Midgley et al., 2013), aberrant EGFR signalling is proposed to play a pathophysiological role in chronic inflammation and organ fibrosis (Vallath et al., 2014, Zhuang and Liu, 2014, Rayego-Mateos et al., 2018, Wang et al., 2019).

Although the biological effects of oxIL-33 signalling remain largely unknown, preliminary results from the Cohen lab suggest that the oxIL-33:EGFR axis impairs the proliferation, migration and differentiation of lung epithelial cells. Given the proposed pathophysiological importance of delayed and inadequate epithelial cell repair in IPF (Selman et al., 2001, Sgalla et al., 2018), it is possible that, once oxidised, IL-33 contributes to the development of IPF via the oxIL-33:EGFR axis. Indirect evidence to support this hypothesis includes the findings that other EGFR ligands (including EGF and TGF α) are elevated in the BALF and lung tissue of IPF patients (Madtes et al., 1999, Emad and Emad, 2007, Hamada et al., 2008). Moreover, the expression of total and phosphorylated EGFR is increased in IPF lungs versus control (Tzouvelekis et al., 2013, Epstein Shochet et al., 2019). *In vitro*, treatment with either EGF or TGF α induces EGFR-dependent fibroblast proliferation and exposure to AREG increases fibroblast motility and invasiveness following injury (Ishii et al., 2006, Zhou et al., 2012, Ding et al., 2016). *In vivo*, bleomycin (BLM) treatment increases EGFR expression in the lungs of rats (Madtes et al., 1999) and TGF α overexpression leads to the development of pulmonary fibrosis in mice (Korfhagen et al., 1994, Hardie et al., 1997, Le Cras et al., 2003, Hardie et al., 2007). Conversely, pharmacological inhibition of EGFR (Rice et al., 1999, Ishii et al., 2006, Hardie et al.,

2008), germline deletion of TGF α (Madtes et al., 1999) and RNA silencing of AGER (Yoon et al., 2016) reduces pulmonary fibrosis in multiple rodent models.

6.2. Aims

Due to its emerging effects on lung epithelial cells and the reported involvement of EGFR signalling in IPF, oxIL-33 may contribute to the development of pulmonary fibrosis. As of yet however the role of the oxIL-33:EGFR axis in the pathogenesis of IPF has not been explored. Although effects on epithelial cells are of interest, the importance of fibroblasts during the development of IPF meant that the overall goal of this chapter was to investigate the effects of oxIL-33 on human lung fibroblasts (HLFs). To do this the specific aims of this chapter were to:

- Generate oxIL-33 and characterise the resulting protein using HUVECs and epithelial cells
- Determine if ST2-independent signalling occurs in HLFs
- Investigate the functional importance of oxIL-33 signalling in HLFs in relation to IPF

6.3. Methods

Methods specific to this chapter are detailed below. For general methods including HLF and A549 cell culture, RNA extraction, reverse transcription, qPCR, protein extraction, western blotting, ELISAs and MSD assays, please refer to the relevant sections in chapter 2. For details of all the materials, kits, antibodies, qPCR primers, media and buffer recipes used in this chapter, please refer to the appendix.

6.3.1. NF-κB translocation assay

6.3.1.1. Principle

Since ST2-dependent IL-33 signalling results in the translocation of NF-κB to the nucleus, a NF-κB translocation assay was used to measure ST2-dependent IL-33 signalling. This assay uses a primary antibody specific to the p65 subunit of NF-κB, a fluorescently labelled (AF488-conjugated) secondary antibody and DNA-binding Hoechst stain. Translocation of NF-κB to the nucleus is visualised using a Gen5 Cytation plate reader and percentage nuclear translocation calculated using the following equation:

$$\% \text{ Nuclear translocation} = \left(\frac{\text{No. AF488}^+ \text{ Hoechst}^+ \text{ nuclei}}{\text{Total No. Hoechst}^+ \text{ nuclei}} \right) \times 100$$

6.3.1.2. Method

HUVECs were seeded at a density of 1×10^4 cells/well into black, clear bottomed, collagen-coated 96 well plates. Once adhered, cells were stimulated in duplicate with increasing concentrations of either reduced IL-33 (redIL-33) or oxidised IL-33 (oxIL-33). After 30 minutes at $37^\circ\text{C} + 5\% \text{ CO}_2$, all media was aspirated and 50 μl /well of 10% formalin added. Cells were fixed at room temperature for 20 minutes before being washed twice with 100 μl /well of PBS. 50 μl /well of permeabilization buffer (see appendix) was subsequently added and cells incubated at room temperature for a further 20 minutes. After washing twice with 100 μl /well of PBS, cells were stained with 50 μl /well of NF-κB p65 antibody (see appendix for antibody clone and dilution) diluted in 10% FCS in PBS. Following 1 hour at room temperature, cells were washed twice with 100 μl /well of PBS and 50 μl /well of secondary antibody mixture (Hoechst stain (1:10000) and an AF488 conjugated anti-rabbit secondary antibody (see

appendix for antibody clone and dilution) in 10% FCS in PBS) added. After 1 hour at room temperature protected from light, cells were washed twice with 100 μ l/well of PBS and 100 μ l/well of PBS added. All plates were then imaged using a Gen5 Cytation plate reader with percentage nuclear translocation calculated and recorded using BioTek Gen5™ Data analysis software.

6.3.2. Phosphorylated EGFR (p-EGFR) homogenous time-resolved fluorescence (HTRF) assay

6.3.2.1. Principle

In the p-EGFR HTRF assay, an Eu3+ Cryptate labelled donor antibody and a d2 labelled acceptor antibody bind to EGFR phosphorylated at tyrosine (Y) 1068. Following excitation of Eu3+ Cryptate with light, the close proximity of the p-EGFR-bound antibodies results in Fluorescence Resonance Energy Transfer (FRET) from the donor to the acceptor antibody (Fig. 6.1). This results in a fluorescent signal at 665 nm which is proportional to EGFR phosphorylation at Y1068 (Cisbio, 2020).

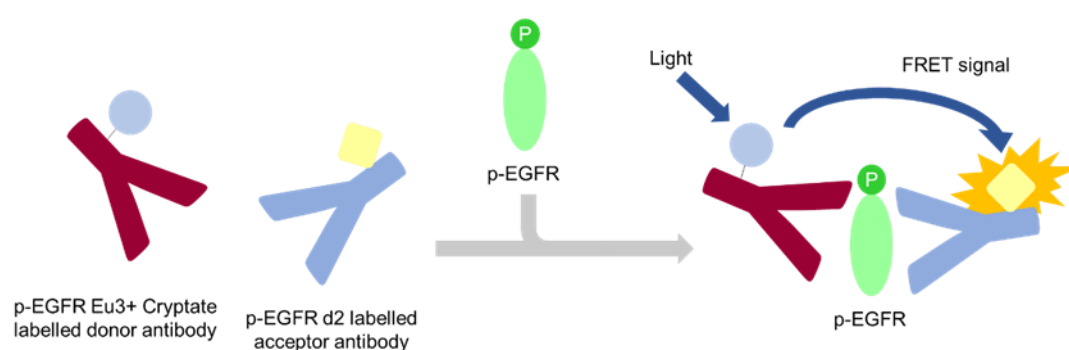


Figure 6.1: Principle of the p-EGFR HTRF assay.

Following binding to p-EGFR, FRET between the donor and acceptor antibody results in fluorescence. Figure adapted from Cisbio (2020).

6.3.2.2. Method

Activation of EGFR was measured using the p-EGFR (Tyr1068) HTRF assay kit from Cisbio according to the manufacturer's instructions. Briefly, A549 cells or non-IPF HLFs were seeded at 5×10^4 cells/well into 96 well plates and allowed to adhere overnight at $37^\circ\text{C} + 5\% \text{CO}_2$. Following serum starvation for 24 hours, cells were stimulated in duplicate with either redIL-33 or oxIL-33. After 10 minutes at $37^\circ\text{C} + 5\% \text{CO}_2$, all media was aspirated and 50 (A549s) or 25 (HLFs) μl /well of HTRF cell lysis buffer (see appendix) added. Plates were shaken at 300 rpm for 30 minutes at room temperature before 16 μl /well of cell lysate or HTRF lysis buffer (acting as the blank control) was transferred to a white 384 well plate. Both the phospho-EGFR Eu^{3+} Cryptate (donor) and Phospho-EGFR d2 (acceptor) antibodies were diluted 1:20 in HTRF assay detection buffer before mixing 1:1 to generate an antibody cocktail. 4 μl /well of this cocktail was added to each sample and the plate sealed and protected from light. After shaking at 300 rpm for 4 hours at room temperature, fluorescence at 620 and 665 nm was measured using an Envision plate reader. All data was blank corrected by subtracting the values obtained for the lysis buffer alone controls. Finally, the extent of EGFR phosphorylation induced by redIL-33 and oxIL-33 was calculated as follows:

$$\text{p-EGFR (Y1068)} = \frac{\text{Signal at 665 nm}}{\text{Signal at 620 nm}} \times 10^4$$

6.3.3. Phospho-kinase arrays

6.3.3.1. Principle

Phospho-kinase arrays are commercially available, membrane based immunoassays which allow phosphorylation at multiple kinase phosphorylation sites to be measured

simultaneously. Following the addition of whole cell lysates, membrane-bound capture antibodies (45 in total, spotted in duplicate onto nitrocellulose membranes) bind their target proteins. Immobilised proteins are subsequently recognised by biotinylated, phospho-specific detection antibodies and visualised using HRP-conjugated streptavidin and chemiluminescent detection reagents. The signal at each capture antibody spot is proportional to the amount of phospho-protein bound (R&D Systems, 2014).

6.3.3.2. Method

7×10^5 HLFs were seeded into 15 cm tissue culture treated dishes and cultured at 37°C + 5% CO₂. Once confluent, cells were serum starved for 24 hours prior to stimulation with 30 ng/ml of either redIL-33 or oxIL-33. After 10 minutes at 37°C + 5% CO₂, protein was extracted according to chapter 2 section 2.7.1 and quantified via BCA assay (see chapter 2 section 2.8.2). To analyse these samples, Phospho-kinase arrays were performed according to the manufacturer's instructions. Briefly, all arrays were blocked with array buffer 1[®] for 1 hour at room temperature. Cell lysates were subsequently diluted in array buffer 1[®] and 200 µg of protein added to each (blocked) array membrane. After incubation overnight at 4°C, all lysates were removed and six 7-minute washes with supplied wash buffer performed. Working concentrations of detection antibody were prepared in array buffer 2/3[®] and 1 ml of detection antibody A or detection antibody B added to the appropriate membranes (i.e. antibody A was added to membrane A and antibody B was added to membrane B). All arrays were incubated at room temperature for 2 hours before six 7-minute washes with wash buffer were performed. HRP-conjugated streptavidin was then diluted 1:2000 in array buffer 2/3[®] and 1 ml added to each array. After 30 minutes at room temperature, six

7-minute washes with wash buffer were performed before 2 ml of Clarity Max™ ECL substrate was added to each array for 3 minutes. Finally, all arrays were visualised using a LI-COR C-DiGit® blot scanner and analysis performed using Image Studio Lite v5.2 software. For each protein/phospho-protein, data is presented as fold change versus unstimulated control.

6.3.4. SDS-PAGE and in-gel protein detection

6.3.4.1. Principle

Ionic interactions between protein amine and sulfonic acid groups enable the binding of InstantBlue® Protein Stain to SDS-PAGE separated protein samples. As previously described by Cohen et al. (2015), the oxidation of IL-33 can be measured by SDS-PAGE and in-gel protein staining. In this assay oxidised IL-33 migrates more rapidly than reduced IL-33 (due to its more compact, disulphide-bonded structure) under non-reducing conditions (i.e. in the absence of β -mercaptoethanol).

6.3.4.2. Method

Recombinant protein samples (see relevant Figure legend for sample details and concentrations) were diluted 3:1 in SDS running buffer $\pm$ 10% β ME and boiled at 95° for 5 minutes. 0.75 μ g of protein/lane was loaded into Bolt™ 12% Bis-Tris Plus gels and SDS-PAGE performed according to chapter 2 section 2.9.2.2.2. Once removed from their cassettes, gels were submerged in 30 ml of InstantBlue® Protein Stain. Following overnight incubation on a rocker, excess protein stain was removed by six 1-hour washes with distilled water. Upon completion, gels were visualised using an

Epson Perfection V700 Photo scanner and images processed using Epson scan software.

6.4. Results

6.4.1. IL-33 oxidises in IMDM media

To investigate whether fibroblasts respond to oxIL-33, a large batch of this particular form of IL-33 was required. Since oxIL-33 is not commercially available, it was necessary to generate a batch of oxidised material ‘in house’.

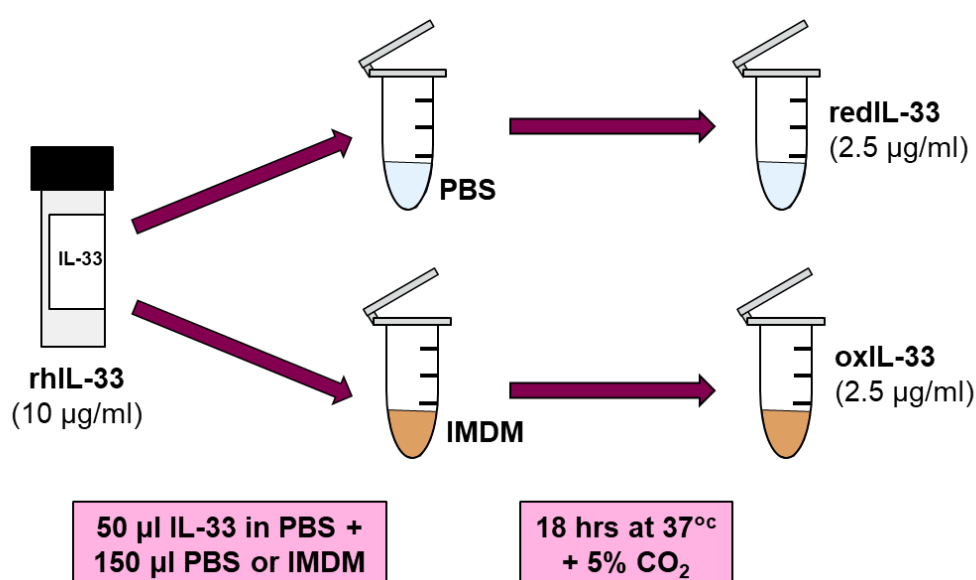


Figure 6.2: The generation of crude oxIL-33 from recombinant IL-33.

Recombinant human IL-33 was reconstituted in PBS to give a stock concentration of 10 µg/ml. 50 µl of this stock solution was transferred into 1.5 ml low binding Eppendorf tubes and 150 µl of either PBS or IMDM media added. For each vial of recombinant IL-33, 9 PBS and 9 IMDM tubes were prepared. After vortexing to mix, all tubes were incubated for 18 hours at 37°C + 5 % CO₂. The contents of all PBS and IMDM tubes were then pooled resulting in large batches of PBS-generated reduced IL-33 (redIL-33) and IMDM-generated oxidised IL-33 (oxIL-33). For all subsequent experiments using these ‘crude’ preps, a stock concentration of 2.5 µg/ml was assumed.

