Alveolar Macrophage heterogeneity in Idiopathic Pulmonary Fibrosis

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Declaration of originality
I declare that all of the work presented in this thesis is my own, and that all else, information, data, results, figures and ideas from another source or from collaborations have been appropriately referenced or acknowledged.

Simon Luk
July 2015

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Abstract

Idiopathic Pulmonary Fibrosis (IPF) involves excess extracellular matrix (ECM) deposition within the lung interstitium, caused by non-resolving chronic inflammation and dysregulated repair. Alveolar macrophage (AMφ) may contribute to IPF through releasing various mediators by different subsets, investigated here in vitro, and ex vivo using a mouse model of bleomycin (BLM)-induced pulmonary fibrosis. The role of foamy AMφ in the reported increased susceptibility of Hermansky Pudlak Syndrome (HPS) 1 mice to BLM-induced pulmonary fibrosis was also assessed.

Novel characterisation studies revealed that terminally differentiated AMφ are inducible into M1-like [nitric oxide synthase 2 (NOS2)hi interleukin (IL)-1βhi IL-12 p40 (total)hi major histocompatibility protein (MHC)-IIhi mannose receptor C, type 1 (MRC1)] or M2-like [Arginase 1 (Arg1)hi Fibronectinhi IGF-1hi MHC-IIlo MRC1+/−] phenotypes following IFN-Υ or IL-13 priming respectively. Lipopolysaccharide (LPS) altered these AMφ subset phenotypes.

AMφ heterogeneity in a novel multiple oropharyngeal dose, BLM-induced pulmonary fibrosis was evaluated from days 7 to 21. Accumulation of M1-like AMφ at day 7, and M1/M2-hybrid AMφ [Arg1hi IL-12 p40 (total)hi fibronectinhi MHC-IIlo MRC1+/−] from days 7 to 21, may promote inflammation and fibrosis respectively. Toll-like Receptor (TLR) 9 messenger ribonucleic acid (mRNA) and TLR2 surface protein, and both TLRs2 and 9 ex vivo activities were increased in BLM-challenged mice from days 7 to 21, suggesting their roles in inflammation and fibrosis.

Foamy AMφ accumulated in BLM-induced pulmonary fibrosis, and their potential role in the reported increased susceptibility to BLM-induced pulmonary fibrosis of HPS1 mice was evaluated. BLM-challenged HPS1 mice (days 7-21) had increased weight loss indicating reduced BLM tolerance from days 7 to 11, but little/no difference in collagen accumulation, suggesting that reduced BLM tolerance is not correlated with increased pulmonary fibrosis.

In conclusion AMφ alter their phenotype in response to their environment that contributes to different stages of BLM-induced pulmonary fibrosis. Reduced BLM tolerance in HPS1 mice is not correlated with increased pulmonary fibrosis.
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Abbreviations

ACK  Ammonium Chloride Potassium
ADA  Adenosine deaminase
ADAM  A Disintegrin And Metalloproteinase
AEC1  Alveolar Epithelial Cell type 1
AEC2  Alveolar Epithelial Cell type 2
ALI  Acute Lung Injury
ALK5  Transforming growth factor β receptor 1 (TGF-βR1)
AMΦ  Alveolar Macrophage
AP  Activator Protein
APCs  Antigen Presenting Cells
APO-AI  Apolipoprotein-AI
Arg-1  Arginase-1
ASC  Apoptosis-associated Speck-like protein containing a CARD
ATP  Adenosine Triphosphate
BAL  Bronchoalveolar Lavage
BALF  Bronchoalveolar Lavage Fluid
Bax  Bcl-2 associated X protein
BCA  B-Cell Attracting chemokine
BCG  Bacillus Calmette-Guérin
Bcl-2  B-cell lymphoma (Bcl)-2
BIR  Baculovirus Inhibitor of apoptosis protein Repeat
BLM  Bleomycin
BLOC  Biogenesis of Lysosome-Related Organelles Complex
BLYS  B lymphocyte Stimulator
BMDM  Blood monocyte derived macrophages
BMI  Body Mass Index
BMMΦ  Bone Marrow Derived Macrophage
BMP  Bone morphogenetic proteins
cAMP  cyclic Adenosine Monophosphate
CARD  Caspase Recruitment Domain
CC2  Cell Conditioner 2
CCL  CC chemokine ligand
CCl₄  Carbon tetrachloride
CCm-Tf  Carboxymethylated transferrin
CCR  CC chemokine receptor
CD  Cluster of Differentiation
cDNA  complementary deoxyribonucleic acid
Chi3l3  Chitinase 3 like 3
CIITA  MHCII Transcriptor Activator
CLMF  Cytotoxic lymphocyte maturation factor
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<tr>
<th>Abbreviation</th>
<th>Full Form</th>
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<td>Cytomegalovirus</td>
</tr>
<tr>
<td>Col1α1</td>
<td>Collagen 1 α 1</td>
</tr>
<tr>
<td>COP</td>
<td>Cryptogenic organising pneumonia</td>
</tr>
<tr>
<td>CpG</td>
<td>Cytidine-phosphate guanosine</td>
</tr>
<tr>
<td>CR</td>
<td>Complement Receptor</td>
</tr>
<tr>
<td>CREB</td>
<td>cAMP Response Element Binding protein</td>
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<td>CSF</td>
<td>Colony Stimulating Factor</td>
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<tr>
<td>CSFR1</td>
<td>CSF-1 receptor</td>
</tr>
<tr>
<td>Ct</td>
<td>Cycle threshold</td>
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<tr>
<td>CTMC</td>
<td>Connective tissue mast cell</td>
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<td>CVD-IP</td>
<td>Interstitial pneumonia with collagen vascular disease</td>
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<tr>
<td>CXCL</td>
<td>CXC chemokine ligand</td>
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<td>CXCR</td>
<td>CXC chemokine receptor</td>
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<tr>
<td>DAMP</td>
<td>Danger Associated Molecular Pattern</td>
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<tr>
<td>DARC</td>
<td>Duffy Antigen Receptor for Chemokines</td>
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<td>DC</td>
<td>Dendritic cells</td>
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<tr>
<td>DC-SIGN</td>
<td>Dendritic Cell- Specific Intercellular adhesion molecule-Grabbing Nonintegrin</td>
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<tr>
<td>DKC</td>
<td>Dyskeratosis congenita</td>
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<tr>
<td>DNA</td>
<td>Deoxyribonucleic acid</td>
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<td>DPBS</td>
<td>Dulbecco’s Phosphate Buffered Saline</td>
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<td>DPI</td>
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<td>Dipeptidyl peptidase-IV</td>
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<td>Diphtheria toxin</td>
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<td>EBV</td>
<td>Epstein-Barr Virus</td>
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<td>ECF</td>
<td>Eosinophilic Chemotactic Factor</td>
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<td>ECM</td>
<td>Extracellular Matrix</td>
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<tr>
<td>EGF</td>
<td>Epidermal growth factor</td>
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<td>Early Growth Response protein</td>
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<td>ELISA</td>
<td>Enzyme linked immunosorbent assay</td>
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<td>Glutamine-leucine-arginine</td>
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<td>ER</td>
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<tr>
<td>ERK</td>
<td>Extracellular regulated kinase</td>
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<tr>
<td>FACS</td>
<td>Fluorescence activated cell sorting</td>
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<tr>
<td>FcγR III</td>
<td>Fc gamma receptor III</td>
</tr>
<tr>
<td>FCS</td>
<td>Fetal Calf Serum</td>
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<tr>
<td>FGF</td>
<td>Fibroblast Growth Factor</td>
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<td>FITC</td>
<td>Fluorescein isothiocyanate</td>
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<tr>
<td>FI ZZ1</td>
<td>Found In Inflammatory Zone</td>
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<td>FOXP3</td>
<td>Forkhead Box P3</td>
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<tr>
<td>FR-β</td>
<td>Folate-receptor-beta</td>
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<tr>
<td>FVC</td>
<td>Forced vital capacity</td>
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<tr>
<td>Galectin-3</td>
<td>Galectin-3</td>
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<td>GCP</td>
<td>Granulocyte Chemotactic Protein</td>
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<tr>
<td>GEF</td>
<td>Guanine Exchange Factor</td>
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<td>GLBD</td>
<td>Giant Lamellar Body Degeneration</td>
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<tr>
<td>GM-CSF</td>
<td>Granulocyte Macrophage colony stimulating factor</td>
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<tr>
<td>gp130</td>
<td>Signal transducing subunit of IL-6R</td>
</tr>
<tr>
<td>Acronym</td>
<td>Definition</td>
</tr>
<tr>
<td>---------</td>
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<tr>
<td>GpC</td>
<td>Guanine-phosphorothiate-cytosine</td>
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<tr>
<td>GR</td>
<td>Glucocorticoid receptor</td>
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<td>GRO-α</td>
<td>Growth regulated alpha protein</td>
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<td>GTP</td>
<td>Guanosine TriPhosphate</td>
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<tr>
<td>GTPase</td>
<td>GTP hydrolase</td>
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<tr>
<td>HA</td>
<td>Hyaluronan</td>
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<tr>
<td>HCl</td>
<td>Hydrochloric Acid</td>
</tr>
<tr>
<td>HDL</td>
<td>High Density Lipoprotein</td>
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<tr>
<td>HEK</td>
<td>Human embryonic kidney</td>
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<tr>
<td>HET-E</td>
<td>Incompatibility locus protein from <em>Posaspora anserine</em></td>
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<tr>
<td>hFLMC</td>
<td>human Fetal Lung Mononuclear Cells</td>
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<tr>
<td>HHV</td>
<td>Human Herpesvirus</td>
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<tr>
<td>HIF</td>
<td>Hypoxia-inducible factor</td>
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<tr>
<td>HIV</td>
<td>Human Immunodeficiency Virus</td>
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<tr>
<td>HLA</td>
<td>Human Leukocyte Antigen</td>
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<td>HMGB</td>
<td>High Mobility Group Box protein 1</td>
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<td>HMW</td>
<td>High molecular weight</td>
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<tr>
<td>Hox</td>
<td>Homeobox</td>
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<td>Hprt1</td>
<td>Hypoxanthine phosphoribosyltransferase 1</td>
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<td>HPS</td>
<td>Hermansky Pudlak Syndrome</td>
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<td>HPSPF</td>
<td>HPS pulmonary fibrosis</td>
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<tr>
<td>HRCT</td>
<td>High-Resolution Computer Tomography</td>
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<tr>
<td>HRE</td>
<td>Hypoxia-response elements</td>
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<td>HSC</td>
<td>Hepatic stellate cells</td>
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<td>HSP70</td>
<td>Heat Shock Protein 70</td>
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<td>hTERT</td>
<td>Human telomerase reverse transcriptase</td>
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<td>hTR</td>
<td>Human telomerase ribonucleic acid (RNA)</td>
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<td>H&amp;E</td>
<td>Hematoxylin and eosin</td>
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<tr>
<td>I-309</td>
<td>CC Chemokine Ligand 1 (CCL1)</td>
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<tr>
<td>IAP</td>
<td>Intracisternal A particle</td>
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<tr>
<td>ICE</td>
<td>IL-1β Converting Enzyme</td>
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<tr>
<td>IFN</td>
<td>Interferon</td>
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<tr>
<td>IFN-γR</td>
<td>IFN-γ Receptor</td>
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<tr>
<td>Ig</td>
<td>Immunoglobulin</td>
</tr>
<tr>
<td>IGF-1</td>
<td>Insulin-like growth factor-1</td>
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<tr>
<td>IIP</td>
<td>Idiopathic Interstitial Fibrosis</td>
</tr>
<tr>
<td>IKKB</td>
<td>IκB Kinase</td>
</tr>
<tr>
<td>IL</td>
<td>Interleukin</td>
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<tr>
<td>IL-10R</td>
<td>IL-10 Receptor</td>
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<td>IL-13R</td>
<td>IL-13 Receptor</td>
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<tr>
<td>IL-1R</td>
<td>IL-1 Receptor</td>
</tr>
<tr>
<td>IL-1R AcP</td>
<td>IL-1R accessory protein</td>
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<td>IL-1ra</td>
<td>IL-1 receptor antagonist</td>
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<tr>
<td>IL-4R</td>
<td>IL-4 Receptor</td>
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<tr>
<td>IL-6R</td>
<td>IL-6 Receptor</td>
</tr>
<tr>
<td>IL-13R</td>
<td>IL-13 Receptor</td>
</tr>
<tr>
<td>IMΦ</td>
<td>Interstitial macrophage</td>
</tr>
<tr>
<td>IMS</td>
<td>Industrial Methylated Spirit</td>
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</table>
iNOS     inducible Nitric Oxide Synthase
i.p.     intraperitoneal
IPF      Idiopathic Pulmonary Fibrosis
I/R      Ischemic/ reperfusion
IRAK     Interleukin-1 Receptor Associated Kinase
IRF      Interferon Regulatory Factor
i.t.     intratracheal
I-TAC    Interferon-inducible T-cell alpha Chemoattractant
IU       International units
IkB      Inhibitor of NFkB
JAK      Janus kinase
JNK      c-Jun N-terminal kinase
KC       Keratinocyte-derived chemokine
LAP      Latency Associated Peptide
LARC     Liver and Activation-regulated Chemokine
LCM      Laser Capture Microdissection
LDL      Low density lipoprotein
LESTR    Leukocyte-derived seven transmembrane receptor
LLC      Large Latent Complex
LMW      Low molecular weight
LPS      Lipopolysaccharide
LRO      Lysosome related organelles
LRR      Leucine Rich Region
LTBP     Latent TGF-β Binding Protein
M1       Classically activated macrophages
M2       Alternatively activated macrophages
MAM      Metastasis Associated Macrophages
MAPK     Mitogen-Activated Protein Kinase
MCP      Macrophage Chemoattractant Protein
M-CSF    Macrophage colony stimulating factor
Mfge8    Milk fat globule epidermal growth factor 8
MFI      Median Fluorescence Intensity
MHC      Major Histocompatibility Complex
MIP      Macrophage Inflammatory Protein
miRNA    Micro RNA
MMP      Matrix Metalloproteinase
MMR      Macrophage Mannose Receptor
MPF      Mouse Pulmonary Fibroblast
MRC1     Mannose receptor C, type 1
mRNA     messenger RNA
MSD      Meso Scale Discovery
MSU      Monosodium Urate
MMC      Mucosal tissue mast cell
MUC5B    Mucin 5B
MYD88    Myeloid Differentiation primary response 88
N/A      Not applicable
NACHT    Domain present in NAIP, CIITA, HET-E and TP1
NADPH    Nicotinamide Adenine Dinucleotide Phosphate
NAP Neutrophil Activating Peptide
NAIP Neuronal Apoptosis Inhibitor Protein
NCF Neutrophilic Chemotactic Factor
NFkB Nuclear Factor κ β
Ng Nanogram
NK Natural Killer cells
NKSF NK cell stimulatory factor
NLR NOD Like Receptors
NLRP3 NLR family, Pyrin domain containing 3
NO Nitric Oxide
NOD Nucleotide-binding Oligomerization Domain
NOS Nitric Oxide Synthase
NOX NADPH Oxidase
NSIP Non-Specific Interstitial Pneumonia
ODN Oligodinucleotides
o.p. oropharyngeal
ORO Oil Red O
oxPc Oxidised phosphatidylcholine
PACE Paired basic Amino acid Cleaving Enzyme
PAF Platelet Activating Factor
PAI-1 Plasminogen Activator Inhibitor-1
PAMP Pathogen associated molecular patterns
PBL Peripheral blood lymphocytes
PCR Polymerase Chain Reaction
PDGF Platelet Derived Growth Factor
PGE Prostagladin E
PHD Propyl Hydroxylase Domain
PI3K phosphoinositide 3 kinase
PRR Pathogen Recognition Receptors
PSR Picrosirius red
PTEN Phosphatase and tensin homologue
PTK Protein Tyrosine Kinase
PYD Pyrin Domain
qRT-PCR quantitative real time PCR
RAG Recombination activation gene
RAGE Receptor for Advanced Glycation End-products
RANTES Regulated upon Activation, Normal T cell Expressed and Secreted
RBC Red Blood Cells
RELM-α Resistin-Like Molecule alpha
RIP-1 Receptor Interacting Protein-1
RNA Ribonucleic Acid
RNA-seq RNA-sequencing
ROI Reactive Oxygen Intermediate
ROS Reactive Oxygen Species
RT-PCR Reverse Transcription-Polymerase Chain Reaction
SAM Scar-associated macrophages
SCF Stem cell factor
SCI Stem cell inhibitor
<table>
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<tr>
<th>Abbreviation</th>
<th>Full Form</th>
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<tr>
<td>SCID</td>
<td>Severe combined immunodeficiency</td>
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<tr>
<td>SDF</td>
<td>Stromal cell derived factor</td>
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<tr>
<td>Shh</td>
<td>Sonic Hedgehog</td>
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<tr>
<td>siRNA</td>
<td>Small interfering RNA</td>
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<tr>
<td>SLC</td>
<td>Small Latent Complex</td>
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<tr>
<td>Smad</td>
<td>Mothers against decapentaplegic homolog</td>
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<td>SNA1</td>
<td>Snail family zinc finger 1</td>
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<tr>
<td>SNP</td>
<td>Single nucleotide polymorphism</td>
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<tr>
<td>SOCS</td>
<td>Suppressor of cytokine signalling</td>
</tr>
<tr>
<td>SPA</td>
<td>Surfactant Protein A</td>
</tr>
<tr>
<td>SPC</td>
<td>Surfactant Protein C</td>
</tr>
<tr>
<td>ssRNA</td>
<td>Single stranded RNA</td>
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<tr>
<td>STAB-1</td>
<td>stabilin-1</td>
</tr>
<tr>
<td>STAT</td>
<td>Signal transducers and activator of transcription</td>
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<tr>
<td>TACE</td>
<td>TNF-α Converting Enzyme</td>
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<tr>
<td>TAK1</td>
<td>TGF-β Activated Kinase 1</td>
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<tr>
<td>TAM</td>
<td>Tumour Associated Macrophages</td>
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<tr>
<td>TANK</td>
<td>TRAF family member-associated NFκB activator</td>
</tr>
<tr>
<td>TBK1</td>
<td>TANK binding kinase 1</td>
</tr>
<tr>
<td>TBP</td>
<td>TATA-Binding Protein</td>
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<td>TCR</td>
<td>T-cell receptor</td>
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<td>TERT</td>
<td>Telomerase Reverse Transcriptase</td>
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<td>TGF</td>
<td>Transforming Growth Factor</td>
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<td>TGF-βR1</td>
<td>TGF-β receptor 1, ALK5</td>
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<td>tGPI</td>
<td>Glycosylphosphatidylinositol</td>
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<td>TH1</td>
<td>T helper lymphocytes type 1</td>
</tr>
<tr>
<td>TH2</td>
<td>T helper lymphocytes type 2</td>
</tr>
<tr>
<td>TIE2</td>
<td>Receptor Tyrosine kinase with Ig-like and EGF-like domains</td>
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<td>TIR</td>
<td>Toll/IL-1 receptor</td>
</tr>
<tr>
<td>TIRAP</td>
<td>TIR domain containing Adaptor Protein</td>
</tr>
<tr>
<td>TLC</td>
<td>Total Lung Capacity</td>
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<td>TLR</td>
<td>Toll Like Receptor</td>
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<td>TNF</td>
<td>Tumour Necrosis Factor</td>
</tr>
<tr>
<td>TNFR</td>
<td>TNF receptor</td>
</tr>
<tr>
<td>tPA</td>
<td>Tissue plaminogen activator</td>
</tr>
<tr>
<td>TP1</td>
<td>Telomerase associated Protein 1</td>
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<td>TRAF</td>
<td>TNF Receptor Associated Factor</td>
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<td>TRAM</td>
<td>TRIF Related Adaptor Molecule</td>
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<td>Treg</td>
<td>Regulatory T lymphocytes</td>
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<td>TIR-domain containing adaptor-inducing interferon-β</td>
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<td>Transient receptor potential cation channel subfamily V member 1</td>
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<td>Tyrosine kinase</td>
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<td>UIP</td>
<td>Usual Interstitial Pneumonia</td>
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<td>uPA</td>
<td>Urokinase</td>
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<td>VCAM</td>
<td>Vascular cell adhesion molecule</td>
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<td>VEGF</td>
<td>Vascular endothelial growth factor</td>
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<td>vWF</td>
<td>von Willebrand Factor</td>
</tr>
<tr>
<td>WT</td>
<td>Wild Type</td>
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<tr>
<td>Abbreviation</td>
<td>Description</td>
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<td>YKL-40</td>
<td>Tyrosine-Lysine-Leucine, 40 kDa</td>
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<td>αT2ib</td>
<td>Anti-TLR2 ER-intrabodies</td>
</tr>
<tr>
<td>α-SMA</td>
<td>α-smooth muscle actin</td>
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<tr>
<td>H$_2$O$_2$</td>
<td>Hydrogen Peroxide</td>
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</tbody>
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1.1 Idiopathic Pulmonary Fibrosis (IPF)

1.1.1 Epidemiology of IPF patients

IPF is a chronic fibrosing interstitial lung disease of unknown etiology that is characterised by a histological pattern of usual interstitial pneumonia (UIP)\(^1\). IPF has a prevalence of 50 per 100,000 population, and mostly occurs in adults over 50 years old\(^2\text{-}^4\). It is more common in men than women (1.5:1)\(^5\text{-}^6\), and 1-4% of patients have a family history of the disease\(^7\text{-}^10\).

1.1.2 Prognosis of IPF patients

IPF has a high incidence of morbidity and mortality, with a median survival of 3 years following diagnosis\(^11\). A worse prognosis is associated with old age (>70 years old), smoking history, low body-mass index (BMI), severe physiological impairment, large radiological extent of disease and pulmonary hypertension. Several variants of IPF pathology have been identified, including a stable or progressive course, an accelerated form of IPF, and acute exacerbation of stable IPF\(^12\).

Many IPF patients have a stable or slowly progressive course that can last for decades. They present with decreased lung volumes and capacities, and hypoxemia at rest that worsens with exercise. A subgroup of IPF patients that are in general male cigarette smokers have a shortened survival, termed accelerated IPF. Accelerated IPF patients have a different clinical course and transcriptional profile compared to the slowly progressive counterparts, despite showing similar signs of lung function, chest imaging and histological findings\(^13\). Altered transcriptional profiles are present mostly in the alveolar epithelial and mesenchymal domains. An example of upregulated genes in the accelerated variant are members of the Mitogen-Activated Protein Kinase (MAPK)-Early Growth Response protein 1 (EGR1) -Heat Shock Protein 70 (HSP70) pathway, which regulate cigarette smoke induced inflammation\(^14\). Acute exacerbation is defined as the rapid deterioration of the disease in the absence of infection, heart failure, or pulmonary embolism\(^15\text{-}^16\). The cause of 50% of acute exacerbations in IPF is unknown\(^17\), perhaps triggered by an occult stressor such as viral infection, microaspiration or ambient pollution\(^18\text{-}^19\). Acute exacerbations affect 5-20% IPF patients, and have >60% mortality during administration to hospital and >90% mortality rate within 6 months of discharge for those who survive\(^15\text{-}^17\) (Figure 1.1).
Figure 1.1 The prognosis of idiopathic Pulmonary Fibrosis (IPF) patients. IPF has a long (months to years) asymptomatic period. Patients consult when the severity of lung lesions reaches a threshold that is sufficient to provoke symptoms. Most patients follow a relatively slow clinical and functional decline (slowly progressive course) after diagnosis. About 10% of these patients present with acute episodes of clinical deterioration (acute exacerbations) that precede or possibly initiate the terminal phase of their disease. A few patients have a short duration of illness with a rapidly progressive course. Smokers may develop emphysema, and have shorter survival when compared to IPF patients alone. Figure adapted from King JE Jr, 2010.

1.1.3 Diagnosis of IPF patients

Definitive diagnosis of IPF patients consists of four criteria based on pulmonary physiology analyses, surgical lung biopsies, and High-Resolution Computer Tomography (HRCT) scanning.

- IPF patients have abnormal pulmonary physiology with evidence of restriction and/or impaired gas exchange.

- Surgical lung biopsies of IPF patients show a pattern of UIP. There is spatial heterogeneity, where normal lung architecture alternates with patchy areas of histologically apparent pulmonary parenchymal fibrosis. The most affected part of the lung is replaced by dense collagen and occasional cystic structures known as microscopic honeycombs. At the leading edge of fibrosis specialised structures known as fibrotic foci can be found, which comprise of pale staining whirls of extracellular matrix (ECM) interspersed with numerous cells of the fibroblast type. Hyperplastic type II alveolar epithelial cells (AEC2s) can be observed overlying the fibrotic foci. Inflammation is absent except in occasional lymphoid follicles found in end stage fibrosis. IPF: UIP also does not contain hyaline membranes, granulomas or organised alveolar exudates.
• HRCT demonstrates a pattern of ‘confident’ or ‘patchy’ IPF, which consists of patchy, predominantly peripheral and subpleural, bibasilar reticular opacification with little to no ground glass opacity, thickened interlobular septae, traction bronchiectasis and subpleural honeycombing.

• All other known causes of interstitial lung disease have been excluded.

1.1.4 Molecular Mechanisms of IPF

1.1.4.1 IPF may arise from a dysregulated wound healing process

IPF is believed to arise as a result of dysregulated wound healing process following alveolar epithelial injury caused by a persistent irritant. It is proposed that initial and repetitive injury occurs to the alveolar epithelium, and most likely causes damage to the type I alveolar epithelial cells (AEC1s) that line the alveolar surface\(^23\). Under homeostatic conditions, these cells play a role in regulating structural mesenchymal cells through cell-cell contact and mediator secretion. When AEC1s are injured or lost, it is thought that the underlying AEC2s undergo hyperplastic proliferation to cover the exposed basement membranes. If this process is inefficient, alveoli can collapse and consolidate. In normal conditions, AEC2 undergo steady state turnover (apoptosis), and the remainder of the cells will spread and differentiate into AEC1s. Under pathological conditions and in the presence of profibrotic mediators, including Transforming Growth Factor (TGF)-\(\beta\) however, fibroblasts accumulate at the site of injury and differentiate into \(\alpha\)-Smooth Muscle Actin (\(\alpha\)-SMA) expressing myofibroblasts that have increased expression of ECM components, including collagen to form specialised structures called fibrotic foci\(^21,22\). AEC2 surrounding the fibrotic foci are hyperplastic and abnormal rather than undergoing normal repair\(^24\), and may relate to cellular stress and failure to regenerate AEC1s lost by injury\(^25\).

Several lines of evidence support the hypothesis that insults to the alveolar epithelium can serve as a trigger of IPF. Early ultrastructural studies by electron microscopy have demonstrated alveolar epithelial injury in lung biopsies from IPF patients\(^26\). Immunohistochemical studies have shown the upregulation of various mediators indicative of alveolar cell apoptosis, including pro-apoptotic Fas, p53 and p21, B-cell lymphoma (Bcl)-2 associated X protein (Bax) and Caspase-3, whereas anti-apoptotic Bcl-2 is downregulated\(^27-30\). BAL from IPF patients also show soluble mediators indicative of increased apoptosis\(^31\). Inhibiting epithelial cell apoptosis with various approaches, including blocking the Fas-Fas ligand pathway and blocking caspase activation, abrogates bleomycin (BLM)-induced pulmonary fibrosis\(^32-35\). On the other hand, direct stimulation of Fas via anti-Fas antibody or injury to type II pneumocytes via a diphtheria toxin (DTR) approach also leads to pulmonary fibrosis\(^36,37\). The various factors that may lead to AEC damage is summarised in figure 1.2.
Epithelial injury may occur through insults from environmental factors, viral infections or genetic mutations in AECs. Environmental factors such as cigarette smoking and microaspiration (asymptomatic aspiration of oropharyngeal secretions or gastric fluids into the lung)\(^1,38,39\) may lead to repeated injury to the lung. Other environmental factors, such as asbestos or silica, may persist in the lungs due to their large size and failure of antigen presenting cells (APCs) to effectively phagocytose and remove them, resulting in prolonged lung injury. Both of these events may lead to pathological chronic inflammation and subsequent dysregulated wound-healing that may ultimately result in pulmonary fibrosis.

Chronic viral infections, mainly herpes virus infections, may contribute to the pathogenesis of IPF\(^38,40-43\). There is correlation between certain latent viral infections, such as Epstein-Barr Virus (EBV), Cytomegalovirus (CMV), Human Herpesvirus (HHV)-7 and HHV-8 and human IPF\(^25,40-42,44-47\). Such viruses are known to infect and remain latent in pulmonary epithelial cells\(^48\), thereby rendering them less able to repair subsequent injury. Infection may also potentially change the transcriptional program of these cells such that they secrete profibrotic factors and recruit inflammatory cells and fibrocytes (bone marrow derived cells that express both hematopoietic and stromal markers) to the lung\(^12\).

Genetic predisposition may also contribute to some forms of familial pulmonary fibrosis as identified in early studies\(^49-52\). Pulmonary fibrosis developed in a subset of patients with defined clinical syndromes, such as Hermansky-Pudlak Syndrome (HPS) and dyskeratosis congenita (DKC)\(^49,53-55\). HPS patients carry autosomal recessive mutations that lead to defective trafficking of intracellular
organelles. HPS associated pulmonary fibrosis (HPSPF) is thought to be caused by defective secretion of pulmonary surfactant by AEC2s that leads to tissue injury, or lysosomal accumulation that leads to aberrant activation of pulmonary macrophages, and will be discussed in greater detail in section 1.4. DKC involves a group of mutations that directly or indirectly affects the functionality of telomerase RNA component (TERC) that extends telomeres. Shortened telomeres may lead to cellular apoptosis or permanent cell cycle damage due to the deoxyribonuclease nucleic acid (DNA) damage response. In the context of IPF, AECs may not be able to self-renew and differentiate effectively following tissue injury\(^56\) and thereby fail to efficiently repair and cover the denuded basement membrane, thus perpetuating the release of pro-fibrotic mediators and promoting fibrosis\(^56\).