Based on methods described in Cohen et al. (2015), a substantial quantity of oxidised IL-33 (oxIL-33) was generated by incubating recombinant IL-33 in IMDM media (Fig. 6.2). Additionally, to control for the effects of prolonged incubation at 37°C, a large batch of PBS incubated reduced IL-33 (redIL-33) was made from the same vial of

recombinant protein (Fig. 6.2). As no further purification process was performed, the resulting batches of reduced and oxidised IL-33 were regarded as 'crude' preparations (preps).

Increased gel migration under non-reducing conditions can be used to differentiate disulphide bonded oxIL-33 from redIL-33 (Cohen et al., 2015). Consequently, to confirm the oxidation statuses of my crude preps, proteins were separated by SDS-PAGE under reducing and non-reducing conditions and IL-33 detected by western blot.

As expected, a predominately single band at ~18 kDa was detected for redIL-33 (Fig. 6.3A) and oxIL-33 (Fig. 6.3B) under reducing conditions. In contrast to redIL-33 (Fig. 6.3A), a significant downward shift in this band was observed for oxIL-33 under non-reducing conditions (Fig. 6.3B) indicating the presence of oxidation-induced disulphide bonds. To validate these findings, both crude preps were serially diluted and analysed using MSD assays specific to either reduced or oxidised IL-33.

A concentration dependent increase in signal was observed when redIL-33, but not oxIL-33, was titrated into the reduced IL-33 MSD assay (Fig. 6.3C). As the trends for redIL-33 and oxIL-33 were reversed when the oxidised IL-33 MSD assay was used (Fig. 6.3D), these results, combined with the western blot data, confirm the oxidation statuses of my crude preps.

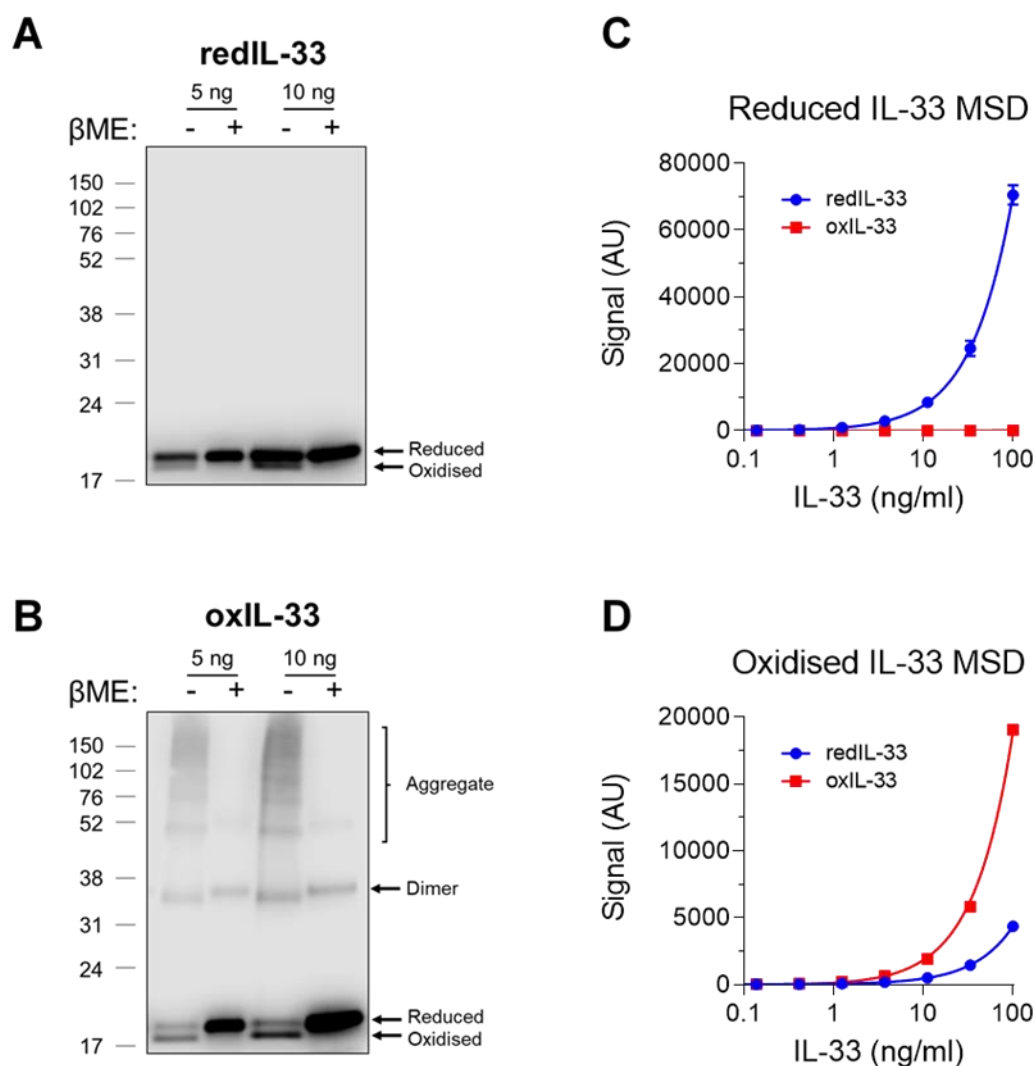


Figure 6.3: Determining the oxidation statuses of redIL-33 and oxIL-33 crude preps.

Crude redIL-33 (**A**) and oxIL-33 (**B**) were diluted 3:1 in SDS running buffer with (+) or without (-) 10% β-mercaptoethanol (βME). After denaturation at 95°C for 5 minutes, 5 and 10 ng of the resulting reduced (βME +) and non-reduced samples (βME -) were separated via SDS-PAGE and detected via IL-33 western blot. Alternatively, crude redIL-33 and oxIL-33 were serially diluted and 'in-house' MSD assays specific for reduced (**C**) and oxidised (**D**) IL-33 performed. Representative western blots from a single experiment are shown and all data is presented as mean ±SD, n=2 independent experiments.

6.4.2. IL-33 loses its ST2-dependent activity following oxidation

Following disulphide bond formation, oxidised IL-33 has been shown to lose its ST2-dependent activity (Cohen et al., 2015). To determine if this was true for my batch of crude oxIL-33, HUVECs were stimulated with increasing concentrations of redIL-33

or oxIL-33 and ST2-dependent signalling assessed via NF κ B translocation assay and IL-6 ELISA.

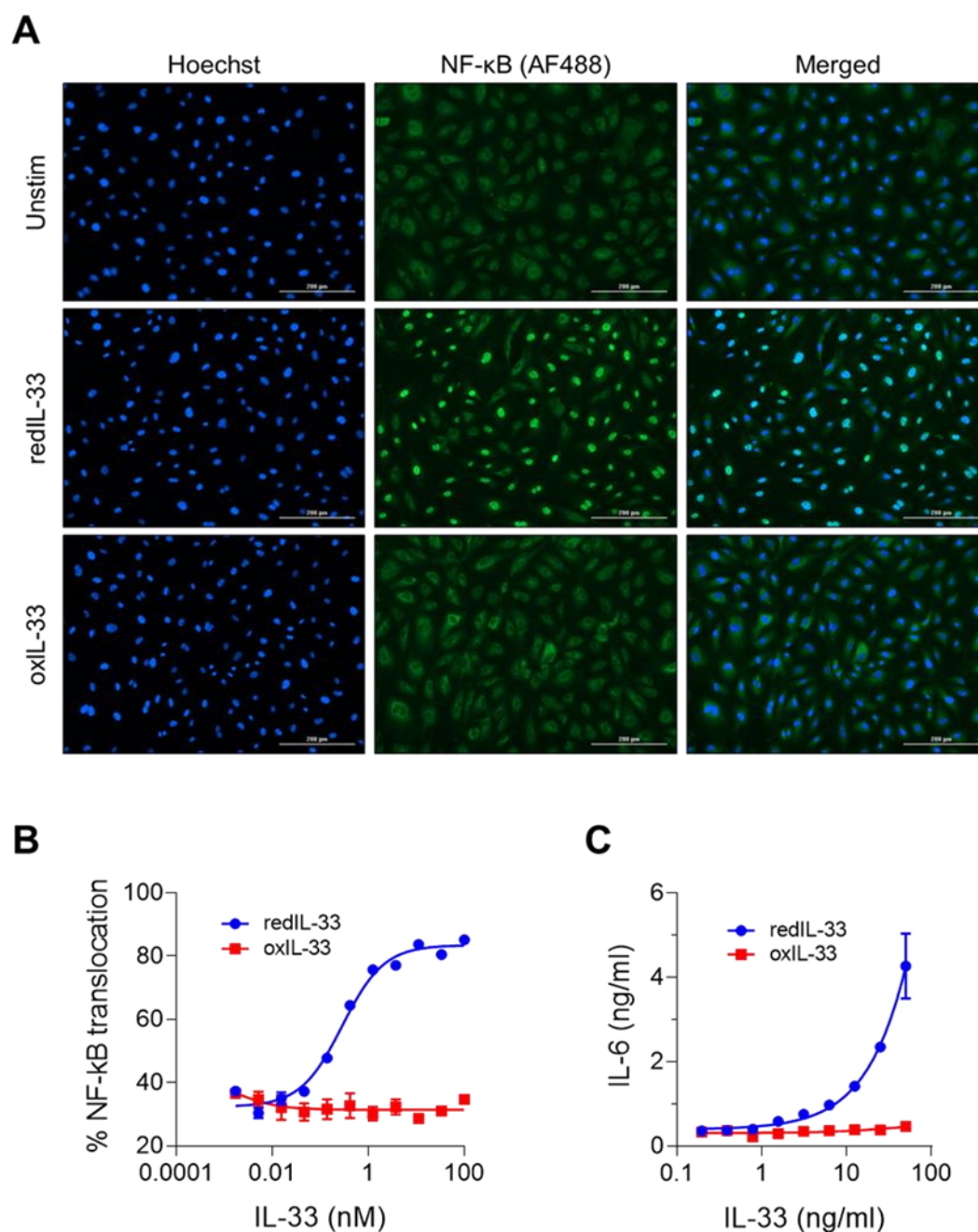


Figure 6.4: Assessing the ST2-dependent activity of crude oxIL-33.

HUVECs were stimulated with increasing concentrations of crude redIL-33 or crude oxIL-33. After 30 minutes, translocation of NF κ B to the nucleus was visualised by immunofluorescence (**A**) and quantified as a percentage (**B**). After 24 hours of stimulation, IL-6 release was measured by ELISA (**C**). Representative images from a single experiment are shown and all data is presented as mean $\pm$ SD, n = 2 independent experiments.

Stimulation with redIL-33 increased the translocation of NFκB to the nucleus (Fig. 6.4A & 6.4B) and induced IL-6 release (Fig. 6.4C) from HUVECs in a concentration-dependent fashion. As oxIL-33 treatment had no such effect on NFκB translocation (Fig. 6.4A & 6.4B) and cytokine release (Fig. 6.4C), these results suggest my batch of crude oxIL-33 has lost its ST2-dependent activity.

6.4.3. IL-33 gains ST2-independent activity in epithelial cells following oxidation

Recent unpublished data suggests that, following oxidation, IL-33 can drive ST2-independent responses in A549 cells and bronchial epithelial cells. As EGFR phosphorylation acts as a readout of such signalling, the ST2-independent activity of my batch of crude oxIL-33 was assessed by stimulating A549 epithelial cells with increasing concentrations of redIL-33 or oxIL-33 and measuring the phosphorylation of EGFR.

When assessed via HTRF assay (Fig. 6.5A) and validated by western blot (Fig. 6.5B – 6.5D), redIL-33 failed to induce EGFR phosphorylation in A549 epithelial cells. In contrast, oxIL-33 treatment increased the phosphorylation of EGFR at tyrosine 992 (Fig. 6.5B & 6.5C) and 1068 (Fig. 6.5A, 6.5B & 6.5D) in a concentration-dependent fashion. Taken together, these results demonstrate that my batch of crude oxIL-33 can successfully induce ST2-independent signalling in epithelial cells.

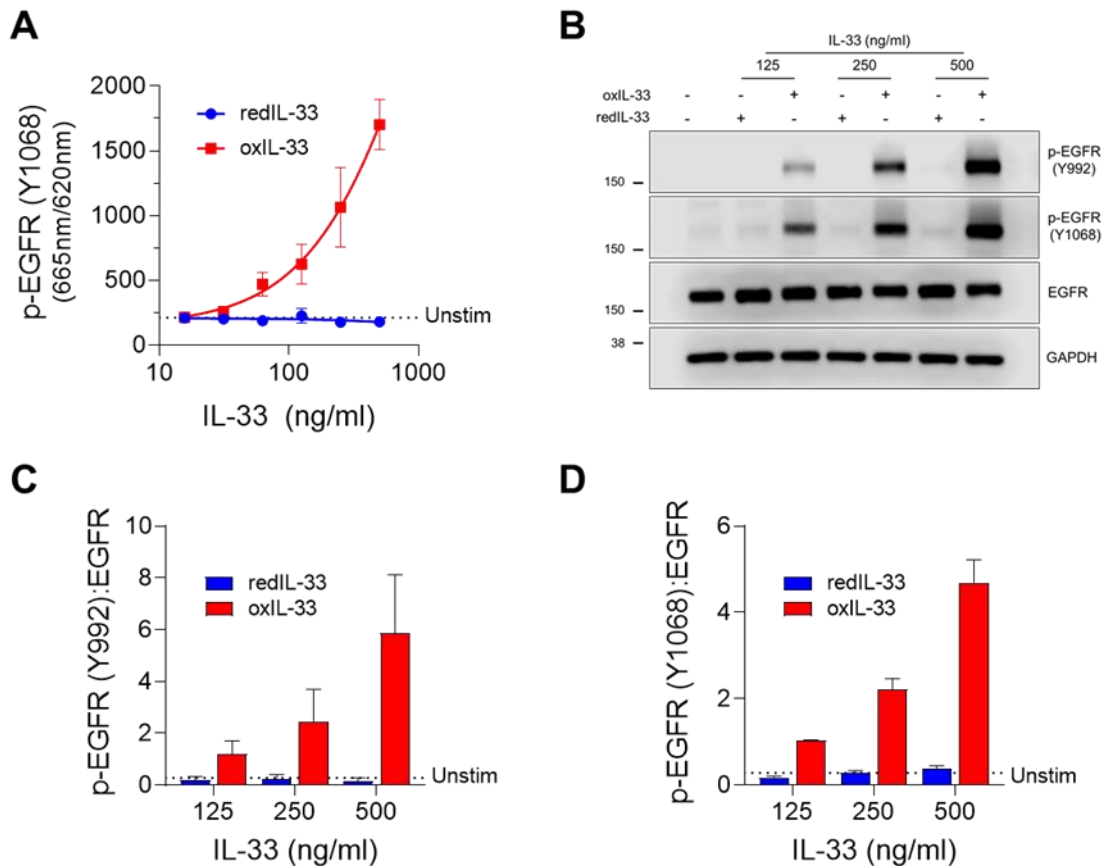


Figure 6.5: Determining the ST2-independent activity of crude oxIL-33.

A549 cells were stimulated with increasing concentrations of crude redIL-33 or crude oxIL-33 for 10 minutes. EGFR phosphorylation at tyrosine 1068 was assessed by HTRF assay (A). A549 cells were treated with 125, 250 or 500 ng/ml of crude redIL-33 or crude oxIL-33. After 10 minute stimulation, 20 μ g/lane of total protein was separated by SDS-PAGE and EGFR phosphorylation measured by western blot (B) and quantified relative to total EGFR at tyrosine 992 (C) and 1068 (D) by densitometry. Representative western blots from a single donor are shown. All data is presented as mean $\pm$ SD, n=2 independent experiments.

6.4.4. oxIL-33 induces EGFR phosphorylation in fibroblasts

As the response of other, non-epithelial cell types to oxIL-33 has never been studied it was unknown whether oxIL-33 has ST2-independent effects on fibroblasts. After demonstrating that my batch of crude oxIL-33 could drive ST2-independent signalling in epithelial cells, the ability of this protein to induce EGFR phosphorylation in HLFs was assessed via HTRF assay and western blotting.

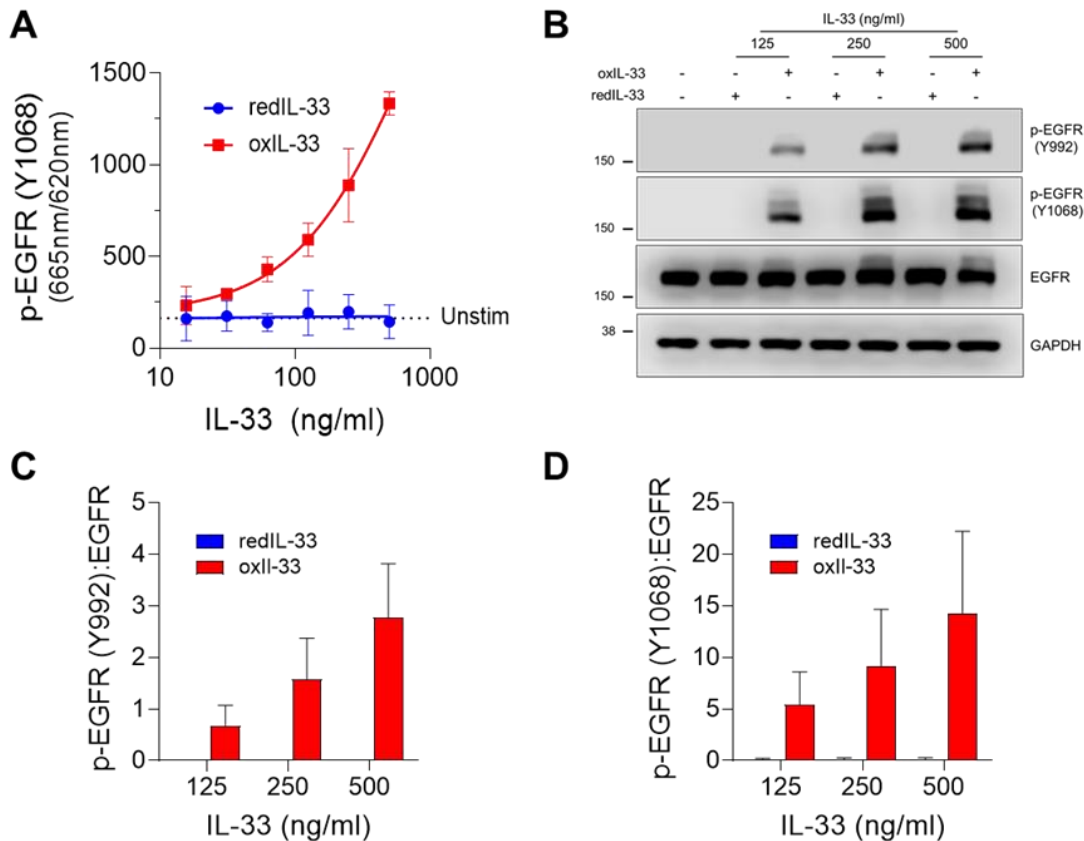


Figure 6.6: The effect of crude oxIL-33 on EGFR phosphorylation in HLFs.

Non-IPF HLFs were stimulated with increasing concentrations of crude redIL-33 or crude oxIL-33 for 10 minutes. EGFR phosphorylation at tyrosine 1068 was assessed by HTRF assay (**A**). Non-IPF HLFs were stimulated with 125, 250 or 500 ng/ml of crude redIL-33 or crude oxIL-33. After 10 minutes, 20 μ g/lane of total protein was separated by SDS-PAGE and EGFR phosphorylation measured by western blot (**B**) and quantified relative to total EGFR at tyrosine 992 (**C**) and 1068 (**D**) by densitometry. Representative western blots from a single donor are shown. All data is presented as mean $\pm$ SD, n=2 independent donors.

Similar to epithelial cells, oxIL-33 treatment induced a concentration-dependent increase in EGFR phosphorylation at tyrosine 1068 (Fig. 6.6A). This finding was validated by western blot (Fig. 6.6B & 6.6D) and confirmed at tyrosine Y992 (Fig. 6.6B & 6.6C). In contrast to oxIL-33, stimulation with redIL-33 had no effect on EGFR phosphorylation. Taken together, these results suggest that oxIL-33 can induce ST2-independent signalling in fibroblasts.

6.4.5. oxIL-33 is unlikely to have direct effects on the pro-fibrotic activity of fibroblasts

After using crude oxIL-33 to establish that ST2-independent signalling occurs in fibroblasts, purified oxIL-33 was generated and its effects on the pro-fibrotic activity of these cells assessed. Specifically, non-IPF HLFs were stimulated with TGF β or oxIL-33 for 24 hours and *ACTA2*, *COL1A1* and *FN1* (fibronectin) gene expression measured by qPCR.

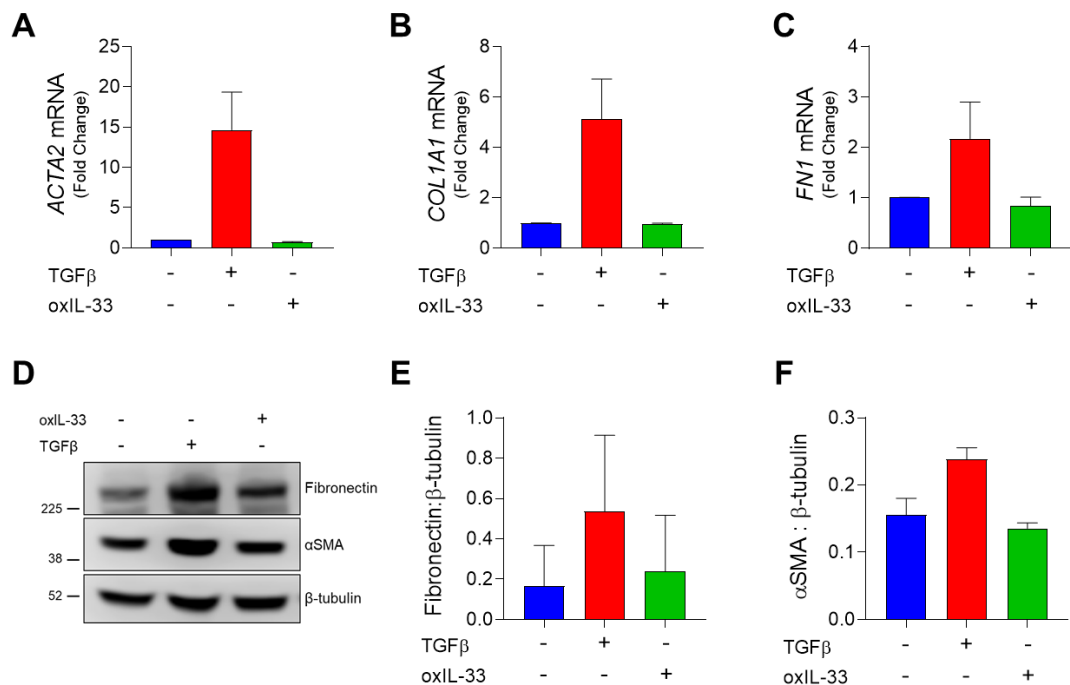


Figure 6.7: The effect of oxIL-33 on the pro-fibrotic activity of HLFs.

Non-IPF HLFs were treated with either 2 ng/ml of TGF β or 30 ng/ml of purified oxIL-33 for 24 hours. The effects of these cytokines on *ACTA2* (A), *COL1A1* (B) and *FN1* (C) gene expression were measured by qPCR. For each donor, all gene expression data was normalised to the unstimulated control condition. Additionally, western blotting was used to assess pro-fibrotic protein expression by HLFs with 20 μ g/lane of total protein loaded (D) and fibronectin (E) and α -smooth muscle actin (α SMA) (F) levels relative to β -tubulin quantified via densitometry. Representative western blots from a single donor are shown and all data is presented as mean $\pm$ SD, n=2 independent donors.

In contrast to TGF β which increased *ACTA2* (Fig. 6.7A), *COL1A1* (Fig. 6.7B) and *FN1* (Fig. 6.7C) levels in HLFs relative to unstimulated control, treatment with oxIL-

33 had no effect on the expression of these genes (Fig. 6.7A – 6.7C). As gene expression was not measured at an earlier time point than 24 hours, it was possible that the effects of oxIL-33 had been missed. To explore this hypothesis, the effect of oxIL-33 on the expression of pro-fibrotic proteins by HLFs was determined.

When assessed by western blotting (Fig. 6.7D), TGF β treatment induced fibronectin (Fig. 6.7E) and α SMA (Fig. 6.7F) expression by HLFs. In contrast, 24-hour stimulation with oxIL-33 failed to increase the levels of these proteins relative to unstimulated control (Fig. 6.7D – 6.7F). Combined with the gene expression data, these results suggest that oxIL-33 alone is unlikely to have direct effects on the pro-fibrotic activity of fibroblasts.

6.4.6. Identifying oxIL-33-induced signalling pathways in fibroblasts

As oxIL-33 treatment had no obvious effects on the pro-fibrotic activity of fibroblasts, the functional importance of oxIL-33 signalling in these cells remained unclear. To address this, HLFs were stimulated with oxIL-33 and phospho-kinase arrays performed to identify the signalling pathways induced by this cytokine.

Despite measuring a total of 45 proteins, only 4 were identified by phospho-kinase array to be upregulated by oxIL-33 (Fig. 6.8A). Compared to unstimulated control, a statistically significant increase in EGFR phosphorylation was observed when HLFs were stimulated with oxIL-33 (Fig. 6.8A & 6.8B). In addition to phospho-EGFR, oxIL-33 treatment significantly increased the expression of phospho-WNK1 (Fig. 6.8A & 6.8C), phospho-STAT3 (Fig. 6.8A & 6.8D), and phospho-PRAS40 (Fig. 6.8A & 6.8E) relative to unstimulated control. There was no obvious difference in the

response of non-IPF and IPF cells to oxIL-33 (Fig. 6.8B – 6.8E) and, consistent with findings in chapter 3, redIL-33 had no effect on the expression of any of the proteins measured by phospho-kinase array (Fig. 6.8A – 6.8E).

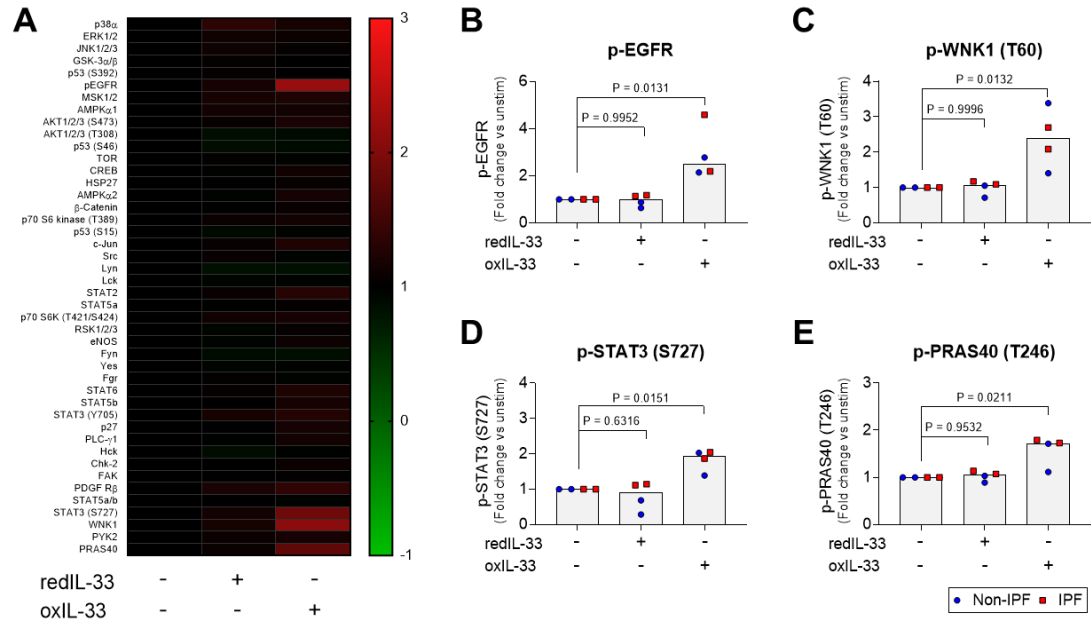


Figure 6.8: Identification of oxIL-33 signalling pathways in HLFs using phospho-kinase arrays. Non-IPF (n=2) and IPF (n=2) HLFs were stimulated with 30 ng/ml of purified redIL-33 or purified oxIL-33. After 10 minutes, protein lysates were generated and analysed by phospho-kinase array. The expression of each phospho-protein following redIL-33 and oxIL-33 treatment was calculated relative to unstimulated control and summarised using heatmaps. A representative heatmap for a single IPF donor (**A**) is shown in addition to the levels of phosphorylated EGFR (**B**), WNK-1 (**C**), STAT3 (Y727) (**D**) and PRAS40 (**E**) for all donors. Non-IPF and IPF donors are shown in blue and red respectively. Where appropriate, bars indicate median values and each data point represents a single donor. Friedman test with Dunn's multiple comparisons test.

To validate these results, HLFs were stimulated with redIL-33 or oxIL-33 and the phosphorylation of EGFR, WNK1, STAT3 and PRAS40 measured by western blot.

Compared to unstimulated control, oxIL-33 significantly increased WNK1 (Fig. 6.9A & 6.9B), EGFR (Fig. 6.9A & 6.9C), STAT3 (Fig 6.9A & 6.9D), and PRAS40 (Fig. 6.9A & 6.9E), phosphorylation in HLFs. The expression of these phospho-proteins was comparable in non-diseased and diseased cells following oxIL-33 treatment (Fig. 6.9B – 6.9E). Combined with the observation that all cells failed to respond to redIL-

33 (Fig. 6.9A – 6.9E), these results are highly consistent with my phospho-kinase array data.