Genetic analysis of IPF patients have since identified mutations in several genes that may be involved in pathogenesis of the disease. A common theme to all mutations identified to date is that they affect genes that are expressed in lung epithelial cells [e.g. Surfactant Protein A (SPA)\(^57\), Surfactant Protein C (SPC)\(^{12,49,58-62}\), and Mucin 5B (MUC5B)\(^{63,64}\)] or lead to molecular changes in AECs [e.g. Telomerase Reverse Transcriptase (TERT)\(^{56,65}\)]. Pulmonary surfactants are released by AEC2s to reduce surface tension within the lung. Missense or short deletion mutations in the SPC gene gives rise to the variant misfolded protein that acts as a persistent irritant through accumulation or complex formation and causes alveolar epithelial injury\(^{12,49,58-62}\). SPC mutations account for ~1% of sporadic IPF\(^61,62\). Missense mutations in the SPA gene give rise to protein instability and retention in the endoplasmic reticulum (ER)\(^57\). Reduced SPA secretion may affect lung homeostasis and lead to tissue injury. MUC5B is essential for the production of mucus, which is a pulmonary lubricant that serves as a chemical barrier. A common single nucleotide polymorphism (SNP) in the MUC5B promoter region is associated with familial interstitial fibrosis and also sporadic IPF\(^{63,64}\). The SNP is present in 34-38% of sporadic IPF patients compared with 9-11% of healthy controls\(^64\). The exact mechanism the variant MUC5B in promoting pulmonary fibrosis is unclear, although two hypotheses have been proposed. First, the variant protein that may alter the local cell environment, perhaps by altering the mucosal host defence and/or local cytokine production, such that these changes affect the capability of bronchiolar or alveolar epithelial cells to self-renew or differentiate in response to injury. Second, the variant MUC5B may also lead to excessive mucus production that may provide a physical barrier that compromises normal epithelial repair of denuded basal lamina\(^63\). Mutations in essential telomerase genes, including human telomerase transcriptase (hTERT), telomerase RNA (hTR), and the associated machinery [e.g. dyskerin gene in DKC] lead to accelerated telomere shortening\(^{65-69}\), and as aforementioned affects the ability of AEC2 to repair efficiently following tissue injury\(^56\). 8-15% of patients with FPF\(^{56,70}\) and 1-3% of patients with sporadic IPF\(^{71,72}\) have one or more telomere-related mutations.
There are two non-exclusive hypotheses on the inducers for such dysregulated wound healing, including pathological chronic inflammation, and also an aberrantly activated epithelial cell mediated process. Pathological chronic inflammation may lead to prolonged tissue injury that results in a dysregulated wound healing process. Immune cells that are recruited to the site of tissue injury, such as monocytes, macrophages, and lymphocytes, accumulate and may drive fibrosis through the extended release of pro-fibrotic mediators that induce the migration, proliferation and activation of fibroblasts. In the aberrantly activated epithelial cell model, it was proposed that injury to the alveolar epithelium directly activated AECs to promote fibrosis through the release of pro-fibrotic mediators and also epithelial-to-mesenchymal (EMT) transition. The mechanisms for chronic inflammation and aberrantly activated epithelial cells in the initiation and progression of IPF will be discussed in greater detail in sections 1.1.4.2 and 1.1.4.3 respectively.
1.1.4.2 The potential role of chronic inflammation in IPF

1.1.4.2.1 Physiological inflammation in wound healing

The wound healing process takes place over three phases; these include (i) the anti-fibrinolytic coagulation cascade to temporarily plug the affected tissue to prevent it from further damage and facilitate the recruitment of inflammatory cells; (ii) physiological inflammation where phagocytes such as macrophages and neutrophils clear cellular debris and invading pathogens by phagocytosis, and leukocytes release pro-inflammatory and pro-fibrotic mediators to promote epithelial and endothelial cell proliferation, and the recruitment and proliferation fibroblasts; and (iii) the repair and resolution phase, where wound closure occurs and tissue injury-induced inflammation is resolved to complete the wound healing process. The various stages of the wound-healing process will be described in greater detail below.

Following tissue injury, damaged endothelial and epithelial cells release pro-inflammatory mediators that initiate the coagulation phase, which triggers blood clot formation and formation of the provisional ECM. Platelets are exposed to ECM components, triggering aggregation, clot formation and haemostasis. The coagulation cascade also provide access to inflammatory cells for tissue entry through basement membrane disruption and neoangiogenesis Platelet degranulation also promotes vasodilation and increased blood vessel permeability, while myofibroblasts and epithelial and endothelial cells secrete matrix metalloproteinases (MMPs), which disrupt the basement membrane allowing the recruitment of inflammatory cells to be recruited to the site of injury. Growth factors, cytokines and chemokines are also produced, which stimulates the recruitment and proliferation of macrophages and neutrophils. Tissue resident macrophages are also activated through binding alarmins with their pathogen recognition receptors (PRRs) and secrete a number of pro-inflammatory mediators [e.g. Tumour Necrosis Factor (TNF)-α, Interleukin (IL)-6]. Tissue-resident mast cells can also be activated through binding endogenous alarmins through their PRRs, or via cross-linking of their cell surface FceRI, by multivalent antigens or complement peptides C3a or C5a. Activated mast cells can release secretory granules containing preformed mediators, including histamine, eosinophil chemotactic factor (ECF) and neutrophilic chemotactic factor (NCF), acid hydrolases (e.g. acid hydrolases, β-glucuronidase, phosphatase) and neutral proteases (e.g. tryptase, chymase) that promote smooth muscle contraction and increased vascular permeability, granulocyte activation and chemotaxis, and cytotoxicity. Mast cells also release heparin sulfate, an anticoagulation and anti-complementary factor that may play a role in the diffusion of mast cell mediators despite the activation of the coagulation pathway. Mast cells can also synthesise secondary mediators in a delayed response, which include leukotrienes, prostaglandins, platelet
activating factor (PAF), and other pro-inflammatory mediators that promotes further vasodilation, chemotaxis of neutrophils, macrophages and lymphocytes. In the inflammatory phase, immune cells are recruited to the site of injury and are involved in the clearance of cellular debris, recruitment and activation of fibroblasts and further angiogenesis. Some of the early responders to inflammation are the phagocytes, including macrophages and neutrophils, which eliminate cellular debris, dead cells and invading organisms by phagocytosis. They can release cytokines and chemokines, which are mitogenic for endothelial cells that begin to surround the injured site. They also help form new blood vessels through the release of pro-angiogenic mediators as endothelial and epithelial cells migrate towards the centre of the wound. In addition, neutrophils are able to release high levels of reactive oxygen species (ROS) and reactive nitrogen species (RNS) to eliminate invading pathogens. Macrophages also release growth factors for fibroblast activation (e.g. TGF-β) and also MMPs for further disruption of the basement membrane for macrophage entry into the site of injury. Neutrophils are the predominant leukocyte involved in the early inflammatory response. However, as these cells release cytotoxic mediators (e.g. ROS and RNS) that may induce collateral damage to the surrounding tissue they have to be eliminated quickly. To this end neutrophils are rapidly negatively regulated by apoptosis and subsequent efferocytosed by macrophages. Efferocytosis of apoptotic neutrophils by macrophages increases TGF-β and CC chemokine ligand (CCL)2 secretion and decreases CXC chemokine ligand (CXCL)8 production, leading to a chemokine shift that favours monocyte recruitment. As neutrophils are depleted from the inflammatory site, blood monocytes continue to accumulate and differentiate into inflammatory macrophages, which complete phagocytosis and destruction of the pathogens and/or irritants. The monocytes and macrophages then emigrate after several days into the local lymph node. During this migratory process, monocytes differentiate into dendritic cells, upregulating human leukocyte antigen (HLA) class II antigen membrane expression, and acquiring costimulatory molecules such as cluster of differentiation (CD)80 and CD86. These cells may then present antigenic peptides to lymphocytes, contributing to the generation of an adaptive immune response. Lymphocytes are then recruited to the site of injury and become activated and begin secreting profibrotic cytokines and growth factors [e.g. TGF-β, IL-13 and platelet derived growth factor (PDGF)], which further activates macrophages and fibroblasts.

In the repair and resolution phase, fibroblasts are activated and differentiate into α-SMA+, collagen-secreting myofibroblasts. Myofibroblasts are able to contract and draw the edges of the wound towards the centre. Epithelial and/or endothelial cells then divide and migrate over the basal layers to regenerate the damaged tissue, which completes the wound healing process. The remainder
myofibroblasts and inflammatory cells then undergo apoptosis to inhibit excessive ECM deposition, and apoptotic cells are then subsequently phagocytosed by macrophages. Physiological inflammation therefore plays an important role in the wound-healing process, including the elimination of invading pathogens through the release of ROS/RNS, clearance of cellular debris, invading pathogens and/or apoptotic cells by phagocytosis, release of cytokines and/or growth factors, to promote inflammation and wound-healing, tissue remodelling proteases (e.g. MMPs/TIMPs) and pro-angiogenic factors (e.g. ELR+ CXC chemokines) to promote tissue remodelling and angiogenesis respectively to allow tissue access by haematopoetic cells, which are recruited by chemokines that are secreted at the site of tissue injury. The role of physiological inflammation in wound healing is summarised in Figure 1.3.
Figure 1.3 The role of physiological inflammation in wound healing. Upon infection and/or tissue injury, pathogens and/or alarmins may trigger an inflammatory response. Tissue-resident macrophages may release a number of pro-inflammatory mediators: chemokines recruit other immune cells, including granulocytes, monocytes, macrophages, and lymphocytes to the site of injury to amplify the inflammatory response. In addition, chemokines may also recruit fibroblasts to promote wound repair. ELR+ chemokines are angiogenic factors that promote neovascularisation and allows the recruitment of hematopoietic cells, including immune cells and fibrocytes (hematopoietic precursor of fibroblasts), to the site of tissue injury. Tissue remodelling proteases, including MMPs and TIMPs, facilitate the access of hematopoietic cells into the site of injury. Cytokines facilitates the inflammatory response and tissue repair through the activation of several hematopoietic and mesenchymal cells. Phagocytes, such as macrophages or neutrophils, may also be able to remove pathogens or alarmins through phagocytosis. In addition, phagocytes may produce reactive oxygen species (ROS) or reactive nitrogen species (RNS) to digest phagocytosed material. Physiological inflammation aids wound healing through the coordinated process in removal of the source of tissue injury, wound repair and return to homeostasis. Persistent irritation may lead to chronic inflammation and dysregulated wound repair that may lead to further tissue injury and fibrosis.
1.1.4.2.2 Pathological inflammation in pulmonary fibrosis

Although physiological inflammation is essential for efficient wound healing, chronic inflammation that arises as a result of persistent irritation, as in the case of pulmonary fibrosis is pathological. In chronic inflammation, the inflammatory repertoire is dominated by macrophages and lymphocytes with little granulocytic presence. The transition from acute to chronic inflammation involves the replacement of short-lived neutrophils with long-lived macrophages and lymphocytes in an IL-6-dependent process. Levels of IL-6 are elevated in several chronic inflammatory diseases, including rheumatoid arthritis, systemic juvenile idiopathic arthritis, ankylosing spondylitis, psoriasis and Crohn’s disease, and elevated levels are correlated with disease activity. IL-6 is also elevated in different types of tissue fibrosis, including IPF, liver fibrosis and systemic sclerosis. IL-6 promotes the transition from acute to chronic inflammation through selectively recruiting monocytes and macrophages, but not neutrophils to the site of injury, through promoting the release of CCL2 but not CXCL8. Transsignalling by IL-6, through the formation of complexes with soluble IL-6 receptors, and subsequent binding to cell surface IL-6 co-receptor gp130 activates endothelial cells and promotes their release of CCL2 and IL-6 but not CXCL8. IL-6 also promotes the apoptosis of neutrophils, and the subsequent efferocytosis of these cells by macrophages also favours the secretion of CCL2 and TGF-β and decreases CXCL8 release as mentioned above. The accumulation and persistent activation of macrophages and lymphocytes lead to the prolonged release of pro-inflammatory and pro-fibrotic mediators, and also tissue remodelling enzymes that results in dysregulated wound healing and ultimately fibrosis. As aforementioned wound healing is negatively regulated following myofibroblast contraction and wound closure, epithelial and/or endothelial cell proliferation and migration to cover the basal layer, followed by myofibroblast apoptosis and subsequent efferocytosis by macrophages. Excessive pro-inflammatory mediators (e.g. TNF-α, IL-1β) may lead to further tissue injury that affects effective wound repair; increased levels of TNF-α may also induce hyperproliferation of epithelial cells that may further propagate the dysregulated wound healing process through promoting fibroblast recruitment, proliferation and activation, fibrocyte recruitment and EMT. The excessive release of pro-fibrotic mediators may result in the disproportionate increase in the recruitment, proliferation and/or activation of fibroblasts, thereby leading to the excessive accumulation of ECM components (e.g. collagen) that results in a fibrotic scar. Excessive release of tissue remodelling enzymes such as MMPs may lead to extensive disruption to the basement membrane and tissue architecture that may affect tissue functionality. MMPs may also cleave and activate other pro-fibrotic zymogens (e.g. TGF-β), thereby further propagating the pro-fibrotic response.
Chronic inflammation has a pivotal role in pathology for most forms of interstitial lung diseases, and may lead to alveolar capillary wall and basement membrane disruption, which may in turn lead to loss of alveoli, dysregulated wound healing and fibrosis. Chronic inflammation may also play a role in IPF, and inflammatory mediators have been found to be elevated in Bronchoalveolar lavage (BAL) and lung biopsies of IPF patients and also murine models of pulmonary fibrosis. TNF-α was found at elevated levels in surgical lung biopsies and serum of IPF and systemic sclerosis associated pulmonary fibrosis patients. IL-1β was found present in chronic inflamed tissues and tissues undergoing fibrogenesis, with accumulation of myofibroblasts and matrix deposition. IL-12 p40 was elevated in silica-, BLM-, or anti-Fas antibody-induced models of pulmonary fibrosis, and are found to be largely produced by alveolar macrophage (AMφ). There are also increased levels of tissue remodelling proteases such as MMPs in IPF patients, including MMP-7 and MMP-9 that are secreted by macrophages.

MMP-7, also known as matrilysin, is among the most highly expressed and have increased gene transcript levels in fibrotic lungs. The levels of MMP-7 in BAL fluid (BALF) are also related to the severity of lung function impairment in IPF. Immunoblot and immunohistochemistry revealed that both pro-and active forms of MMP-7 were increased in levels within the BALF and lung respectively. Immunohistochemistry showed that pro-MMP-7 localised to bronchial epithelial cells and AMφ, whereas active MMP-7 was found in hyperplastic epithelial cells and AMφ respectively. In a subsequent cross-linked carboxymethylated transferrin-(CCm-Tf) bioassay, BALF from IPF patients had greater CCm-Tf degrading activity than healthy controls. This has been accredited to increased MMP bioactivity in IPF patients, as a mixture of serine protease (aprotinin and elastinal) and MMP inhibitors (1, 10-phenanthroline), but not serine proteinase inhibitors alone, inhibited CCm-Tf degradation. From these studies however it is not clear which MMP(s) is/are contributing to increased CCm-Tf-degrading bioactivity. Further inhibition studies with specific MMP neutralising antibodies will have to be carried out to deduce the particular MMPs that have increased bioactivity in IPF patients.

MMP-9/gelatinase B is upregulated in both human and experimental lung fibrosis, and an increased gene and protein expression was observed in AMφ and fibroblasts isolated from IPF patients compared to normal controls. Levels of an elastin fragment that is generated exclusively by MMP-9 and/or MMP-12 cleavage of elastin was found to be increased in BALF of IPF patients, suggesting the increased bioactivities of these proteases. The increased levels of the active form of MMP-9 in BALF were reported to be greatest in patients with rapidly progressive IPF. Further animal studies have identified the importance of these mediators in the initiation
and/or progression of experimental pulmonary fibrosis, which will be discussed in greater detail later (Section 1.2.3.1).

1.1.4.2.3 Debate on the contributions of pathological inflammation on pulmonary fibrosis

Despite the apparent role of inflammation in the initiation and progression of murine models of pulmonary fibrosis, its participation in the human disease has been challenged over the recent years, primarily because treatment of IPF with anti-inflammatory drugs, including corticoids and glucocorticoids, have been largely ineffective\textsuperscript{113}. In addition, there is no histological difference between early and late stage IPF with respect to inflammation\textsuperscript{114}. Furthermore, long term overexpression of the anti-inflammatory cytokine IL-10 in bitransgenic mice that carry a doxycycline inducible IL-10 transgene was also able to induce pulmonary fibrosis in the absence of inflammation. Lung specificity of IL-10 overexpression was achieved by the introduction of the IL-10 transgene into the in the Clara cell secretory protein promoter, a protein that is uniquely expressed by alveolar epithelial cells\textsuperscript{115}. These observations have led to the proposal of an epithelial injury-driven fibrosis model that is inflammation-independent. In this model, AECs, rather than immune cells, are proposed to be central to the fibrotic response. AECs that become activated following tissue injury by alarmins can facilitate the initiation and progression of fibrosis through the release of pro-fibrotic mediators, EMT or recruitment of fibrocytes in the absence of inflammation. This will be discussed in greater detail in Section 1.1.4.3.

Inflammation may be required for the initiation but not the progression of fibrosis. In a study of idiopathic interstitial pneumonia (IIP), of which the most common form is IPF\textsuperscript{116}, surgical lung biopsies present with intralobar and interlobar heterogeneity of Non-Specific Interstitial Pneumonia (NSIP) that is associated with chronic inflammation, and also UIP that is associated with fibrosis\textsuperscript{117}. Interestingly, a gradual increase in mean age from NSIP only (mean age, 53.1 years), to NSIP and UIP (mean age, 57 years), to UIP only (mean age, 63.3 years) in patients was observed\textsuperscript{117}, indicating that UIP may be a result of unresolved NSIP. Inflammation may be required for the recruitment of inflammatory cells, such as macrophages, into the lungs where they accumulate. These cells display plasticity and are able to adapt their phenotype to their surrounding environment. Within a dysregulated wound healing environment, these may contribute to pulmonary fibrosis through the release of pro-fibrotic mediators such as TGF-\(\beta\)\textsuperscript{118}, fibronectin\textsuperscript{119} and PDGF\textsuperscript{120}. Interestingly these cells are also responsive to glucocorticoid induction to display a wound-healing/ pro-fibrotic phenotype, with increased production of mediators such as IL-10 and TGF-\(\beta\)\textsuperscript{121}. Glucocorticoid treatment may therefore worsen rather than protect from pulmonary fibrosis.
Finally, ongoing inflammation may be required for the progression of fibrosis. As aforementioned above, animal studies have revealed that inflammatory mediators, such as IL-1β\textsuperscript{122} and IL-12 p40\textsuperscript{100,123–125}, are required for the progression of pulmonary fibrosis. The lack of beneficial effects of anti-inflammatory glucocorticoid and corticosteroid therapies in IPF patients\textsuperscript{113} may be due to their reduced response to these mediators, rather than inflammation being non-essential. IPF patients were reported to have lower glucocorticoid receptor (GR) content than normal controls\textsuperscript{126} and the messenger Ribonucleic Acid (mRNA) levels of GR-α, the predominant isoform of the receptor that binds steroids and is able to mediate the transactivation or transrepression of target genes, is decreased in IPF patients as compared to other steroid sensitive interstitial lung diseases, such as sarcoidosis and cryogenic organising pneumonia (COP)\textsuperscript{127}.

1.1.4.3 The potential role of aberrantly activated AECs in IPF

Following the lack of efficacy of IPF patients to anti-inflammatory therapy, an inflammation-independent pathway resulting in pulmonary fibrosis was proposed instead. In the epithelial injury model, it was hypothesised that aberrantly activated AECs, rather than inflammatory cells, were responsible for the recruitment and activation of fibroblasts that are involved in dysregulated wound healing that ultimately drives the initiation and progression of pulmonary fibrosis. There is strong evidence that indicates that AECs are the primary source of mediators that function as chemokines or mitogens for mesenchymal cells, including PDGF, TGF-β, and TNF-α and Endothelin-1\textsuperscript{103}.

AECs in IPF patients also strongly express CXC chemokine receptor (CXCR) 4 ligand, CXCL12\textsuperscript{128}, which may be responsible for the observed recruitment of fibrocytes into the lungs of IPF patients\textsuperscript{128} through their CXCR4 receptor. Fibrocytes are a unique subpopulation of leukocyte characterised by their expression of both hematopoietic [Cluster of Differentiation (CD)45, CD34] and mesenchymal cell markers (collagen 1, fibronectin)\textsuperscript{129} that may contribute to the fibroblast pool.

In addition, there may be abnormal alveolar epithelial and mesenchymal cross talk, which may lead to EMT. In EMT, epithelial cells acquire mesenchymal properties through which they increase their capability to move and to synthesize interstitial matrix\textsuperscript{103,130}. Several evidence have supported the role of EMT as a source of IPF myofibroblasts. There is co-localisation of epithelial cells (pro-surfactant proteins) and mesenchymal (α-SMA, N-Cadherin) markers in AECs from IPF lungs\textsuperscript{11,131}. There is also an upregulation of typical epithelial proteins (Keratin-18) in IPF fibroblasts\textsuperscript{132}. AEC2 from IPF patients also have an increased gene expression of mesenchymal proteins and potential regulators of EMT\textsuperscript{113}. Moreover, there is an increase in EMT drivers in hyperplastic AECs from lungs of IPF patients, including Snail family zinc finger 1 (SNA1) and TWIST\textsuperscript{134–136}. SNA1 is a transcription factor that facilitates TGF-β induced EMT\textsuperscript{134,135}; high levels of TWIST in IPF patients is often
associated with the presence of the herpesvirus, EBV, and may therefore facilitate EBV induced EMT and fibrosis. The potential role of aberrantly activated AECs in the initiation and progression of pulmonary fibrosis, through the recruitment and activation of fibroblasts, is summarised in figure 1.4.

1.1.4.4 Dysregulation of tissue repair and the Nicotinamide Adenine Dinucleotide Phosphate (NADPH) Oxidase (NOX)4 pathways in IPF

The activation of embryological pathways are essential for the tissue repair process. There is increasing evidence that persistent irritation to the lung leads to dysregulation of some embryological pathways, including Wnt/Wingless, phosphatase and tensin homologue (PTEN), Sonic hedgehog (Shh) and bone morphogenetic proteins (BMPs), which may play a role in the abnormal behaviour of AECs and perhaps fibroblasts in IPF. The dysregulation of the NOX4 redox signalling pathway has also been reported to participate in the pathogenesis of IPF.

1.1.4.4.1 Wnt/Wingless pathway

The Wnt ligands comprise a large family of proteins that are essential to the morphogenetic processes. Results from several studies have indicated an overexpression of members of the Wnt/wingless pathway in the lungs of IPF patients. In addition, there is an accumulation of...
β-catenin, a signal transducer of the Wnt signalling pathway, in AECs and fibroblasts, suggesting that the Wnt–β-catenin pathway is switched on in both cell types.\textsuperscript{146}

### 1.1.4.4.2 PTEN

PTEN is crucial for development. In adults, PTEN participates in the regulation of physiological processes such as cell polarity, proliferation and apoptosis.\textsuperscript{147} In IPF patients, myofibroblasts within the fibrotic foci downregulate PTEN expression, which may protect them from apoptosis.\textsuperscript{148} The interaction between β1-integrin, a transmembrane receptor required for cell-cell or cell-ECM interactions, and collagen in normal fibroblasts activates PTEN, which is also a negative growth regulator; this negative feedback is however defective in fibroblasts in IPF patients.\textsuperscript{149}

### 1.1.4.4.3 Shh

Shh is an essential morphogen for patterning during embryogenesis. This developmental ligand allows the evasion of apoptosis and cell cycle arrest and promotes proliferation in cells. In the lungs of IPF patients an elevated level of Shh expression was observed, particularly in epithelial cells lining the honeycomb cysts.\textsuperscript{150,151} Shh overexpression may contribute to AEC2 hyperplasia observed in the lungs of IPF patients.

### 1.1.4.4.4 BMPs

BMPs belong to the TGF-β superfamily and have an essential role in embryonic and postnatal development.\textsuperscript{152} In adults, reactivating the expression of BMP antagonists can contribute to the progression of some chronic degenerative diseases, such as IPF,\textsuperscript{153} liver,\textsuperscript{154} and renal fibrosis and cervical cancer.\textsuperscript{155,156} BMP-4 is one of the key morphogens during embryonic lung development.\textsuperscript{158} BMP-4 inhibits the proliferation of human pulmonary fibroblasts and during embryogenesis induces the proliferation and differentiation of pulmonary epithelial cells. Gremlin is a strong BMP antagonist that can associate with BMP-2, -4 and -7 and inhibit their binding to cell surface receptors.\textsuperscript{160} Increased expression of gremlin-1 has been reported in fibroblasts in lungs affected by IPF.\textsuperscript{42} Increased concentrations of gremlin-1 could attenuate the mothers against decapentaplegic homolog (Smad)1/5/8 phosphorylation mediated by BMP-4 signalling in the lungs, leading to increased TGF-β1 induced EMT and reduced myofibroblast apoptosis.\textsuperscript{161–163}

In conclusion, some of embryological pathways are activated during normal tissue repair, but this process must be tissue specific and temporarily modulated. On the other hand, sustained activation of these programmes as a result of dysregulated wound healing may contribute to the pathology of IPF.
1.1.4.4.5 NOX4

NOX4 is a member of the NADPH oxidases, a group of transmembrane multiprotein complexes found in the plasma membrane and phagosomes, which generates superoxide anions by transferring electrons from NADPH across the membrane to molecular oxygen\(^ {164}\). Accumulating evidence highlights the involvement of NOX-dependent redox signalling, in particular NOX4\(^ {165-171}\), in the profibrotic responses mediated by TGF-\(\beta\) signalling\(^ {165}\). TGF-\(\beta\)1 induced the increased gene expression of NOX4, but not other NOX family members, in human fetal lung mesenchymal cells (hFLMCs). Hydrogen peroxide (H\(_2\)O\(_2\)) generated by NOX4 induction is found to promote TGF-\(\beta\)1-induced differentiation of hFLMC into \(\alpha\)-SMA+ myofibroblasts. Knockdown of NOX4 expression by small interfering RNA (siRNA) inhibited TGF-\(\beta\)1-induced H\(_2\)O\(_2\) production and hFLMC differentiation into myofibroblasts. Removal of H\(_2\)O\(_2\) by exogenous catalase activity also mimicked such TGF-\(\beta\)1 inhibitory activity\(^ {168}\). H\(_2\)O\(_2\) may promote the conversion of latent TGF-\(\beta\) into its active form, enhancing the phosphorylation and activation of the TGF-\(\beta\) receptor (TGF-\(\beta\)R) TGF-\(\beta\)R1/ALK5 and/or the downstream signalling molecule Smad2/3, and increasing the activation of the transcription factors c-Jun N-terminal kinase (Jnk) and p38\(^ {172}\).

NOX4 has been shown to play a role in pulmonary fibrosis. There is evidence showing increased NOX4 mRNA and protein levels in pulmonary fibroblasts isolated from IPF patients, and the NOX4 expression level correlates with expressions of procollagen I and \(\alpha\)-SMA\(^ {142}\). NOX4 was observed in immunohistochemistry staining to be highly expressed in the fibrotic foci of IPF patients\(^ {168}\). NOX4 was also found to mediate TGF-\(\beta\)1 signalling in human mesenchymal cells isolated from IPF patients that lead to fibronectin and \(\alpha\)-SMA gene and protein expression, collagen secretion, and proliferation. These processes were inhibited by NOX4 siRNA\(^ {168}\). In BLM-induced pulmonary fibrosis in mice, NOX4 was induced between days 7 to 21 post-challenge, and was found to accumulate around remodelled alveolar structure at day 14\(^ {168}\). Knockdown of NOX4 by siRNA at day 0 post BLM challenge reduced fibrosis that is observed by reduced staining for collagen, \(\alpha\)-SMA and decreased acid soluble collagen in whole lung homogenate at days 14 and 21\(^ {168}\). Therapeutic treatment of BLM-challenged mice at day 8 with diphenyleneiodonium (DPI), a NOX/flavoenzyme inhibitor, protected mice from pulmonary fibrosis at day 21\(^ {168}\). NOX4 knockout mice were also protected from BLM-induced pulmonary fibrosis, which was attributable to reduced TGF-\(\beta\) induced epithelial cell apoptosis\(^ {173}\). Oral treatment with a NOX4 inhibitor attenuated BLM-induced pulmonary fibrosis in rats and TGF-\(\beta\)-induced induction of procollagen and \(\alpha\)-SMA expression in human pulmonary fibroblasts\(^ {174}\). Knockdown of NOX4 by siRNA similarly attenuated fluorescein isothiocyanate (FITC)-induced pulmonary fibrosis in mice\(^ {168}\).
1.1.4.5 The role of immune cells in IPF

The pathogenesis of IPF, including potential pathological chronic inflammation-induced injury, fibrosis, and tissue remodelling, may be mediated by cells of the immune system that accumulate in the lungs of IPF patients, including macrophages, mast cells, T lymphocytes and B lymphocytes, through their release of various mediators.

1.1.4.5.1 Macrophages

AMφ accumulate in the BAL and lung tissue of IPF patients and contribute to pulmonary fibrosis through the release of various pro-inflammatory and pro-fibrotic mediators. AMφ have been found to be essential for the progression and/or resolution of BLM-induced pulmonary fibrosis in mice challenged intratracheally, as depletion of pulmonary macrophages by liposomal chondrate or DTR in CD11c-DTR mice at the active or established fibrotic phases (day 14 or 21), or resolution phase (days 42-46) reduced or prolonged fibrosis at days 32 or 56 respectively. These distinct roles may be contributed by various subpopulations of AMφ, which will be described in greater detail later (section 1.2.3.2). AMφ can be replenished by proliferation within the lung or recruitment of circulating monocytes. However, it is unclear which source is responsible for the increased AMφ population in pulmonary fibrosis. Ly6c<sup>hi</sup> monocytes generated from bone marrow in vitro were found to exacerbate BLM-induced pulmonary fibrosis and promote the generation of Ym1<sup>+</sup> M2-like macrophages in mice when injected intravenously during the progressive fibrotic phase (day 21). On the other hand, adoptive transfer experiments of Ly6C<sup>hi</sup> pro-inflammatory circulating monocyte precursors isolated from donor CD45.1 mice intravenously injected into recipient CD45.2 mice during the established fibrotic phase (day 21) of BLM-induced pulmonary fibrosis did not lead to an accumulation of CD45.1+ cells in the recipient lung, suggesting that Ly6c<sup>hi</sup> cells do not directly contribute to the M2-like AMφ population during pulmonary fibrosis at day 28. It may be possible that these Ly6c<sup>hi</sup> monocytes indirectly induce M2-like AMφ in the lung during pulmonary fibrosis through the release of a number of M2-inducing mediators, such as IL-4, IL-13, IL-10, and TGF-β.

Ly6c<sup>-</sup> monocytes may also participate in pulmonary fibrosis through their release of the pro-angiogenic mediator vascular endothelial growth factor (VEGF) to promote fibroblast differentiation into myofibroblast and promote collagen deposition. The human equivalent of both murine Ly6c<sup>hi</sup> and Ly6c<sup>-</sup> monocytes, CD14<sup>hi</sup>CD16<sup>lo</sup> CC chemokine receptor (CCR)2<sup>+</sup> and CD14<sup>hi</sup>CD16<sup>hi</sup>CCR2<sup>+</sup> monocytes respectively, were found to be elevated in IPF patients and correlated with disease progression, although their exact role in pathology remains to be determined. It is possible that these monocyte subsets play a similar role to their mouse counterparts in biasing towards M2 polarisation and angiogenesis respectively.
1.1.4.5.2 Mast cells

Mast cells are tissue resident innate immune cells that exerts its effects through acute degranulation of preformed mediators (e.g. histamine, tryptase, chymase) and also the release of synthesised cytokines and chemokines (e.g. IL-13, CCL2, CCL5) in a delayed response. Two different type of mast cells have been identified in mice based on their production of the serine proteases, chymase and tryptase, and their anatomical positions. In mice mast cells secreting both tryptase and chymase are found in connective tissue of intestinal submucosa, peritoneal cavity and surrounding blood vessels and skin, and are known as connective tissue mast cells (CTMCs); mast cells secreting chymase only are found in the mucosal tissue of lungs and intestine, and are named mucosal tissue mast cells (MMCs)\(^1\). In humans mast cells are classified into two groups based on their secretion of chymases or tryptases, including MCTC that secretes both chymases and tryptases, and MC\(_T\) that secretes tryptase only. MCTC and MC\(_T\) in humans correspond to CTMC and MMC in mice respectively\(^2\,3\).