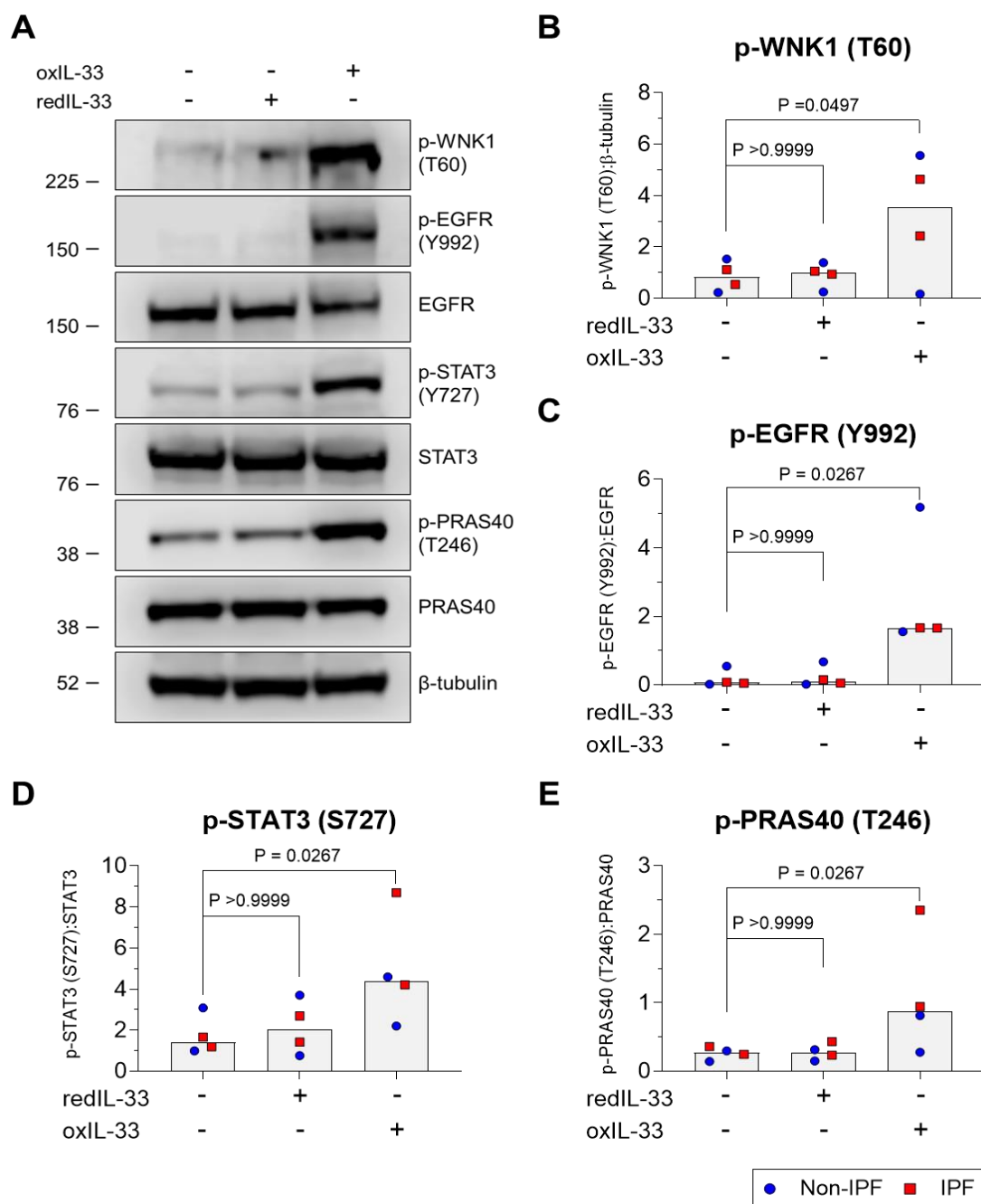


Figure 6.9: Validation of phospho-kinase array hits via western blot.

Non-IPF (n=2) and IPF (n=2) HLFs were stimulated with 30 ng/ml of purified redIL-33 or purified oxIL-33. After 10 minutes, 20 µg/lane of total protein was separated by SDS-PAGE and the effect of these cytokines on the phosphorylation of WNK1 at threonine 60, EGFR at tyrosine 992, STAT3 at serine 727 and PRAS40 and threonine 246 assessed by western blot (A) and quantified relative to β-tubulin (B), EGFR (C), STAT3 (D) and PRAS40 (E) by densitometry. Representative blots from a single non-IPF donor are shown. Non-IPF and IPF donors are shown in blue and red respectively. For all densitometry data, bars indicate median values and each data point represents a single donor. Friedman test with Dunn's multiple comparisons test.

Although all proteins identified by the phospho-kinase array were successfully validated by western blot it was possible that false negatives had occurred. Given that PRAS40 is a substrate of AKT (Kovacina et al., 2003, Wiza et al., 2012), HLFs were stimulated with oxIL-33 and the phosphorylation of AKT assessed by western blot.

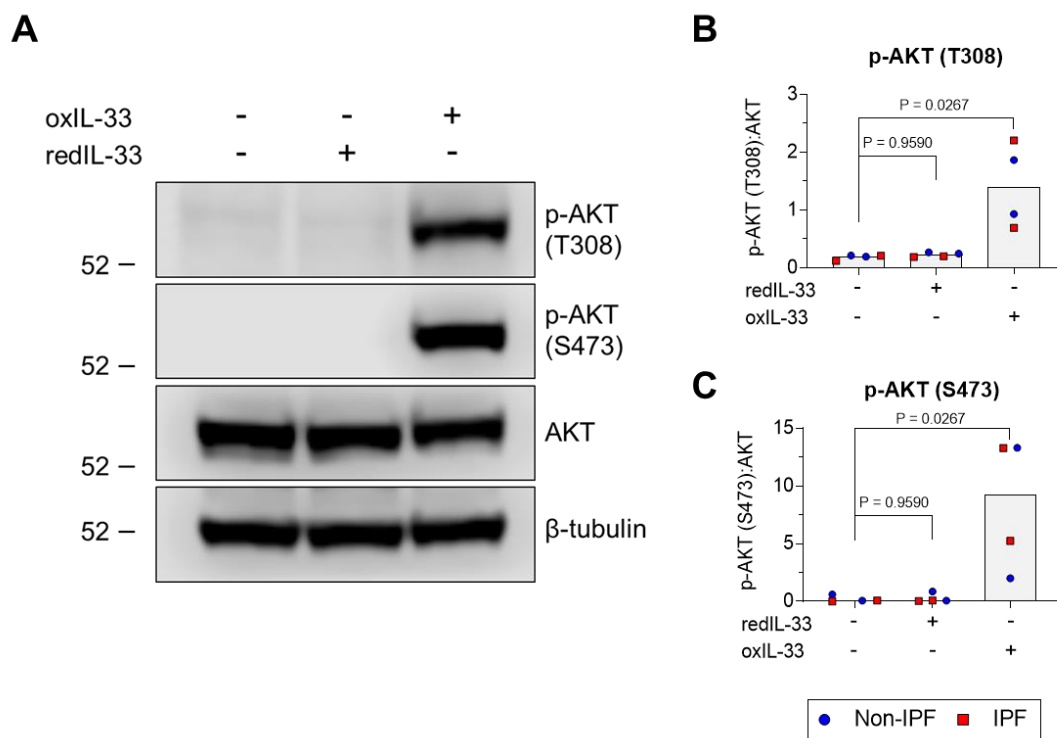


Figure 6.10: The effect of oxIL-33 on AKT phosphorylation in HLFs.

Non-IPF (n=2) and IPF (n=2) HLFs were treated with 30 ng/ml of purified redIL-33 or purified oxIL-33 for 10 minutes. 20 µg/lane of total protein was separated by SDS-PAGE and AKT phosphorylation at this time point assessed by western blot (A) and quantified relative to total AKT at threonine 308 (B) and serine 473 (C) by densitometry. Representative blots from a single non-IPF donor are shown. Non-IPF and IPF donors are shown in blue and red respectively. For all densitometry data, bars indicate median values and each data point represents a single donor. Friedman test with Dunn's multiple comparisons test.

Unlike redIL-33 which had no effect, oxIL-33 increased AKT phosphorylation in HLFs (Fig. 6.10A). Compared to unstimulated control, this increase was statistically significant at threonine 308 (Fig. 6.10B) and serine 473 (Fig. 6.10C) and was evident for both non-diseased and diseased cells (Fig. 6.10B & 6.10C). Combined with my validated kinase array data, these results suggest that oxIL-33 may induce PI3K/AKT/mTOR signalling in fibroblasts.

6.4.7. oxIL-33 signalling in fibroblasts is EGFR-dependent

To determine if oxIL-33 signalling in fibroblasts was EGFR-dependent, HLFs were treated with oxIL-33 in the presence of an α EGFR antibody. EGFR, AKT and PRAS40 phosphorylation were then measured by western blot.

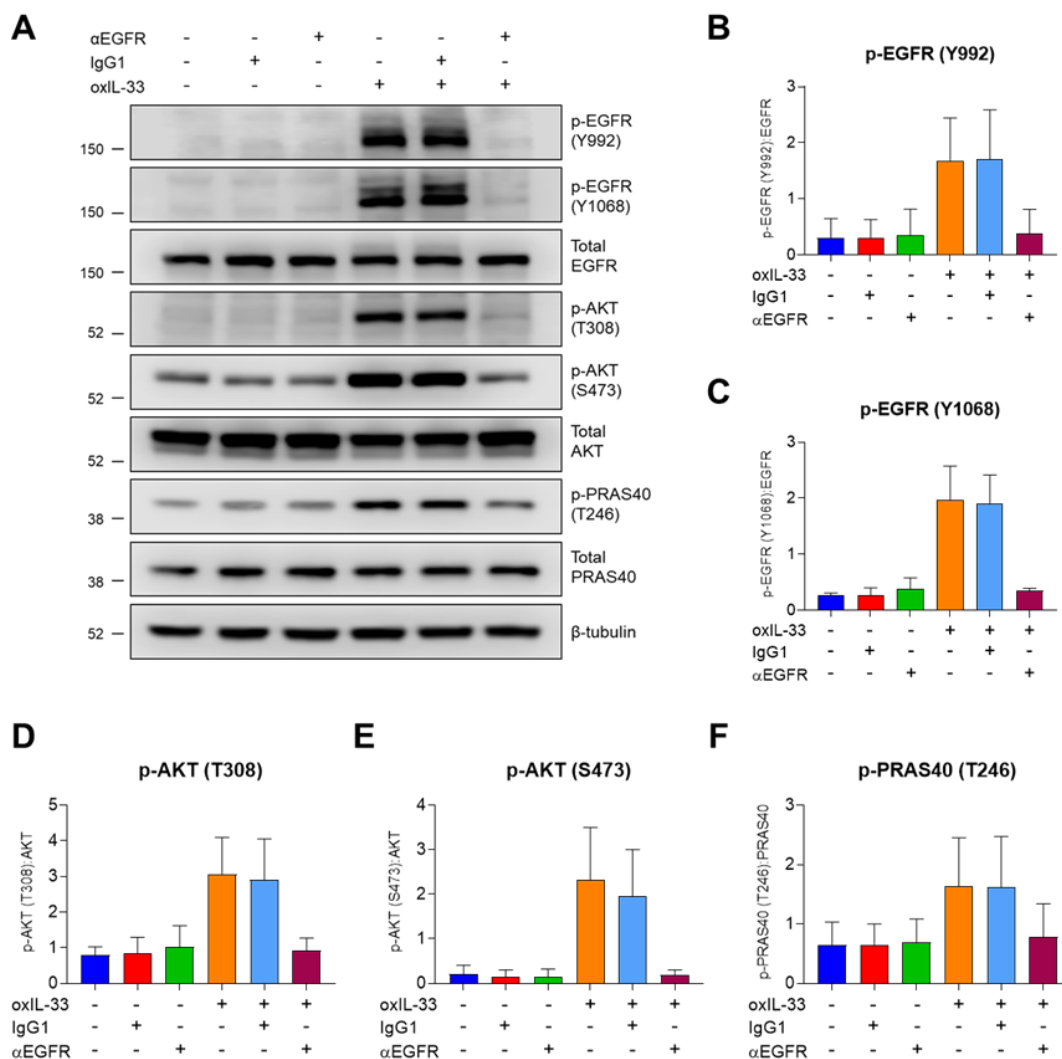


Figure 6.11: The effect of inhibiting EGFR on oxIL-33 signalling in HLFs.

10 μ g/ml of an α EGFR or mouse IgG1 isotype control antibody was added to non-IPF HLFs. After 30-minute preincubation with these antibodies, cells were stimulated with 500 ng/ml of purified oxIL-33 for 10 minutes. 20 μ g/lane of total protein was separated by SDS-PAGE and oxIL-33-induced EGFR, AKT and PRAS40 phosphorylation assessed by western blotting (**A**). The effect of the α EGFR antibody on the phosphorylation of; EGFR at tyrosine 992 (**B**) and tyrosine 1068 (**C**), AKT at threonine 308 (**D**) and serine 473 (**E**) and PRAS40 at threonine 246 (**F**), was quantified relative to total EGFR, AKT and PRAS40 via densitometry. Representative blots from a single donor are shown and all data is presented as mean $\pm$ SD, n=2 independent donors.

Compared to each of the unstimulated control conditions (i.e. no antibody, IgG1 and α EGFR alone), oxIL-33 treatment induced EGFR, AKT and PRAS40 phosphorylation in HLFs (Fig. 6.11A – 6.11F). Pre-incubation with the α EGFR antibody inhibited oxIL-33-induced EGFR phosphorylation at tyrosine 992 (Fig. 6.11A & 6.11B) and 1068 (Fig. 6.11A & 6.11C) and ablated AKT phosphorylation at threonine 308 (Fig. 6.11A & 6.11D) and serine 473 (Fig. 6.11A & 6.11E). In contrast, treatment with the isotype control antibody had no effect. As an identical trend was observed at threonine 246 of PRAS40 (Fig. 6.11A & 6.11F), these findings suggest that oxIL-33 signalling in fibroblasts is EGFR-dependent.

6.4.8. Using AKT phosphorylation as a readout of TGF β -induced IL-33 release from fibroblasts

TGF β treatment increases IL-33 expression by fibroblasts (see chapter 3 section 3.4.2). To date however I have not been able to detect IL-33 in the supernatants collected from these cells. As this could be due to multiple issues with the ELISA kit used (Scott et al., 2018b), a surrogate readout of IL-33 release from fibroblasts was desired. Recently, Woodcock et al. (2019) demonstrated that 12-hour TGF β treatment induces AKT phosphorylation in HLFs. Since IL-33 levels peak 8 hours post TGF β treatment (see chapter 3, section 3.4.2), it was hypothesised that AKT phosphorylation could act as a biological readout of IL-33 release from fibroblasts. To test the feasibility of this and assess the reproducibility of Woodcock et al.'s findings, HLFs were treated with TGF β and the expression of phosphorylated AKT and cell-associated IL-33 measured over time.