There are several evidence suggesting the role of mast cells in pulmonary fibrosis. There is an accumulation of mast cell specific products in the BAL of IPF patients, including histamine\(^4\) and basic fibroblast growth factor (FGF)\(^5\). The level of tryptase in BAL also predicted a poorer outcome in IPF patients\(^6\). An accumulation of mast cells was also observed in the BAL and lung tissue of IPF patients. MC\(_T\) are most frequently found in the healthy lung\(^7\), but in IPF patients there was an increase in the ratio of M\(_{TC}\) as compared to M\(_T\) in BAL\(^8\), suggesting that M\(_{TC}\) may have a more significant role in pathology. There was also a 10-fold increase in the number of interstitial mast cells in IPF patients as compared to control subjects\(^9\). Immunohistological analysis revealed that mast cells accumulated in the connective tissue directly adjacent to the lumen of small airways and fibrotic foci in IPF patients and appeared to be degranulating\(^10,11\). Elevated c-kit+ mast cells positive for both chymase and tryptase (i.e. M\(_{TC}\)) were also observed in the lungs of IPF patients\(^12\). In addition, an increased TGF-β expression in mast cells found in the alveolar parenchyma, and the density and percentage of M\(_{TC}\) correlated positively with the degree of fibrosis and negatively with patient lung function\(^9\).

Rodent models have also provided clues about the potential role of mast cells in pulmonary fibrosis. WBBF1-W/Wv mice, which are deficient in mast cells through a mutation in the mast cell survival and proliferation factor [stem cell factor (SCF)] receptor c-kit, were protected from BLM-induced pulmonary fibrosis\(^13\). However, this protection was lost when mast cells were restored\(^14\), indicating a role for mast cells in the initiation of pulmonary fibrosis. SCF was reported to be elevated on fibroblast membranes in IPF patients, and the co-culture of fibroblasts from IPF patients prolonged mast cell proliferation and survival\(^15\). On the contrary, the genetic or pharmacological inhibition of
SCF abolished BLM-induced pulmonary fibrosis in mice, again indicating a role for mast cells in the pathology of pulmonary fibrosis\textsuperscript{195}.

1.1.4.5.3 T lymphocytes

T lymphocytes accumulate within the BAL\textsuperscript{196,197} and lung tissue\textsuperscript{198–201} of IPF patients, and are activated following antigen presentation by APCs\textsuperscript{201,202}. Both CD4\textsuperscript{+} and CD8\textsuperscript{+} T-cells are present in the lung tissue of IPF patients, although CD8+ cells represent the majority. In the BAL the CD4/CD8 ratio is more variable where some patients present with greater numbers of CD4\textsuperscript{+} T-cells, whereas others present with more CD8\textsuperscript{+} T-cells 196. IPF patients with CD4/CD8 greater than one is correlated with a higher percentage of lymphocytes, a lower percentage of neutrophils in BALF and a better response to steroid treatment with respect to transfer factor for carbon monoxide (TLCO)\textsuperscript{196}. However, the role of T lymphocytes in pulmonary fibrosis remains unclear due to the discrepancies observed in animal models of pulmonary fibrosis. Genetic depletion of T lymphocytes in athymic nude mice, or all lymphocytes in severe combined immunodeficiency (SCID) mice and recombination activation gene (RAG) mice, or systemic depletion of T lymphocytes with neutralising antibodies either had no effect on\textsuperscript{203–206}, protected from\textsuperscript{207,208} or amplified\textsuperscript{209} pulmonary fibrosis. However, the presence of diverse T-lymphocyte subsets in pulmonary fibrosis would suggest that these are not the primary cause for the initiation of pathology, but recruited to the lung by a chemokine gradient and and activated by autoantigens (e.g. annexin-1, periplakin, HSP70\textsuperscript{210–212}) at the site of tissue injury, resulting in secondary complications and facilitating disease progression. Indeed mice expressing a transgenic T-cell receptor (TCR)-\(\beta\) gene, which prevents effective recognition of antigens (other than a single epitope of hen-egg lysozyme), when instilled with BLM presented no difference in the inflammatory or fibrotic response compared with control mice, suggesting that the initiation of fibrogenesis involves an antigen-independent mechanism\textsuperscript{213}. Various CD4\textsuperscript{+} T-lymphocyte subsets may release different mediators that contribute to different aspects of the disease. T\(_{h1}\) and T\(_{h2}\) cells may contribute to tissue injury and fibrosis through the release of pro-inflammatory mediators [e.g. Interferon (IFN)-\(\gamma\) and TNF-\(\alpha\)], or pro-fibrotic mediators (e.g. IL-4, IL-13 and TGF-\(\beta\)) respectively\textsuperscript{214}. T\(_{h17}\) cells also appear to be critical to BLM or IL-1\(\beta\) induced pulmonary fibrosis through their production of IL-17\textsuperscript{215–217}. Furthermore another subset of cells, namely CD4\textsuperscript{+} CD25\textsuperscript{+} Forkhead box Protein 3 (FOXP3)+ regulatory T cells (T\(_{reg}\)), have been found to have different roles at different stages of pulmonary fibrosis\textsuperscript{218}. T\(_{reg}\) cells may promote fibrosis through their direct or indirect release of the pro-fibrotic mediator TGF-\(\beta\)\textsuperscript{214}. Indeed co-culture studies have identified the induction of TGF-\(\beta\) expression in epithelial cells by T\(_{reg}\) cells\textsuperscript{218}. T\(_{reg}\) cells has also been shown to inhibit fibrocyte recruitment via suppression of fibroblast growth factor (FGF)-9 expression in a lung-specific, doxycycline-inducible TGF-\(\beta\) overexpression model of pulmonary fibrosis in mice\textsuperscript{219}.
1.1.4.5.4 B lymphocytes

B lymphocytes may also play a role in IPF: highly abnormal intrapulmonary B-cell aggregates\textsuperscript{201,220} and an overexpression of immunoglobulin genes\textsuperscript{104} were found in IPF patients. Potentially pathogenic immune complexes have been detected in the sera, BAL and pulmonary parenchyma of IPF patients\textsuperscript{212,221,222}. Diverse circulating immunoglobulin (Ig) G antibodies are present in more than 80\% of IPF patients\textsuperscript{210-212,222-227} that has been linked to disease severity and/or poor prognosis\textsuperscript{210-212,224}. The role of B lymphocytes in pulmonary fibrosis was controversial due to the lack of effect of lymphocyte depletion in SCID or RAG mice in animal models of pulmonary fibrosis described earlier\textsuperscript{204,205}. However, plasma B-lymphocyte stimulating factor (BLyS), an obligate factor for B cell survival and differentiation was significantly greater in IPF patients than normal controls, and the concentration of circulating BLyS is inversely correlated with patient outcome\textsuperscript{228}. BLyS was also found to be central to BLM- and IL-17-induced pulmonary fibrosis\textsuperscript{229}.

B lymphocytes may contribute to pulmonary fibrosis by the expression of tissue bound or circulating antibodies. Tissue bound antibodies can cause cytotoxicities through antibody-dependent cellular cytotoxicity (ADCC), where natural killer (NK) cells are recruited and/or activated by the Fc portion of IgG1 and IgG3 through their Fc\textsubscript{\gamma}RIII receptor (Fc\textsubscript{\gamma}RIII)\textsuperscript{230-233}. Tissue-bound antibodies can also direct release of pro-inflammatory, pro-fibrotic or vasoactive mediators by complement activation\textsuperscript{233}, and/or act as APCs to T lymphocytes to promote phagocytosis\textsuperscript{233,234}.

B lymphocytes also produce autoantibodies that promote a pro-inflammatory response in target cells through neutralisation of anti-inflammatory proteins, or formation of pro-inflammatory auto-antigens. Autoantibodies that recognise endogenous Annexin-1, periplakin and HSP70 are elevated in IPF patients, and the levels of these have been linked to more severe disease and poor prognosis\textsuperscript{210-212}. Annexin-1 is a calcium and phospholipid binding protein produced by AECs and AM\textsubscript{\phi}, and has anti-inflammatory properties through its inhibition of phospholipase A\textsubscript{2}, the first enzyme in the metabolism of the pro-inflammatory eicosanoids\textsuperscript{235}. Neutralisation of Annexin-1 leads to increased inflammation in pulmonary fibrosis that may increase tissue injury. Moreover, inhibition of Annexin-1 abrogates the inhibitory glucocorticoid effects on neutrophil extravasation and may again lead to an exacerbation of the pro-inflammatory response\textsuperscript{236}. Periplakin is a small protein of the plakin family that is produced by bronchial and alveolar epithelial cells\textsuperscript{211}, and is localised to desmosomes and intermediate filaments\textsuperscript{237}; inhibition of periplakin with autoantibodies inhibits alveolar epithelial repair \textit{in vitro}\textsuperscript{211}. Extracellular HSP70 is an alarmin that acts as an intracellular messenger by binding to specific cell surface receptors that transduce signals and modulate inflammatory responses\textsuperscript{238,239}. HSP autoantibodies can augment production of pro-inflammatory
mediators\textsuperscript{238,240,241} by cross-linking the cell surface receptor complexes or after gaining access to intracellular auto-antigens via lipid rafts.

1.1.4.6 Hypoxia in IPF
In IPF the continuous proliferation and activation of fibroblasts and excessive deposition of collagen within the alveolar airspaces results in scarred non-functional airspaces and subsequent hypoxia\textsuperscript{5,242\textendash 247}. Progressive fibrosis clinically correlates with worsening hypoxia, and increasing desaturation during exercise has been found to be a significant predictor of subsequent mortality\textsuperscript{248}. This will eventually lead to death by asphyxiation. Experimental evidence suggests that tissue hypoxia may play a role in the progression of IPF\textsuperscript{249\textendash 254}, and hypoxic lung tissue is associated with pulmonary fibrosis\textsuperscript{255}. Another study has found that lactic acid levels, a mediator generated by cells in response to hypoxia, are high in IPF lung tissue\textsuperscript{256}. Hypoxia exerts its effects via the transcription factors hypoxia-inducible factor (HIF)-1\textalpha and HIF-2\textalpha\textsuperscript{257,258}. Hypoxia may contribute to fibrosis through promoting cell proliferation, such as fibroblasts found in IPF patients\textsuperscript{259} via expression of the microRNA (miR)-210 that represses the c-myc (a transcription factor with a role in cellular proliferation) inhibitor, MNT. Indeed MNT levels are reduced in IPF fibroblasts, and knockdown of miR-210 increased MNT levels. Overexpression of MNT also inhibited hypoxia-induced IPF fibroblast proliferation. Hypoxia-mediated increase in miR-210 and fibroblast proliferation are downstream of HIF-2\textalpha, as silencing of HIF-2\textalpha attenuates both of these processes\textsuperscript{259}. Hypoxia also induced a profibrotic macrophage phenotype in MH-S cells (a mouse alveolar macrophage cell line\textsuperscript{260}) in vitro\textsuperscript{261}. Treatment of MH-S cells increased the production of activated PAI-1 that is attenuated through siRNA silencing of HIF-1\textalpha expression\textsuperscript{261}. PAI-1 is a pro-fibrotic mediator that is found at elevated levels in the lung tissue of mice with BLM-induced pulmonary fibrosis\textsuperscript{262}. Overexpression of PAI-1 augmented BLM-induced pulmonary fibrosis\textsuperscript{263}, whereas PAI-1 deficient mice had increased fibrinolysis, reduced collagen accumulation and prolonged survival\textsuperscript{264}. Activated PAI-1 is an indirect inhibitor of plasminogen activation into plasmin, which mediates the cleavage of a multitude of substrates, such as cytokines, MMPs, and fibrin. PAI-1 inhibition of fibrinolysis may promote fibrosis through cellular recruitment through sustaining the fibrin clot\textsuperscript{265}. HIF-1\textalpha also mediates the TGF-\beta\textsubscript{1} induced production of the pro-fibrotic mediator PDGF-AA in MH-S cells. As HIF-1\textalpha is a downstream effector of hypoxia, it is possible that this process is also inducible by hypoxia alone\textsuperscript{261}.

1.2 Macrophages
Macrophages are innate immune cells that are derived from bone marrow precursors. Mature macrophages localise in tissues and constitute to the mononuclear-phagocyte or reticuloendothelial system. In general, macrophages are characterised morphologically by an enlarged horse-shoe
shaped nucleus, significant rough surfaced ER, and large number of mitochondria or ER. They are motile cells, and appear at sites of inflammation within 24-48 hours. They are relatively long-lived, and exhibit continuous secretory activity during inflammatory processes, allowing them to destroy a range of cells, antigens and pathogens. Macrophages are also highly phagocytic to a number of substances, including viruses, bacteria, effete red blood cells (RBCs), tissue and debris and some tumour cells. Macrophages play a variety of roles in an organism’s biology, including development, homeostasis, tissue repair and immune defence against pathogens. Resident macrophages regulate tissue homeostasis by acting as sentinels and responding to changes in tissue physiology and also environmental challenges. In the event of tissue injury or infection, macrophages are recruited from the monocyte reservoirs of blood, spleen and bone marrow, and perhaps from tissue progenitors or local proliferation.

Within the alveolar space, tissue resident AMφ act as the first line of defence against invading pathogens. AMφ are lung-specific long-lived cells, with a turnover rate of around 40% per year. These cells have adapted to deal with factors unique to the lung environment that is reflected by their ability to respond to airborne irritants and microbes through increased chemotaxis, phagocytosis, cytotoxicity and release of reactive oxygen and nitrogen intermediates, Prostaglandin E (PGE), TNF-α and IFN. Critically, AMφ have adapted to default to a relatively quiescent state so as to minimize damage to the lung tissue in response to external stimuli. AMφ secrete latent TGF-β that is activated by αvβ6 integrin expressed on adjacent injured epithelial cells to limit inflammation and protect from alveolar epithelial damage. At the same time, AMφ may also suppress inflammation by efferocytosis of apoptotic cells. In chronic lung diseases such as IPF, AMφ are defective in their ability to resolve inflammation and homeostasis and therefore allowing tissue injury to persist. This will be discussed in greater detail in section 1.2.3.

Macrophages are able to carry out these diverse roles through altering their phenotype in response to signals in their immediate environment to generate heterogeneous specialised subpopulations. Several signals have been identified through in vitro experiments, which will be described below.

### 1.2.1 Heterogeneity of macrophages to environmental signals

Macrophages have phenotypic heterogeneity and are engaged in a diverse range of activities including pro- and anti-inflammatory activities, immunogenic and tolerogenic activities and tissue destruction and tissue repair activities. This plastic expression profile of macrophage is influenced by the nature, concentration and length of exposure to the inducing signals in their immediate environment. Two types of signals have been reported to alter macrophage phenotype, including priming signals (e.g. cytokines, growth factors, immune complexes, hypoxia) that influence
the inflammatory potential and the wound-healing response in macrophages, and also stimulatory signals (e.g. PRR ligands) that have strong inflammatory effects\textsuperscript{282}.

Several macrophage subsets have been characterised \textit{in vitro} through the distinct priming and/or stimulation of bone marrow derived macrophages (BMM\textsubscript{φ})\textsuperscript{283} in mice and monocyte derived macrophages (MDM) in humans that can broadly be classified into two groups dependent on their inflammatory potential. ‘Classical activation’ of macrophages, also known as M1 macrophages, are characterised as those that have an elevated inflammatory potential, with enhanced microbicidal capacity and increased secretion of pro-inflammatory mediators\textsuperscript{284,285}, and includes macrophages that have been primed with the Th1 cytokine IFN-\textgamma alone or in combination with stimulation from PRR ligands, and the growth and survival factor macrophage colony stimulating factor (M-CSF).

‘Alternative activation’ of macrophages, also known as M2 macrophages, have a reduced inflammatory potential and were proposed to play an important role in the immune response to parasites, allergy, wound healing and tissue remodelling\textsuperscript{281,282}. M2 macrophages are characterised as those primed with the Th2 cytokines IL-4 or IL-13, the growth and survival factor granulocyte macrophage colony stimulating factor (GM-CSF), immune complexes in the presence of PRR stimulation, or the resolution mediators IL-10 or TGF-\beta. These macrophages are also known as M2a, M2b, or M2c macrophages respectively\textsuperscript{283}; M2b and M2c macrophages were also referred to as M2-like macrophages owing to their functional similarities to the prototypical M2a macrophages. Priming with the macrophage growth and survival factor GM-CSF were also reported to promote a M2-like phenotype. The different nomenclature, priming and/or stimulatory signals, associated mediators and function for distinct macrophage subsets pre-defined \textit{ex vivo} is summarised in table 1.1.
Table 1.1 Summary of macrophage subsets derived *in vitro*. M1 macrophages are induced by IFN-γ priming and/or TNF-α or LPS stimulation. M1 macrophages promote a TH1 antimicrobial response that is targeted towards defence against intracellular pathogens, but may also play a role in tumour resistance and delayed type hypersensitivity. M1 macrophages exert their effects through the release of a number of cytotoxic reactive oxygen species (ROS), reactive nitrogen species (RNS), pro-inflammatory cytokines, chemokines and tissue remodelling proteases. M2 macrophages are those induced by IL-4/IL-13 priming, and promote a TH2 response that is targeted towards extracellular pathogens, including parasites and also allergens. M2 macrophages may also play a role in wound healing through the release of a number of growth factors and extracellular matrix (ECM) components that recruit and activate fibroblasts. M2-like macrophages include macrophages that are primed by a deactivating signal, such as immune complexes (IC), interleukin (IL)-10, transforming growth factor (TGF)-β or glucocorticoids, and then stimulated by a PRR ligand (e.g. LPS). M2-like macrophages have a high expression of the anti-inflammatory cytokine IL-10, and a low expression of the pro-inflammatory cytokine IL-12 p70, and may therefore play a role in immunoregulation. M2-like macrophages may also secrete growth factors that are involved in wound healing. Deactivated macrophages are those primed by anti-inflammatory mediators only, and may play a role in tissue homeostasis. Arg-1, Arginase-1; CCL, CC chemokine ligand; CD, Cluster of Differentiation; IC, immune complex; IFN, interferon; IL, interleukin; IGF-1, insulin-like growth factor-1; Ig-H3, TGF-β induced protein; TGF-β, transforming growth factor-β; TNF-α, tumour necrosis factor-α; LPS, Lipopolysaccharide; NOS2; Nitric Oxide Synthase 2; MHC, Major Histocompatibility Complex; MMP, Matrix Metalloproteinase; MRC1, Mannose Receptor C, type 1. N/A: Not applicable.
The priming or stimulatory signals have only a temporary effect on macrophage phenotype. In other words, macrophages will eventually revert to their original, functional status after the cytokine signalling ceases. For example, the \textit{in vitro} or \textit{in vivo} treatment of macrophages with cytokines alter their functional response pattern to lipopolysaccharide (LPS). However, if the cytokines are washed away after incubation and macrophages are then maintained in the absence of cytokines for 1-2 days before LPS stimulation, the functional response is usually identical to macrophages that have not been pre-stimulated with cytokines\textsuperscript{286}. This reversibility of macrophage phenotype thereby allowing them the flexibility to adapt their phenotype to the ever changing microenvironment.

Macrophages also display a high level of plasticity in their phenotype, and demonstrate reversibility in their polarisation state. Macrophages display distinct functions when primed with IFN-\(\gamma\), IL-12, IL-4, or IL-10 \textit{in vitro}, and additional functional patterns are displayed depending on whether or not the cytokine is present alone or with other cytokines, and whether or not the cytokine is added before or together with the activating stimulus LPS. For example, IL-10 is able to promote IL-4–induced Arginase protein expression in mouse BMM\(\phi\). Sequential treatment of macrophages with different cytokines results in a progression through multiple functional phenotypes. For example, treatment of mouse BMM\(\phi\) and peritoneal macrophages with IL-10 following IFN-\(\gamma\) polarisation reduced IL-12 protein levels upon LPS stimulation\textsuperscript{287}.

Furthermore, macrophage polarisation in tumour associated macrophages (TAM) have been shown both \textit{in vitro} and \textit{in vivo} to be reversible. TAM adopt a M2-like phenotype (Arg-1\textsuperscript{hi} IL-10\textsuperscript{hi}) that is dependent on NF\(\kappa\)B activation via the IL-1R and MYD88 signalling pathways. Co-culture of macrophages with ID8 cells, a mouse ovarian cancer cell line, was able to generate M2-like TAM \textit{in vitro}. However, inhibition of NF\(\kappa\)B activation through impairment of IKK\(\beta\) production, an upstream signalling mediator of NF\(\kappa\)B, in IKK\(\beta\)/\(\sim\) mice, biases BMDM co-cultured with ID8 cells away from M2-like polarisation (Arg-1\textsuperscript{hi} IL-10\textsuperscript{hi}) towards M1-like polarisation (IFN-\(\gamma\)\textsuperscript{hi} IL-12 p70\textsuperscript{hi} NOS2\textsuperscript{hi})\textsuperscript{288}.

IL-12 p70 secretion was found to be defective in M2-like TAMs isolated from human and mice tumours, which was attributed towards increased production of IL-10. Inhibition of IL-10 with neutralising antibodies restored the defective capacity of TAMs to produce IL-12 p70\textsuperscript{289}. These results indicate that macrophages display a high level of plasticity, and are able to adapt their phenotype to their changing microenvironment.

In light of the temporal effects of signals and plasticity of macrophages, the M1-M2 classification of macrophage subsets \textit{in vitro} is therefore, likely to be an oversimplification of the actual \textit{in vivo} phenotype, where a multitude of inducing signals, including both M1 and M2 stimuli, are present. Macrophages are subject to at least four levels of recognition/ response, including one where the monocyte survives and acquires mature phenotypes, a level where macrophage interacts with
immune cells (NK and T helper cells, eosinophils and basophils), a level where macrophages deals with the pathogen and a resolution phase, each of which are capable of shaping macrophage phenotype\textsuperscript{290}. The different signals that may influence macrophages at various stages of development and immune response is summarised in table 1.2. These signals may have agonistic or antagonistic effects to one another such that a spectrum, rather than mutually exclusive, distinct M1 or M2 macrophage phenotypes are thought to be present \textit{in vivo}. For example in smoking and non-smoking COPD patients, AM\textsubscript{Φ} expressed a mixture of some M1 [pro-inflammatory mediators (TNF-α, IL-1β, and IL-12 p70), complement receptor (CR)-3] and M2 [CD209/dendritic cell- specific intercellular adhesion molecule-grabbing nonintegrin (DC-SIGN)] macrophage-associated markers\textsuperscript{291}, although it is unclear whether or not these represent distinct subpopulations or hybrid phenotypes.

<table>
<thead>
<tr>
<th>Growth and survival factors</th>
<th>Lineage determining cytokines (Conventional maturation)</th>
<th>Survival, recruitment and retention</th>
<th>Other</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>M-CSF, GM-CSF</td>
<td>Adhesion molecules, chemokines</td>
<td>VitD3, Retinoic acid, PPR gamma ligands</td>
</tr>
<tr>
<td>Lymphoid and myeloid cytokines</td>
<td>Classical and alternative activation</td>
<td>Pro-inflammatory cytokines</td>
<td>Anti-inflammatory cytokines</td>
</tr>
<tr>
<td></td>
<td>IFN-γ, IL-4, IL-13</td>
<td>TNF, IL-6, IL-1β</td>
<td>IL-10, TGF-β</td>
</tr>
<tr>
<td>Interaction with pathogens</td>
<td>Direct interaction</td>
<td>Humoral</td>
<td></td>
</tr>
<tr>
<td></td>
<td>PRRs</td>
<td>IgG, IgE, IgA</td>
<td>Complement, Lectins, Ficolins</td>
</tr>
<tr>
<td>Resolution</td>
<td>Systemic mechanisms</td>
<td>Local mechanisms</td>
<td></td>
</tr>
<tr>
<td></td>
<td>IL-10, TGF-β</td>
<td>ECM Proteoglycans, ATP and sugar nucleoCdes, Resolvins, Maresins</td>
<td></td>
</tr>
</tbody>
</table>

Table 1.2 Different mediators at various stages of macrophage development and immune response may influence macrophage phenotype. Growth and survival factors released during monocyte maturation into macrophages, lymphoid and myeloid cytokines released during an immune response, direct or indirect activation of macrophages through PRR stimulation or the release of humoral mediators as a result of pathogen interaction, and resolution phase mediators may act together to alter macrophage phenotype. ATP, adenosine triphosphate; ECM, extracellular matrix; GM-CSF, granulocyte macrophage-colony stimulating factor; IFN-γ, interferon gamma; Ig, immunoglobulin; IL, interleukin; M-CSF, macrophage stimulating factor; PRR, pathogen recognition receptor; TGF-β, transforming growth factor-beta; TNF-α, tumor necrosis factor-alpha; vitamin D3 (VitD3). This table is adapted from Martinez FO and Gordon S, 2014\textsuperscript{290}.

For the sake of simplicity however, macrophages subsets derived \textit{in vitro} will be referred to as M1, M2 and M2-like, and their phenotype will be discussed below. Macrophages also play various roles in tissue fibrosis, including IPF that may be mediated by different subpopulations. The role of macrophages in various diseases, including fibrosis, and the participation of various subsets in IPF specifically will be discussed further in sections 1.2.2 and 1.2.3 respectively.
1.2.1.1 M1 ‘classically activated’ macrophages

M1 ‘classically-activated’ macrophages develop following exposure to IFN-γ and/or TNF-α or LPS, which induces TNF-α production. M1 macrophages increase the production of pro-inflammatory cytokines, including IL-1β, TNF-α, IL-12 p70 and IL-6, and chemokines, including CCL15/ Macrophage Inflammatory Protein (MIP)-5, CCL20/MIP-3α and CXCL13/ B-Cell Attracting chemokine (BCA). M1 macrophages also increase the surface expression of antigen presenting proteins, including MHC-II and the co-stimulatory proteins CD80 and CD86, and have increased antigen presenting capabilities to T-cells. M1 macrophages also facilitate a type I immune response through the release of CXCL11/ Interferon-inducible T-cell alpha Chemottractant (I-TAC), which recruit NK cells and Th1 cells through CXCR3. M1 macrophages also show increased phagocytosis of microorganisms, and enhanced production and secretion of MMPs, such as MMP-7 and MMP-9. MMPs enable macrophage to gain access to the injured tissue during inflammation through basement membrane disruption, but excessive release or unregulated production results in tissue damage. In addition, M1 macrophages have increased microbicidal activity by restriction of iron and other nutrients for microorganisms, acidification of phagosomes, synthesis of reactive oxygen intermediates (ROI) and Nitric Oxide (NO) through the increased expression of Nitric Oxide Synthase (NOS) 2/inducible NOS (iNOS).

1.2.1.2 M2 or M2-like macrophages

M2 ‘alternatively activated’ macrophages were originally identified in BMMφ exposed to IL-4/IL-13, and are so-called to distinguish them from the ‘classically-activated’ M1 macrophages. Other related subsets, closely resembling each other but distinct in function, have since been identified, and are referred to as M2-like macrophages. These M2-like macrophages include those primed with anti-inflammatory mediators, such as immune complexes, glucocorticoids, prostaglandin (PG) E2, IL-10, and TGF-β that are subsequently stimulated by a TLR ligand, and are characterized by their high expression of IL-10 and low expression of IL-12 p70.

1.2.1.2.1 M2 macrophages

M2 macrophages generated by IL-4 or IL-13 priming increase the expression of scavenger receptors, such as mannose receptor C, type 1 (MRC1)/ Macrophage Mannose Receptor (MMR)/ CD206 in humans and mice. They are considered wound-healing macrophages, and can play a role in physiological and pathological tissue remodelling. M2 macrophages increase fibrogenic mediators, such as fibronectin 1 and matrix associated protein beta Ig-H3. They also release mediators required for tissue repair and proliferation, including coagulation factor XIII, insulin-like growth factor (IGF)-1. In addition, M2 macrophages increase the expression of arginase-1 (Arg-1), an enzyme that converts arginine into L-proline, the precursor for collagen synthesis.
M2 macrophages also facilitate a type II immune response and are responsible for defence against parasites. M2 macrophages produce the chemokines CCL13/Macrophage Chemoattractant Protein (MCP)-4, CCL8/MCP-2 and CCL26/eotaxin-3, which recruit eosinophils, basophils and some polarized TH2 cells through the activity of CCR3. However, M2 macrophages have poor antigen-presentation skills and exhibit increased release of iron and clearance of apoptotic cells and ECM components.

1.2.1.2 M2-like macrophages
M2-like macrophages are a group of macrophage subpopulations that are characterised by their high levels of IL-10 production, and the requirement for two stimuli, an anti-inflammatory priming signal followed by a TLR stimulatory signal, to induce their anti-inflammatory activity. The primary signals include antibody immune complexes, glucocorticoids, PGE2, TGF-β and IL-10, adenosine and apoptotic cells, which has little or no stimulatory function of its own. However, when combined with a second stimulus, such as TLR ligand (e.g. LPS), the two signals reprogramme macrophages to produce IL-10. In addition, M2-like macrophages reduced levels of IL-12 p35 and p40 production. IL-10 may suppress an inflammatory response through the ligation of the IL-10 receptor (IL-10R) complex, which is a heterotetramer composed of two ligand-binding α-subunits (IL-10Rα) and two signal transducing β-subunits (IL-10Rβ), and activation of a signalling cascade involving Janus kinase (JAK)1, tyrosine kinase (TYK)2, and signal transducers and activator of transcription (STAT)3. IL-10 induces the inactivation of macrophages and dendritic cells (DC), with a resulting inhibition of inflammatory cytokine secretion and inhibition of the expression of MHC-II and co-stimulatory molecules. IL-10 also inhibits the maturation of DCs from monocyte precursors and limits the ability of macrophages to kill intracellular organisms, partly by inhibiting their production of TNF. IL-10 also suppresses a T, response by inhibiting the production of its primary inducer IL-12 p70 by macrophages and DCs. In addition, IL-10 induced IL-10 producing CD4+ Treg, which may amplify its immunosuppressive response.

As a result of their high IL-10 production, M2-like macrophages are considered to be anti-inflammatory and may play an immunoregulatory role to limit inflammation at the later stages of immune response. M2-like macrophages may be the subpopulation that produces TGF-β in addition to IL-10, although this has not been shown rigorously due to the overlap in markers between M2 and M2-like macrophages.

There are subtle differences in M2-like macrophages induced by different priming signals. M2-like macrophages generated by priming with immune complexes and TLR stimulation resemble M1 macrophages more than M2 macrophages, and have increased expression of NOS2 but low levels of Arg-1, and increased expression of the antigen presenting co-stimulatory molecules CD80 and CD86.
While these M2-like macrophages produce high levels of anti-inflammatory IL-10, they are not anti-inflammatory per se as they also produce pro-inflammatory cytokines, including TNF-α, IL-6 and IL-1β. M2-like macrophages generated from immune complex and TLR stimulation have been reported to favour the development of a type II adaptive immune response. These macrophages may also play a role in immunoregulation through the recruitment of Treg through the release of CCL1/I-309.

On the other hand, M2-like macrophages generated from priming with anti-inflammatory mediators, including IL-10, TGF-β and glucocorticoids have inhibited secretion of pro-inflammatory mediators, including TNF-α, IL-6, IL-12 p70 and IL-18. There is also a decreased antigen presenting capabilities by these cells, with a reduced expression of MHC-II, CD80 and CD86. Similar to M2 macrophages, M2-like macrophages induced by IL-10 also have increased surface and intracellular expression of MRC1.

### 1.2.2 The role of macrophages in disease

Following tissue damage resulting from injury or infection, inflammatory monocytes (Ly6c+ in mice) are recruited from the circulation and differentiate into macrophages that migrate into the affected tissues. These recruited macrophages are usually pro-inflammatory and M1-like in the early stages of a wound-healing process, secreting a variety of inflammatory mediators including TNF-α, IL-1 and NO, which activate antimicrobial defence mechanisms, including oxidative processes that contribute to the killing of invading organisms. These macrophages also secrete IL-12 and IL-23, which direct the differentiation and expansion of Th1 and Th17 cells that help drive inflammatory responses forward. Although these inflammatory macrophages are beneficial in that they facilitate the clearance of invading organisms, they also trigger collateral tissue damage arising from the toxic activities of ROS and RNS and of Th1 and Th17 cells. If the inflammatory response is not quickly controlled, it can become pathogenic and contribute to disease progression, as is seen in many chronic inflammatory and autoimmune diseases, including atherosclerosis, asthma, inflammatory bowel disease, rheumatoid arthritis, and fibrosis. To counteract the tissue damaging potential of the inflammatory macrophage response, macrophages undergo apoptosis or switch to an anti-inflammatory M2 and/or M2-like phenotype that dampens the pro-inflammatory response, while facilitating wound healing. These regulatory macrophages usually produce ligands that are associated with development such as WNT ligands that are essential for tissue repair.

There may be several contributors to the switch in macrophage phenotype. Anti-inflammatory mediators, such as IL-10 and TGF-β may be produced to negatively regulate the inflammatory response in tissue injury and promote a M2-like phenotype. Efferocytosis of apoptotic neutrophils induce macrophages to produce IL-4 and IL-13 that acts in an autocrine manner to promote the M2
phenotype in a mouse peritonitis model\textsuperscript{345}. Prolonged LPS stimulation in vitro may also induce tolerance in peritoneal macrophages to dampen the pro-inflammatory M1-like phenotype and promote the expression of M2-like associated proteins\textsuperscript{346}. These factors have a major impact on many chronic diseases. In the presence of persistent irritation, these homeostatic and reparative functions can be subverted, resulting in casual association of macrophages with various disease states, including fibrosis, obesity and cancer\textsuperscript{268}.

Macrophages have pleiotropic roles in tissue fibrosis, including the initiation, progression and resolution of fibrosis. In a carbon tetrachloride (CCl\textsubscript{4})-induced model of liver injury, repeated injury (3 month in mice) leads to extensive scarring that is induced by the transdifferentation of hepatic stellate cells (HSCs) into myofibroblasts\textsuperscript{347}. Following cessation of CCl\textsubscript{4} injections, the scarring resolves completely\textsuperscript{347}. Selective depletion of CD11b\textsuperscript{+} scar-associated macrophages (SAM) using DTR in CD11b-DTR mice during peak fibrosis at 12 weeks reduced the accumulation of ECM components and loss of myofibroblasts in the liver following CCl\textsubscript{4}-induced injury, indicating that SAM may play a role in the progression of liver fibrosis. On the other hand, depletion of SAM in the recovery phase attenuated matrix degradation, particularly perisinusoidal fibrosis\textsuperscript{348}. In a single intratracheal dose model of BLM-induced pulmonary fibrosis, depletion of CD11c\textsuperscript{+} AM\textsubscript{φ} with liposomal chondrate, or with DTR in CD11c-DTR mice during active (day 21) or established (day 28) fibrogenesis reduced collagen deposition observed at day 32. On the other hand, depletion of pulmonary macrophages with liposomal chondrate during the recovery phase (days 42-46) slows down the removal of collagen and the resolution of fibrosis observed at day 56\textsuperscript{349}.

Macrophages may contribute to the initiation and progression of fibrosis through the release of a number of mediators that promote inflammation, and fibrosis and tissue remodelling respectively (discussed further in section 1.2.3.1). Macrophages may also regulate fibrosis through the phagocytosis of apoptotic or necrotic cells, which inhibits or promotes inflammation respectively\textsuperscript{350,351}. Efferocytosis of apoptotic cells may be either pro- or anti-fibrotic. In liver fibrosis, ingestion of apoptotic hepatocytes increase TGF-β secretion in macrophages, which may promote fibrosis\textsuperscript{302,350-352}. On the other hand, clearance of apoptotic myofibroblasts, hepatocytes, and cellular debris removes the stimuli inducing TGF-β\textsuperscript{347,353}. Efferocytosis of necrotic cells may also be pro- or anti-fibrotic. Efferocytosis of erythrocytes by macrophages or Kupffer cells promotes oxidative stress, inflammation and fibrosis by depositing iron derived from haemoglobin in the liver\textsuperscript{354}. Conversely, efferocytosis of apoptotic hepatocytes by macrophages or Kupffer cells reduced the inflammatory response and shortened the course of tissue injury, thereby preventing fibrosis in a CCl\textsubscript{4}-induced liver injury model\textsuperscript{355}. Macrophage-mediated efferocytosis of apoptotic cholangiocytes has also been shown to promote the reversal of biliary fibrosis\textsuperscript{356}. AM\textsubscript{φ} from IPF patients have lower
rates of efferocytosis as compared to controls from other forms of interstitial lung disease in a yet unknown mechanism. This may reduce the clearance rate of apoptotic and/or necrotic cells and prolong cellular activation\textsuperscript{357}, thereby promoting dysregulated wound healing and fibrosis. Macrophages may also aid in the resolution of fibrosis through the secretion of collagen degrading mediators, such as milk fat globule epidermal growth factor 8 (mfge8)\textsuperscript{358}.

1.2.3 The role of macrophages in IPF

Macrophages may play a role in IPF. The study of clinical lung biopsies has shown an accumulation of macrophages around fibrotic lesions in IPF patients\textsuperscript{359}. Gene and protein expression analyses showed elevated levels of pro-inflammatory (e.g. TNF-\(\alpha\), IL-1\(\beta\), IL-12 p40, IL-6, CCL2, CCL3, CCL4, CCL5, CXC1, CXCL2, CXCL4, CXCL8), pro-fibrotic (e.g. fibronectin, TGF-\(\beta\)) and tissue remodelling mediators (e.g. MMPs) in AM\(\phi\) from IPF patients, which may lead to tissue injury and fibrosis respectively. The function of these macrophage derived mediators in IPF is described in greater detail below in section 1.2.3.1. Depletion of macrophages at the active fibrotic phase (day 14) with liposomal chlondrate in C57BL/6 mice, or diphtheria toxin in CD11c-DTR mice, attenuated collagen accumulation in a mouse model of BLM-induced pulmonary fibrosis, suggesting a causative rather than bystander role of macrophages in pulmonary fibrosis\textsuperscript{349}.

As aforementioned in section 1.2.1, macrophages have been shown to differentiate into heterogenous phenotypes in response to various priming and stimulatory signals \textit{in vitro}. It is possible that these different macrophage subsets may contribute to various aspects of pulmonary fibrosis: M1 macrophages may promote tissue injury through the release of various pro-inflammatory mediators, ROS, and RNS. M2 and M2-like macrophages may promote tissue fibrosis through the release of pro-fibrotic mediators. These will be discussed in greater detail in section 1.2.3.2.

1.2.3.1 Macrophage-derived mediators in pulmonary fibrosis

Macrophages have been shown to release a number of mediators, including cytokines, chemokines, matrix remodelling proteins and also ECM components that may contribute to the pathogenesis of pulmonary fibrosis, several of which that are assessed in this thesis will be described in detail below.

TNF-\(\alpha\)

TNF-\(\alpha\) is a pleiotropic inflammatory cytokine that is predominantly produced by activated macrophages\textsuperscript{360}. TNF-\(\alpha\) is produced in its 27 kDa transmembrane precursor form, but can be cleaved by the metalloprotease TNF-\(\alpha\) Converting Enzyme (TACE)/ A Disintegrin And Melloproteinase (ADAM)17 into a soluble 17 kDa form\textsuperscript{361}. Both transmembrane and soluble forms can bind as homotrimers to TNF receptors (TNFR), including a ubiquitously expressed TNFR1 (55kDa) and a hematopoietic cell restricted TNFR2 (75kDa), which activate the Nuclear Factor \(\kappa\) B (NF\(\kappa\)B), MAPK
and/or apoptotic signalling pathways. Soluble forms of TNFRI and TNFRII can be released to neutralise TNF-α activity. TNF-α has been associated with a number of responses, including regulation of cell proliferation, and mediation of the local inflammatory response. The pleiotropic effects of TNF-α may be mediated through extensive cross-talk between different signalling pathways, the type of cell they stimulate, and/or concurrent stimulation with other cytokines.

Lung specific overexpression in mice by introduction of the TNF-α transgene into the SPC promoter, a pulmonary surfactant expressed exclusively by AEC2, induced pulmonary fibrosis. TNF-α may contribute to pulmonary fibrosis through inducing promoting tissue injury, fibroblast proliferation and/or propagating chronic inflammation.

However, TNF-α may also protect from pulmonary fibrosis. TNF-α have been shown to inhibit collagen synthesis in myofibroblasts, and have a role in aiding epithelial cell recovery.

Intratracheal instillation of TNF-α at the 3rd and 4th week following BLM-challenge induced resolution of pulmonary fibrosis that included reduction of collagen accumulation, improved lung function and lung architecture. On the other hand, fibrosis was prolonged in TNF-α-/- knockout mice. The contrasting effects of TNF-α to pulmonary fibrosis may be due to its different contributions to various stages of pulmonary fibrosis, where it acts to promote the initiation but prevent the progression of pulmonary fibrosis.

IL-1β

IL-1β is a member of the IL-1 gene family that is an important group of cytokines involved in acute and chronic inflammation. IL-1β is produced by a wide variety of cells, including macrophages, in response to inflammatory agents, infections, or microbial endotoxins. IL-1β is produced as an inactive zymogen, and the 17kDa bioactive form is released after proteolytic cleavage by IL-1β converting enzyme (ICE) or the Nucleotide-binding Oligomerization Domain (NOD)-Like Receptors (NLR) family, Pyrin domain containing 3 (NLRP3) inflammasome. IL-1β exerts its effects through binding to IL-1 receptor (IL-1R)1, which then associates with IL-1R accessory protein (IL-1R3/ IL-1R AcP) to form a high affinity receptor complex that is competent for signal transduction. IL-1R2 has high affinity for IL-1β but function as a decoy receptor and negatively regulates IL-1β activity. IL-1 receptor antagonist (IL-1ra) also functions as a competitive antagonist by preventing IL-1β from interacting with IL-1R.

IL-1β may contribute to pulmonary fibrosis through its promotion of inflammation and fibrosis. Transient overexpression of IL-1β within rat lungs by adenoviral gene transfer after intranasal administration induced pulmonary fibrosis. This was accompanied by an increase in TNF-α and IL-6 protein levels and evidence of increased acute lung injury (ALI), suggesting that IL-1β may contribute
to elevated fibrotic response by inducing more severe tissue injury\textsuperscript{375}. In addition, IL-1\textbeta promotes
the production of the angiogenic chemokines, including CXCL1 (also known as keratinocyte-derived
chemokine, KC) and CXCL2 (also known as MIP-2), which are murine homologues of CXCL8/IL-
8/melanoma growth stimulating activity (GRO)-\alpha\textsuperscript{376}. On the other hand, lung-specific IL-1\textbeta
overexpression also induced an increase in the pro-fibrotic mediators TGF-\beta and PDGF, suggesting
that IL-1\textbeta may also participate in progressive fibrotic changes\textsuperscript{375}.

Histological analyses revealed that continual inhibition of IL-1\textbeta, by administration of with IL-1ra
through an interperitoneal osmotic minipump at day 2, prevented hydroxyproline accumulation in
the lung and pulmonary damage in BLM- or silica-challenged mice at day 15 (active fibrosis), but had
no effect on cellular composition within BAL, suggesting a causative role between IL-1\textbeta induced
inflammation and subsequent fibrosis\textsuperscript{122}. Furthermore, treatment of BLM- or silica-challenged mice
with IL-1ra at day 25 (established fibrosis) also reduced hydroxyproline content within the lung,
indicating a role for IL-1\textbeta in established fibrosis\textsuperscript{122}.

**IL-12 p40**

IL-12 (IL-12p70)/cytotoxic lymphocyte maturation factor (CLMF)/ NK cell stimulatory factor (NKSF) is
a heterodimer composed of two disulphide-linked subunits of 35 (p35) and 40 kDa (p40) that are
encoded by two separate genes. These two subunits combine to form the bioactive heterodimer of
70-75 kDa (IL-12 p70)\textsuperscript{377,378}. IL-12 p70 is highly expressed by activated APCs, and is a key cytokine for
induction of a Th1 immune response, primarily acting on T lymphocytes and NK cells\textsuperscript{379}. IL-12p70
exerts its effects through binding to the IL-12 receptor complex that is a heterodimer composed of
the IL-12R-\beta1 and IL-12R-\beta2 subunits. In addition to the dimerization with IL-12 p35 to form the
bioactive IL-12 p70, IL-12 p40 can also form homodimers that act as antagonists to IL-12 p70 through
its ability to compete binding to IL-12 receptor IL-12R-\beta1. In addition, IL-12 p40 can also
heterodimerise with a 19kDa polypeptide (IL-23 p19) to form IL-23, which is reported to have similar
functions to IL-12 p70\textsuperscript{380}.

IL-12 p40 has been reported to be elevated in the serum of IPF patients\textsuperscript{125}. IL-12 p40 play a critical role
in murine models of pulmonary fibrosis: Neutralisation of IL-12 p40 with antibodies (day 0) attenuated
BLM-induced pulmonary fibrosis\textsuperscript{102,124} and IL-1\textbeta-induced pulmonary fibrosis\textsuperscript{124}. On the other hand, the
administration of recombinant IL-12 p70 attenuated BLM-induced pulmonary fibrosis\textsuperscript{381}. In a silica-
induced model of pulmonary fibrosis, IL-12 p40\textsuperscript{-/-} mice exhibited limited inflammation and fibrosis,
whereas administration of recombinant IL-12 p40 restored pulmonary fibrosis in IL-12 p40\textsuperscript{-/-} mice and
exacerbated pulmonary fibrosis in wild type (WT) mice. On the other hand, IL-12 p35\textsuperscript{-/-} mice had well-
developed inflammation and fibrosis following silica challenge. These results indicate that IL-12 p40,
but not IL-12 p35 or IL-12 p70, was responsible for the initiation of silica-induced pulmonary fibrosis. It was observed that IL-12 p35−/- mice had significantly higher IL-12 p40 levels in mouse lungs following silica challenge than WT controls, and was correlated with an increased pulmonary macrophage infiltration. Administration of recombinant IL-12 p40 into IL-12 p40−/- mice restored macrophage accumulation following silica challenge, whereas in WT controls there was an increased macrophage influx. IL-12 p40 is chemotactic for macrophages in vitro and in vivo, and was proposed to promote the initiation of silica-induced pulmonary fibrosis through the macrophage recruitment.

IL-12 p40 may also complex with IL-23 p19 to play a significant role in the initiation of BLM-induced pulmonary fibrosis, and IL-23 p19−/- mice have reduced collagen accumulation following BLM challenge. IL-23 may promote the differentiation of IL-17 producing T\(_{h}17\) cells, and IL-17A can mediate BLM or IL-1\(\beta\) induced pulmonary fibrosis. The pro-fibrotic roles of IL-12 p40 mentioned here are deduced from animal models of pulmonary fibrosis; further studies will have to be carried out to deduce their involvement in IPF.

**IL-6**

IL-6 is a pleiotropic 22-28 kDa cytokine that plays important roles in the acute phase reaction, inflammation, hematopoiesis, bone metabolism, and cancer progression. IL-6 signals through a cell surface heterodimeric receptor complex composed of a ligand binding subunit, IL-6 Receptor (IL-6R\(\alpha\)) and a signal transducing subunit (gp130). IL-6 binds to IL-6R\(\alpha\), which then triggers the association with gp130, and gp130 dimerization. Soluble forms of IL-6R\(\alpha\) are generated by both alternative splicing and proteolytic cleavage. In a mechanism known as trans-signalling, complexes of soluble IL-6 and IL-6R\(\alpha\) can elicit responses from cells expressing gp130 but not IL-6R\(\alpha\) on their cell surface. Trans-signalling allows a wider range of cell types to respond to IL-6, as the expression of gp130 is ubiquitous, whereas that of IL-6R\(\alpha\) is predominantly restricted to hepatocytes, monocytes and resting lymphocytes. Soluble splice forms of gp130 block trans-signalling from IL-6/IL-6R\(\alpha\) but not from other cytokines using gp130 as a co-receptor. IL-6, along with TNF-\(\alpha\) and IL-1, drives the acute inflammatory response and the transition from acute inflammation to either acquired immunity under normal conditions, or chronic inflammatory disease when dysregulated, including that observed in obesity, insulin resistance, inflammatory bowel disease, arthritis, sepsis and atherosclerosis. IL-6 can also act as an anti-inflammatory molecule, as in skeletal muscle where it is secreted in response to exercise. In addition, IL-6 enhances hematopoietic stem cell proliferation and the differentiation of T\(_{h}17\) cells, memory B cells and plasma cells. IL-6 and soluble IL-6R\(\alpha\) are elevated in systemic sclerosis and liver cirrhosis, correlating with disease severity. In the kidneys, heart and skin, IL-6 also promotes collagen production. In the lungs, IL-
6 is important in airway remodelling in asthma and induces the conversion of fibroblasts to myofibroblasts, and it promotes pancreatitis-associated lung injury.

IL-6 may also play a role in interstitial pulmonary fibrosis: IL-6 levels were increased in the BALF of IPF, sarcoidosis and systemic sclerosis with interstitial lung disease patients. IL-6 levels in BALF is correlated with worse prognosis in sarcoidosis patients, and is increased in patients requiring steroid therapy or having progressive disease. Serum IL-6 levels is predictive of early functional decline and mortality in interstitial lung disease associated with systemic sclerosis. Adenosine is a purine-signalling nucleoside that is generated in excess during cellular stress and damage and has been implicated in pulmonary fibrosis, where transgenic mice deficient in the enzyme responsible for adenosine catabolism, adenosine deaminase (ada), spontaneously develop pulmonary fibrosis. In a model of adenosine-induced pulmonary fibrosis in ada mice, genetic or pharmacological ablation of IL-6, in ada mice or by anti-IL-6 antibodies respectively, resulted in the attenuation of inflammation and fibrosis. Genetic mutant mice of the IL-6 signal-transducing subunit gp130 with deregulated STAT1/3-signalling (gp130ΔSTAT) attenuated, whereas those with deregulated extracellular regulated kinase (ERK)1/2 signalling (gp130757F) amplified BLM-induced pulmonary fibrosis in mice, indicating that IL-6 promotes pulmonary fibrosis in a STAT-3 dependent manner. Immunhistochemistry also showed excessive STAT3 phosphorylation in the lungs of IPF patients. IL-6 may mediate the progression of pulmonary fibrosis through trans-signalling: blockade of IL-6 trans-signalling by sequestration of soluble IL-6Rα by addition of soluble gp130 (day 19 to day 32 daily) attenuated BLM-induced pulmonary fibrosis in vivo, whereas activation of IL-6 trans-signalling with a combination of IL-6 and soluble IL-6Rα induced proliferation and ECM (collagen and fibronectin) production in normal and IPF fibroblasts in vitro.

In summary, there are increased levels of a number of pro-inflammatory mediators in human patients with interstitial lung diseases, and also rodent models of pulmonary fibrosis. In murine models of pulmonary fibrosis, pro-inflammatory mediators may play a role in initiating ALI-induced fibrosis, especially when overexpressed within the lung at non-physiological levels in transgenic mice. This may contribute to the lack of resolution of tissue injury, leading to progressive cycles of tissue injury and dysregulated wound healing, and ultimately fibrosis. The receptors for regulation and function of macrophage-derived proinflammatory cytokines that are elevated in pulmonary fibrosis are summarised in table 1.2. It should be noted however that therapeutic treatment targeting some of these pro-inflammatory cytokines (e.g. TNF-α neutralising antibody etanercept) did not reverse pathology in IPF patients, such that they are unlikely to be key mediators in the established active fibrotic response. On the other hand, therapeutic treatment of others (e.g. IL-1β
and IL-6) attenuated pathology in rodent models of pulmonary fibrosis, and may therefore play a role in the progression as well as initiation of fibrosis.
### Table 1.3 Macrophage-derived cytokines elevated in pulmonary fibrosis

<table>
<thead>
<tr>
<th>Receptor</th>
<th>Regulation</th>
<th>Function</th>
</tr>
</thead>
<tbody>
<tr>
<td>TNF-α</td>
<td>TNFR1/TNFRI homotrim homotrimer released from membrane by ADAM-17; sequestered by soluble forms of TNFR1 and TNFR2</td>
<td>Drives the acute inflammatory response and the transition from acute inflammation to either acquired immunity under normal conditions, or chronic inflammatory disease when dysregulated.</td>
</tr>
<tr>
<td>IL-1β</td>
<td>IL-1R1+IL-1R3 heterodimer activation by proteolytic cleavage by ICE and NLRP3; sequestration by IL1R2 and/or IL-1ra.</td>
<td>Drives the acute inflammatory response and the transition from acute inflammation to either acquired immunity under normal conditions, or chronic inflammatory disease when dysregulated; promotes the production of angiogenic mediators (e.g. CXCL1, CXCL2, and CXCL8), induction of pro-fibrotic mediators (e.g TGF-β and PDGF).</td>
</tr>
<tr>
<td>IL-12p40</td>
<td>IL-12R-β1 + IL-12R-β2 heterodimer IL-12p40 can form homodimers to inhibit IL-12p70.</td>
<td>Subunit of IL-12 p70 and IL-23 heterodimer; IL-12 p40 also acts as a chemoattractant to monocytes and macrophages.</td>
</tr>
<tr>
<td>IL-6</td>
<td>IL-6Rα + gp130 heterodimer soluble splice forms of gp130 block IL-6 trans-signalling.</td>
<td>Drives the acute inflammatory response and the transition from acute inflammation to either acquired immunity under normal conditions, or chronic inflammatory disease when dysregulated; enhances hematopoietic stem cell proliferation and the differentiation of T(\text{α}17) cells, memory B cells and plasma cells; promotes collagen production in kidneys, heart and skin; airway remodelling in asthma; fibroblast differentiation into myofibroblasts; anti-inflammatory in skeletal muscle in response to exercise.</td>
</tr>
</tbody>
</table>

Table 1.3 Macrophage-derived cytokines elevated in pulmonary fibrosis. (ADAM)-17, A Disintegrin And Melloproeinase/ TNF-α Converting Enzyme (TACE); CXCL, CXC chemokine ligand; gp130, glycoprotein 130; ICE, IL-1β converting enzyme; IL-1β, interleukin-1β; IL-6, interleukin-6; IL-12p40, interleukin-12 p40 subunit; IL-1R, interleukin-1 receptor; IL-1Ra, interleukin-1 receptor antagonist; NLRP3, Nucleotide-binding Oligomerization Domain (NOD)-Like Receptors (NLR) family, Pyrin domain containing 3; PDGF, platelet derived growth factor; TGF-β, Transforming Growth Factor-β; T\(\text{α}17\): IL-17+ T Helper lymphocytes; TNF-α, Tumour Necrosis Factor-α; TNFR, TNF receptor.
The CC chemokines are a family of chemoattractant that have two conserved adjacent cysteines near their amino terminus. This family of chemokines may attract a variety of immune cells in response to tissue injury and inflammation. Several CC chemokines that are produced by AMφ have been implicated in pulmonary fibrosis, including CCL2, CCL3, CCL4, CCL5, and CCL20, which will be described in detail below.

CCL2

CCL2, also known as MCP-1, is best known as a chemotactic agent for mononuclear cells. CCL2 is predominantly produced by monocytes and macrophages, but can also be produced by fibroblasts, glioma cells, smooth muscle cells, endothelial cells, lymphocytes and mononuclear phagocytes either constitutively or upon mitogenic stimulation. In addition to its chemotactic role, CCL2 also induces enzyme and cytokine release by monocytes, NK cells and lymphocytes, and histamine release by basophils that express its receptor CCR2. In addition, CCL2 promotes Th2 polarization in CD4+ T cells. CCL2-mediated recruitment of monocytes to the site of inflammation is proposed to play a role in atherosclerosis, multiple sclerosis and allergic asthma. CCL2 is upregulated in breast cancer and promotes tumour progression by recruiting pro-inflammatory TAM, promoting angiogenesis, and increasing the migratory and invasion-related properties of tumour cells.

CCL2 has been implicated in pulmonary fibrosis, and CCL2 mRNA and protein were strongly expressed in the lungs of IPF patients. CCL2 levels in serum and BALF were also elevated in IPF patients. CCR2-/- mice were protected from BLM- and FITC-induced pulmonary fibrosis and CCL2 gene therapy attenuated BLM-induced pulmonary fibrosis. CCL2 may play a role in pulmonary fibrosis through the recruitment and activation of mononuclear cells. CCL2 may also promote fibrosis through the direct and indirect activation of fibroblasts through the induction of TGF-β, leading to ECM generation. In addition, CCL2 may recruit and activate a subset of circulatory bone marrow-derived CD45+ CD13+ collagen1+ CCR2+ CD34- fibrocytes to the lungs through their surface CCR2 expression in pulmonary fibrosis. Indeed the recruitment of fibrocytes by CCL2 via their cell surface CCR2 expression has been demonstrated in a mouse model of FITC-induced pulmonary fibrosis. CCR2-/- mice had attenuated FITC-induced pulmonary fibrosis and fewer number of lung fibrocytes isolated, whereas these were restored in recipient CCR2-/- mice following bone marrow transplantation from donor CCR2+/- mice, indicating that CCR2 is required for the initiation of FITC-mediated tissue injury and fibrocyte recruitment respectively. Furthermore, fluorescent-labelled CCR2+ fibrocytes generated ex vivo, administered via an intravenous route were found to migrate to...
FITC-injured lung air spaces in CCR2−/− mice in vivo. Isolated lung fibrocytes also expressed CCR2 and migrated towards CCL2 in vitro, and CCL2 is able to induce collagen production in fibrocytes422.

CCL3

CCL3, also known as MIP1α, is closely related to CCL4/ MIP1β. CCL3 is expressed by a variety of hematopoietic cells, fibroblasts, smooth muscle cells and epithelial cells423. CCL3 exerts its effects through interactions with CCR1, CCR3 and CCR5423. CCR3 promotes the chemoattraction, adhesion to activated vascular endothelium, and cellular activation of many hematopoietic cell types including activated T cells, NK cells, neutrophils, monocytes, immature dendritic cells and eosinophils423–426.

CCL3 can also act as a stem cell inhibitor (SCI) that inhibits the proliferation of hematopoietic progenitor cells427. CCL3 also contributes to tumour metastasis and the pro-inflammatory component of viral infection, rheumatoid arthritis, and hepatitis428–431, although it can also suppress the replication of Human Immunodeficiency Virus (HIV)432. CCL3 can also promote hyperalgesia (increased sensitivity to pain) by sensitizing sensory neurons to transient receptor potential cation channel subfamily V member 1 (TRPV-1) mediated noxious stimulation433.

CCL3 is elevated in the BALF of IPF434,435, sarcoidosis434, and mustard-gas induced pulmonary fibrosis patients436. Immunohistochemistry has revealed that CCL3 is predominantly produced by lung macrophages, including AMφ and interstitial macrophages (IMφ), and also by interstitial fibroblasts434. Interestingly, there is an increased CCL3 production by BAL immune cells from IPF and sarcoidosis patients with progressive but not stable disease, indicating that CCL3 may be involved in active fibrosis437. Indeed elevated levels of CCL3 in BAL is also predictive of a poor outcome in IPF438. BALF from IPF or sarcoidosis patients has been found to be chemotactic for monocytes but not neutrophils, but this was reduced when pre-incubated with monoclonal rabbit anti-human CCL3 antibodies434. CCL3 may therefore participate in active fibrosis through the recruitment of monocytes. In a mouse model of BLM-induced pulmonary fibrosis, CCL3 gene and protein expression were elevated from day 1 to at least day 21 post-challenge, and was detected in infiltrating macrophages and granulocytes as determined by immunofluorescent staining at day 7. CCL3−/− mice had reduced collagen accumulation and fewer infiltrating macrophage and fibrocytes, indicating that CCL3 plays a role in the initiation of pulmonary fibrosis and macrophage and fibrocyte recruitment respectively439.

CCL4

CCL4, also known as MIP-1β, is secreted at sites of inflammation by activated leukocytes, lymphocytes, vascular endothelial cells, and pulmonary smooth muscle cells423,440. CCL4 is also secreted from activated monocytes as a heterodimer with CCL3441–443. The first two N-terminal
amino acids can be cleaved from human CCL4 by CD26/Dipeptidyl peptidase-IV (DPPIV)\textsuperscript{442,443}. Both the full length and truncated forms of CCL4 can exert their biological activity through CCR5, and the truncated form additionally interacts with CCR1 and CCR2. CCL4 attracts a number of immune cells, including monocytes, macrophages, NK cells and T lymphocytes to sites of microbial infection as well as pathological inflammation, including allergic asthma and ischemic myocardium\textsuperscript{444,445}. CCL4 is elevated in the BALF of IPF\textsuperscript{435} and mustard-gas induced pulmonary fibrosis patients\textsuperscript{436}. CCL4 may recruit immune cells to the site of fibrosis, however this hypothesis remains to be confirmed.

**CCL5**

CCL5, also known as Regulated upon Activation, Normal T cell Expressed and Secreted (RANTES), plays a primary role in the inflammatory immune response by its recruitment and activation of leukocytes\textsuperscript{446–448}. CCL5 is secreted by a number of cell types at the site of inflammation, including T cells, macrophages\textsuperscript{449} and also AEC\textsuperscript{450}, and exerts its effects through binding to CCR1, CCR3, CCR4 and CCR5\textsuperscript{451,452}. Sequestration of CCL5 by the cytomegalovirus protein US28 can impair the inflammatory response\textsuperscript{453}. In humans, the first two N-terminal amino acids can be cleaved from CCL4 by CD26/ DPPIV, generating a protein that acts as a chemotaxis inhibitor and blocks M-tropic HIV-1 infection of monocytes\textsuperscript{454}. Oligomerisation of CCL5 on glycosaminoglycans is required for CCR1-mediated leukocyte adhesion and activation and also interaction with the chemokine CXCL4/Platelet Factor (PF)\textsuperscript{4455–459}. The deposition of CCL5 on vascular endothelial cells is crucial for monocyte adhesion to the damaged vasculature, but CCL5 oligomerisation is not required for the extravasation of adherent leukocytes\textsuperscript{457–459}. CCL5 is upregulated in breast cancer and promotes tumour progression by recruiting pro-inflammatory TAM, promoting angiogenesis, and increasing the migratory and invasion-related properties of tumour cells\textsuperscript{415}.

CCL5 may play a role in pulmonary fibrosis: CCL5 mRNA and protein levels were elevated in BALF of sarcoidosis\textsuperscript{460,461}, interstitial pneumonia with collagen vascular disease (CVD-IP)\textsuperscript{461} and IPF patients\textsuperscript{461}. In sarcoidosis patients, CCL5 was localised to CD68+ pulmonary macrophages, and there was significant correlation between CCL5 mRNA and protein expression and CD45RO+ memory T-lymphocyte and total lymphocyte numbers, indicating that CCL5 may mediate T-lymphocyte influx in pathology\textsuperscript{460}. In addition, CCL5 is a chemokine for mast cells that may promote pulmonary fibrosis through the release of a number of pro-fibrotic mediators.

In summary, CCL chemokines may promote the initiation and progression of pulmonary fibrosis through the recruitment of various hematopoetic cells that are involved in pathogenesis, such as circulating monocytes, fibrocytes and macrophages. It should be noted however that there is considerable redundancy in chemokine expression, in that multiple chemokine ligands bind to the
same receptor, and most chemokines bind multiple receptors, which may pose a challenge when trying to establish the relative importance in disease pathology and designing potential therapeutic approaches for targeting chemokine signalling pathways. The alternative nomenclature, receptors for, and function of macrophage-derived CC-chemokines that are elevated in pulmonary fibrosis are summarised in table 1.3.