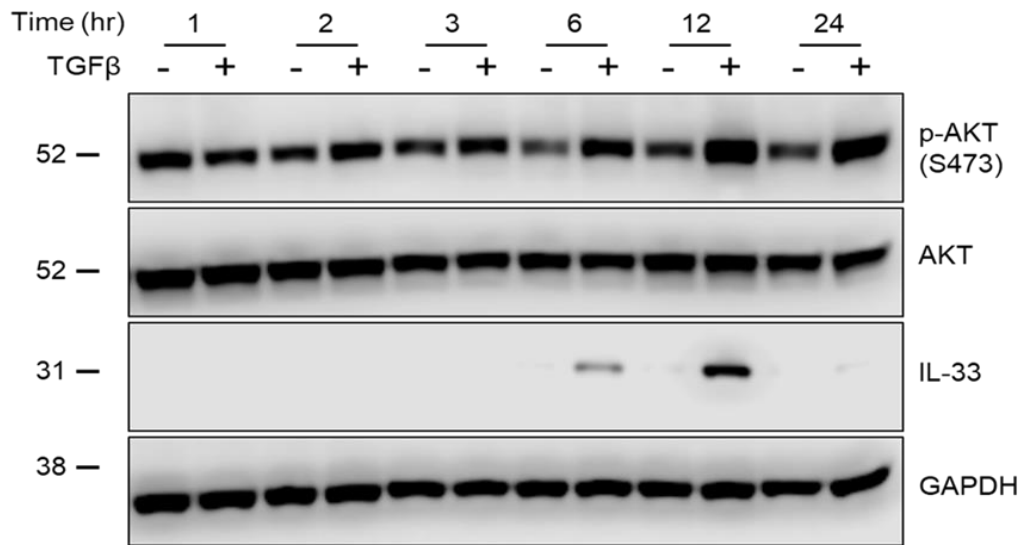
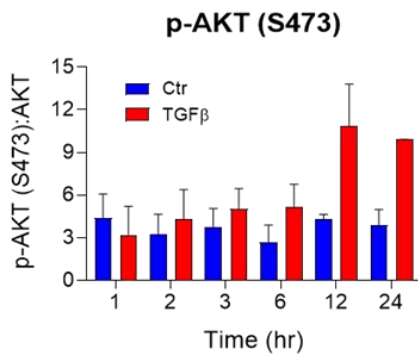
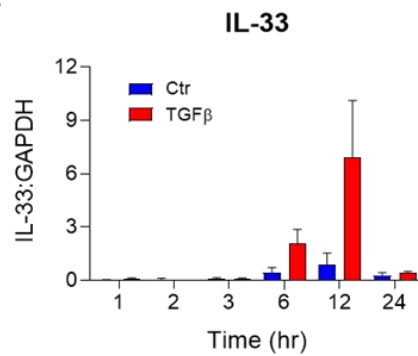
A**B****C**

Figure 6.12: The effect of TGFβ on AKT phosphorylation and IL-33 expression by HLFs over time.

Non-IPF HLFs were stimulated with 2 ng/ml of TGFβ and protein lysates generated at the intervals indicated. 20 μg/lane of total protein was separated by SDS-PAGE and the expression of phospho-AKT (phosphorylated at serine 473) and IL-33 assessed by western blot (A) and quantified relative to total AKT (B) and GAPDH (C) respectively. Representative western blots from a single donor are shown and all data is presented as mean ± SD, n=2 independent donors.

Following TGFβ treatment, phospho-AKT expression peaked at 12 hours and remained high after 24 hours (Fig. 6.12A & 6.12B). Cell-associated IL-33 expression increased at 6 hours, peaked at 12 hours and returned to baseline after 24 hours (Fig. 6.12A & Fig. 6.12C). Since IL-33 expression preceded AKT phosphorylation, it was plausible that IL-33 release was involved in the induction of AKT downstream of TGFβ. To determine if TGFβ-induced IL-33 release from HLFs contributes to AKT

phosphorylation at 12 hours, an α IL-33 antibody shown to block IL-33 oxidation (Fig. 6.13) was used. Specifically, HLFs were stimulated with TGF β in the presence of α IL-33 antibody or isotype control. After 12 hours, AKT phosphorylation at threonine 308 and serine 473 was assessed by western blot (Fig. 6.14A).

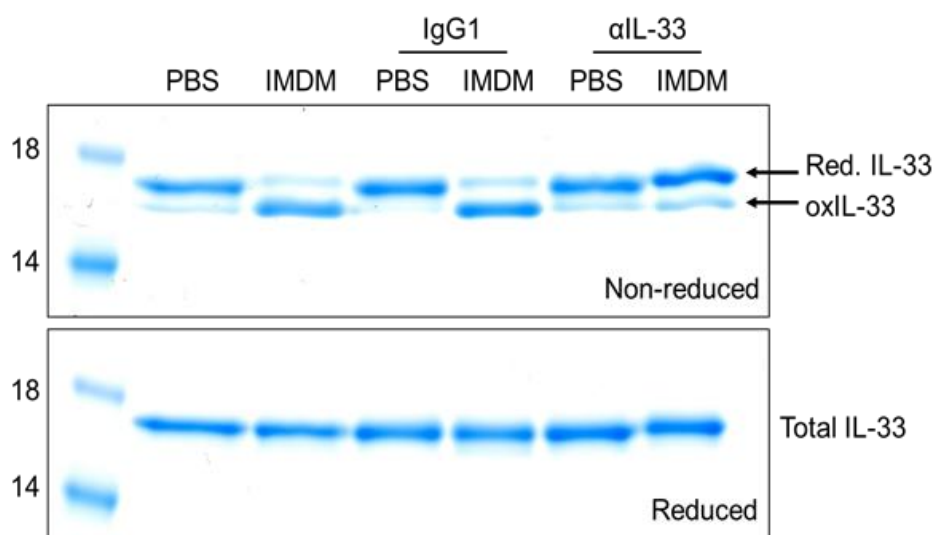


Figure 6.13: The effect of the α IL-33 antibody on the oxidation of IL-33.

0.7 μ M of α IL-33 antibody or human IgG1 isotype control (both from AstraZeneca) was added to 1.4 μ M of recombinant IL-33 to give a volume of 50 μ l. After adding 150 μ l of PBS or IMDM to the appropriate antibody-IL-33 mixtures, all 1.5 ml low binding Eppendorf tubes were incubated at 37°C for 18 hours. All PBS and IMDM generated proteins were separated by SDS-PAGE under reducing and non-reducing conditions and proteins visualised using InstantBlue® Protein Stain.

In the presence of the IgG1 isotype control, 12-hour TGF β treatment induced AKT phosphorylation at threonine 308 (Fig. 6.14B & 6.14C) and serine 473 (Fig. 6.14C & 6.14D) in HLFs. Despite preventing the oxidation of recombinant IL-33 (Fig. 6.13) the α IL-33 antibody failed to reduce TGF β -induced phospho-AKT levels in HLFs relative to isotype control (Fig 6.14B - 6.14D). Although assessment of AKT phosphorylation at 24 hours is desirable (to confirm that the α IL-33 antibody has no effect at later time points), these preliminary results suggest that TGF β -induced AKT phosphorylation is unlikely to be mediated by secreted IL-33 and potentially imply that TGF β does not induce IL-33 release from fibroblasts.

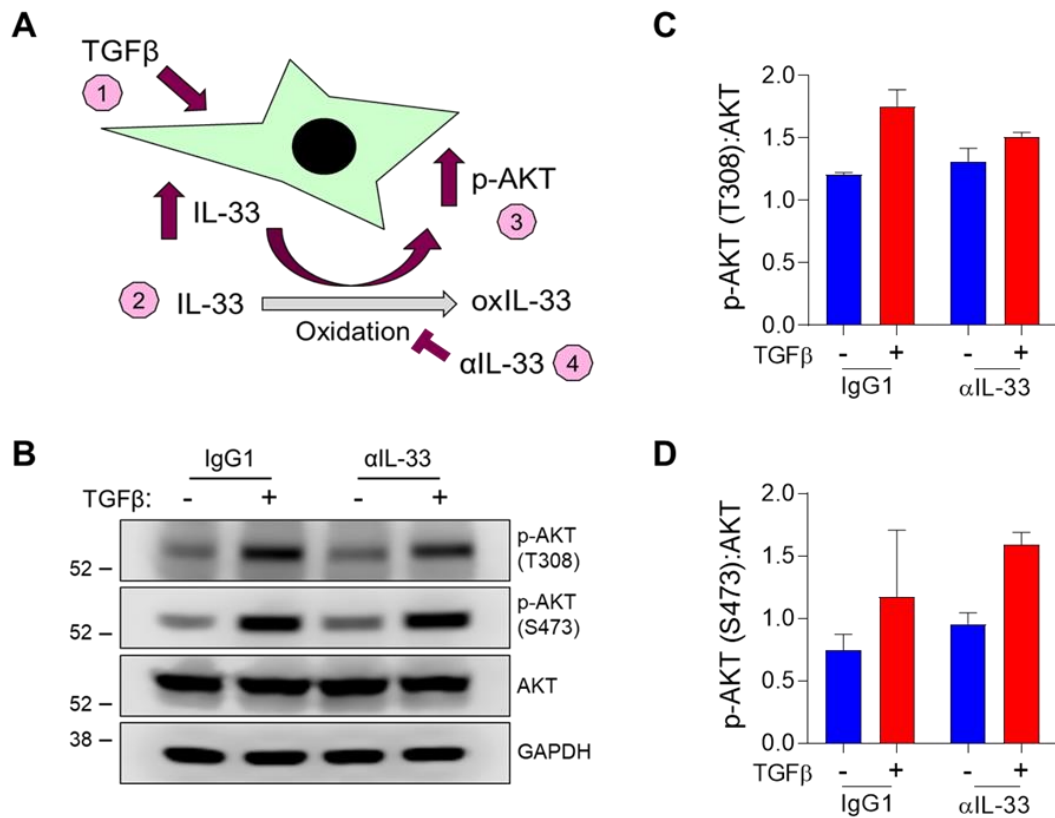


Figure 6.14: The effect of an α IL-33 antibody on TGF β -induced AKT phosphorylation at 12 hours.

To test the hypothesis that AKT phosphorylation can be used as a readout of TGF β -induced IL-33 release from HLFs, 1 μ g/ml of an α IL-33 antibody or IgG1 isotype control was added to non-IPF HLFs and cells stimulated with 2 ng/ml of TGF β (A). After 12 hours, 20 μ g/lane of total protein was separated by SDS-PAGE and AKT phosphorylation measured by western blot (B). The effect of the α IL-33 antibody on AKT phosphorylation at threonine 308 (C) and serine 473 (D) was quantified relative to total AKT by densitometry. Representative western blots from a single donor are shown and all data is presented as mean $\pm$ SD, n=2 independent donors.

6.5. Discussion

Unpublished data from the Cohen group suggests that oxIL-33 signals via EGFR in epithelial cells. Although previous studies suggest that EGFR signalling may promote pulmonary fibrosis, the novelty of the oxIL-33:EGFR axis means that the role of oxIL-33 during the development of IPF has not been evaluated. In particular, the effects of oxIL-33 on fibroblasts have never been studied. Here I show that IL-33 readily oxidises in media, loses its ST2-dependent activity and gains ST2-independent activity in both epithelial cells and fibroblasts. Despite failing to increase the pro-fibrotic activity of fibroblasts directly, oxIL-33 appeared to activate PI3K/AKT/mTOR signalling in an EGFR-dependent fashion. Although further work is required to confirm these findings, it is important to realise that PI3K/AKT/mTOR signalling promotes the proliferation and survival of ECM-secreting fibroblasts (Nho and Hergert, 2014, Im et al., 2015, Mercer et al., 2016, Romero et al., 2016). Given the importance of these cells in the pathogenesis of IPF (see chapter 1, section 1.1.6.2.2), it is tempting to speculate that oxIL-33 may play a pathophysiological role in the development of IPF.

To establish if oxIL-33 has ST2-independent effects on fibroblasts, a batch of crude protein was generated according to the protocol outlined in Cohen et al. (2015). When assessed by western blot and MSD assay, overnight incubation in IMDM resulted in the oxidation of IL-33. Consistent with the observations of Cohen et al. (2015), my IMDM-generated crude prep failed to induce ST2-dependent NF κ B signalling in HUVECs. As stimulation of A549 cells with the same protein resulted in a concentration-dependent increase in EGFR phosphorylation, it was concluded that my

crude oxIL-33 prep displayed the biochemical and biological characteristics necessary to accurately assess whether ST2-independent signalling occurs in fibroblasts.

In contrast to redIL-33, treatment with crude oxIL-33 induced EGFR phosphorylation in HLFs. As comparable results were obtained by HTRF assay and western blot, it demonstrates that my results are highly reproducible. Moreover, the concentration-dependent effect of oxIL-33 at both tyrosine 992 and 1068 suggests that the response of HLFs to this cytokine is not an artefact of using oxIL-33 at a single concentration or assessing its effects at one phospho-site in isolation. Given their demonstrated expression of EGFR (Fig. 6.5, 6.7, 6.8, 6.9 & 6.10), my novel discovery that HLFs can respond to oxIL-33 fits well with what is currently known about the oxIL-33:EGFR axis in epithelial cells. Since multiple other cell types implicated in the pathogenesis of IPF (such as macrophages, endothelial cells and smooth muscle cells) express EGFR (Reyfman et al., 2019, Schiller et al., 2019, Vieira Braga et al., 2019), the effects of oxIL-33 on these cell types represents an exciting avenue of further investigation.

24-hour stimulation with oxIL-33 failed to increase the expression of *ACTA2*, *COL1A1* and *FN1* by HLFs. As the expression of other IL-33 responsive genes peaks 6 hours post treatment (Gautier et al., 2016), it is possible that this result is due to the time point used. Although time course experiments identical to those performed in chapter 3 are required to exclude this possibility, the failure of oxIL-33 to increase α SMA and fibronectin protein expression after 24 hours suggests that oxIL-33 alone is unlikely to have direct effects on the pro-fibrotic activity of fibroblasts. However, as EGF synergises with TGF β to increase the expression of *ACTA2* by rabbit corneal keratocytes and human dermal fibroblasts (He and Bazan, 2008, Simpson et al., 2010), it is possible that a similar relationship exists between oxIL-33 and TGF β . To test this

hypothesis, experiments with non-IPF and IPF HLFs could be performed to establish whether oxIL-33 synergises with TGF β to increase the pro-fibrotic activity of these cells.

EGFR phosphorylation results in the recruitment of PI3K to the plasma membrane and the subsequent conversion of PIP₂ to PIP₃. This in turn leads to the activation of PDK1 and the phosphorylation of AKT at T308 (Alessi et al., 1997, Stokoe et al., 1997, Wee and Wang, 2017) (Fig. 1.6). Since oxIL-33 induced EGFR phosphorylation at Y992 and Y1068 and AKT phosphorylation at T308, these results indicate that oxIL-33 activates PI3K/AKT signalling in fibroblasts.

As previously described in chapter 1 section 1.1.6.4, the serine-threonine protein kinase mTOR associates with multiple proteins to form two distinct complexes. Referred to as mTORC1 and mTORC2, the activity of mTOR within these complexes is tightly regulated (Saxton and Sabatini, 2017, Liu and Sabatini, 2020). In addition to being regulated by TSC2 phosphorylation, mTORC1 can be activated by AKT-mediated phosphorylation of the negative regulator PRAS40 (Kovacina et al., 2003, Sancak et al., 2007, Vander Haar et al., 2007, Wiza et al., 2012) (Fig. 1.6). As oxIL-33 induces PRAS40 phosphorylation in HLFs, it suggests that oxIL-33 activates mTORC1 (mTOR) in these cells. Although further experiments are required to confirm this, my results regarding EGFR, AKT and PRAS40 phosphorylation collectively suggest that oxIL-33 induces PI3K/AKT/mTOR signalling in fibroblasts.