<table>
<thead>
<tr>
<th>CC Chemokines</th>
<th>Other names</th>
<th>Receptor</th>
<th>Function</th>
</tr>
</thead>
<tbody>
<tr>
<td>CCL2</td>
<td>MCP-1</td>
<td>CCR2</td>
<td>Chemoattraction of monocytes, macrophages and fibrocytes.</td>
</tr>
<tr>
<td>CCL3</td>
<td>MIP-1α</td>
<td>CCR1, CCR3, CCR5</td>
<td>Chemoattraction, adhesion to activated vascular endothelium, and cellular activation of many hematopoietic cell types including fibrocytes, monocytes, activated T lymphocytes, NK cells, neutrophils, immature dendritic cells and eosinophils</td>
</tr>
<tr>
<td>CCL4</td>
<td>MIP-1β</td>
<td>CCR1, CCR2, CCR5</td>
<td>Chemoattraction of monocytes, macrophages, NK cells and T-lymphocytes.</td>
</tr>
<tr>
<td>CCL5</td>
<td>RANTES</td>
<td>CCR1, CCR3, CCR4, CCR5</td>
<td>Recruitment and activation of leukocytes.</td>
</tr>
</tbody>
</table>

Table 1.4 Macrophage-derived CC chemokines elevated in pulmonary fibrosis. CCL, CC chemokine Ligand; CCR, CC chemokine Receptor; LARC, Liver and Activation-regulated Chemokine; MCP-1, Monocyte Chemoattractant Protein-1; MIP, Macrophage Inflammatory Protein; NK cells, Natural Killer cells.
CXC chemokines

The CXC chemokines are a family of chemoattractant that have two conserved cysteines separated by one amino acid (X) near their amino terminus. In mammals, CXC chemokines can be separated into two functional subgroups, those with a conserved glutamine-leucine-arginine (ELR) immediately preceding the first cysteine of the CXC motif (ELR⁺) and those without (ELR⁻). ELR⁺ CXC chemokines are chemoattractive to neutrophils, and have an angiogenic role; ELR⁻ CXC chemokines on the other hand are chemoattractive to lymphocytes and have an angiostatic role⁴⁶².

While the role of granulocytes in pulmonary fibrosis is unclear, aberrant angiogenesis has been associated with chronic inflammation and chronic fibroproliferative disease. The metabolic demands of tissue undergoing proliferative, reparative and hyperplastic changes are extremely high and require a proportionally greater capillary blood supply as compared to normal tissue, which is provided by aberrant angiogenesis⁴⁶². The existence of aberrant angiogenesis was originally identified by Turner-Warwick, who examined the lungs of patients with widespread interstitial fibrosis and demonstrated aberrant angiogenesis linking systemic and pulmonary microvasculatures and evidence of vascular remodelling in areas of fibrosis⁴⁶³. Aberrant angiogenesis (neoangiogenesis) is also observed within fibrotic tissue and surrounding the fibrotic foci in BLM-induced pulmonary fibrosis in mice⁴⁶⁴. In IPF patients, there is also an imbalance of ELR⁺ and angiostatic ELR⁻ CXC chemokines, with elevated levels of angiogenic CXCL8 and reduced expression of the angiostatic CXCL10⁴⁶⁵. The ELR⁺ CXC chemokines induce angiogenesis through promoting endothelial cell survival and proliferation. These effects are exerted through the binding of CXC chemokines to their respective CXC receptors: CXCR1 is bound by CXCL6 and CXCL8 only, whereas CXCR2 is bound by all ELR⁺ CXC chemokines⁴⁶⁶. Endothelial cells express both CXCR1 and CXCR2 on their cell surface⁴⁶⁶–⁴⁶⁸. However, ELR⁺ chemokine mediated angiogenesis is through binding and activation of CXCR2 but not CXCR1⁴⁶²,⁴⁶⁶,⁴⁶⁷,⁴⁶⁹. NIH3T3 (a mouse fibroblast cell line) transfected with CXCR2, but not CXCR1, was migratory towards CXCL8⁴⁶⁹. Human microvascular endothelial cells respond to CXCL8 with rapid stress fiber assembly, chemotaxis, enhanced proliferation, and phosphorylation of ERK1/2 related to CXCR2 activation. Blocking the function of CXCR2 by specific neutralizing antibodies or inhibiting downstream signalling using specific inhibitors of ERK1/2 and PI3K impaired CXCL8-induced stress fibre assembly, chemotaxis and endothelial tube formation in endothelial cells⁴⁷⁰. In a rat corneal micropocket assay for detecting angiogenesis, ELR⁺ chemokine mediated angiogenesis was abolished in CXCR2⁻/⁻ mice, and also CXCR2⁺ mice in the presence of CXCR2-neutralizing antibodies⁴⁶⁶. CXC chemokines may play a pro-fibrotic role in BLM-induced pulmonary fibrosis through promoting angiogenesis via CXCR2. Blockade of CXCR2 with a CXCR2 antagonist, DF1262, dosed twice daily from days 0 to 16, reduced collagen deposition following BLM challenge and von Willebrand Factor (vWF),
a marker for angiogenesis levels at day 16\textsuperscript{471}. Several angiogenic ELR\textsuperscript{+} chemokines, including CXCL1, CXCL2 and CXCL8, which are produced by macrophages, which will be discussed in greater detail below. The role of CXCL12, an ELR\textsuperscript{-} CXC chemokine that plays an important role in the recruitment of fibrocytes in pulmonary fibrosis will also be discussed.

**CXCL1**

CXCL1 is expressed in monocytes\textsuperscript{472}, macrophages\textsuperscript{473}, neutrophils, fibroblasts\textsuperscript{474,475}, epithelial\textsuperscript{473} and endothelial cells\textsuperscript{476,477}. CXCL1 exerts its bioactivities through the ligation of CXCR2, and is a potent neutrophil attractant and activator\textsuperscript{478,479}. CXCL1 has important roles in inflammation, and was found to be involved in monocyte arrest on atherosclerotic endothelium\textsuperscript{480,481} and may also pay a pathophysiological role in Alzheimer’s disease\textsuperscript{403}. CXCL1 also promotes angiogenesis\textsuperscript{482}, which have important roles in wound healing\textsuperscript{483}, and tumourigenesis\textsuperscript{482,484}.

CXCL1 was elevated in ada\textsuperscript{−/−} mice\textsuperscript{485} that spontaneously develop pulmonary fibrosis\textsuperscript{405}, and gene expression analysis and immunohistochemistry revealed that CXCL1 was predominantly produced by AM\textsuperscript{φ}. Lung extracts from ada\textsuperscript{−/−} mice showed increased angiogenic activity in corneal micropocket angiogenesis assay as compared to ada\textsuperscript{+/+} controls, but this was abrogated by inhibition of CXCL1 or its receptor CXCR2 with neutralising antibodies\textsuperscript{485}, suggesting that CXCL1 is responsible for angiogenesis in a mouse model of pulmonary fibrosis. Further studies will have to be carried out to study the effects of CXCL1 inhibition on angiogenesis and also pulmonary fibrosis in ada\textsuperscript{−/−} mice \textit{in vivo}.

**CXCL2**

CXCL2 is expressed in monocytes and macrophages. CXCL2 exerts its effects through binding to CXCR2, and is chemotactic for neutrophils\textsuperscript{472,486} and hematopoietic stem cells\textsuperscript{487}, and may also play a role in angiogenesis\textsuperscript{462}.

In a BLM-induced pulmonary fibrosis in mice, CXCL2 was directly correlated with total lung hydroxyproline levels, a measure of collagen deposition\textsuperscript{488}. Depletion of CXCL2 in BLM-challenged mice attenuated pulmonary fibrosis that was entirely attributed to a reduction in angiogenesis in the lung\textsuperscript{488}.

**CXCL8**

CXCL8, also known as IL-8, Granulocyte Chemotactic Protein (GCP) 1 and Neutrophil Activating Peptide (NAP) 1, is produced by macrophages, epithelial cells, airway smooth muscle cells\textsuperscript{489}, endothelial cells\textsuperscript{490,491} and fibroblasts. CXCL8 can associate into a homodimer or a heterodimer with CXCL4/ PF4\textsuperscript{492}, and can also interact with matrix and cell surface glycosaminoglycans\textsuperscript{493}. The
bioactivity of CXCL8 is regulated by truncations by multiple proteases at the N-terminal, by CXCL8 citrullination by Arg 5 (N-terminal to the ELR motif)\textsuperscript{494}, and by the decoy receptor Duffy Antigen Receptor for Chemokines (DARC)\textsuperscript{495}. CXCL8 effects are mediated by CXCR1/ IL-8RA, which is also used by CXCL6, and through CXCR2/ IL-8RB, which is used by multiple ELR+ chemokines\textsuperscript{496}. CXCR1 and CXCR2 associate into functional homodimers and heterodimers with each other\textsuperscript{497}. CXCL8 promotes neutrophil adhesion to the vascular endothelium, and migration to sites of inflammation\textsuperscript{498}, and triggers antimicrobial activation of neutrophils through CXCR1\textsuperscript{499}. CXCL8 also binds to Serpin A1/ α1 Antitrypsin, which prevents CXCL8 interaction with CXCR1\textsuperscript{500}. CXCL8 is upregulated in atherosclerotic lesions and other cardiac pathologies where it exacerbates inflammatory tissue damage\textsuperscript{501}. In addition, it induces VEGF expression\textsuperscript{502}, vascular endothelial cell proliferation\textsuperscript{503}, angiogenesis and tumour cell invasiveness\textsuperscript{504,505}.

**CXCL12**

CXCL12/stromal cell derived factor (SDF)-1/Pre-B cell growth Stimulatory Factor is an ELR- CXC chemokine that acts via its receptor CXCR4/leukocyte-derived seven transmembrane receptor (LESTR)/ fusin\textsuperscript{506} and serves as a chemoattractant for fibrocytes, monocytes, T-lymphocytes, pro and pre B-lymphocytes but not neutrophils\textsuperscript{507}.

In IPF patients, CXCL12 levels are increased in the BAL, lung tissue and plasma. Immunohistochemistry revealed that this chemokine was primarily expressed by hyperplastic AECs lining the fibrotic foci, but were also found in AMϕ and endothelial cells\textsuperscript{128}. Increased CXCL12 was also correlated with poorer lung function in IPF patients. CXCL12 is thought to contribute to IPF through their recruitment of circulating fibrocytes via CXCR4, which may then further differentiate into collagen-secreting fibroblasts\textsuperscript{128}. Indeed immunofluorescence revealed that CXCR4+ procollagen-I+ fibrocytes were found to accumulate in the lungs of IPF patients\textsuperscript{128}. Human peripheral blood CD45+ collagen I+ CXCR4+ fibrocytes undergo chemotaxis in response to CXCL12 \textit{in vitro}. These cells, when injected into the tail vein of BLM-challenge SCID mice at day 4 is found to migrate to the injured lung by day 8, presumably mediated by CXCL12. Mouse CD45+ collagen I+ CXCR4+ fibrocytes were also found to accumulate from day 2, at maximal levels at day 8, to at least day 20 post BLM-challenge in C57BL/6 mice. In addition, daily administration of CXCL12 neutralizing antibody from day 0 blocked CD45+ collagen I+ CXCR4+ fibrocyte recruitment (but not other CXCR4-expressing cells, e.g. monocytes, lymphocytes) and attenuates fibrosis in BLM-challenged mice at day 16. This indicates that CXCL12 mediated recruitment of fibrocytes is crucial for the initiation of BLM-induced pulmonary fibrosis\textsuperscript{508}. The alternative nomenclature, receptors for, and function of macrophage-derived CC-chemokines that are elevated in pulmonary fibrosis are summarised in table 1.4.
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Table 1.5 Macrophage-derived CXC chemokines elevated in pulmonary fibrosis. ELR, Glutamine-Leucine-Arginine; CXCL, CXC chemokine Ligand; CXCR, CXC chemokine Receptor.

<table>
<thead>
<tr>
<th>CXC chemokines</th>
<th>ELR</th>
<th>Receptor</th>
<th>Function</th>
</tr>
</thead>
<tbody>
<tr>
<td>CXCL1</td>
<td>+</td>
<td>CXCR2</td>
<td>Neutrophil recruitment, angiogenesis.</td>
</tr>
<tr>
<td>CXCL2</td>
<td>+</td>
<td>CXCR2</td>
<td>Neutrophil recruitment, angiogenesis.</td>
</tr>
<tr>
<td>CXCL8</td>
<td>+</td>
<td>CXCR1, CXCR2</td>
<td>Neutrophil recruitment, angiogenesis.</td>
</tr>
<tr>
<td>CXCL12</td>
<td>-</td>
<td>CXCR4</td>
<td>Fibrocyte, monocyte, lymphocyte recruitment.</td>
</tr>
</tbody>
</table>

MMPs

MMPs belong to the metzincin superfamily of metalloproteinases, and they play key roles in ECM catabolism, activation and inactivation of cytokines, chemokines, growth factors, and other proteinases at the cell surface and within the ECM. MMPs are involved in many physiological processes including embryonic development, morphogenesis, tissue remodelling, cell growth, migration and apoptosis, and pathological conditions such as inflammation, rheumatoid arthritis, osteoarthritis, cardiovascular diseases, nephritis, chronic wounds, pulmonary diseases, cancer and fibrosis. MMPs are produced and released in their inactive zymogen form, and have to be subsequently cleaved by other proteinases, including other MMPs, into their bioactive state.

MMP-7 null mice are protected from BLM-induced pulmonary fibrosis, suggesting the importance of this protease in initiating pathology. It can promote pulmonary fibrosis through actions on its various bioactive substrates, including TGF-β, osteopontin and MMPs. MMP-7 is able to release latent TGF-β and thereby promote fibroblast growth, survival and collagen synthesis. MMP-7 may also cleave and activate osteopontin to induce fibroblast migration and proliferation. Furthermore, MMP-7 may promote epithelial cell migration by reducing the affinity of α2β1 integrin. In addition, MMP-7 may activate other pro-fibrotic proteases, including itself, pro-MMP1, -2 and -9. MMP-9 is implicated in basement membrane disruption.

Fibronectin

Fibronectin is a large modular glycoprotein that is found as a polymeric fibrillar network in the ECM, as a soluble disulphide-linked dimeric protomers in plasma and other types of body fluid. The protein subunits is made up of three types of homologous structural repeats termed fibronectin type 1, type II and type III repeats. Fibronectin is a ligand for fibrin, heparin, chondroitin sulfate, collagen/gelatin, and many integrin receptors. It is involved in multiple cellular processes including cell adhesion/migration, blood clotting, morphogenesis, tissue repair and cell signalling.

There was increased fibronectin levels detected in the BALF of IPF patients. AMφ isolated from IPF patients also had increased production of fibronectin ex vivo as compared to normal controls. IPF AMφ supernatant was found to recruit human lung fibroblasts ex vivo, but depletion of fibronectin
with gelatin-bound Sepharose beads from AMφ supernatant abolished this observation, suggesting that fibronectin is chemoattractive to fibroblasts\textsuperscript{519}. Indeed fibronectin is a major component of the fibrotic clot, and may also act as a scaffold for fibroblast migration\textsuperscript{520,521}.

**TGF-β**

The TGF-β family consists of three isoforms, including TGF-β1, TGF-β2 and TGF-β3\textsuperscript{522-524}, which are highly pleiotropic cytokines that are secreted by all cells\textsuperscript{525,526}. TGF-β acts as a cellular switch to regulate processes such as immune function, proliferation and EMT\textsuperscript{527,528}. Targeted deletions of these genes in mice show that each TGF-β isoform has some non-redundant functions\textsuperscript{529}. TGF-β1 is involved in homeostasis and endothelial differentiation; TGF-β2 affects the development of cardiac, lung, craniofacial, limb, eye ear and urogenital systems; TGF-β3 influences palatogenesis and pulmonary development\textsuperscript{529}.

TGF-β is initially synthesised as a 390 amino acid inactive zymogen (pre-pro-TGF-β) consisting of an N-terminal signal peptide (29 amino acids), a central pro-region called the latency associated peptide (LAP) (249 amino acids) and a C-terminal consisting of the active form of TGF-β (112 amino acids)\textsuperscript{530,531}. The N-terminal signal peptide is first removed by proteolysis, and the resulting pro-TGF-β then homodimerises through the formation of three disulphide bonds\textsuperscript{532}. The covalent bonds between the LAP and mature TGF-β in the pro-TGF-β homodimer are then cleaved by furin/paired basic amino acid cleaving enzyme (PACE) to form the small latent complex (SLC). In the SLC, the connection between LAP and mature TGF-β are still maintained by non-covalent interactions\textsuperscript{533,534}. LAP acts as a chaperone to TGF-β to maintain its latency through conformational changes and also non-covalent interactions that prevents TGF-β from binding its receptor\textsuperscript{535}. The SLC may interact with latent TGF-β binding protein (LTBP) via disulphide bonds to form the large latent complex (LLC). The LLC can then be secreted into the extracellular space, where they anchor via the LTBP onto ECM components, such as fibronectin. Active TGF-β can be released through the proteolytic cleavage by proteases (e.g. MMP2, MMP9, plasmin), ROS and FGF-2, or by conformational change in the LLC through the binding to integrins\textsuperscript{536-538}. Active TGF-β can then exert its effects through binding the TGF-βR heterotetrameric complex consisting of two TGF-βR1 and TGF-βR2\textsuperscript{528,539,540} either via Smad-dependent\textsuperscript{541-543} or Smad-independent [e.g. MAPK, small Guanosine TriPhosphate (GTP)-binding proteins, protein tyrosine kinase (PTK), NK-κB and Wnt/β-catenin] pathways\textsuperscript{544-553}.

The TGF-β1 isoform are found at elevated levels in the lung tissue of IPF patients around the fibrotic foci\textsuperscript{118,554}, indicating that TGF-β1 may play an important role in pulmonary fibrosis\textsuperscript{555}. Immunohistochemistry has identified AECs and AMφ as the predominant source for TGF-β1 in IPF patients\textsuperscript{118,554}. TGF-β1 is elevated in the BALF and lung tissue of mice with BLM-induced pulmonary fibrosis, and treatment with daily intratracheal administration of a peptide inhibitor to TGF-β from
day 2 of the early inflammatory phase, or from day 10 of the early fibrotic phase, reduced collagen accumulation\textsuperscript{556}. A protective effect towards BLM-induced pulmonary fibrosis was also observed in other rodent models when treated with TGF-\(\beta\) inhibitors, including anti-TGF-\(\beta\) neutralising antibodies, decorin and soluble TGF-\(\beta\)R receptors\textsuperscript{557–560}. On the other hand, adenovector-mediated gene transfer of active TGF-\(\beta\)\textsubscript{1} induced prolonged pulmonary fibrosis in rat lung that is characterised by extensive deposition of ECM proteins, including collagen, fibronectin and elastin, and an accumulation of myofibroblasts\textsuperscript{561}.

TGF-\(\beta\) may play a role in dampening the immune response that may cause tissue damage and loss of homeostasis following tissue injury. However following persistent injury, prolonged TGF-\(\beta\) elevation may contribute to pulmonary fibrosis via several mechanisms, including the promotion of EMT\textsuperscript{131}, fibroblast migration\textsuperscript{561}, proliferation\textsuperscript{561}, differentiation into myofibroblasts\textsuperscript{562}, ECM (procollagen and fibronectin) expression in myofibroblasts\textsuperscript{563}, and AEC apoptosis\textsuperscript{564}.

### 1.2.3.2 The potential role of macrophage subsets in IPF

Macrophages may play a role in tissue injury and fibrosis through the release of a number of mediators, as described in the above section (section 1.2.3.1) in IPF patients. These different roles may be contributed by different macrophage subsets in response to signals from their immediate environment (Figure 1.5). Previous studies have suggested that M1 macrophages may participate in inflammation-induced tissue injury through its release of various ROS, RNS, pro-inflammatory cytokines, chemokines and tissue remodelling proteases. M2 and M2-like macrophages may contribute to the wound healing process, and if dysregulated the progression of pulmonary fibrosis through their release of pro-fibrotic mediators (Figure 1.5). On the other hand, M1, M2 and/or M2-like macrophages may also contribute to tissue repair and the resolution of fibrosis through the release of anti-fibrotic mediators\textsuperscript{278}. However, as forementioned in section 1.2.1, tissue macrophages interact with a number of priming or stimulatory signals, such that the phenotype of macrophage found \textit{in vivo} are unlikely to be strictly associated with a distinct \textit{in vitro} defined subset, but rather exist within the M1-M2 polarisation spectrum. It is therefore more likely than not that pro-inflammatory and pro-fibrotic macrophages observed in pulmonary fibrosis are M1- and M2-biased, rather than strictly adhering to a distinct subset.

Despite the shortfall on describing the complexity of macrophage heterogeneity \textit{in vivo}, the M1-M2 paradigm provides a simple starting point for the assessment of complex macrophage polarisation \textit{in vivo}. Previous studies of macrophage heterogeneity in IPF have based their classification along the M1-M2 paradigm, presumably due to the lack of technology to identify complexities in macrophage phenotypes. These include single-cell analysis to identify macrophage heterogeneity within a patient.
sample, or genomic and proteomic analysis to identify subtle differences between in vivo and in vitro derived macrophage subsets.

In the following section, these previous studies on macrophage heterogeneity will be summarised in the context of the M1-M2 paradigm, with respect to the pro-inflammatory M1 macrophages, and pro-fibrotic M2 and M2-like macrophages as described in section 1.2.1. It should be noted that the macrophage-subsets described below are unlikely to be mutually exclusive and may coexist in the form of hybrid macrophages.

1.2.3.2.1 The potential role of M1 macrophages in IPF

There has been one study indicating the presence of M1 macrophages in pulmonary fibrosis: immunostaining analyses with anti-human or anti-mouse folate-receptor-beta (FR-β) monoclonal antibody revealed that FR-β positive macrophages were present in the fibrotic areas of IPF patients and mice with BLM-induced pulmonary fibrosis respectively. These macrophages are likely to be M1 macrophages, as they have a pro-inflammatory phenotype and produce TNF-α and oxygen radicals. Treatment by ablation of FR-β expressing macrophages by intranasal administration of a recombinant immunotoxin, consisting of Ig heavy and light chain Fv portions of an anti-mouse FR-β

Figure 1.5 The potential contributions of various macrophage subsets to pulmonary fibrosis. M1 macrophages may promote inflammation-induced tissue injury and the initiation of pulmonary fibrosis through the release of various reactive oxygen species (ROS), reactive nitrogen species (RNS), pro-inflammatory cytokines, chemokines and tissue remodelling proteases. M2 and M2-like macrophages may contribute to the wound healing process, but may promote the progression of fibrosis when this process becomes dysregulated when the irritation is persistent. Conversely, M1, M2 and M2-like macrophages also have the potential to resolve fibrosis through their release of anti-fibrotic mediators and phagocytosis of ECM components. Figure adapted from Boorsma CE, 2013.
monoclonal antibody and truncated Pseudomonas exotoxin A, dosed from day 3 every other day, increased survival and reduced hydroxyproline (collagen precursor) and fibrosis in BLM-challenged mice at day 21. Immunohistochemical analysis also revealed decreased numbers of TNF-α, CCL2 and CCL12 producing cells in the immunotoxin-treated group. The authors concluded that FR-β-positive macrophages have a critical role in the initiation of pulmonary fibrosis, possibly through their release of pro-inflammatory mediators. However, the pro-inflammatory role of FR-β positive macrophages in BLM-induced pulmonary fibrosis has to be confirmed by further flow cytometric analysis of pulmonary macrophages co-stained for FR-β and the aforementioned pro-inflammatory mediators. FR-β positive pulmonary macrophages can also be isolated by fluorescence activated cell sorting (FACS) and the levels of their protein products analysed by enzyme linked immunosorbent assay (ELISA) or meso scale discovery (MSD). M1 macrophages may contribute to inflammation induced tissue injury through the production of various pro-inflammatory mediators, including TNF-α, IL-1β and IL-12 p40, ROS and NO, and MMPs (MMP-7 and MMP-9), leading to fibrosis development. TNF-α may contribute to pulmonary fibrosis through inducing promoting fibroblast proliferation, AEC1 apoptosis and/or propagating chronic inflammation. IL-1β may induce the release of other pro-inflammatory mediators (e.g. TNF-α and IL-6) to amplify inflammation, pro-angiogenic mediators (e.g. CXCL1, CXCL2, CXCL8) to promote neovascularisation and also pro-fibrotic mediators (e.g. TGF-β and PDGF) to promote fibrosis. IL-12 p40 may act as a chemoattractant for macrophages as observed in vitro and in vivo. MMP-7 may promote epithelial cell migration by reducing the affinity of α2β1 integrin and activate other pro-fibrotic proteases, including itself, pro-MMP1, -2 and -9. MMP-9 is implicated in basement membrane disruption.

However, other evidence challenges the role of M1 macrophages in established fibrosis. TNF-α is also shown to inhibit collagen synthesis in myofibroblasts, and have a role in aiding epithelial cell recovery and treatment of TNF-α in established fibrosis contributes to resolution of BLM-induced pulmonary fibrosis in mice. The pleiotropic effects of TNF-α observed in pulmonary fibrosis may result from different types of cells present at various stages of pulmonary fibrosis. TNF-α promotes inflammation-induced tissue injury, fibroblast proliferation and subsequent fibrosis in the inflammatory phase; on the other hand, fibroblast are activated into myofibroblasts in the fibrotic phases and their collagen-secreting activities are inhibited by TNF-α in a negative feedback loop.

Depletion of tissue macrophages and/or circulating inflammatory monocytes during the inflammatory phase of in vivo models does not affect the onset or degree of fibrosis developing later on. On the other hand, it is equally possible that pro-inflammatory mediators that are associated with M1
macrophages in the inflammatory phase are also released at a low level by M1/M2 hybrid macrophages in the fibrotic phases.

1.2.3.2.2 The potential role of M2 macrophages in IPF

There is a body of evidence demonstrating that M2 macrophages accumulate in the lungs of IPF patients and also mouse models of pulmonary fibrosis. Markers found on or produced by M2 macrophages have been found to be increased in IPF patients, including Galectin-3 (Gal-3)\(^{568,569}\), CCL18\(^{570}\), Chitinase 3 like 3 (Chi3l3)/ Tyrosine-Lysine-Leucine, 40 kDa (YKL-40)\(^{571}\), CD163\(^{572}\), IGF-I and PDGF\(^{573-575}\). M2 markers are also increased in murine models of pulmonary fibrosis, including Arg-1\(^{576}\), Found In Inflammatory Zone (FIZZ1)/ Resistin-Like Molecule alpha (RELM-α)\(^{577,578}\), and MRC1\(^{337,579}\). There is also an increase in the M2-inducing cytokine IL-13 in IPF patients compared to controls, and AM\(\Phi\) isolated from IPF patients produce more IL-13 than that from control lungs\(^{580}\). IL-13 may therefore participate in promoting the M2 macrophage phenotype in IPF patients. M2 macrophages may play a pro-fibrotic role through the release of their various associated mediators. For example, Arg-1 promotes the conversion of arginine into orthinine\(^{581}\), the precursor of proline that is required for collagen synthesis could support fibrosis\(^{582}\). FIZZ1 induces the differentiation and survival of myofibroblasts\(^{583}\), as well as increase ECM production in fibroblasts that would favour the development of fibrosis\(^{577}\). IGF-I and PDGF drive the proliferation of fibroblasts and their transformation to ECM-producing myofibroblasts\(^{120}\). M2 macrophages are able to secrete the ECM component fibronectin\(^{119}\); Fibronectin is a major component of the fibrotic clot, and may also act as a scaffold for fibroblast migration\(^{520,521}\).

Alternatively other studies have suggested that M2 macrophages have an anti-fibrotic role. For example, mannose receptors and the glycoprotein mfges8 are responsible for the uptake of ECM components, and both can attenuate fibrosis in different mouse models\(^{358,584}\). Mannose receptors are associated with M2 macrophages, which may suggest that this subset may play a role in the resolution of fibrosis. These mechanisms for the clearance of ECM components may serve as a negative feedback mechanism for M2 macrophages to prevent excessive ECM accumulation. In the presence of a persistent irritant however, it is possible that the pro-wound healing activities of M2 macrophages may become dysregulated in a frustrated effort to repair damaged tissue, which is able to overcome negative feedback activities and lead to excessive ECM accumulation and fibrosis.

1.2.3.2.3 The potential role of M2-like macrophages in IPF

M2-like macrophages share many M2-associated proteins (e.g. Arg-1, FIZZ1, MRC1), but are distinguished by their high levels of IL-10 production. M2-like macrophages contribute to immunoregulation, matrix deposition, and tissue remodelling, and may be important during the
transition from inflammation towards wound healing following lung injury. Elevated levels of IL-10 have been observed in several fibrotic diseases, including IPF\textsuperscript{125,585,586} and systemic sclerosis patients with interstitial lung disease\textsuperscript{572}. The role of IL-10 in pulmonary fibrosis remains elusive. Some studies suggest that it has a protective role: IL-10 is an anti-inflammatory cytokine, and can suppress T-cell activation\textsuperscript{587}, inhibit T cell activity\textsuperscript{320}, and also suppress TNF-α and ROS production in macrophages following LPS stimulation\textsuperscript{588}. IL-10 is essential for the regulation of acute and chronic inflammation: in IL-10-deficient mice infected with rapidly replicating pathogens, such as \textit{Trypanosoma cruzi} and \textit{Toxoplasma gondii}, the mice typically die rapidly but not from excessive growth of the pathogens, but of a massive inflammatory response\textsuperscript{589,590}. In the context of pulmonary fibrosis, IL-10 may prevent further tissue injury and fibrosis\textsuperscript{591}. However, this hypothesis is challenged by another study, where IL-10 knockout mice showed increased BLM-induced inflammation but not fibrosis\textsuperscript{592}. On the other hand, prophylactic and therapeutic gene delivery of IL-10 attenuated BLM-induced pulmonary fibrosis in mice through inhibiting the production of TGF-β in the lung, suggesting an anti-fibrotic role for IL-10\textsuperscript{593}. IL-10 can have a pro-fibrotic role: long term lung-specific overexpression of IL-10 in mice induced pulmonary fibrosis through fibrocyte recruitment and M2-like macrophage activation through a CCR2/CCL2 axis\textsuperscript{594}. M2-like macrophages may also be producers of the pro-fibrotic cytokine TGF-β, which may promote fibrocyte recruitment, and fibroblast activation/differentiation into the ECM-producing myofibroblasts\textsuperscript{554}.

In conclusion, there is evidence for both the contribution and inhibition by different macrophage subsets, including M1-, M2-, or M2-like macrophages, of pulmonary fibrosis. These pleiotropic effects of these macrophage subsets to promote or inhibit pulmonary fibrosis are more likely than not to represent the complexity in macrophage activation \textit{in vivo}, with respect to the plasticity of macrophages to respond to multiple signals to give rise to hybrid macrophages, and the adaption of macrophages phenotype to the changing environment during disease progresson in IPF (i.e. temporal heterogeneity of IPF).

\subsection*{1.2.4 The role of PRRs in IPF}

Macrophages express a variety of PRRs to detect endogenous alarmins released by apoptotic or necrotic cells, or exogenous pathogens entering the lung. Several PRRs that can be found on macrophages are increased in BAL cells (including macrophages, T and B lymphocytes) from IPF patients, including TLR2\textsuperscript{595}, TLR3\textsuperscript{596}, TLR7, TLR9\textsuperscript{595,596} and NLRP3 inflammasome\textsuperscript{230,597}. TLR2 is upregulated in immune cells (macrophages, lymphocytes) and AECs in BLM-induced pulmonary fibrosis in mice\textsuperscript{598}. NLRP3 has also been implicated in various mouse models of pulmonary fibrosis\textsuperscript{230,597}.
1.2.4.1 TLRs

TLRs are type I transmembrane glycoproteins which are structurally characterized by extracellular leucine-rich repeats (LRRs) and Toll/IL-1 receptor (TIR) signalling domains and includes 10 members in humans and 12 members in mice. TLRs can be activated by highly conserved pathogen associated molecular patterns (PAMPs) of bacteria, viruses, fungi, and parasites, and also by danger associated molecular patterns (DAMPs) of endogenous ligands or alarmins, such as chromatin-IgG complexes, hyaluronan fragments (HA) or High Mobility Group Box 1 (HMGB1). TLR ligation following the binding of their respective ligands is signalled through a number of TIR-domain containing adaptors, such as Myeloid Differentiation primary response 88 (MyD88), TIR domain containing Adaptor Protein (TIRAP), TIR-domain containing adaptor-inducing interferon-β (TRIF), and TRIF Related Adaptor Molecule (TRAM), and can be categorised into MYD88-dependent and MYD88-independent pathways. All TLRs, with the exception of TLR3, signal through the MYD88-dependent pathway, whereas TLR3 and TLR4 signal through the MYD88-independent, TRIF-dependent pathway. TLR1, TLR2, TLR4, and TLR6 recruit TIRAP, which serves as an adaptor between the TIR domain of TLRs and MyD88, while TLR5, TLR7, TLR9, TLR10, TLR11 and TLR12 can recruit MyD88 directly. TLR3 recruits TRIF directly, but TLR4 recruits TRIF indirectly through TRAM. The recruitment of these adaptor proteins trigger a signal cascade of signalling molecules that ultimately activate transcription factors, including NF-κB and IRFs. These transcription factors induce the expression of various inflammatory cytokines, type I IFNs, and chemokines. NF-κB is a central regulator of immune responses involved in cell proliferation and survival and induces the expression of many cytokine and chemokine genes including IL-2, IL-6, IL-12, MCP-1, and TNF-α.

In the MYD88-dependent pathway, MYD88 recruits the Interleukin-1 Receptor Associated Kinase (IRAK) family of protein kinases that phosphorylates and activates the E3 ubiquitin protein ligase TNF Receptor Associated Factor 6 (TRAF6). TRAF6 induces the activation of TGF-β Activated Kinase 1 (TAK1) through K63-linked polyubiquitination. NF-κB, Activator Protein (AP)-1 and cyclic Adenosine Monophosphate (cAMP) Response Element Binding protein (CREB) is then activated by the IkB Kinase (IKK) complex and MAPK, respectively.

In the TRIF-dependent pathway induced by TLR3 ligation, the N-terminal domain of TRIF interacts with TRAF6 while the C-terminal domain of TRIF interacts with Receptor Interacting Protein (RIP)-1 and activates TAK1, both of which can activate NF-κB, resulting in the expression of inflammatory cytokines. TLR3 ligation also induces the expression of type I interferons via IRF3. TRIF recruits TRAF3 and the IKK-related kinase TRAF family member-associated NFκB activator (TANK) Binding Kinase 1 (TBK1), which then phosphorylates and activates the transcription factor IRF3.
TLR7 and TLR9 engagement induces the secretion of inflammatory cytokines through the activation of NF-κB via MyD88. However, TLR7 and TLR9 can also induce the expression of type I IFNs through the activation of IRF7, which is phosphorylated by IRAK1, IRAK4, or IKKα, and then translocated to the nucleus where it induces the transcription of IFN-α.\textsuperscript{616}
Binding Kinase 1 (TBK1), which then phosphorylates and activates the transcription factor IRF3 to induce the expression of type I IFNs.

Figure 1.6 Toll Like Receptor (TLR) 2, TLR3, TLR4, TLR7, TLR8 and TLR9 signalling pathways. TLR2 binds to di- or tri-acetylated lipoproteins when complexed with TLR1 or TLR6 respectively. TLR2 heterodimers are also able to bind alarmins, including High Mobility Group Box protein 1 (HMGB1) and low molecular weight (LMW) Hyaluronan (HA) fragments. TLR3 binds to double stranded Ribonucleic Acid (dsRNA) expressed by viruses. TLR4 binds to Lipopolysaccharide (LPS), and also to HMGB1 and LMW HA fragments. TLR7 and 8 bind to single stranded RNA (ssRNA) and TLR9 bind to CpG-oligodinucleotide (CpG-ODN) from viruses. TLR9 also binds to alarmins such as histones. The alarmin HMGB1 is also able to act as a molecular chaperone to TLR2, TLR4 and TLR9 ligands, thereby enhancing their responses. TLR2, 4, 7, 8 and 9 are able to signal through the Myeloid Differentiation 88 (MYD88) pathway. Activation of the MYD88 pathway results in the increased gene expression of pro-inflammatory genes. TLR2 recruits MYD88 indirectly through TIR domain containing Adaptor Protein (TIRAP), whereas TLR7, 8 and 9 are able to recruit MYD88 directly. MYD88 is then able to recruit the interleukin-1 receptor associated kinase (IRAK) family of protein kinases, including IRAK1, IRAK2, and IRAK3 that recruits and activates the E3 ubiquitin ligase Tumour Necrosis Factor Receptor Associated Factor Associated Factor 6 (TRAF6). TRAF6 modulates the activation of TGF-β Activated Kinase 1 (TAK1) through K63-linked polyubiquitination. TAK1 can then initiate a phosphorylation cascade via the IκB Kinase (IKK) complex to activate NFXb, or via the Mitogen Activated Protein Kinase (MAPK) pathway to activate cyclic Adenosine Monophosphate Response Element Binding protein (CREB) or Adaptor Protein 1 (AP1). The phosphorylation and activation of MAPK kinase (MKK) 3 or 6 leads to the activation of CREB via p38, whereas that of MKK 4 or 7 leads to the activation of AP1 via c-Jun N-terminal Kinase (JNK). TLR7 and TLR9 can also induce the expression of type I IFNs through the activation of Interferon Regulatory Factor (IRF)7, which is phosphorylated by IRAK1, IRAK4, or IKKa, and then translocated to the nucleus where it induces the transcription of IFN-α. TLR3 and endocytosed TLR4 are also able to signal through a MYD88-independent pathway via the adaptor protein TIR-domain containing adaptor inducing IFN-β (TRIF) instead. TLR3 recruits TRIF directly, but TLR4 recruits TRIF indirectly through TRAM. TRIF recruits Receptor Interacting Protein-1 (RIP-1), which in turn activates TAK1, thereby integrating with the MYD88-dependent pathway to activate pro-inflammatory genes. TRIF also recruits TRAF3 and the IKK-related kinase TRAF family member-associated NFXb activator (TANK) Binding Kinase 1 (TBK1), which then phosphorylates and activates the transcription factor IRF3 to induce the expression of type I IFNs.
TLR2

TLR2 is an extracellular membrane protein involved in the recognition of a wide range of PAMPs originating from bacteria, parasites and viruses\(^{617}\). These include lipoproteins from bacteria, peptidoglycan and lipoteichoic acid from Gram-positive bacteria, lipoarabinomannan from mycobacteria, zymosan from fungi, Glycosylphosphatidylinositol-anchored mucin-like glycoprotein (tGPI-mucin) from \textit{Trypanosoma cruzi} and the haemagglutinin protein from measles viruses. TLR2 usually forms heterodimers with TLR1 or TLR6. TLR1-TLR6 heterodimers recognise triacylated lipopeptides from Gram-negative bacteria and mycoplasma, whereas TLR2-TLR6 heterodimers recognise diacylated lipopeptides from Gram-positive bacteria and mycoplasma\(^{618}\). TLR2 is thought to induce mainly the production of inflammatory cytokines but not type I IFN by macrophages and DCs. On the other hand, it can trigger type I IFN production by inflammatory monocytes in response to infection with vaccinia virus\(^{619}\), which suggests a cell specific role in TLR2 in antiviral responses. This process requires the internalization of TLR2, as blockade of endocytosis with the actin-depolymerizing drug cytochalasin D or blockade of endosomal maturation with chloroquine abrogates IFN-\(\beta\) production. However, nucleic acids that usually trigger the production of type I IFN do not participate in TLR2 activation\(^{619}\).

TLR2 is involved in the pathogenesis in a mouse model of BLM-induced pulmonary fibrosis. TLR2\(^{-/-}\) mice were protected from BLM-induced inflammation and subsequent pulmonary fibrosis. This was confirmed by prophylactic administration of an anti-TLR2 neutralizing antibody in BLM-challenged WT mice, suggesting a role for TLR2 in the inflammatory response that precedes onset of fibrosis\(^{620}\). Therapeutic treatment of BLM-challenged WT mice at the late inflammatory (day 7) or early fibrotic phases attenuates pulmonary fibrosis, suggesting a role for TLR2 beyond inflammation and in active fibrosis\(^{620}\). TLR2 is also elevated in immune cells, including AM\(\phi\), granulocytes, lymphocytes, and AECs in BLM-induced pulmonary fibrosis\(^{598}\).

TLR2 is able to recognize various alarmins, including low molecular weight (LMW) HA fragments and HMGB1. High molecular weight (HMW) (>500 kDa) HA is a nonsulfated glycosaminoglycan composed of repeating polymeric disaccharides D-glucuronic acid and \(N\)-acetyl-D-glucosamine linked by a glucuronidic \(\beta\) (1-3) bond\(^{621,622}\). HA is a major ECM component that is conserved from simple organisms such as bacteria\(^{623,624}\) to complex eukaryotes\(^{625}\). HA has multiple functions, such as space filling hydration, lubrication of joints, and provision of a matrix through which cells can migrate\(^{626}\). HA is actively produced during tissue injury, tissue repair, and wound healing\(^{623,627}\). During tissue injury, HA may be broken down into LMW fragments (10-500 kDa) that may function as alarmins to elicit an immune response by promoting angiogenesis\(^{602,603}\) and inflammation\(^{604,605,628}\).
For example, LMW HA induces pro-inflammatory chemokine production, including CCL2, CCL3 and CCL4 in MH-S cells and also CCL2 in renal tubular epithelial cells, whereas LMW HA fragments accumulates during tissue injury in IPF patients and in the BLM-induced pulmonary fibrosis, and may contribute to chronic inflammation in IPF.

HMGB1 is an abundant and highly conserved non-histone chromosomal protein that is present in almost all eukaryotic cells that acts as a transcription factor-like protein to regulate the expression of several proteins, such as the GR. HMGB1 binds without sequence specificity to the minor groove of DNA, causing local distortions in the DNA structure. In addition, HMGB1 associates with high affinity to DNA with highly bent structures, such as four-way junctions and cisplatin-modified DNA. HMGB1 is thought to be associated with active chromatin, and have crucial roles in nuclear events including transcription, DNA replication and recombination, either by remodelling chromatin and nucleosome structure, or through direct interactions with transcription factors, including Homeobox (Hox), steroid hormone receptors, p53-p73 transcriptional complexes, NFκB and TATA-Binding Protein (TBP), and recombination factors such as V(D)J recombinase.

Upon tissue injury or infection, extracellular HMGB1 acts as a delayed mediator of inflammation. HMGB1 release occurs considerably later (12-18 hours) than secretion of the classical pro-inflammatory mediators TNF (2 hours) and IL-1 (4-6 hours) following LPS administration to generate endotoxemia in mice. Administration of HMGB1-specific antibodies protects against the lethal effects from LPS, even when administration is delayed after the peak levels of TNF and IL-1.

HMGB1 is passively released by necrotic cells and actively released by activated macrophages, neutrophils and mature DCs in response to the presence of elevated level of pro-inflammatory mediator and oxidative stress. Extracellular HMGB1 can activate immune cells, including macrophages, through the ligation of TLR2, TLR4 and Receptor for Advanced Glycation End-products (RAGE). HMGB1 can also act as a molecular chaperone to the ligands of TLRs 2, 4 and 9 and augment the stimulatory responses of their respective TLRs by promoting ligation of these receptors. HMGB1 may bind and accumulate multiple TLR ligands, thereby simultaneously activating multiple TLRs and amplifying the pro-inflammatory response. Alternatively, the presentation of TLR ligands on the surface of HMGB1 may also make them more accessible to receptors on target cells and thereby promote binding.

In IPF, HMGB1 is upregulated in BALF. During acute exacerbation, macrophages and injured epithelial cells also have an increased expression of HMGB1. HMGB1 contributes to BLM-induced pulmonary fibrosis, and treatment by intraperitoneal injection of an anti-HMGB1 neutralising antibody at day 5 attenuated inflammation and fibrosis at day 21.
TLR7 and TLR9

TLR7 and TLR9 are intracellular nucleotide-activated PRRs important for detecting viral infection. TLR7 is located in the cytoplasm and recognizes single stranded Ribonucleic Acid (ssRNA) derived from RNA viruses such as vesicular stomatitis virus, influenza A virus and human immunodeficiency virus\textsuperscript{617,655}. TLR7 also recognizes synthetic poly (U) RNA and certain siRNA\textsuperscript{656}. TLR9 are found on the endosomal membrane recognizes unmethylated 2ʹ-deoxyribo (cytidine-phosphate guanosine) (CpG) DNA motifs that are frequently present in bacteria and viruses but rare in mammalian cells. Synthetic CpG oligonucleotides (ODN) function as TLR9 ligands and drive strong T_{H}1 responses\textsuperscript{617}. Recently it has been reported that TLR9 can recognize endogenous histones released by necrotic cells, which act as alarmins during sterile inflammation in liver injury\textsuperscript{657}. In addition, TLR9 can interact with HMGB1, and thereby promote TLR9-mediated pro-inflammatory responses by acting as a molecular chaperone to its ligand CpG-ODN\textsuperscript{611}. Stimulation of TLR7 and/or TLR9 results in the release of pro-inflammatory cytokines, and also of type I IFNs (IFN-\alpha and IFN-\beta), both of which are important in the anti-viral response\textsuperscript{618}.

Chronic viral infections, mainly herpes virus infections, may contribute to the pathogenesis of IPF\textsuperscript{38,40–43}. There is correlation between certain latent viral infections, such as EBV, CMV, HHV-7 and HHV-8 and human IPF\textsuperscript{25,40–42,44–47} (section 1.1.4.1). Such viruses can infect and remain latent in pulmonary epithelial cells\textsuperscript{48}, thereby rendering them less able to repair subsequent injury. Infection may also potentially change the transcriptional programme of these cells such that they secrete profibrotic factors and recruit inflammatory cells and fibrocytes (bone marrow derived cells that express both hematopoietic and stromal markers) to the lung. TLR7 and TLR9 may therefore promote chronic inflammation and induce tissue injury in pulmonary fibrosis through their anti-viral responses.

TLR9 appears to play a pathogenic role in IPF. Rapidly progressing IPF patients have elevated TLR9 expression compared to slowly progressing IPF patients and normal controls. In a xenograft mouse model of IPF, fibroblasts from rapid progressors induced more severe fibrosis than slow progressors in response to TLR9 activation. CpG-ODN also induced EMT in a human epithelial cell line\textsuperscript{658}.

However, in another study, TLR9 stimulation was protective of pulmonary fibrosis in mice. TLR9\textsuperscript{77} BALB/c mice had increased collagen deposition in gamma herpes virus-induced exacerbation of BLM-induced pulmonary fibrosis, and CpG-ODN abrogated BLM-induced pulmonary fibrosis in C57BL/6 mice. This may occur through the induction of IFN-\beta production by fibroblasts\textsuperscript{659,660}, which inhibit their proliferation\textsuperscript{661}.
1.2.4.2 NLRP3 inflammasome

NLRP3 is a cytosolic PRR consisting of 3 domains, including a LRR responsible for ligand binding at its C terminus, a central oligomerization domain (domain present in Neuronal Apoptosis Inhibitor Protein (NAIP), MHCII Transcriptor Activator (CIITA), HET-E (incompatibility locus protein from Posospora anserine) and Telomerase associated Protein 1 (TP1)) (NACHT domain), and an effector domain at the N-terminus. In humans, common effector proteins include the Pyrin Domain (PYD), Caspase Recruitment Domain (CARD) and Baculovirius Inhibitor of apoptosis protein Repeat (BIR).

Under healthy cellular conditions, NLRP3 is auto-repressed by internal interactions between the NACHT domain and LRRs. In the presence of exogenous pathogens or endogenous alarmins binding to the LRRs, this auto-repression is removed and the NACHT domain is exposed. This allows NLRP3 monomers to oligomerise through their NACHT domains, and recruit the adaptor molecule, Apoptosis-associated Speck-like protein containing a CARD (ASC) through PYD-PYD interactions. ASC in turn recruits pro-caspase 1 through CARD-CARD interactions. Pro-caspase 1 clustering on oligomerised NLRP3 results in caspase 1 auto-activation and caspase 1-dependent processing of cytoplasmic targets, including the zymogens of several pro-inflammatory cytokines, including pro-IL-1β and pro-IL-18.

NLRP3 inflammasomes may contribute to BLM-induced lung injury and fibrosis. As mentioned above, ASC is the adaptor molecule for the recruitment of the NLRP3 inflammasome substrate pro-caspase 1 and knockout of ASC in ASC−/− mice may effect NLRP3 inflammasome activation of pro-caspase 1, and subsequent downstream activation of pro-IL-1β and pro-IL-18. ASC−/− mice have reduced IL-1β and IL-6 levels in BALF in the early inflammatory phase (day 1) compared to wild type controls when challenged with BLM. There was a reduction in lymphocyte accumulation, collagen deposition and matrix turnover proteases (pro-MMP-2, pro-MMP-9, MMP-9, TIMP-1) in the lung of NLRP3−/− mice at the early fibrotic phase (day 14) following BLM challenge compared to wild type controls. The NLRP3 inflammasome contribute to silicosis and asbestosis, and NLRP3−/− mice have decreased inflammation and fibrosis as compared to wild type controls. The NLRP3 inflammasome may promote fibrosis through the uptake of uric acid crystals released from BLM-induced DNA damage. This PRR may also be activated by ROS generated by macrophages and neutrophils and extracellular Adenosine Tri-Phosphate (ATP) and drive IL-1β activation through a caspase-1 dependent mechanism. The activation of the pro-inflammatory IL-1β may contribute to tissue injury through the induction of the release of other pro-inflammatory cytokines such as TNF-α and IL-6. IL-1β may also promote neovascularization through the release of –CXC- chemokines, including CXCL1, CXCL2 and CXCL8. Moreover, IL-1β may promote fibrosis through the release of pro-fibrotic mediators such as TGF-β and PDGF.
Figure 1.7 The Nucleotide-binding Oligomerization Domain (NOD)-Like Receptors (NLR) family, Pyrin domain containing 3 (NLRP3) inflammasome pathway. Under healthy cellular conditions, NLRP3 is auto-repressed by internal interactions between the domain present in Neuronal Apoptosis Inhibitor Protein (NAIP), MHCII Transcriptor Activator (CIITA), HET-E (incompatibility locus protein from Posaspora anserine) and Telomerase associated Protein 1 (TP1) (NACHT domain) and Leucine Rich Regions (LRRs). In the presence of exogenous pathogens, such as bacteria or viruses, or endogenous alarmins, such as reactive oxygen species (ROS), Adenosine TriPhosphate (ATP) and uric acid, binding to the LRRs, this auto-repression is removed and the NACHT domain is exposed. This allows NLRP3 monomers to oligomerise through their NACHT domains, and recruit the adaptor molecule, Apoptosis-associated Speck-like protein containing a Caspase Recruitment Domain (CARD) (ASC) through PYD-PYD interactions. ASC in turn recruits pro-caspase 1 through CARD-CARD interactions. Pro-caspase 1 clustering on oligomerised NLRP3 results in caspase 1 auto-activation and caspase 1-dependent processing of cytoplasmic targets, including the zymogens of several pro-inflammatory cytokines, including pro-IL-1β and pro-IL-18.
1.3 BLM-induced pulmonary fibrosis in mice

BLM is the most frequently applied method and therefore the best characterised experimentally-induced pulmonary fibrosis\textsuperscript{670,671}. Originally used as a chemotherapeutic agent, BLM was discovered to cause pulmonary fibrosis in some cancer patients as a side effect. The incidence of interstitial pulmonary fibrosis is directly linked to the dose of BLM administered and the length of treatment. This indicates that there is a multiple injury threshold effect directly linked to BLM, and pulmonary fibrosis is not caused by cancer per se. The lung is thought to be particularly susceptible to BLM due to the lack of the BLM inactivating enzyme, BLM hydrolase, that converts BLM into the less active deamidoBLM (dBLM), which has only 1\% efficiency as the parent compound in producing oxygen radicals, DNA strand scissions, tumour cytotoxicity or pulmonary fibrosis in animal models\textsuperscript{672}. Indeed, lung extracts from C57BL/6 mice was revealed to be less efficient than that from BALB/c mice in the catabolism of BLM into dBLM, signifying a lower BLM hydrolase activity in the lungs of these mice\textsuperscript{673}. Since then BLM-induced pulmonary fibrosis has been used in multiple animal models, including mice, rats, guinea pigs, hamsters, rabbits, dogs and primates; mice however remains the most common\textsuperscript{670,671}. Several delivery routes are employed, including intratracheal, intraperitoneal, subcutaneous, intravenous, and inhalational\textsuperscript{310,670}. Regardless of the delivery route, BLM induces cell injury through the induction of DNA breaks, the generation of free radicals and the induction of oxidative stress\textsuperscript{671}. This is followed by cell necrosis and apoptosis, with subsequent inflammation and development of fibrosis\textsuperscript{670}.

When delivered systematically (intravenously, intraperitoneally or subcutaneously) the initial site of injury is the pulmonary vascular endothelium, which is thought to reflect similar occurring in humans affected by BLM-induced pneumonitis\textsuperscript{674}. With this delivery route BLM first causes endothelial cell damage, before gaining access to the alveolar epithelium to induce damage. In contrast, with lung-specific delivery, BLM directly induces damage to AECs\textsuperscript{670,671,675}. The delivery of BLM directly to the airways can take place via several administration methods, including oropharyngeal route, intratracheal injections with or without surgical neck cutdown\textsuperscript{422,675}, injections of dry powder\textsuperscript{676}, or endotracheal intubation\textsuperscript{677}.

BLM can induce fibrosis in a relatively short time period, from 2-4 weeks in an intratracheal model to 4-12 weeks in a systemic delivery model. The histopathology is not fully consistent with UIP (e.g. lack of fibrotic foci hyperplastic epithelium, and temporal heterogeneity), and pulmonary fibrosis arising from the single-dose BLM model has widely been reported to resolve spontaneously over time, although there has been conflicting reports\textsuperscript{420,675,678–681}. The response to BLM-induced pulmonary fibrosis is strain-dependent, with C57BL/6 being more susceptible than BALB/c mice, possibly due to their difference in expression of BLM hydrolase. BLM can be delivered as a single-dose or multiple-
doses. Multiple dosing is thought to mimic the repetitive or chronic injury that induces pulmonary fibrosis and provide a more representative model than single dosing. In an endotracheal intubation model, single dosing with 0.04 U BLM increased collagen accumulation indicating fibrosis from the second week following dosing. Similar to that observed in a single dose intratracheal model, UIP was not observed and there was a lack of AEC2 hyperplasia, fibrotic foci, and also the presence of neutrophilic inflammation. However, in multiple dosing (0.04 U BLM every other week for eight doses), analysed two weeks after the last dose, neutrophilic inflammation is reduced as compared to a single dose. AEC hyperplasia is also prominent at the sites of fibrosis, and fibrosis and AEC hyperplasia remains conspicuous by 20-30 weeks after the final dose of BLM. Histopathological changes are also more similar to UIP that is observed in human IPF, and features including AEC2 hyperplasia, formation of fibrotic foci and reduced neutrophilic inflammation were present. Active fibrotic remodelling also persisted 10 weeks after the last BLM dose.

There has been much debate over the translatable nature of the BLM model to clinical IPF due to several reasons. In the single-dose models of BLM-induced pulmonary fibrosis, fibrosis does not fully recreate UIP, in that there is a lack of AEC2 hyperplasia, formation of fibrotic foci and sustained neutrophilic inflammation. Fibrosis is also self-limiting after 28 days following the last dose, and may spontaneously resolve after 5-6 weeks. Moreover, the mechanism and kinetics of BLM-induced pulmonary fibrosis appears to differ from IPF. BLM-induced pulmonary fibrosis is dependent on tissue injury from an initial inflammatory phase, and pathogenesis represents acute lung injury (ALI), whereas in IPF only low levels of inflammation is found. On the other hand, IPF may be the result of pathological inflammation caused by the presence of a persistent irritant that have since subsided (section 1.1.4.2). There is also a rapid (2-3 weeks) onset of pulmonary fibrosis in mice as compared to a chronic (decades) onset in humans. Finally, there is a strain-dependent difference in response to BLM-induced fibrosis, primarily due to difference in expression of the inactivating enzyme BLM hydroxylase across strains. To date there has been only one study investigating the potential variation in pulmonary toxicity of BLM chemotherapy on germ cell cancer patients with BLM hydroxylase genetic variation, the deletion of a 1450A→G polymorphic site that should decrease BLM hydrolase activity. However, results did not show a difference in pulmonary toxicity between patients with the different genotypes. It is possible that this particular polymorphism has only subtle effects that is masked by normal renal clearance of BLM. Further studies looking into the variation of susceptibility to BLM-induced pulmonary toxicity in other forms of BLM hydrolase polymorphisms will have to be carried out.

Despite these differences in the histopathology and initiation of pulmonary fibrosis, it should be highlighted that a recent study using gene set enrichment analyses (GSEA) have revealed that murine
BLM-induced pulmonary fibrosis at the progressive and established fibrotic phases (days 14 and 21) shared a number of commonly altered signatures with IPF patients, including cell-cycle related genes [e.g. cell cycle division proteins (CDCs), cyclin-dependent kinases (CDKs)], fibrosis-related pathways gene sets (PDGF-, integrin, and syndecan) and other pro-fibrotic mediators (MMPs, collagen, and TGF-β)\(^\text{686}\). These results suggest that for the purpose of investigating the mechanism of pathogenesis for IPF, the murine BLM-model is somewhat translatable.

In addition, BLM-induced pulmonary fibrosis has the advantage over other models of pulmonary fibrosis in that BLM produces relatively uniform pathology and experiments are repeatable, BLM can be delivered via the nasal cavity through a number of different routes (intranasal, intraperitoneal, oropharyngeal) such that it is more clinically relevant, and pulmonary fibrosis develops quickly (2-4 weeks). Indeed, BLM-induced pulmonary fibrosis has been widely used and accepted in literature to identify and study the involvement of a large number of mediators in IPF and is the best characterised model for pulmonary fibrosis\(^\text{670,683,687}\).
<table>
<thead>
<tr>
<th></th>
<th>IPF</th>
<th>Mouse (single i.t. dose)</th>
<th>Mouse (multiple endotracheal dose)</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Cause</strong></td>
<td>Unknown (Hypothesized to be caused by persistent irritation to alveolar epithelium)</td>
<td>BLM injury to alveolar epithelium</td>
<td>BLM injury to alveolar epithelium</td>
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<tr>
<td><strong>Onset of pulmonary fibrosis</strong></td>
<td>Decades$^{683,684}$</td>
<td>2-4 weeks$^{683,684}$</td>
<td>2-4 weeks$^{677}$</td>
</tr>
<tr>
<td><strong>ALI</strong></td>
<td>No$^{683,684}$</td>
<td>Yes$^{683,684}$</td>
<td>No$^{677}$</td>
</tr>
<tr>
<td><strong>Self-limiting fibrosis</strong></td>
<td>No$^{683,684}$</td>
<td>Yes (after 28 days)$^{683,684}$</td>
<td>No$^{677}$</td>
</tr>
<tr>
<td><strong>Resolution</strong></td>
<td>No$^{683,684}$</td>
<td>Yes (after 5-6 weeks)$^{683,684}$</td>
<td>No$^{677}$</td>
</tr>
<tr>
<td><strong>Survival</strong></td>
<td>No (3-5 years)</td>
<td>Yes (Beyond 6 weeks)$^{683,684}$</td>
<td>Yes (Beyond 6 weeks)$^{677}$</td>
</tr>
<tr>
<td><strong>Lung Function</strong></td>
<td>Decline$^{688}$</td>
<td>Decline$^{689}$</td>
<td>?</td>
</tr>
<tr>
<td>$D_lC0$</td>
<td>$\downarrow$, $^{688}$</td>
<td>$\downarrow$, $^{689}$</td>
<td>?</td>
</tr>
<tr>
<td>$FVC$</td>
<td>$\downarrow$, $^{688}$</td>
<td>$\downarrow$, $^{689}$</td>
<td>?</td>
</tr>
<tr>
<td>Airway resistance</td>
<td>$\uparrow$, $^{688}$</td>
<td>$\uparrow$, $^{689}$</td>
<td>?</td>
</tr>
<tr>
<td>Tissue elastance</td>
<td>$\downarrow$, $^{688}$</td>
<td>$\downarrow$, $^{689}$</td>
<td>?</td>
</tr>
<tr>
<td>Sustained neutrophilic inflammation</td>
<td>No$^{683,684}$</td>
<td>Yes$^{683,684}$</td>
<td>No$^{677}$</td>
</tr>
<tr>
<td>Histopathology (UIP)</td>
<td>Yes$^{683,684}$</td>
<td>No$^{683,684}$</td>
<td>No$^{677}$</td>
</tr>
<tr>
<td>AEC2 hyperplasia</td>
<td>Yes$^{683,684}$</td>
<td>No$^{683,684}$</td>
<td>Yes$^{677}$</td>
</tr>
<tr>
<td>Fibrotic foci</td>
<td>Yes$^{683,684}$</td>
<td>No$^{683,684}$</td>
<td>Somewhat$^{677}$</td>
</tr>
<tr>
<td>Temporal heterogeneity</td>
<td>Yes$^{683,684}$</td>
<td>No$^{683,684}$</td>
<td>No$^{677}$</td>
</tr>
</tbody>
</table>

Table 1.6 Comparison between IPF in human patients versus single and multiple dose BLM-induced pulmonary fibrosis in mice. ALI, Acute Lung Injury; $D_{lC0}$, Diffusion of Lung Carbon monoxide; FVC, Forced Vital Capacity; UIP, Usual Interstitial Pneumonia.
1.4 Hermansky Pudlak Syndrome Pulmonary Fibrosis (HPSPF)

HPS is a collection of autosomal recessive genetic disorders that is characterized by abnormal biogenesis and/or function of lysosome related organelles (LROs) found in specialised secretory cells, including melanosomes, platelets, epithelial cells and macrophages. Several forms of HPS, including HPS1, HPS2 and HPS4 are associated with interstitial pulmonary fibrosis that, similar to IPF patients, present with UIP. It is therefore possible that HPSPF and IPF share common pathways in pathogenesis leading to the development of pulmonary fibrosis. Notably in HPSPF patients there is an accumulation of giant lamellar bodies in AEC2 and also foamy macrophages that are engorged with cholesterol crystals. The altered appearances of AEC2 and macrophages may be the result of defective lysosomal transport in HPS patients, which affects the release of pulmonary surfactants and cholesterol efflux respectively. It is possible that such defects result in persistent irritation that ultimately leads to pulmonary fibrosis. Pulmonary surfactants reduce surface tension within the lung and are exclusively released by AEC2; defective pulmonary surfactant release may therefore lead to prolonged tissue injury. Excessive cholesterol crystal accumulation in foamy macrophages may induce a pro-inflammatory phenotype that result in chronic inflammation and subsequent tissue fibrosis. Spontaneous mutations in HPS1 and HPS2 have arisen in C3HeB/FeJ mice, but have since been backcrossed onto the more well-studied C57BL/6 strain. Similar to their human counterparts, naïve HPS1 and HPS2 single mutants have increased numbers of foamy AEC2 and AMφ. However, they do not spontaneously develop pulmonary fibrosis, but are reported to have an increased susceptibility to BLM-induced pulmonary fibrosis. It would be interesting to investigate whether or not the accumulation of foamy macrophages and/or in naïve HPS1 and HPS2 mice contributes to their increased pathology compared to their WT counterparts. On the other hand, HPS1/HPS2 double mutant mice are reported to spontaneously develop pulmonary fibrosis from 9 months of age. These mice may therefore be a useful model for the study of naturally occurring interstitial pulmonary fibrosis. The background of HPS mutations, HPSPF, and also mouse models of HPS will be discussed further below.