Due to time constraints and issues with the availability of purified protein, I was unable to explore the functional effects of oxIL-33 on fibroblasts. However, since PI3K/AKT/mTOR signalling induces fibroblast proliferation (Mercer et al., 2016), an

interesting avenue of investigation would be to stimulate HLFs with oxIL-33 and assess changes in cell number via MTT and thymidine incorporation assays. Moreover, as this pathway inhibits autophagy and promotes fibroblast survival (Nho and Hergert, 2014, Im et al., 2015, Romero et al., 2016), the effect(s) of oxIL-33 on these processes could be investigated via LC3 western blot (the conversion of LC3-I to LC3-II is a readout of autophagy) and CellTiter-Blue® viability assay.

In addition to components of mTOR signalling, AKT has multiple other substrates (Franke, 2008, Manning and Toker, 2017). Indeed, treatment with oxIL-33 induced the phosphorylation of WNK1 in HLFs. By modulating the activity of several cation-chloride cotransporters this serine-threonine kinase plays an important role in the regulation of blood pressure and cell volume (Bazúa-Valenti and Gamba, 2015, Shekarabi et al., 2017, Hadchouel et al., 2016). Although mutations in WNK1 are implicated in disorders such as familial hyperkalemic hypertension syndrome (Wilson et al., 2001) and hereditary sensory and autonomic neuropathy type II (Lafreniere et al., 2004), the role of WNK1 during the fibrotic response remains unknown. However, since WNK1 phosphorylation can inhibit autophagy (Gallolu Kankanamalage et al., 2017) it is possible that oxIL-33 contributes to the reduction in fibroblast autophagy reported in IPF and promotes the survival of these cells in collagen rich environments (Patel et al., 2012, Nho and Hergert, 2014, Im et al., 2015, Sosulski et al., 2015, Romero et al., 2016).

Treatment with oxIL-33 induced the phosphorylation of STAT3 at S727. Unlike phosphorylation at Y705 which results in translocation to the nucleus (Huynh et al., 2019), mTOR mediated phosphorylation at S727 results in the localisation of STAT3 to the mitochondria and endoplasmic reticulum (ER) (Gough et al., 2009, Wegrzyn et

al., 2009, Avalle et al., 2019). In addition to regulating the expression of mitochondrial-encoded genes (Gough et al., 2009, Wegrzyn et al., 2009), the phosphorylation of STAT3 at S727 has been shown to prevent the release of calcium ions from the ER. As this inhibits H₂O₂ and menadione induced apoptosis in mouse embryonic fibroblasts (Avalle et al., 2019), it is feasible that oxIL-33 may contribute to the development of IPF by promoting STAT3-mediated fibroblast survival.

Despite being useful tools, it is important to realise that the phospho-kinase arrays used in this chapter have multiple limitations which may have affected my results. Firstly, since these arrays are relatively low throughput, I only assessed the effects of oxIL-33 on HLFs after 10 minutes. As the phosphorylation of some of the proteins included on the arrays takes longer than 10 minutes to occur (Tremblay and Marette, 2001, Wang et al., 2007), it is possible that I have not identified all of the signalling pathways activated by oxIL-33. For example, the use of this single early time point could explain why phosphorylated p70S6K (a substrate of activated mTOR– see chapter 1, section 1.1.6.4) was not identified despite oxIL-33 appearing to induce PI3K/AKT/mTOR signalling in HLFs. Secondly, although no false positives were apparent, false negatives were evident. Indeed, the ability of oxIL-33 to activate AKT was only discovered due to the identification of its substrate PRAS40. As the failure to identify phospho-AKT was likely due to issues regarding the sensitivity and/or specificity of the primary antibodies used, it highlights the possibility that other oxIL-33 mediated signalling pathways (in addition to the PI3K/AKT/mTOR axis), might have been missed. Finally, it is important to realise that by using the arrays I only assessed the effects of oxIL-33 on 45 selected proteins. Although the proteins analysed are involved in a wide range of signalling pathways, it is possible that I have missed the effects of

oxIL-33 on other pathways not assessed by the array. To address these issues and gain a more comprehensive insight into the functional effects of oxIL-33 on fibroblasts, time course experiments should be performed and the effects of oxIL-33 assessed by bulk RNA-seq and validated by western blot.

Recently, reduced IL-33 has been shown to signal via a novel receptor complex of EGFR and ST2 in mouse T_H2 cells (Minutti et al., 2017). Despite differences in species, cell type and IL-33 oxidation status, Minutti et al.'s (2017) findings raise the possibility that a co-receptor may be required for EGFR-dependent oxIL-33 signalling in HLFs. To investigate this hypothesis, HLFs could be treated with oxIL-33 and immunoprecipitated with EGFR. After elution, any resulting protein complexes could be digested and analysed by mass spectrometry. Any proteins identified by this technique could be validated by co-immunoprecipitation (Co-IP) and their ability to facilitate oxIL-33 signalling in fibroblasts assessed via blocking antibodies and siRNA.

Due to observations by Woodcock et al. (2019), I hypothesised that AKT phosphorylation could be used as a biological readout of TGF β -induced IL-33 release from fibroblasts. Consistent with earlier reports (Woodcock et al., 2019) phospho-AKT levels in HLFs peaked 12 hours post TGF β stimulation and remained elevated after 24 hours. Despite IL-33 expression being induced by TGF β , treatment with an α IL-33 antibody (shown to inhibit the oxidation of IL-33 in IMDM) failed to reduce AKT phosphorylation at 12 hours. Although these results potentially suggest that TGF β does not induce IL-33 release from fibroblasts, the use of TGF β at a single relatively high concentration means that any synergistic effects on AKT phosphorylation may have been missed. Additionally, given the relatively small assay

window observed between unstimulated and TGF β treated cells, it is possible that the phospho-AKT assay was not sensitive enough to detect the potentially low concentrations of IL-33 released from TGF β stimulated HLFs. When more sensitive IL-33 ELISAs and/or MSD assays are available they should be used to validate the results presented in this chapter and definitively determine whether TGF β induces IL-33 release from fibroblasts.

Alternative splicing and proteolytic cleavage have both been shown to alter the ST2-dependent activity of IL-33 (Lefrançois et al., 2012, Lefrançois et al., 2014, Gordon et al., 2016b). Although further investigation is required, it is possible that these mechanisms might also affect the ability of IL-33 to elicit ST2-independent responses. As I only used oxIL-33 generated from recombinant, *E. coli* derived, IL-33 (Serine 112 - Threonine 270), my results may not be applicable to endogenous protein. Given that mechanical injury induces IL-33 release (Cayrol and Girard, 2009) and that PI3K/AKT/mTOR signalling induces fibroblast migration and proliferation (Hsu et al., 2017, Xiao et al., 2017, Demirci et al., 2018), scratch wound assays could be used to address this possibility.

Competition for receptors and receptor internalisation and degradation regulates cellular responses to EGFR ligands (Tomas et al., 2014). Although the affinity of oxIL-33 for EGFR versus other ligands requires further investigation, it is plausible that at high concentrations, oxIL-33 may outcompete other EGFR ligands. Moreover, if oxIL-33 promotes the internalisation and degradation of EGFR it is feasible that canonical EGFR signalling will be disrupted by oxIL-33. Since canonical EGFR signalling has been shown to promote pulmonary fibrosis (Korfhagen et al., 1994, Hardie et al., 1997, Rice et al., 1999, Le Cras et al., 2003, Ishii et al., 2006, Hardie et al., 2007, Hardie et

al., 2008, Yoon et al., 2016) it is possible that, by disrupting EGFR-mediated responses, oxIL-33 may play a protective rather than pathophysiological role in IPF. However, given that the pharmacological inhibition of EGFR has been shown by some groups to drive fibrotic responses in the lungs (Inoue et al., 2003, Suzuki et al., 2003, Teramoto et al., 2003, Takano et al., 2004), it is also possible that oxIL-33-mediated disruption of canonical EGFR signalling would promote pulmonary fibrosis. Consequently, further work is required to 1) elucidate the role of canonical EGFR signalling during the development of IPF and 2) establish the effect(s) of oxIL-33 on canonical EGFR signalling in the context of IPF.

Crosstalk between multiple cell types is essential for the development of IPF (Barkauskas and Noble, 2014, Martinez et al., 2017, Barratt et al., 2018). Moreover, the expression of EGFR can be altered by repeated passaging *in vitro* and influenced by the tissue microenvironment *in vivo* (Wee and Wang, 2017, William et al., 2017). It is therefore difficult to know whether my results with cultured HLFs are translatable to human disease. To address this issue with other cytokines, methods to generate human precision cut lung slices (PCLS) have been devised (Alsafadi et al., 2017, Gerckens et al., 2019, Liu et al., 2019a). As detailed in Chapter 5, I developed and optimised a method to generate human PCLS. Unfortunately due to time constraints I did not have the opportunity to evaluate the effects of purified oxIL-33 in the PCLS system. Future experiments with purified oxIL-33 (and reagents that block the oxidation of endogenous IL-33) are therefore desired to determine the pro-fibrotic activity of this cytokine in a complex species-relevant system.

As the oxIL-33 used in my experiments was generated by overnight incubation in tissue culture media, the physiological relevance of my results is debatable. However,

given that: 1) oxIL-33 was detected in the BALF of *Alternaria* challenged mice (Cohen et al., 2015), 2) oxIL-33 can be measured in the sputum of severe asthmatics (Cohen et al., 2015) and 3) oxidative stress is implicated in the development of BLM-induced fibrosis and IPF (Bargagli et al., 2009, Kliment and Oury, 2010, Yan et al., 2017, Fois et al., 2018, Huang et al., 2020b), it is reasonable to believe that oxIL-33 exists in the lungs of BLM-treated mice and IPF patients and has ST2-independent effects. To test this hypothesis, oxIL-33 levels in the BALF of BLM-treated mice and IPF patients should be measured and the effects of oxIL-33 on BLM-induced fibrosis in wildtype and ST2 knockout mice investigated.

In summary, IL-33 oxidises in media and induces ST2-independent signalling in fibroblasts. Despite having no direct effect on the pro-fibrotic activity of these cells, oxIL-33 appears to activate PI3K/AKT/mTOR signalling in fibroblasts. As PI3K/AKT/mTOR signalling promotes the proliferation and survival of ECM-secreting fibroblasts *in vitro*, it is tempting to speculate (given the pathophysiological importance of these cells in IPF) that oxIL-33 may contribute to the development of IPF. A considerable amount of further work is required however to confirm my findings and understand the cellular and molecular mechanisms by which the oxidised form of IL-33 may modulate pulmonary fibrosis.

Chapter 7: Summary, conclusions and future directions

7. Summary, conclusions and future directions

7.1. Summary and conclusions

IPF is a chronic, progressive ILD characterised by the excessive deposition of extracellular matrix by fibroblasts and an irreversible loss of lung function (Martinez et al., 2017, Lederer and Martinez, 2018, Yanagihara et al., 2019). With a median survival time of 3-5 years from diagnosis, the prognosis for IPF patients is worse than all but two types of cancer (Vancheri et al., 2010, Hambly et al., 2017, Fernández Fabrellas et al., 2018). Despite the incidence of IPF continuing to increase the aetiology and pathophysiology of this deeply debilitating ILD remains unclear (Hutchinson et al., 2015, Lederer and Martinez, 2018). Currently only two drugs, pirfenidone and nintedanib are licensed to treat IPF (Hewitt and Maher, 2019). Since both drugs fail to halt disease progression and have potentially use limiting side effects (Richeldi et al., 2014, Valeyre et al., 2014, Ogura et al., 2015), the need to better understand the pathogenesis of IPF has never been greater. Recently, emerging evidence from patient-based studies and animal models suggests that the cytokine, IL-33, may play an important pathophysiological role during the development of IPF (Tajima et al., 2003, Luzina et al., 2012, Luzina et al., 2013, Li et al., 2014b, Gao et al., 2016, Xu et al., 2016a, Lee et al., 2017, Fanny et al., 2018, Zhao et al., 2018, Majewski et al., 2019b). Despite this, the cellular and molecular mechanisms by which IL-33 exerts its reported pro-fibrotic effects remain poorly understood. Consequently, the overall aim of this thesis was to investigate the role of IL-33 in the development of IPF.

Given the importance of fibroblasts in the development of IPF, it was hypothesised that IL-33 exerts some of its reported fibrogenic effects via this cell type. To assess whether IL-33 directly increases the pro-fibrotic activity of fibroblasts, HLFs were stimulated with recombinant IL-33 and the expression of multiple genes measured. Surprisingly, treatment with recombinant IL-33 had no effect on the expression of pro-inflammatory (*IL6* and *CXCL8*) and pro-fibrotic genes (*ACTA2* and *COL1A1*) by non-IPF and IPF HLFs. Despite contradicting earlier reports using rat cardiac (Zhu and Carver, 2012) and murine lung fibroblasts (Kurokawa et al., 2011), my results are consistent with those of Yagami et al. (2010), with HLFs failing to respond to recombinant IL-33 as they lack the IL-33 receptor ST2. Although experiments with IPF lung tissue (i.e. *in situ* hybridisation and IHC) are required to confirm that fibroblasts do not express ST2 *in situ*, my *in vitro* results suggest that, during the development of IPF, IL-33 is unlikely to have direct, ST2-dependent effects on the pro-fibrotic activity of fibroblasts.

Since HLFs failed to express ST2 *in vitro* it was concluded that, if IL-33 acts as a major pro-fibrotic mediator in IPF, alternative cell types likely mediate the effects of this cytokine during the fibrotic response. To test this hypothesis, a complex biological system was required. Although IL-33 has previously been reported to promote bleomycin (BLM)-induced pulmonary fibrosis (Li et al., 2014b, Gao et al., 2016, Fanny et al., 2018, Zhao et al., 2018), blockade of IL-33 signalling during the inflammatory rather than the fibrotic phase of this model meant that the true pro-fibrotic activity of IL-33 in this system was unclear. To differentiate between the pro-inflammatory and pro-fibrotic effects of IL-33 *in vivo*, I administered blocking reagents (either an α ST2 antibody or a ST2-Fc fusion protein) during the fibrotic phase

of the BLM mouse model. Despite having biological effects suggestive of target engagement, neither the α ST2 antibody nor the ST2-Fc fusion protein reduced established BLM-induced fibrosis. I therefore propose that, when signalling via ST2, IL-33 does not act as a major pro-fibrotic mediator in the BLM mouse model of pulmonary fibrosis.

Due to species-specific differences in IL-33 expression (Liew et al., 2016, Cayrol and Girard, 2018) it was possible that my findings from the BLM mouse model of pulmonary fibrosis would not translate fully to human disease. To validate my *in vivo* findings in a complex species-relevant system, a method to generate human PCLS was successfully developed and the ability of recombinant IL-33 to induce fibrotic changes in human PCLS assessed. In contrast to TGF β , IL-33 stimulation had no effect on *ACTA2*, *COL1A1*, *FN1* and fibronectin expression by human PCLS. Although dose response and time course experiments are desirable to confirm this result in additional donors, this observation suggests that IL-33 is unable to induce fibrotic changes in human PCLS. Taken together, the data in chapters 3-5 of this thesis implies that the IL-33:ST2 axis is unlikely to play a key pathophysiological role in IPF.