1.4.1 HPS

HPS is primarily found in Northwest Puerto Ricans in the form of a HPS-1 mutation, with a prevalence of one in 1,800, and accounts for ~50% cases in non-Puerto Rican cases. Occurrence in other nationalities is extremely rare, affecting 1: 500,000 to 1,000,000.

HPS patients may experience a variety of symptoms, including oculocutaneous albinism, a bleeding diathesis secondary to platelet storage disease, lysosomal accumulation of ceroid lipofuscin, and pulmonary fibrosis. These are thought to result from defective organelle transport in melanosomes,
platelet dense granules and lysosomes respectively. The occurrences of these different symptoms is variable between patients, but are generally dependent upon HPS subtype caused by the mutation of different HPS genes.\textsuperscript{690,700}

There are eight known HPS human genes, which code for proteins that mostly function in membrane and protein trafficking in LROs. Many of these HPS proteins associate with one another to form multiple protein complexes, namely the AP-3 complex, or the Biogenesis of Lysosome-Related Organelles Complex (BLOC)-1, BLOC-2 and BLOC-3. Mutations of HPS genes results in a defect in these complexes that leads to dysfunctional LRO biogenesis and/or transport.\textsuperscript{692}

1.4.2 HPSPF

Pulmonary fibrosis is associated with several subtypes of HPS, including HPS1, HPS2, and HPS4. HPS1 and HPS4 encode for components of the BLOC-3 complex, which has recently been reported to be a Guanine Exchange Factor (GEF), and may play a role in activation and membrane localization of Ras GTP hydrolases (GTPases) (Ras-32 and Ras-38) that is required for organelle biogenesis and enzyme trafficking.\textsuperscript{701} Dysfunction of the BLOC-3 complex leads to defects in organelle cargo trafficking and production, and is associated with pulmonary fibrosis in adults.\textsuperscript{702–704} HPS-2 encodes for the $\beta_3A$ subunit of the AP-3 complex, which regulates biogenesis of lysosome related organelles and modulates intracellular trafficking of proteins to late endosomes, lysosomes, and lysosome-related organelles. Dysfunction of the AP-3 complex leads to defects in lysosome biogenesis and intracellular protein trafficking, and may result in pulmonary fibrosis in children and young adults.\textsuperscript{705}

Although the exact link between defective organelle transport and pulmonary fibrosis of HPSPF is unclear, several hypotheses have been proposed.\textsuperscript{705} Increased number and size of lysosomes may lead to their increased fragility followed by more severe lung abnormalities including lung cell necrosis. Reduced secretion of lysosome contents may also disrupt normal lung physiology.\textsuperscript{706} For example, pulmonary surfactant, in lysosomes or lamellar bodies, is regularly secreted from type II cells to the air-liquid interface to reduce surface tension.\textsuperscript{707,708} In macrophages, the accumulation of cholesterol crystals, either by increased uptake of modified low density lipoprotein (LDL) through their scavenger receptors, or reduced/defective cholesterol efflux, may also lead to the formation of foamy macrophages.

Foamy macrophages have been characterised to have a pro-inflammatory phenotype in atherosclerosis and HPS1 patients.\textsuperscript{709} Human macrophages that were stimulated in vitro with minimally oxidised LDL induced the release of pro-inflammatory cytokines, including TNF, IL1 and IL-6 in a CD14-TLR4-MD2-dependent manner.\textsuperscript{710} Stimulation of human macrophages with moderately oxidised LDL promoted the release of pro-inflammatory chemokines, including CCL2, CCL5, CXCL1 in
a CD36 dependent manner. Cholesterol crystals in foamy macrophages may also directly activate the NLRP3 inflammasome, resulting in IL-1β and IL-18 activation. Human foamy macrophages generated by oxLDL challenge has also been reported to secrete tissue remodelling mediators such as MMP-9. Therefore, foamy macrophages can promote both inflammation and tissue remodelling, perhaps as a frustrated effort to remove excess lipids from tissue. Pro-inflammatory foamy macrophages may function in tissue injury associated with chronic inflammation. On the other hand, there has been one report of in vitro generated foamy macrophages that had a pro-fibrotic M2-like phenotype, with increased protein expression of IL-10 and TGF-β, which may play a role in tissue fibrosis. Indeed, foamy macrophages accumulate in IPF patients, and also HPS1 patients.

HPSPF resembles IPF in terms of disease progression, and its UIP histopathology; however, HPSPF is characterized by giant lamellar body formation in AEC2s, resulting in AEC2 swelling.

1.4.3 Mouse models of HPSPF

There are fifteen mouse HPS genes, eight of which are human orthologues. Mutations of such mouse orthologues have been reported as models to represent the corresponding human condition. Three HPS mouse models have been used extensively to study HPSPF, including pale ear (ep; HPS1) mice carrying a double recessive mutation in the HPS1 gene, pearl (pe; HPS2) mice carrying a double recessive mutation in the HPS2 gene, and the HPS1 and HPS2 crossed, double recessive, double mutant (HPS1/2).

1.4.3.1 Pale ear (HPS1) mice

The HPS1 mutations originally arose spontaneously in the C3HeB/FeJ strain, but have since been maintained in the more well-studied C57BL/6 strain. Two different types of HPS1 mutation have been observed, including the insertion of an intracisternal A particle (IAP) at the end of exon 19 of the HPS1 gene in HPS1 mice, or a 23bp insertion, 3 bp deletion on exon 17 in the HPS1 (6J) mice. The nomenclature of mouse models for various types of HPS mutations, the identity of the affected protein, the intracellular transport complex they form, and their corresponding function is summarised in table 1.6. The phenotypes of different HPS mutations in humans and mice are summarised in table 1.7.

Adult HPS1 mice present with ears, paws and tails that are pale in colour, although the pigmentation of the coat and eyes are almost normal. HPS1 mice exhibit an abnormal respiratory phenotype: There are progressive cytoplasmic foamy changes in AEC2 from P0 to 24 months, increasing in both affected cell number and degree of severity as mice age. There is also marked swelling and degenerative changes of AEC2s in so called Giant Lamellar Body Degeneration’ (GLBD) in aged mice.
Alveolar lamellar bodies are also found to be increased in size, and often fused to each other in aged mice. There is an enlargement of air spaces, and also mild lymphocytic infiltration of the alveolar septa in some aged mice. HPS1 mice also display basal inflammatory dysregulation with elevated IL-12 p40 levels in BAL and constitutive AMφ activation that parallels abnormalities that have been reported in HPS patients. However in HPS1 mice, unlike human HPS1 patients pulmonary fibrosis does not spontaneously occur. However, HPS1 mice have increased susceptibility to BLM-induced pulmonary fibrosis, including reduced survival, increased collagen accumulation as determined by Masson’s trichrome staining and Sircol assay at day 7 post BLM-challenge, and elevated levels of TGF-β and also IL-12 p40 in the lungs of HPS2 mice from day 3 to day 7.

1.4.3.2 Pearl (HPS2) mice

The HPS2 mutation originally occurred spontaneously in the C3H/HeJ strain, but are now maintained as a congenic mutant onto C57BL/6 mice. Three different types of mutations have been observed, including HPS2, with a 793 bp tandem duplication of exons 17 to 23, HPS2 (8J), a 107 bp deletion in exon 20; and HPS2 (9J), an 867 bp deletion in exons 8-14 of the HPS2 gene in HPS2 mice.

Adult HPS2 mice present with a hypopigmented phenotype as compared to wild type controls, with a brown-grey coat colour, and also lighter ears, tails and paws. HPS2 mice also exhibit premature death, with less than 40% (as compared to 100% of WT C57BL/6 control mice) surviving for more than 2 years. HPS2 mice also have an abnormal respiratory phenotype. They have basal inflammatory dysregulation with elevated IL-12 p40 and MIP-1Y levels in BAL and constitutive AMφ activation that parallels abnormalities that have been reported in HPS patients. There is a moderately honeycombed structure within the lung, and moderate air space enlargement. In three out of eight aged mice surviving beyond 2 years, lung haemorrhage was also observed. Similar to HPS1 mice, HPS2 mice do not spontaneously develop pulmonary fibrosis, but display increased susceptibility to BLM-induced pulmonary fibrosis, including reduced survival, increased collagen accumulation as determined by Masson’s trichrome staining and Sircol assay at day 7 post BLM-challenge, elevated levels of TGF-β and also IL-12 p40 in the lungs of HPS2 mice from day 3 to day 7, a rise in the release of TGF-β and IL-12 p40 by AMφ at day 1, and also increased AEC2 apoptosis as determined by TUNEL assay from as early as five hours post BLM-challenge.

There are some explanations for this discrepancy in spontaneous pulmonary fibrosis between mice and humans. There are fifteen HPS genes in mice compared to eight in humans, and some of these genes may carry some redundancy to HPS1. HPS1 and HPS2 mice also carries different types of
mutations compared to their respective human orthologues, which may have less negative effects on functional activity than its human counterparts. Although HPS1 and HPS2 single mutant mice do not spontaneously develop pulmonary fibrosis, they have increased susceptibility to single intratracheal dose BLM-induced pulmonary fibrosis (at day 7)\textsuperscript{696}, and are therefore invaluable models to study the contributing factors that may augment pathology.

1.4.3.3 HPS1 and HPS2 double mutant mice

Double mutant HPS mice may be useful because epistatic effects between different mutant genes may amplify mutant physiological abnormalities, although it may be difficult to dissect the contributions to each gene mutation to the phenotype. The fact that each mouse model is maintained as a congenic mutant on the C57BL/6J background enables genetically controlled \textit{in vivo} tests for interaction of these 2 important genes\textsuperscript{706}.

Mutants homozygous for both HPS1 and HPS2 genes displayed a lighter coat colour than either single parental single mutant and light red eye colour in both new-born and adult animals. HPS1/2 double mutants also have more severe respiratory defects than each of the single mutants\textsuperscript{697,719}. Unlike other HPS single mutants, levels of lung lysosomes were also elevated in the HPS1/2 double mutant, suggesting abnormalities in LROs, such as lamellar bodies in AEC2\textsubscript{s}\textsuperscript{706}. AEC2\textsubscript{s} and the lamellar bodies are engorged with surfactant\textsuperscript{697,719}. Extensive surfactant abnormalities observed in these mice were correlated with early lysosomal stress, ER stress and marked AEC2 apoptosis\textsuperscript{719}.

Mutant lung also accumulate excessive autofluorescent pigment, and air spaces of mutant lungs contain age-related elevations of inflammatory cells and foamy macrophages. Spontaneous and slowly progressive pulmonary fibrosis was observed in HPS1/2 double mutant mice, with subpleural onset at 3 months and extensive fibrosis by 9 months\textsuperscript{719}. HPS1/2 mutants are therefore a suitable genetic model for interstitial pulmonary fibrosis.
### Table 1.7 Hermansky-Pudlak Syndrome (HPS) Subtypes and Corresponding Mouse Strains

<table>
<thead>
<tr>
<th>Human subtype</th>
<th>Mouse strain</th>
<th>Gene</th>
<th>Complex</th>
<th>Identity</th>
<th>Function</th>
</tr>
</thead>
<tbody>
<tr>
<td>HPS1</td>
<td>Pale ear</td>
<td>HPS1</td>
<td>BLOC-3</td>
<td>GEF</td>
<td>Activation and membrane localization of Ras GTPase</td>
</tr>
<tr>
<td>HPS2</td>
<td>Pearl</td>
<td>AP3B1</td>
<td>AP-3</td>
<td>Subunit for adaptor protein</td>
<td>Cargo selection and vesicle trafficking to the lysosome</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td>complex</td>
<td></td>
</tr>
<tr>
<td>HPS3</td>
<td>Cocoa</td>
<td>HPS3</td>
<td>BLOC-2</td>
<td>?</td>
<td>Cargo selection and vesicle trafficking to the lysosome</td>
</tr>
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<td>HPS4</td>
<td>Light ear</td>
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<td>BLOC-3</td>
<td>GEF</td>
<td>Activation and membrane localization of Ras GTPase</td>
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<td>HPS5</td>
<td>Ruby eye-2</td>
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<td>BLOC-2</td>
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<tr>
<td>HPS6</td>
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<td>BLOC-2</td>
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<tr>
<td>HPS7</td>
<td>Sandy</td>
<td>DTNBPI1/ BLOC1S8</td>
<td>BLOC-1</td>
<td>Rab-GAP, binds α- and β- dystrobrevin, myospryn</td>
<td>Exocytosis of glutamate in neuronal cells</td>
</tr>
<tr>
<td>HPS8</td>
<td>Reduced pigmentation</td>
<td>BLOC1S3</td>
<td>BLOC-1</td>
<td>Rab-GAP</td>
<td>Cargo selection and vesicle trafficking to the lysosome</td>
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<tr>
<td>?</td>
<td>Pallid</td>
<td>Pldn/ BLOC1S6</td>
<td>BLOC-1</td>
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<td>Muted</td>
<td>mu/ BLOC1S5</td>
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<td>Rab-GAP</td>
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<tr>
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<td>Cappuccino</td>
<td>cno/ BLOC1S4</td>
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<td>Rab-GAP</td>
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<tr>
<td>?</td>
<td>Buff</td>
<td>VPS33A</td>
<td>Class C VPS complex</td>
<td>?</td>
<td>Vacuole to vacuole fusion, vacuole protein sorting</td>
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<td>?</td>
<td>Gunmetal</td>
<td>RABGGTA</td>
<td>RabGGTase</td>
<td>RabGGPase</td>
<td>Adds lipophilic prenyl groups to carboxyl terminus of rab proteins</td>
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<td>?</td>
<td>Mocha</td>
<td>AP3D1</td>
<td>AP-3</td>
<td>Subunit for adaptor protein</td>
<td>Cargo selection and vesicle trafficking to the lysosome</td>
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<tr>
<td>?</td>
<td>Subtle grey</td>
<td>Skc7a11</td>
<td>Skc7a11-Slk3a2 (4F2hc)</td>
<td>Cystine/ glutamate exchanger (xCT)</td>
<td>Regulate the ration of pheomelanin and melanin synthesized in melanocytes, and affects cellular response to oxidative stress</td>
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<tr>
<td>?</td>
<td>Ashen</td>
<td>RAB27A</td>
<td>RAB27A-MYOSA-MLPH</td>
<td>GTPase</td>
<td>Transport melanosomes within melanocytes</td>
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</tbody>
</table>

Table 1.7 Hermansky-Pudlak Syndrome (HPS) subtypes and corresponding mouse strains. 4F2hc, 4F2 cell-surface antigen heavy chain; AP-3, Adaptor Protein complex-3; BLOC, Biogenesis of Lysosomal related Organelles Complex; BLOC1S, BLOC1 subunit; cno, Cappuccino; DTNBPI1, Dystrophin Binding Protein-1; GEF, Guanine Exchange Factor; GAP, GTPase Activating Protein; GTPase, Guanine Triphosphate (GTP) hydrolase; HPS, Hermansky Pudlak Syndrome; MLPH, Melanophilin; mu, muted; Myo5A, Myosin 5A; Pldn, Pallidin; PABGGTA, Rab geranylgeranyl transferase-α; slc, soluble carrier; VPS, Vacuolar Protein Sorting; VPS33A, VPS-associated protein 33A; xCT, Cytisine/ glutamate exchanger.
### Chapter 1 | General Introduction

<table>
<thead>
<tr>
<th>Human</th>
<th>Mouse</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>HPS1</strong></td>
<td>Pulmonary fibrosis(^{703}), reduced visual acuity, horizontal nystagmus, oculocutaneous albinism(^{690}), prolonged bleeding time(^{690}), IBS in large intestine(^{690}).</td>
</tr>
<tr>
<td><strong>HPS2</strong></td>
<td>Susceptibility to respiratory illnesses, neutropenia(^{725}), mild conductive hearing loss, haemorrhage, failure to thrive(^{725}), oculocutaneous albinism, platelet defects, immunodeficiency(^{726}).</td>
</tr>
<tr>
<td><strong>HPS3</strong></td>
<td>Reduced visual acuity, ocular albinism, platelet dysfunction (excessive bruising and epistaxis), with mild extra-ocular symptoms compared to HPS1(^{730-732}).</td>
</tr>
<tr>
<td><strong>HPS4</strong></td>
<td>Restrictive pulmonary fibrosis, granulomatous colitis, epistaxis, bruising, and in female patients menorrhagia(^{703}).</td>
</tr>
<tr>
<td><strong>HPS5</strong></td>
<td>Nystagmus, reduced visual acuity, bruising, menorrhagia, metrorrhagia, absent platelet dense bodies, elevated cholesterol levels(^{733,734}).</td>
</tr>
<tr>
<td><strong>HPS6</strong></td>
<td>Oculocutaneous albinism, epistaxis and bleeding(^{734}).</td>
</tr>
<tr>
<td><strong>HPS7</strong></td>
<td>Oculocutaneous albinism, easy bruisability, a bleeding tendency, mild shortness of breath with exertion, decreased lung compliance, but otherwise normal pulmonary function tests and HRCT(^{736}).</td>
</tr>
<tr>
<td><strong>HPS8</strong></td>
<td>Incomplete oculocutaneous albinism and mild platelet dysfunction (easy bruising, hematomas after venesection, frequent epistaxis and prolonged bleeding)(^{737}).</td>
</tr>
</tbody>
</table>

Table 1.8 Disease phenotypes in HPS patients and HPS mouse models. Ck1d, Caesin Kinase 1, delta.
1.5 Hypothesis of thesis

The main hypothesis of this thesis is that AMφ are able to respond to signals in their immediate environment, including priming and stimulatory signals, to differentiate into different functional subpopulations that may play dissimilar roles. The specific chapter hypotheses are listed below.

The hypothesis of Chapter 3 is that AMφ are able to respond to various priming and/or stimulation signals to form distinct subsets, in a similar fashion to that observed in BMMφ. Stimulatory signals on PRRs may have a modulatory effect on gene and protein expression of AMφ that have been polarised by a priming signal.

The hypothesis of Chapter 4 is that AMφ may alter their gene and protein expression in response to external priming and stimulatory signals and contribute differently to different stages of pulmonary fibrosis. Foamy macrophages are formed following the accumulation of cholesterol crystals, either by excessive uptake of modified LDL or by reduced cholesterol efflux, and may promote a pro-inflammatory and/or a pro-fibrotic phenotype in macrophages. Foamy macrophages accumulate in HPS1 patients and also naïve HPS1 mice, and it is hypothesised that these cells contribute to the increased susceptibility to BLM-induced pulmonary fibrosis observed in HPS1 mice.

The hypothesis of Chapter 5 is that PRRs expressed on AMφ may be stimulated by endogenous alarmins that are released following tissue injury, and may alter AMφ gene and/or protein expression to promote fibrosis.

1.6 Aim of the thesis

The main aim of the thesis is to assess the heterogeneity of AMφ and investigate the contributions of heterogeneous macrophage populations on pathophysiology in murine models of pulmonary fibrosis. The chapter aims are listed below.

The aim of Chapter 3 is to investigate the heterogeneity of AMφ in vitro in response to various priming and stimulatory signals.

The aim of Chapter 4 is to evaluate AMφ heterogeneity ex vivo isolated from a mouse model of BLM-induced pulmonary fibrosis. The potential role of foamy macrophages in increased susceptibility to BLM-induced pulmonary fibrosis will also be evaluated in HPS1 mice. HPS1 mice, but not HPS1/2 mice were used as the latter cannot be successfully bred despite several attempts.

The aim of Chapter 5 is to identify the contributions of PRRs on AMφ heterogeneity in BLM-induced pulmonary fibrosis in mice.

The specific background and detailed aims are described in the separate results chapters.
Chapter 2
Materials and Methods
Chapter 2 | Materials and Methods

2.1 Materials

Table 2.1 Cell culture reagents used in thesis.

<table>
<thead>
<tr>
<th>Product</th>
<th>Company</th>
<th>Catalogue number</th>
</tr>
</thead>
<tbody>
<tr>
<td>RPMI-1640 (Glutamax)</td>
<td>Gibco</td>
<td>72400-021</td>
</tr>
<tr>
<td>Fetal Bovine Serum (Heat Inactivated)</td>
<td>Gibco</td>
<td>10500</td>
</tr>
<tr>
<td>Sodium Pyruvate</td>
<td>Gibco</td>
<td>11360</td>
</tr>
<tr>
<td>β-mercaptoethanol</td>
<td>Gibco</td>
<td>315350-010</td>
</tr>
<tr>
<td>Geneticin</td>
<td>Gibco</td>
<td>10131</td>
</tr>
<tr>
<td>DPBS</td>
<td>Gibco</td>
<td>14190-094</td>
</tr>
<tr>
<td>Penicillin/Streptomycin</td>
<td>Gibco</td>
<td>15140-122</td>
</tr>
<tr>
<td>Polymyxin B sulfate salt</td>
<td>Sigma-Aldrich</td>
<td>P-4932-1MU</td>
</tr>
<tr>
<td>Cell Dissociation Buffer</td>
<td>Sigma-Aldrich</td>
<td>5914-100ML</td>
</tr>
<tr>
<td>0.25% Trypsin (1X), Phenol Red</td>
<td>Gibco</td>
<td>15050-065</td>
</tr>
<tr>
<td>Ammonium chloride (NH4Cl)</td>
<td>Sigma-Aldrich</td>
<td>A9434-500G</td>
</tr>
<tr>
<td>Potassium bicarbonate (KHCO3)</td>
<td>Sigma-Aldrich</td>
<td>237205-100G</td>
</tr>
<tr>
<td>Ethylenediaminetetraacetic acid disodium salt dihydrate</td>
<td>Sigma-Aldrich</td>
<td>E4884-100G</td>
</tr>
<tr>
<td>BD-Falcon Cell strainer (70 μm)</td>
<td>VWR</td>
<td>21008-952</td>
</tr>
<tr>
<td>0.4% Trypan Blue solution</td>
<td>Sigma-Aldrich</td>
<td>T8154-20ML</td>
</tr>
<tr>
<td>Corning® CoStar® culture plates (48-well, flat bottom)</td>
<td>Sigma-Aldrich</td>
<td>CL S3548-100EA</td>
</tr>
<tr>
<td>Corning® CoStar® culture plates (96-well, flat bottom)</td>
<td>Sigma-Aldrich</td>
<td>CL S3997-50EA</td>
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<tr>
<td>Recombinant mouse IFN-γ</td>
<td>R&amp;D Systems</td>
<td>485-MI-100</td>
</tr>
<tr>
<td>Recombinant mouse IL-4</td>
<td>R&amp;D Systems</td>
<td>404-ML-050</td>
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<tr>
<td>Recombinant mouse IL-13</td>
<td>R&amp;D Systems</td>
<td>413-ML-025</td>
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<tr>
<td>Recombinant mouse TGF-β</td>
<td>R&amp;D Systems</td>
<td>7666-MB-005</td>
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<tr>
<td>Recombinant M-CSF</td>
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<td>416-ML</td>
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<tr>
<td>LPS-EB Ultrapure</td>
<td>Invivogen</td>
<td>tlr3-pelps</td>
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<tr>
<td>Pam2CSK4</td>
<td>Invivogen</td>
<td>tlr1-pm2s-1</td>
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<tr>
<td>ODN1585</td>
<td>Invivogen</td>
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Table 2.2 Reagents used for RNA extraction and reverse transcription.

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<tr>
<th>Product</th>
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<tbody>
<tr>
<td>Rneasy Micro Kit</td>
<td>Qiagen</td>
<td>74004</td>
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<tr>
<td>Trizol *</td>
<td>Life Technologies</td>
<td>15596</td>
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<tr>
<td>Chloroform</td>
<td>Sigma-Aldrichs</td>
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<tr>
<td>Taqman® Reverse Transcription Reagents</td>
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Table 2.3 Primers used for qRT-PCR analysis.

<table>
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<th>Product</th>
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<tr>
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<td>Life Technologies</td>
<td>Mm00475988_m1</td>
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<tr>
<td>Hprt-1</td>
<td>Life Technologies</td>
<td>Mm00446968_m1</td>
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<td>IL-1β</td>
<td>Life Technologies</td>
<td>Mm00434228_m1</td>
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<td>IL-6</td>
<td>Life Technologies</td>
<td>Mm00446190_m1</td>
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<td>NLRP3</td>
<td>Life Technologies</td>
<td>Mm00840904_m1</td>
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<td>NOS2</td>
<td>Life Technologies</td>
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<td>TLR2</td>
<td>Life Technologies</td>
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<td>TLR4</td>
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<td>TLR7</td>
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<td>Mm00446590_m1</td>
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<td>TLR9</td>
<td>Life Technologies</td>
<td>Mm00446193_m1</td>
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<td>TNF</td>
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<td>Mm00443260_g1</td>
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</table>

Table 2.4 Reagents used for protein level analysis.

<table>
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<tr>
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</thead>
<tbody>
<tr>
<td>Mouse Fibronectin ELISA Kit</td>
<td>AbCam</td>
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<td>Mouse TH1/TH2 9-plex ultra sensitive kit</td>
<td>MSD</td>
<td>K15013C-2</td>
</tr>
<tr>
<td>V-Plex proinflammatory panel 1 (mouse) kit</td>
<td>MSD</td>
<td>K15048D-2</td>
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</table>

Table 2.5 Reagents used for flow cytometr analysis.

<table>
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<tbody>
<tr>
<td>Mouse Serum</td>
<td>Sigma</td>
<td>M5905-5ML</td>
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<tr>
<td>BD CellFix™</td>
<td>BD BioSciences</td>
<td>340181</td>
</tr>
<tr>
<td>BD™ CompBeads</td>
<td>BD BioSciences</td>
<td>552845</td>
</tr>
<tr>
<td>BD Cytofix/ Cytoperm Cell Fixation/ Permeabilisation Kit</td>
<td>BD BioSciences</td>
<td>554714</td>
</tr>
<tr>
<td>FcR blocking reagent, mouse</td>
<td>Miltenyi Biotec</td>
<td>130-092-575</td>
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Table 2.6 Detection antibodies and corresponding isotype controls used for flow cytometric analyses.

<table>
<thead>
<tr>
<th>Detection Antibodies</th>
<th>Product</th>
<th>Clone</th>
<th>Company</th>
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<tr>
<td>Anti-Mouse CD11c PE</td>
<td>N418</td>
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<tr>
<td>Anti-mouse CD11c-APC/Cy7</td>
<td>N418</td>
<td>Biolegend</td>
<td>117324</td>
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<tr>
<td>Anti-mouse IA/IE-Pacific Blue</td>
<td>M5.114 15.2</td>
<td>Biolegend</td>
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<tr>
<td>Anti-mouse IA/IE-FITC</td>
<td>M5.114 15.2</td>
<td>Biolegend</td>
<td>107606</td>
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</tr>
<tr>
<td>Anti-mouse MMR (CD206)-PerCP/Cy5.5</td>
<td>C068C2</td>
<td>Biolegend</td>
<td>141715</td>
<td></td>
</tr>
<tr>
<td>Anti-Mouse CD282 (TLR2) eFluor® 660 (Alexa Fluor® 647 Replacement)</td>
<td>6C2</td>
<td>eBiosciences</td>
<td>50-9021-80</td>
<td></td>
</tr>
<tr>
<td>Mouse TLR4 Allophycocyanin MAb (Clone 267518), Rat IgG2A</td>
<td>267518</td>
<td>R&amp;D</td>
<td>FAB2759A</td>
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<tr>
<td>Rabbit Anti-TLR7 antibody</td>
<td>Polyclonal</td>
<td>AbCam</td>
<td>ab13732</td>
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<tr>
<td>Goat polyclonal Secondary Antibody to Rabbit IgG - Fc (DyLight® 650)</td>
<td>Polyclonal</td>
<td>AbCam</td>
<td>ab96986</td>
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<tr>
<td>Monoclonal Anti-mouse NLRP3-APC</td>
<td>768319</td>
<td>R&amp;D</td>
<td>IC7578A</td>
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<tr>
<td>Rat Anti-Mouse CD289 (TLR9) Biotin</td>
<td>M9.D6</td>
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<tr>
<td>Streptavidin APC</td>
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<table>
<thead>
<tr>
<th>Isotype controls</th>
<th>Armenian Hamster IgG Isotype Control PE</th>
<th>HTK888</th>
<th>Biolegend</th>
<th>400908</th>
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<tbody>
<tr>
<td></td>
<td>Armenian Hamster, IgG-APC/Cy7</td>
<td>HTK888</td>
<td>Biolegend</td>
<td>400927</td>
</tr>
<tr>
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<td>Pacific Blue™ Rat IgG2b, κ Isotype Ctrl Antibody</td>
<td>RTK4530</td>
<td>Biolegend</td>
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<tr>
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<td>FITC Rat IgG2b, κ Isotype Ctrl Antibody</td>
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<td>Biolegend</td>
<td>400606</td>
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<td>Rat IgG2a κ-PerCP/Cy5.5</td>
<td>RTK2758</td>
<td>Biolegend</td>
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<td>Rat IgG2b Isotype Control eFluor® 660 (Alexa Fluor® 647 Replacement)</td>
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<td>eBioscience</td>
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</tr>
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<td></td>
<td>Rat IgG2A Allophycocyanin Isotype Control, Rat IgG2A</td>
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<td>R&amp;D</td>
<td>IC006A</td>
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<td>Rabbit Control IgG</td>
<td>Polyclonal</td>
<td>AbCam</td>
<td>ab37373</td>
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<td></td>
<td>Rat IgG2A control Biotin</td>
<td>eBR2a</td>
<td>eBiosciences</td>
<td>13-4321-85</td>
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Table 2.7 Reagents used for histology analysis,

<table>
<thead>
<tr>
<th>Product</th>
<th>Company</th>
<th>Catalogue number</th>
</tr>
</thead>
<tbody>
<tr>
<td>Wright-Giemsa Stain Set for Hema-Tek® 1-, 1000 and 2000</td>
<td>Sigma-Aldrich</td>
<td>WGHT-1SET</td>
</tr>
<tr>
<td>Hematoxylin solution, Harris modified</td>
<td>Sigma-Aldrich</td>
<td>HHS128-4L</td>
</tr>
<tr>
<td>Aqueous Eosin (1%)</td>
<td>Leica</td>
<td>3801590BBE</td>
</tr>
<tr>
<td>Sirius Red F3B</td>
<td>BDH laboratory supplies</td>
<td>341492F</td>
</tr>
<tr>
<td>Cell Conditioning 2 (CC2)</td>
<td>Roche</td>
<td>950123</td>
</tr>
<tr>
<td>Rat anti-mouse CD68 antibody [FA11]</td>
<td>AbCam</td>
<td>Ab53444</td>
</tr>
<tr>
<td>Polyclonal Rabbit anti-Rat Immunoglobulin</td>
<td>Dako</td>
<td>E0468</td>
</tr>
<tr>
<td>Discovery DAB Map Detection Kit (RUO)</td>
<td>Roche</td>
<td>760-124</td>
</tr>
<tr>
<td>Ethanol</td>
<td>Sigma-Aldrich</td>
<td>458600-2SL</td>
</tr>
<tr>
<td>Oil Red O</td>
<td>Sigma-Aldrich</td>
<td>O0625-100G</td>
</tr>
<tr>
<td>Mayer’s hematoxylin</td>
<td>AbCam</td>
<td>ab128990</td>
</tr>
</tbody>
</table>
2.2 Methods

2.2.1 Animals

All procedures were carried out in accordance with Home Office guidelines (Animal Scientific Procedures of 1986) [Home office project license number: 70/7336; Home Office personal license number: 70/24209]. Male C57BL/6 mice were 8-10 weeks old, weight-matched (23-27 g), purchased from Charles River.