Several forms of IL-33 have been described (see chapter 1 section 1.2.6). Recently, IL-33 was discovered to oxidise in the extracellular environment and, in doing so, undergo a considerable conformational change (Cohen et al., 2015). Although this conformational change confers loss of ST2 binding and downstream signalling (Cohen et al., 2015), recent unpublished data from the Cohen group suggests that oxidised IL-33 (oxIL-33) can signal through EGFR and impair the proliferation, migration and differentiation of lung epithelial cells. Given the proposed pathophysiological importance of delayed and inadequate epithelial cell repair in IPF (Selman et al., 2001,

Sgalla et al., 2018) and the reported involvement of EGFR signalling in pulmonary fibrosis (Korfhagen et al., 1994, Hardie et al., 1997, Rice et al., 1999, Le Cras et al., 2003, Ishii et al., 2006, Hardie et al., 2007, Hardie et al., 2008, Yoon et al., 2016), it was hypothesised that the oxidised form of IL-33 may contribute to the development of IPF. To address this possibility, the effects of oxIL-33 on HLFs were assessed. Despite failing to increase the pro-fibrotic activity of HLFs directly, oxIL-33 appeared to activate PI3K/AKT/mTOR signalling in an EGFR-dependent fashion. Since PI3K/AKT/mTOR signalling has been shown to promote the proliferation and survival of ECM-secreting fibroblasts *in vitro* (Nho and Hergert, 2014, Im et al., 2015, Mercer et al., 2016, Romero et al., 2016), it is tempting to speculate (given the central role of fibroblasts in the fibrotic response) that oxIL-33 may play a pathophysiological role in IPF. Much further work is required however to confirm my initial results, elucidate the mechanisms by which oxIL-33 may modulate pulmonary fibrosis and verify the physiological relevance of the oxIL-33:EGFR axis in IPF.

7.2. Future directions

The data presented in this thesis raises the possibility that, once oxidised, IL-33 may promote pulmonary fibrosis via the novel oxIL-33:EGFR axis. To test this hypothesis and confirm that oxIL-33 induces PI3K/AKT/mTOR signalling in HLFs, multiple experiments could be performed. Firstly, animal models and patient samples could be used to verify the physiological relevance of the oxIL-33:EGFR axis in IPF. For instance, the effects of oxIL-33 in the BLM mouse model of pulmonary fibrosis could be assessed and the levels of oxIL-33 in the BALF of IPF patients measured. Furthermore, the ability of oxIL-33 to induce fibrotic changes in human PCLS could be determined. Secondly, the functional effects of oxIL-33 signalling in fibroblasts

could be investigated and the ability of oxIL-33 to induce PI3K/AKT/mTOR signalling in HLFs confirmed. Specifically, HLFs could be stimulated with purified oxIL-33 and the proliferation and survival of these cells assessed via MTT assay, thymidine incorporation assay, LC3 western blot and CellTiter-Blue® viability assay. If oxIL-33 was found to increase the proliferation and/or survival of HLFs, these experiments could be repeated in the presence of PI3K/AKT/mTOR inhibitors (i.e. Rapamycin, Omipalisib, Wortmannin and Borussertib) and the ability of oxIL-33 to induce PI3K/AKT/mTOR signalling in fibroblasts confirmed. Additionally, bulk RNA-seq time course experiments could be performed to comprehensively assess the effects of oxIL-33 on fibroblasts and establish whether additional signalling pathways are induced following oxIL-33 treatment. Thirdly, the effects of oxIL-33 on other cell types implicated in the pathogenesis of IPF could be investigated. For example, the ability of oxIL-33 to inhibit the proliferation and differentiation of AEC2s, modulate EMT and induce EGFR phosphorylation in alveolar and monocyte derived macrophages could be explored. Finally, the ability of oxIL-33 to synergise with known pro-fibrotic mediators could be assessed. In particular, HLFs could be treated with increasing concentrations of both TGFβ and oxIL-33 and the expression of fibrotic genes and proteins measured by qPCR and western blotting.

Although the results from such experiments are somewhat controversial (Ali et al., 2011, Choi et al., 2012, Shao et al., 2014, Gautier et al., 2016, Chen et al., 2019, Rana et al., 2019), intranuclear IL-33 has been shown to regulate gene expression by endothelial cells and mesenchymal stem cells. Given the nuclear pattern of IL-33 expression in IPF lung tissue and the apparent failure of (unstimulated and TGFβ treated) HLFs to release IL-33, IL-33 siRNA knockdown experiments were conducted

to assess the intracellular role of IL-33 in fibroblasts. Since I was unable to process the cDNA samples from these experiments (due to COVID-19-related disruption of ongoing lab work) an important future step would be to analyse these stored samples (using custom TaqMan™ gene expression array cards) and establish whether IL-33 modulates the pro-fibrotic activity of HLFs via an intracellular rather than an extracellular mechanism.

In addition to IL-33, TSLP and IL-25 are released following cell damage and/or death (Allakhverdi et al., 2007, Liu et al., 2018). Elevated in the EBC, BALF and lung tissue of IPF patients (Luzina et al., 2012, Datta et al., 2013, Luzina et al., 2013, Hams et al., 2014, Lee et al., 2017, Xu et al., 2019, Majewski et al., 2019b, Majewski et al., 2019a), IL-33, TSLP and IL-25 have been shown to have overlapping biological functions (Divekar and Kita, 2015, Vannella et al., 2016, An et al., 2020). Interestingly, blockade of all three cytokines was required to reduce *Schistosoma mansoni*-induced liver fibrosis and house dust mite-induced lung fibrosis. Since individual blockade of IL-33, TSLP and IL-25 had no effect on fibrosis in either of these models (Vannella et al., 2016), it is possible that some of my results - especially my *in vivo* findings - are due to the redundant and overlapping functional activities of these cytokines. To test this hypothesis, antibodies or fusion proteins against IL-33, TSLP and IL-25 should be used alone or in combination during the fibrotic phase of the BLM mouse model and their effects on established pulmonary fibrosis assessed.

7.3. Concluding remarks

IPF is a chronic, progressive ILD with limited treatment options. Despite being implicated in the development of IPF, the role of IL-33 during the fibrotic response remains unclear. The majority of the *in vitro*, *in vivo* and *ex vivo* data presented in this thesis suggests that, when signalling via ST2, IL-33 is unlikely to play a pathophysiological role in IPF. However, as the oxidised form of IL-33 appears to signal via a novel, potentially pro-fibrotic pathway in fibroblasts, it is possible that, once oxidised, IL-33 promotes pulmonary fibrosis via the oxIL-33:EGFR axis. Although much further work is required to establish if the oxIL33:EGFR axis represents a novel therapeutic target in IPF, the data presented in this thesis provides an interesting avenue for further investigation.

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8. References

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Appendix

9. Appendix

9.1. Materials

9.1.1. Reagents and general lab supplies

<u>Material</u>	<u>Supplier</u>	<u>Cat. No.</u>
0.9% Saline	Sigma-Aldrich	S8776
1.5 ml Protein LoBind Eppendorf Tubes	Eppendorf	022431081
15cm Dish	Nunc	168381
200 Stainless steel beads (5 mm diameter)	Qiagen	69989
20X Bolt™ MOPS SDS Running Buffer	Invitrogen™	B0001
24 wells plates	Costar®	3524
3,3',5,5'-Tetramethylbenzidine (TMB) Liquid Substrate	Sigma-Aldrich	T0440
3',3'-Diaminobenzidine tablets (DAB)	Sigma-Aldrich	D4418
384 well white round bottomed plates	Corning	4513
3M Vetbond Tissue Adhesive	3M	14695B
4X Bolt™ LDS Sample Buffer	Invitrogen™	B0007
6 well plates	Costar®	3516
96 well black collagen-I coated plates	Greiner Bio-One	655956
96 well Flat Clear Bottom White Polystyrene TC-treated Microplates	Corning®	3610
96 well plates	Costar®	3596
Acid Fuchsin	Sigma-Aldrich	F8129
Acrylamide/Bis-acrylamide, 30% solution	Sigma-Aldrich	A3699

Agarose, II™, for biotechnology (Low melting point agarose)	VWR	0815-100G
Aluminum potassium sulfate dodecahydrate	Sigma-Aldrich	A7210
Amersham™ ECL™ Rainbow™ Marker - Full range	Sigma-Aldrich	GERPN800E
Ammonium persulfate (APS)	Sigma-Aldrich	A3678
Aniline Blue	Sigma-Aldrich	415049
Biebrich scarlet	VWR	ICNA021548558
Bleomycin sulfate	Kyowa Hakko	
Blotto, non-fat dry milk	Santa Cruz	sc-2324
β-mercaptoethanol	Sigma-Aldrich	M3148
Bolt™ 10%, Bis-Tris, 1.0 mm, Mini Protein Gel, 12-well	Invitrogen™	NW00102BOX
Bolt™ 12%, Bis-Tris, 1.0 mm, Mini Protein Gel, 12-well	Invitrogen™	NW00122BOX
Bolt™ 4 to 12%, Bis-Tris, 1.0 mm Mini Protein Gel, 12-well	Invitrogen™	NW04122BOX
Bolt™ 4 to 12%, Bis-Tris, 1.0 mm, Mini Protein Gel, 15-well	Invitrogen™	NW04125BOX
Bovine Serum Albumin (BSA)	Sigma-Aldrich	A2153
Bromophenol blue	Sigma-Aldrich	B0126
Cell lysis Buffer (10x)	Cell Signaling Technology®	9803
Chloramine T	Sigma-Aldrich	402869
Chloroform	Sigma-Aldrich	C2432
Citric acid	Sigma-Aldrich	251275
Clarity Max™ Western ECL Substrate	BIO-RAD	1705062
Clarity™ Western ECL Substrate	BIO-RAD	1705061
Collection Microtube Caps	Qiagen	19566
Collection Microtubes (racked, 10 x 96)	Qiagen	19560

cOmplete™, Mini Protease Inhibitor Cocktail	Roche	44693124001
Deoxynucleotide triphosphates (dNTPs)	Promega	U1240
Direct Red 80 (Sirius Red)	Sigma-Aldrich	365548
Disposable Skin Biopsy Punch	KAI MEDICAL	BP-80F
Distilled Water (dH ₂ O)	Gibco™	15230089
DPBS, no calcium, no magnesium (PBS)	Gibco™	14190094
DPX Mountant	Sigma-Aldrich	06522
Dulbecco's Modified Eagle's Medium (DMEM)	Sigma-Aldrich	D5546
ECL™ detection reagent	Sigma-Aldrich	RPN2209
Ehrlich's solution	Sigma-Aldrich	03891
Endothelial Cell Growth Medium	PromoCell	C-22010
Endothelial Cell Growth Medium Supplement Mix	PromoCell	C-39215
Eosin Y	Sigma-Aldrich	230251
Ethanol	Fisher Scientific	10428671
Ferric Chloride	Sigma-Aldrich	157740
Fetal Bovine Serum (FBS)	Sigma-Aldrich	F0804
Formaldehyde solution (37%)	Sigma-Aldrich	252549
Formalin solution, neutral buffered, 10%	Sigma-Aldrich	HT501128
Freeze dried skimmed milk	Premier foods	
Glacial Acetic Acid (100%)	Sigma-Aldrich	1.00063
Glycerol	Fisher Scientific	10618294
Glycine	Fisher Scientific	G/0800/48
Growth Medium Supplement Pack	PromoCell	C-39170
Haematoxylin	Sigma-Aldrich	H3136
Halt™ Protease and Phosphatase Inhibitor Cocktail, EDTA-free (100x)	Thermo Scientific™	78445

HCl	Fisher Scientific	H/1200/PB17
Heparin Solution	Sigma-Aldrich	H5152
High-Capacity RNA-to-cDNA™ Kit	Applied Biosystems™	4388950
Hoechst 33342, Trihydrochloride, Trihydrate	Invitrogen™	H3570
Honeywell Fluka™ Sulfuric acid solution, 1M	Honeywell	35276-1L
Horse Serum	Sigma-Aldrich	H0146
Hydrogen Peroxide (30%)	Sigma-Aldrich	H-1009
IMDM	Gibco™	21980032
InstantBlue® Coomassie Protein Stain	Abcam	ab119211
Isopropyl alcohol	Fisher Scientific	P/7500/17
KAPA SYBR® Green FAST Master Mix (2x)	Roche	KK4601
L-Glutamine	Sigma-Aldrich	G7513
Lipofectamine™ RNAiMAX Transfection Reagent	Invitrogen™	13778075
MaxiSorp™, F96 wells per plate (for ELISA)	Nunc	735-0199
Mega Base Molds 2 (Histology Base Mould)	Leica Biosystems	3803085E
Methanol	Sigma-Aldrich	32213
M-MLV Reverse Transcriptase (Supplied with 5x Buffer)	Promega	M5313
MSD standard multi-array plates	MSD (Meso Scale Discovery)	L15XA-3
N,N,N',N'-Tetramethylethylenediamine (TEMED)	Sigma-Aldrich	T9281
Nuclease free water	Qiagen	1039498
ON-TARGETplus Human IL33 siRNA	Dharmacon	L-015122-01-0005
ON-TARGETplus Non-targeting Control Pool	Dharmacon	D-001810-10-05

Penicillin-Streptomycin	Sigma-Aldrich	P4333
Phenylmethylsulfonyl fluoride (PMSF)	Roche	10837091001
Phosphomolybdic acid	Sigma-Aldrich	HT153
Phosphotungstic acid	Sigma-Aldrich	HT152
PhosSTOP™	Roche	4906837001
Picric acid solution	Sigma-Aldrich	P6744
Primer for cDNA Synthesis (Oligo DT)	Sigma-Aldrich	10814270001
Purified oxIL-33	AstraZeneca	
Purified redIL-33	AstraZeneca	
PYREX® Test Tube Without Rim	Pyrex®	1622/02M
rhIL-33	R&D	3625-IL-010
rhTGFβ	R&D	240-B-010
RNAlater®	Sigma-Aldrich	R0901
RNasin® Ribonuclease Inhibitor	Promega	N2118
RPMI 1640 Medium, GlutaMAX™ Supplement	Gibco™	61870010
Small Airway Epithelial Cell Growth Medium	PromoCell	C-21270
Sodium Chloride	Fisher Scientific	S/3160/60
Sodium dodecyl sulfate (SDS)	Sigma-Aldrich	L3771
Sodium iodate	Sigma-Aldrich	S4007
SuperScript™ IV Reverse Transcriptase	Invitrogen™	18090050
SYBR® Green qPCR primers	Eurofins	
TaqMan Probes	Applied Biosystems™	
TaqMan™ Fast Advanced Master Mix	Applied Biosystems™	4444557
Trans-4-hydroxyl-1-proline	Sigma-Aldrich	H54409

Trans-Blot Turbo Midi 0.2 µm Nitrocellulose Transfer Packs	BIO-RAD	1704159
Trichloroacetic acid	Sigma-Aldrich	T6399
Tris(Hydroxymethyl) Methylamine, Biological Buffer (Tris base)	Fisher Scientific	T/P630/60
Tri-sodium citrate dihydrate	Sigma-Aldrich	S1804
Triton™ X-100	Roche	11332481001
TRIzol™ Reagent	Invitrogen™	15596018
Trypan Blue solution	Sigma-Aldrich	T8154
Tween®20	Sigma-Aldrich	P1379
Ultrapure LPS	InvivoGen	tlrl-3pelps
VECTASTAIN® Elite ABC-HRP Kit, Peroxidase (Standard)	Vector Laboratories	PK-6100
Xylene	Fisher Scientific	28975325

9.1.2. qPCR Primers

9.1.2.1. SYBR® Green qPCR primers

Table 9.1: SYBR Green qPCR primer sequences

Species	Gene	Primer	Sequence 5'→ 3'
Human	<i>ACTA2</i>	Forward	TGTGCTGGACTCTGGAGATG
		Reverse	GACAATCTCACGCTCAGCAG
	<i>β2M</i>	Forward	AATCCAAATGCGGCATCT
		Reverse	GAGTATGCCTGCCGTGTG
	<i>COL1A1</i>	Forward	CCAGCAAATGTTCTTTTTG
		Reverse	AAAATTTCACAAGTCCCCATC
	<i>CXCL8</i>	Forward	ATGACTTCCAAGCTGGCCGTGGCT
		Reverse	TCTCAGCCCTCTTCAAAAATTCTC
	<i>IL6</i>	Forward	CAATAACCACCCCTGACCCA
		Reverse	GCGCAGAATGAGATGAGTTGTC
	<i>IL33</i>	Forward	CCTGTCAACAGCAGTCTACT
		Reverse	TTGGCATGCAACCAGAAGTC
	<i>ST2</i>	Forward	AGCTGACGTGAAGGAAGAGG
		Reverse	AGGTTTAGCTATGTCTCTCCAGA
Mouse	<i>HPRT</i>	Forward	CCAGCAGGTCAGCAAAGAACT

		Reverse	TGAAAGACTTGCTCGAGATGTCA
		Forward	CACATTGAGCATCCAAGGAA
	<i>IL33</i>	Reverse	AACAGATTGGTCATTGTATGTACTCAG
	<i>ST2 (IL1RL1)</i>	Forward	TCTGTGGAGTACTTTGTTTACC
		Reverse	TCTGCTATTCTGGATACTGCTTTC

9.1.2.2. TaqMan™ assays

Table 9.2: TaqMan assay IDs

Species	Gene	Assay ID
Human	<i>ACTA2</i>	Hs00426835_g1
	<i>β2M</i>	Hs00187842_m1
	<i>COL1A1</i>	Hs00164004_m1
	<i>FN1</i>	Hs01549976_m1

9.1.3. Antibodies

Table 9.3: Details of antibodies used

Antibody	Clone	Supplier	Cat. No.	Dilution/ Conc.
Akt (pan) Rabbit mAb	C67E7	Cell Signaling Technology®	4691S	1:1000
αSMA Rabbit pAb	ab5694	Abcam	ab5694	1:500
EGF Receptor Rabbit mAb	C74B9	Cell Signaling Technology®	2646S	1:1000
Fibronectin Rabbit pAb	ab2413	Abcam	ab2413	1:5000
Goat anti-Rabbit IgG (H+L) Secondary Antibody, DyLight 488	35552	Invitrogen™	35552	1:400
Goat Anti-Rabbit Immunoglobulins/HRP	P0448	Dako	P0448	1:3000
Goat IgG HRP-conjugated Antibody	HAF109	R&D Systems	HAF109	1:1000
Human EGFR Mouse mAb	LA1	Merck	05-101	10 µg/ml
Human IgG1 Isotype Control mAb	N/A	AstraZeneca	N/A	1 µg/ml
Human IL-33 Goat pAb	AF3625	R&D Systems	AF3625	1:750
Human IL-33 mAb	N/A	AstraZeneca	N/A	1 µg/ml
IgG1 Isotype Control Mouse mAb	MAB002	R&D Systems	MAB002	10 µg/ml
IL-33 Mouse mAb	Nessy-1	Abcam	ab54385	1:300
Mouse IgG1 Isotype Control mAb	N/A	AstraZeneca	N/A	10 mg/kg
Mouse IL-33 Goat pAB	AF3626	R&D Systems	AF3626	1:1000
Mouse ST2-Fc	N/A	AstraZeneca	N/A	10 mg/kg
Mouse αST2 mAb	N/A	AstraZeneca	N/A	10 mg/kg
NFκB p65 Polyclonal Antibody	PA5-16545	Invitrogen™	PA5-16545	1:500
Phospho-Akt (Ser473) XP® Rabbit mAb	D9E	Cell Signaling Technology®	4060S	1:1000
Phospho-Akt (Thr308) Rabbit mAb	244F9	Cell Signaling Technology®	4056S	1:1000
Phospho-EGF Receptor (Tyr1068) XP® Rabbit mAb	D7A5	Cell Signaling Technology®	3777S	1:1000

Phospho-EGF Receptor (Tyr992) Rabbit pAb	2235	Cell Signaling Technology®	2235S	1:1000
Phospho-PRAS40 (Thr246) Rabbit mAb	C77D7	Cell Signaling Technology®	2997S	1:1000
Phospho-Stat3 (Ser727) Rabbit pAb	9134	Cell Signaling Technology®	9134	1:1000
Phospho-WNK1 (T60) Rabbit mAb	MAB4720	R&D Systems	MAB4720-100	1:500
PRAS40 XP® Rabbit mAb	D23C7	Cell Signaling Technology®	2691S	1:1000
Recombinant GAPDH Rabbit mAb	EPR16884	Abcam	ab181603	1:10000
Recombinant Vimentin Rabbit mAb	EPR3776	Abcam	ab92547	1:5000
ST2/IL-33R Goat pAb	AF523	R&D Systems	AF523	1:750
Stat3 Rabbit mAb	79D7	Cell Signaling Technology®	4904S	1:1000
β-tubulin Rabbit pAb	ab6046	Abcam	ab6046	1:2000

9.1.4. Kits

<u>Kit</u>	<u>Supplier</u>	<u>Cat. No.</u>
Direct-zol™ RNA Miniprep	Zymo Research	R2053
Human IL-33 DuoSet ELISA	R&D Systems	DY3625B
Human IL-6 DuoSet ELISA	R&D Systems	DY206
Human IL-8/CXCL8 DuoSet ELISA	R&D Systems	DY208
Human ST2/IL-33R DuoSet ELISA	R&D Systems	DY523B-05
LDH-Glo™ Cytotoxicity Assay	Promega	J2381
Mouse IL-33 DuoSet ELISA	R&D Systems	DY3626
Mouse ST2/IL-33R DuoSet ELISA	R&D Systems	DY1004
NucleoSpin® RNA extraction kit	Macherey-Nagel	740955
Phospho-EGFR (Tyr1068) cellular kit	Cisbio	64EG1PEH
Pierce™ BCA Protein Assay Kit	Thermo Scientific™	23225
Proteome Profiler Human Phospho-Kinase Array Kit	R&D Systems	ARY003B
RAL DIFF-QUIK™	Siemens Healthineers	10736130
V-PLEX Proinflammatory Panel 1 Human Kit	MSD (Meso Scale Discovery)	K15049D-2

V-PLEX Proinflammatory
Panel 1 Mouse Kit

MSD (Meso Scale
Discovery) K15048D-2

9.1.5. Lab equipment

Equipment

7900HT Fast Real-Time PCR machine
Criterion™ Blotter
Envision Plate reader
Epson Perfection V700 Photo scanner
FLUOstar® Omega microplate reader
Gen5 Cytation plate reader
GeneAmp® PCR System 9700
Leica VT1200 S vibratome
LI-COR C[®]-DiGit Blot Scanner
Mini Gel Electrophoresis Tank
Mini-PROTEAN® Tetra Electrophoresis Cell
tank
Mx3000P® qPCR machine
NanoDrop™ 1000 spectrophotometer
NanoDrop™ 2000 spectrophotometer
Nikon 90i light microscope
OMNI Tissue Homogenizer
ProFlex™ PCR system
SECTOR® Imager 6000

TissueLyser II
Trans-Blot® Turbo™ Transfer system

Supplier

Applied Biosystems®
Bio-Rad Laboratories
PerkinElmer®
Epson
BMG LABTECH
BioTek Instruments
Applied Biosystems®
Leica Biosystems
LI-COR Biosciences
Life Technologies
Bio-Rad Laboratories

Stratagene
Thermo Scientific
Thermo Scientific
Nikon
OMNI International
Applied Biosystems®
MSD (Meso Scale
Discovery)
Qiagen
Bio-Rad Laboratories

9.1.6. Software

Software

Developer

BioTek Gen™ Data analysis software

BioTeK®

CaseViewer,

3DHISTECH Ltd.

Epson Scan

Epsom

GraphPad Prism 8

GraphPad Software Inc.

Image Studio™

LI-COR Biosciences

ImageJ

National Institutes of Health

MSD DISCOVERY WORKBENCH® software

MSD (Meso Scale
Discovery)

MxPro

Stratagene

SDS 2.4

Applied Biosystems

9.2. Media, buffer, western blot gels and histology stain recipes

9.2.1. Media recipes

Media

Recipe

L-glutamine supplemented
DMEM

DMEM plus 4mM L-glutamine

Serum free DMEM

DMEM plus 4mM L-glutamine, 100U/ml
Penicillin and 100µg/ml Streptomycin

Serum free RPMI

RPMI plus 100U/ml Penicillin and 100µg/ml
Streptomycin

Serum supplemented
DMEM

DMEM plus 10% FBS, 4mM L-glutamine,
100U/ml Penicillin and 100µg/ml Streptomycin

Serum supplemented
RPMI

RPMI plus 10% FBS, 100U/ml Penicillin and
100µg/ml Streptomycin

Supplemented Endothelial
Cell Growth Medium

Endothelial Cell Growth Medium plus 0.02 ml/ml
FBS, 0.0004 ml/ml Endothelial Cell Growth

Supplemented Small Airway Epithelial Cell Growth Medium	Supplement, 0.1 ng/ml EGF, 1 ng/ml bFGF, 90 µg/ml Heparin and 1 µg/ml Hydrocortisone Small Airway Epithelial Cell Growth Medium plus 2.5 mg/ml BSA, 0.004 ml/ml BPE, 10 ng/ml EGF, 5 µg/ml Insulin, 0.5 µg/ml Hydrocortisone, 0.5 µg/ml Epinephrine, 6.7 ng/ml Triiodo-L-thyronine, 10 µg/ml Transferrin, 0.1 ng/ml Retinoic Acid, 100U/ml penicillin and 100 µg/ml streptomycin
Transfection media	DMEM plus 10% FBS and 4mM L-glutamine

9.2.2. Buffer recipes

<u>Buffer</u>	<u>Recipe</u>
10 mM Sodium citrate buffer	2.94 g Tri-Sodium citrate dehydrate, 1000 ml dH ₂ O and 0.5 ml Tween 20 Adjusted to pH 6.0 with concentrated HCl
10x Running buffer	24.2 g Tris base, 144 g glycine, 10 g SDS and 1000 ml dH ₂ O
10x TBST	24.2 g Tris base, 87.6 g NaCl, 1000 ml dH ₂ O and 10 ml Tween®20 Adjusted to pH 7.4 - 7.6 with concentrated HCl
10x Transfer buffer	24.2 g Tris base, 144 g glycine and 1000 ml dH ₂ O
Transfer buffer	200 ml 10 x transfer buffer, 1400 ml dH ₂ O and 400 ml methanol
4x Laemmli buffer	5 ml 0.5 M Tris HCl (pH 6.8), 4 ml 100% Glycerol, 4 ml 10% SDS, 40 mg Bromophenol blue and 7 ml dH ₂ O
Buffer 1 for resolving gel	18.5 g Tris base, 50 ml dH ₂ O and 4 ml 10% SDS Adjusted to pH 8.8 with concentrated HCl before dH ₂ O added to give a total volume of 100 ml
Buffer 2 for stacking gel	6 g Tris base, 60 ml dH ₂ O and 4 ml 10% SDS Adjusted to pH 6.8 with concentrated HCl before dH ₂ O added to give a total volume of 100 ml

Cell lysis buffer	100 µl Cell lysis buffer (10x), 100 µl PhosSTOP™, 100 µl cOmplete™, Mini Protease Inhibitor Cocktail, 20µl PMSF and 680µl dH ₂ O
ELISA Wash Buffer	0.05% Tween®20 in PBS
HTRF cell lysis buffer	2.5 ml HTRF Lysis buffer (4x), 100 µl Blocking reagent (100x) and 7.4 ml dH ₂ O
LDH assay buffer	200mM Tris-HCl (pH 7.3), 10% glycerol and 1% BSA
MSD Read Buffer T®	10 ml Read Buffer T® (4x) and 10 ml dH ₂ O
MSD Wash buffer	0.05% Tween®20 in PBS
PBS-T	0.05% Tween®20 in PBS
Permeabilization buffer	0.1% Triton X-100 in PBS
Reagent diluent	1% BSA in PBS
Tissue lysis buffer	100 µl Cell lysis buffer (10x), 10 µl Halt™ Protease and Phosphatase Inhibitor Cocktail, EDTA-free (100x) and 890µl dH ₂ O
Stop Solution	1M Sulfuric acid solution

9.2.3. Western blot gel recipes

<u>Gel type</u>	<u>Recipe</u>
10% Resolving gel	6.66 ml 30% Bis-acrylamide, 5.2 ml Buffer 1, 7.92 ml dH ₂ O, 200µl, 10% APS and 20 µl TEMED
Stacking gel	1.3 ml 30% Bis-acrylamide, 2.5ml Buffer 2, 6.1 ml dH ₂ O, 50µl 10% APS and 10 µl TEMED

9.2.4. Histology stain recipes

<u>Stain</u>	<u>Recipe</u>
1% Acetic Acid solution	1 ml Glacial acetic acid and 99 ml dH ₂ O
Acid-Alcohol	350 ml ethanol, 145 ml dH ₂ O and 0.5 ml 12 N HCl
Acidified Water	5 ml Glacial acetic acid and 1 l dH ₂ O

Aniline Blue solution	2.5g Aniline blue, 2 ml Glacial acetic acid and 100 ml dH ₂ O
Biebrich Scarlet-Acid Fuchsin solution	90 ml 1% Biebrich scarlet in dH ₂ O, 10 ml 1% Acid fuchsin in dH ₂ O and 1ml Glacial acetic acid
Bouin's solution	75 ml Picric acid (saturated), 25 ml 37% Formaldehyde and 5 ml Glacial acetic acid
Eosin stock solution	10 g Eosin Y, 800 ml 95% ethanol and 200 ml dH ₂ O
Eosin working solution	250 ml Eosin stock solution, 750 ml 80% ethanol and 5 ml Glacial acetic acid
Mayer's Haematoxylin	50 g Aluminum potassium sulfate dodecahydrate, 1 g Haematoxylin, 0.2g Sodium iodate, 1g Citric acid and 1 l dH ₂ O
Phosphotungstic Phosphomolybdic Acid	25 ml 5% Phosphomolybdic acid and 25 ml 5% Phosphotungstic acid
Sirius Red solution	500 mg Direct Red 80, 500 ml in Picric acid (saturated)
Solution A	1g Haematoxylin in 100 ml 95% Ethanol
Solution B	4 ml 30% Ferric chloride in dH ₂ O, 1 ml 12 N HCl and 95 ml dH ₂ O
Weigert's Haematoxylin	1:1 Solution A and Solution B