The breeding pair of HPS1 mice were purchased from Jackson Laboratory (Maine) and the colony was bred and maintained in-house. HPS1 mice were genotyped by Transnetyx (Tennessee, United States). 8-12 week old male and female HPS1 mice were used for studies.

2.2.2 Multiple oropharyngeal dose BLM challenge of mice

WT C57BL/6 male mice or male or female HPS1 mice were challenged three times by oropharyngeal instillations of 50μl of 0.13U BLM sulfate (Kyowa Nippon, Kayakin Co Ltd.) or saline controls at 72 hour intervals on Days -7, -4 and -1. Mice were culled on days 7, 14 and 21 post-challenge, which include time points in the ALI phase, early fibrotic phase and the progressive fibrotic stage respectively (Figure 2.1).

![Figure 2.1 Diagram illustrating time course of pathogenesis in multiple oropharyngeal dose BLM-induced pulmonary fibrosis. Mice were dosed via an oropharyngeal route at 72 hour intervals at days -7, -4 and -1. Mice were culled at days 7, 14 and 21 for analysis at the late inflammatory, active fibrotic and established fibrotic phases respectively. O.p., oropharyngeal; U, international units.](image-url)
2.2.3 Histology

For histology analysis of C57BL/6 mice challenged with multiple oropharyngeal dose of saline or BLM (chapter 4), the right lobe of C57BL/6 mouse lungs was tied off for RNA analysis, and the left lobe was inflated by 0.5 ml 10% formalin, and then removed for histology analysis. The formalin inflated left lobe was then immersed in 10% formalin overnight for further fixation. The fixed left lobe was then dissected longitudinally into four pieces and embedded into paraffin. Embedded tissues were then cut into 3μm slides sections for immunohistological staining. Stained slides were viewed and analyzed on ImagePro (Mediacy).

For histological comparison of saline or BLM-challenged HPS1 and WT C57BL/6 mice, both left and right lungs were inflated with 1 ml (total) Optimal Cutting Temperature (OCT) and snap frozen in liquid nitrogen; 10μm sections were cut for histology analysis.

2.2.3.1 Haematoxylin and Eosin (H&E) Staining

H&E staining was automated by the Leica Discovery XT multistainer. Tissue slides were first deparaffinized at 60°C by heating for 10 minutes, and then washed three times with xylene for 5 minutes each. Tissue slides were then hydrated by three washes in 100% Industrial Methylated Spirit (IMS) for 1 minute each, once in 70% IMS for another minute, and then twice in water for 30 seconds each. Tissue slides were then stained with Harris Haematoxylin for 5 minutes, and then washed in water for another 5 minutes. Non-specific Haematoxylin staining was then removed from tissue slides through differentiation in acid alcohol (99% of 70% ethanol, 1% concentrated Hydrochloric Acid (HCl)). Tissue slides were then stained for 2 minutes in eosin, and excess stain was washed off with water. Tissue slides were then dehydrated by one wash in water for 5 minutes, one wash in 70% IMS for 30 seconds, and then two washes in 100% IMS for 1 minute each. Slides were then cleared of alcohol by three xylene washes, twice for one minute and once for two minutes. A cover slip was then mounted onto the H&E stained tissue slide.

2.2.3.2 Picrosirius Red (PSR) Staining

PSR staining was automated by the Leica Discovery XT multistainer. Tissue slides were first deparaffinized at 60°C by heating for 10 minutes, and then washed three times with xylene for 5 minutes each. Tissue slides were then hydrated by three washes in 100% IMS for 1 minute each, once in 70% IMS for another minute, and then twice in water for 30 seconds each. Tissue slides were then stained with PSR stain for 1 hour. Non-specific PSR stain was then removed by three HCl (0.01M) washes for 5 seconds each, followed by a 5 second rinse in water. Tissue slides were then stained with Harris Haematoxylin for 5 minutes, and then washed in water for another 5 minutes. Non-specific Haematoxylin staining was then removed from tissue slides through differentiation in acid alcohol.
(99% of 70% ethanol, 1% concentrated HCl). Tissue slides were then dehydrated by one wash in water for 5 minutes, one wash in 70% IMS for 30 seconds, and then two washes in 100% IMS for 1 minute each. Slides were then cleared of alcohol by three xylene washes, twice for one minute and once for two minutes. A cover slip was then mounted onto the PSR stained tissue slide.

2.2.3.3 CD68 Staining

CD68 staining was automated by Ventana Discovery XT. In brief, tissue slides were deparaffinized and pretreated with mild Cell Conditioner 2 (CC2) for renaturation of protein molecules. Tissue slides were then stained with 2 μg/ml Rat anti-CD68 (AbCam) for 12 hours, followed by Biotinylated Rabbit anti-Rat antibody (DAKO) and Discovery DAB MAP Detection Kit (RUO) (Roche) for 32 minutes. The amount of staining was quantified by positive pixel count of brown stain by ImageScope (Aperio).

2.2.3.4 ORO staining

0.25% stock solution of ORO stain was made by dissolving 25 mg of ORO powder (Sigma) into 100 ml 100% isopropanol. Stock solution was kept at 4°C until further use. For working solution, 6 ml of 0.25% ORO stock was dissolved in 4 ml dH2O and filtered with 0.2 μm filter paper.

Prior to ORO staining, cells were fixed in 10% formalin for 10 min, and then permeabilised in 60% isopropanol for another 10 min. Fixed and permeabilised cells were then stained with ORO for 15 min; excess ORO stain was washed off with distilled water. ORO stained cells were subsequently stained for cell nuclei with Mayer’s Haematoxylin (AbCam) for 5 min; excess Mayer’s haematoxylin stain was then washed off with distilled water.

BAL cells on microscope slides were scanned using Aperio ScanScope XT (Leica Biosystems) and visualised on ImageScope (Leica Biosystems). AMφ in 96-well plates were visualised by scanning with Zeiss Axio Observer Z1 inverted microscope (Zeiss).
2.2.4 BAL cells isolation
AMΦ were isolated from BAL of C57BL/6 mice. Mice were sacrificed by intraperitoneal (i.p.) 10% pentobarbital overdose. Lungs were lavaged twice with 0.5 ml sterile Dulbecco’s Phosphate Buffered Saline (DPBS) (Gibco) through an intratracheal catheter. A total of 1.0 ml saline was instilled and ~0.8 ml was recovered from each mouse.

2.2.5 Total and Differential Cell Count
100μl was aliquoted from the 1ml sample in DPBS for total cell count, which was determined by manual counting of 0.5% trypan blue stained cells using a hemocytometer. It was noted that around 3-5 X 10^4 BAL cells were recoverable per naïve mouse.

Another 100μl was then aliquoted from the 1ml sample in DPBS for differential cell count. BAL cells were mounted onto microscope slides by cytospin. Differential cell count was determined by manual counting of BAL cells stained with Wright-Giemsa stain (Hematek, Sigma-Aldrich). 300 cells were counted per field, and at least 3 fields were counted. AMΦ predominated the BAL cell population at ~90%, with minimal granulocytic or lymphocytic infiltration.

Flow cytometry was also possible for differential cell count through staining for CD11c+ AMΦ, Ly6G+ granulocytes, CD3+ T-lymphocytes and B220+ B-lymphocytes, but was not used in these studies due to limited amount of BAL cells retrievable from naïve mice.

2.2.6 AMΦ enrichment
BALF was pooled together and passed through a 70μm cell strainer (BD Falcon™) to remove cell clumps, and then washed in Ammonium Chloride Potassium (ACK) lysis buffer (0.15M NH4Cl, 1.0mM KHCO₃ and 0.1mM Na₂EDTA) (Sigma-Aldrich) for lysis of contaminating RBCs. Cells were then washed equal volume of macrophage complete media [RPMI-1640 Glutamax™ media (Gibco) supplemented with 10% Fetal Calf Serum (FCS) (Biosera) for growth factors to promote cell survival, 1mM Sodium Pyruvate (Lonza) for additional nutrients for cell culture, 0.05mM of β-Mercaptoethanol (Gibco) as a reducing agent to prevent the buildup of toxic oxygen radicals in cell culture, 1mM of penicillin/streptomycin (Gibco) as antibiotics, and 50μg/ml Geneticin as an antifungal agent (Gibco) ]. 10% FCS inhibits the lysis reaction by the ACK lysis buffer. Cells were then centrifuged at 500 g for 10 minutes at 4°C. The supernatant was discarded, and the cell pellet was resuspended in 2 ml of macrophage media. 100μl was aliquoted from the 2 ml sample in macrophage complete media for cell count, which was determined by manual counting of 0.5% trypan blue stained cells using a hemocytometer.
BAL cells were resuspended at $2 \times 10^5$ cells/ml, and $1 \times 10^5$ cells (0.5 ml) were then seeded per well on a 48 well plate (Corning®) and allowed to enrich for 90 minutes by plastic adherence. Non-adherent cells were then removed and fresh macrophage complete media was added. Inspection by light microscopy revealed that adherent cells were macrophages.

Magnetic-activated cell sorting (MACS) is also possible for the immune separation of AMφ, either by positive enrichment of CD11c+ AMφ or by negative elimination of Ly6G+ granulocytes, CD3+ T-lymphocytes, and B220+ B-lymphocytes. However, MACS was not used in these studies as adherent AMφ apredominate BAL cells in naïve mice (~90%) and there is little contamination of other cell populations. Moreover, MACS involves a number of adherence and washing steps that may lead to cell loss, and not be an ideal method of enrichment for AMφ where very few BAL cells (~3-5x10^4 cells/mouse) were retrievable to begin with.

### 2.2.7 Bone marrow isolation

Bone marrow was isolated from BAL of C57BL/6 mice. Mice were sacrificed by intraperitoneal (i.p.) 10% pentobarbital overdose. Bone marrow was isolated from the femur and tibia of mice by flushing with DPBS. In brief, the ends of each femur/tibia were cut, and a 27AG needle was used to flush out bone marrow cells (until bones appear white). Cell clumps were then broken up by pipetting with syringe, and the cell suspension was then passed through a 100 μm filter to remove contaminants (e.g. mouse fur). The cell suspension was then centrifuged for 500 g for 5 minutes; supernatant was discarded and the cell suspension was resuspended in ACK lysis buffer (0.15M NH₄Cl, 1.0mM KHCO₃ and 0.1mM Na₂EDTA) (Sigma-Aldrich) for 10 minutes at room temperature for the lysis of contaminating RBCs. Cells were then mixed with an equal volume of macrophage complete media to inhibit further lysis from the ACK lysis buffer, and centrifuged at 500g for 10 min. The supernatant was discarded, and the cell pellet was then resuspended in 10 ml of macrophage complete media (see section 2.2.6). 100μl was aliquoted from the 10 ml sample in macrophage complete media for cell count, which was determined by manual counting of 0.5% trypan blue stained cells using a hemocytometer.

### 2.2.8 BMMφ generation

On day 0, $1.5 \times 10^6$ bone marrow cells were seeded at 3 ml per well of a 6-well plate (i.e. $0.5 \times 10^6$ cells/ml). Bone marrow cells were supplement cells with 100 ng/ml M-CSF (R&D Systems) and incubated at 37°C, at 5% CO₂. On day 4, 3ml of fresh macrophage complete medium supplemented with 100 ng/ml M-CSF was added without disturbing the old media. This was to replenish M-CSF within the media and allow non-adherent undifferentiated bone marrow cells to mature into BMMφ. On day 8, macrophage complete media was aspirated to remove non-adherent cells, and adherent BMMφ...
were harvested for \textit{ex vivo} priming and/or stimulatory studies. 3 ml 0.5\% trypsin was added to BMM\(\phi\) at 3 ml per well and incubated for 15 minutes at 37°C. BMM\(\phi\) were lifted off the wells by gentle scrapping and further trypsin activity was inhibited by the addition of 3 ml of macrophage complete media. The cell suspension was centrifuged at 4°C, 500 g for 10 minutes. The supernatant was discarded and the cell pellet was resuspended in 2 ml of macrophage media. 100\(\mu\)l was aliquoted from the 2 ml sample in macrophage complete media for cell count, which was determined by manual counting of 0.5\% trypan blue stained cells using a hemocytometer.

BMM\(\phi\) were resuspended at 2 x 10^5 cells/ml, and 1x10^5 cells (0.5 ml) were seeded per well on a 48 well plate (Corning\({}\)) and allowed to enrich overnight by plastic adherence. Non-adherent cells were then removed and fresh macrophage complete media was added. Inspection by light microscopy revealed that adherent cells were macrophages.

\textbf{2.2.9 Ex vivo priming and stimulation of BMM\(\phi\)/AM\(\phi\) for mRNA and protein level analyses}

For the generation of M1- or M2-like macrophage subsets, 1 x 10^5 BMM\(\phi\) or AM\(\phi\)/ well of a 48-well plate were primed with either media control (macrophage complete media, section 2.2.6), 20ng/ml IFN-\(\gamma\) (R&D Systems), 10 ng/ml IL-4 (R&D Systems) or 10 ng/ml IL-13 (R&D Systems) for 18 hours, and then cultured with or without 10 ng/ml LPS-EB (Invivogen), for another 4 hours for gene expression analysis or for 24 hours for measurements of protein levels in the AM\(\phi\) supernatant.

For the priming of AM\(\phi\) with lung-fibrosis associated signals, 1 x 10^5 AM\(\phi\)/ well a 48-well plate were serum-starved for 24 hours, and then primed with either media control (macrophage complete media, section 2.2.6), 10 ng/ml TGF-\(\beta\)1 (R&D Systems) or subject to hypoxia (1% O\(_2\), 5% CO\(_2\)) for 18 hours, and then cultured with or without 10 ng/ml LPS-EB (Invivogen), for another 4 hours for gene expression analysis, or for 24 hours for measurements of protein levels in the AM\(\phi\) supernatant.

Total RNA extraction, reverse transcription, and quantitative real time polymerase chain reaction (qRT-PCR) for gene expression analysis is described in greater detail at sections 2.2.11, 2.2.12 and 2.2.13 respectively. Measurements of proteins of interest in culture supernatant is described in greater detail in sections 2.2.14 (fibronectin enzyme linked immunosorbent assay (ELISA) and 2.2.15 [Meso-Scale Discovery (MSD)].
2.2.10 Ex vivo stimulation of AMφ from saline or BLM-challenged mice for mRNA and protein level analyses

AMφ isolated from saline or BLM-challenged mice were stimulated by media, 10 ng/ml Pam2CSK4 (P2C) (Invivogen), 3μM of CpG-ODN (A) (ODN1585, Invivogen) or CpG-ODN (A) Control (ODN1585c, Invivogen) for 24 hours, and AMφ supernatant was collected for protein expression analysis. Measurements of proteins of interest in culture supernatant is described in greater detail in sections 2.2.14 (fibronectin enzyme linked immunosorbent assay (ELISA) and 2.2.15 [Meso-Scale Discovery (MSD)].

2.2.11 Total RNA extraction

Total RNA was extracted using RNeasy® micro kit (Qiagen). For RNA isolation from cell culture, 1x10^5 cells were lysed in 350 μl buffer RLT, which contains the chaotropic agent and protein denaturant guanidium thiocyanate. Guanidium thiocyanate can also denature RNase and thereby inhibit RNA degradation. Lysed cells in RLT buffer were then mixed with 350 μl 70% isopropanol for RNA precipitation. The 700 μl mixture was then added onto an RNeasy MinElute spin column and centrifuged for 30 seconds at 10,000 g and room temperature. RNA precipitate was captured onto the silica membrane of the MinElute spin column, and the flowthrough was discarded. The RNeasy MinElute spin column was then washed by 350 μl buffer RW1, which contains guanidium and ethanol, and was able to efficiently remove biomolecules such as carbohydrates, proteins and fatty acids, while RNA larger than 200 bases remain bound to the column. The RNeasy MinElute spin column was then centrifuged for 30 seconds at 10,000 g and room temperature, and the flowthrough was discarded. 10 μl stock DNase1 (reconstituted from adding 550 μl of RNase-free water to 1 vial of lyophilized DNase1 powder) was then diluted by 70 μl buffer RDD into 80 μl working solution, which was then loaded onto the RNeasy MinElute spin column, mixed by inversion and incubated for 15 minutes at room temperature, for the digestion of contaminating DNA. The RNeasy MinElute spin column was subsequently washed with 350 μl buffer RW1, and centrifuged for 30 seconds at 10,000 g and room temperature. The flowthrough and the 2 ml collection tube was discarded, and a new collection tube was applied to the RNeasy column. The RNeasy MinElute spin column was then washed by 500 μl working buffer RPE (stock RPE + 4 volumes of 100% ethanol) that contains ethanol, and centrifuged for 30 seconds at 10,000 g and room temperature. The RNeasy MinElute spin column was subsequently washed by 500 μl 80% ethanol, and centrifuged for 5 minutes at 10,000 g and room temperature. The flowthrough and the 2 ml collection tube were discarded, and a new collection tube was applied to the RNeasy column. The RNeasy MinElute spin columns were then centrifuged for 5 minutes at 10,000 g and room temperature, with their lids open to dry the columns to remove residual ethanol that may affect downstream
applications. The collection tubes were then discarded, and the RNeasy MidElute spin columns were placed onto new 1.5 ml Eppendorf tubes. RNA was then eluted by adding 15 μl of RNase-free water onto the RNeasy MidElute spin column, and centrifuging for 30 seconds at 10,000 g and room temperature. Eluted RNA was subsequently placed on ice and 1 μl sample was used to test for purity by evaluating optical density (230nm: organic compounds [e.g. ethanol]; 260 nm: nucleic acids; 280 nm: proteins) using Nanodrop (Thermoscientific). RNA samples had 260/230 and 260/280 ratios of around 1.8 and 2.0 respectively indicating that they were free of organic acid or protein contaminants.

For RNA extraction from lung tissue, OCT-inflated lungs (see section 2.2.3) were homogenised using the automated homogeniser PrecellLys at 2 x 3 x 500 reps. RNA was then isolated from lung homogenates using phenol-chloroform extraction. In brief, lung homogenates were mixed with 1 ml Trizol® (Life Technologies) (contains guanidium thiocyanate and phenol). 200μl of choloroform was added to each 1ml Trizol/ lung homogenate mixture and shaken vigorously for 15 seconds. The homogenates were then left to stand at room temperature for 2 min, before centrifuged at 4°C at 10,000 x g for 10 min. The upper aqueous layer containing the RNA was then isolated and further purified using RNeasy MinElute spin columns as described above, whereas the lower layer containing the organic phase was discarded.

2.2.12 Reverse transcription

RNA was then reverse transcribed by reverse transcription-polymerase chain reaction (RT-PCR) using the Taqman® reverse transcription kit (Life Technologies). In brief for a 10μl RT-PCR solution, the RT-PCR reaction mix required 1μl of 10X RT buffer to maintain solution PH, 22 μl of 25 mM MgCl₂ (working concentration: 5.5 mM) to provide Mg²⁺ cations to aid negatively charged deoxynucleotides (dNTPs) into binding RNA and assembling into complementary deoxyribonucleic acid (cDNA), 20μl of 2,500 μM dNTPs (working concentration: 500 μM) as substrates for reverse transcriptase to synthesise cDNA, 5 μl of 5μM random hexamers (working concentration: 2.5 μM) as primers for reverse transcriptase, 2 μl of 20U/μl RNase inhibitor (working concentration: 0.4 U/μl) to prevent RNA degradation, 2.5μl of 5μl/μl MultiScribe™ reverse transcriptase [a recombinant moloney murine leukemia virus (rMoMuLV) reverse transcriptase that has been optimised for TaqMan-based assays] (working concentration: 1.25 U/μl) to catalyse reverse transcription, 20 ng/μl RNA sample as template for reverse transcription, and DNase and RNase-free water to make up total volume to 100 μl. To minimize pipetting errors between samples, a reverse transcriptase master mix containing RT-buffer, MgCl₂, dNTPs, random hexamers, RNase inhibitor and MultiScribe™ reverse transcriptase was used, whereas the volumes of RNA samples and DNase/RNase water used was adjusted per
sample depending on RNA concentration. The volumes, stock and working concentrations for various components of the reverse transcription mix are listed in Table 2.8.

<table>
<thead>
<tr>
<th>Component</th>
<th>Stock conc.</th>
<th>Units</th>
<th>Final conc.</th>
<th>Vol. (ul)</th>
</tr>
</thead>
<tbody>
<tr>
<td>TaqMan RT buffer</td>
<td>10</td>
<td>X</td>
<td>1</td>
<td>10.00</td>
</tr>
<tr>
<td>MgCl₂</td>
<td>25</td>
<td>mM</td>
<td>5.5</td>
<td>22.00</td>
</tr>
<tr>
<td>dNTPs</td>
<td>2500</td>
<td>uM each</td>
<td>500</td>
<td>20.00</td>
</tr>
<tr>
<td>Random hexamers *</td>
<td>50</td>
<td>uM</td>
<td>2.5</td>
<td>5.00</td>
</tr>
<tr>
<td>RNase Inhibitor</td>
<td>20</td>
<td>U/ul</td>
<td>0.4</td>
<td>2.00</td>
</tr>
<tr>
<td>MultiScribe™ Reverse Transcriptase</td>
<td>50</td>
<td>U/ul</td>
<td>1.25</td>
<td>2.50</td>
</tr>
<tr>
<td><strong>SUB TOTAL</strong></td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>61.50</td>
</tr>
<tr>
<td>DNase/RNase-free water</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>36.50</td>
</tr>
<tr>
<td>RNA **</td>
<td>500</td>
<td>ng/ul</td>
<td>20</td>
<td>2.00</td>
</tr>
<tr>
<td><strong>TOTAL</strong></td>
<td></td>
<td></td>
<td></td>
<td>100</td>
</tr>
</tbody>
</table>

Table 2.8 Reverse Transcription master mix using Taqman® reverse transcription kit. Sample ribonucleic acid (RNA) isolated from cells using the RNeasy® kit (Qiagen) is added to the reverse transcription master mix in the above concentrations and then subsequently reverse transcribed using polymerase chain reaction (PCR). NTP, nucleotide triphosphate.
The reverse transcription mix was then loaded onto a thermocycler with the following settings: preheat lid to 95°C, 25°C for 10 minutes to allow primer annealing to RNA sample, 48°C for 30 minutes to allow reverse transcription by MultiScribe™ reverse transcriptase at its optimal temperature, 95°C for 5 minutes to denature MultiScribe™ reverse transcriptase to terminate reverse transcription, and cooling to 4°C. The thermocycle regimen for RT-PCR is summarised in table 2.9.

<table>
<thead>
<tr>
<th>Preheat Lid</th>
<th>Procedure</th>
<th>90°C</th>
<th>min</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>Annealing</td>
<td>25</td>
<td>10</td>
</tr>
<tr>
<td>2</td>
<td>Extension</td>
<td>48</td>
<td>30</td>
</tr>
<tr>
<td>3</td>
<td>Reverse Transcriptase</td>
<td>95</td>
<td>5</td>
</tr>
<tr>
<td></td>
<td>Denaturation</td>
<td></td>
<td></td>
</tr>
<tr>
<td>4</td>
<td>Pause</td>
<td>4</td>
<td>Pause</td>
</tr>
</tbody>
</table>

Table 2.9 Thermocycle for reverse transcription (RT)-PCR.

2.2.13 Quantitative real time PCR (qRT-PCR)

qRT-PCR was performed on cDNA that is generated using Taq fast universal 2x PCR Master Mix (Applied Biosystems) and Taqman primer and probes assay (Applied Biosystems). For a 20 μl reaction, 1μl of cDNA generated from RT-PCR was mixed with 10 μl of 2x PCR Master Mix (Life Technologies), 1 μl of Taqman primer and probes (Life Technologies) and 8 μl of DNase/RNase-free water (Gibco). All reactions were performed in triplicate using a 7500 Fast Real-time PCR system (Applied Biosystems). The qRT-PCR cycles is listed in table 2.10. The relative units were calculated from a standard curve generated from a control sample by plotting the average of triplicate log dilutions against the cycle threshold. Expression data for all genes were normalized to the housekeeping gene Hypoxanthine phosphoribosyltransferase 1 (Hprt1). qRT-PCR results were analyzed on GraphPad Prism (La Jolla, CA).
<table>
<thead>
<tr>
<th>Holding</th>
<th>Denaturation</th>
<th>Temperature (°C)</th>
<th>Time (min: sec)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Cycling Stage (40 cycles)</td>
<td>Denaturation</td>
<td>95</td>
<td>00:03</td>
</tr>
<tr>
<td>Cycling Stage (40 cycles)</td>
<td>Amplification</td>
<td>60</td>
<td>00:30</td>
</tr>
</tbody>
</table>

Table 2.10 Thermocycle in ABI7500 FAST (Life Technologies) for qRT-PCR.

2.2.14 Fibronectin Enzyme Linked Immunosorbent Assay (ELISA)

Fibronectin ELISA was obtained from AbCam (ab108849) and carried out according to manufacturer’s instructions with the following exceptions: Fibronectin Standard range was set at 0.78 to 50.00 ng/ml, and chromogen substrate incubation was for 4 minutes only instead of the 10 minutes recommended in the protocol due to colour saturation of the standard curve.

2.2.15 Meso-Scale Discovery (MSD)

Mouse TH1/TH2 9-Plex Ultra-Sensitive Kit (Part number: 15013C-2) [IFN-Υ, IL-1β, IL-2, IL-4, IL-5, IL-10, IL-12 p40 (total), CXCL1, and TNF-α] and custom Mouse 10-plex MSD (IL-13, IL-6, IL-1β, CCL2, CCL3, CCL4, CCL5, CCL20, CXCL2, and mMMP-9) was obtained from Meso-Scale Diagnostics LLC. MSD was carried out according to manufacturer’s instructions.

2.2.16 AMφ subsets generated ex vivo for flow cytometric analysis

For flow cytometric analysis in naïve mice, 3 x 10^4 AMφ were cultured in Corning Costar® 6 Well Clear Flat Bottom Ultra Low Attachment Multiple Well Plates to prevent binding and preserve cell surface proteins (Corning). For M1-associated MHC-II (IA/IE) analysis, AMφ were primed with media or 20 ng/ml of IFN-Υ for 24 hours. For M2-associated MRC1 analysis, AMφ were primed with media or 10 ng/ml of IL-13 for 72 hours. AMφ were then collected by gentle scraping followed by centrifugation of culture media, and stained for CD11c, MHC-II and MRC1 as described later.

2.2.17 Flow Cytometry

For each antibody-stained sample analysed, there is an unstained control (without staining) and an isotype control (consisting of isotype control antibodies for every different type of detection antibody used), which act as negative controls for background signals and non-specific antibody binding respectively (i.e. 3 tubes per sample analysed). For each tube, cells were resuspended at 1x10^4 cells per 100 μl Fluorescence Activated Cell Sorting (FACS) analysis buffer (DPBS + 0.1% FCS), 1% of Fc Receptor (FcR) block (Miltenyi Biotech) and 5% mouse serum per 100μl reaction. FcR block was added...
to prevent non-specific binding of the Fc (constant) region of detection antibodies/isotype controls to cell surface FcR that may generate a false positive signal. Mouse serum was added to prevent non-specific binding of the variable region of detection antibodies/isotype controls. For extracellular proteins, cells were stained with the appropriate antibodies for 30 minutes in the dark at 4°C by the appropriate antibodies. BAL cells were then washed in 2 ml DPBS and centrifuged at 500 g for 10 minutes at 4°C. The supernatant was then discarded and BAL cells were fixed and permeabilised with 300 μl BD Cytofix/Cytoperm™ (BD Biosciences) for 20 minutes in the dark at 4°C. BAL cells were then washed with 2 ml BD Perm/Wash™ (BD Biosciences). For extracellular staining only, stained cells are then resuspended in 500 μl 1 x Cell Fix (BD Biosciences) (diluted 1:10 from 10x stock solution), and kept in the dark at 4°C until further analysis.

For intracellular staining, permealised BAL cells were stained with primary or directly conjugated antibodies for 30 minutes in the dark at 4°C, and then washed with 2 ml BD Perm/Wash™ (BD Biosciences). This step was repeated for staining with secondary antibodies. Stained cells were then resuspended in 500 μl 1 x Cell Fix (BD Biosciences), and kept at 4°C until further analysis. Details for the concentrations of individual antibodies used will be described further in the text.

Anti-Rat/anti-Hamster IgK compensation kit (BD Biosciences) was used on the day of flow cytometric analysis to compensate for fluorophore spectral overlap in multi-colour staining. The set consists of two populations of microparticles, including the BD CompBeads Anti-Rat/Hamster Ig, κ particles (positive control), which bind any rat or hamster κ light chain-bearing immunoglobulin, and the BD CompBeads Negative Control (FBS) Particles (negative control), which has no binding capacity. When mixed together with a fluorochrome-conjugated rat or hamster antibody, the BD-CompBeads provide distinct positive and negative (background fluorescence)-stained populations that can be used to set compensation levels manually. Anti-Rat/anti-Hamster IgK compensation kit (BD Biosciences) was prepared according to manufacturer’s instructions. In brief, 60 μl of BD CompBeads Anti-Rat/Hamster Ig, κ particles and 60 μl of BD CompBeads Negative Control (FBS) particles were added to 100 μl FACS buffer (+0.1 % FBS), and 20 μl of pre-diluted antibody stock (where final concentration in 220 μl is equivalent to that used in experiments) was subsequently added. This process was carried out separately for every different type of fluorophore that was used in multicolour experiments. The BD CompBeads Anti-Rat/Hamster Ig, κ particles/BD CompBeads Negative Control (FBS) particles/antibody mixture was then incubated in the dark at 4°C for 30 minutes. The mixture was then washed with 2 ml FACS buffer and centrifuged at 500 g for 10 minutes. The supernatant containing unbound antibodies was discarded, and the pellet containing Compbeads was resuspended in 500 μl FACS buffer.
FACS data was read by FACS CANTO II (BD Biosciences). Prior to recording data for samples, compensation for spectral overlap was determined from the pre-stained BD CompBeads Anti-Rat/Hamster Ig, κ particles/ BD CompBeads Negative Control (FBS) particles using the compensation setup on the FACS CANTO II software (BD Biosciences). FACS data was analyzed by Flowjo (Tree Star Inc). Cell populations were identified using sequential gating strategy (see results) and the expression of the protein of interest was presented as median fluorescence intensity (MFI) and % positive with respect to isotype controls.

2.2.18 Statistical analysis
All statistical analysis was carried out using GraphPad Prism 6.0 software (GraphPad software, CA, USA). Normality distribution was evaluated by visual inspection of data. When comparing two groups unpaired two-tailed t-tests (followed by Welch’s correction test for non-equal standard deviations) and Mann-Whitney tests were used for parametric and non-parametric datasets, respectively. When comparing more than two groups, One-way ANOVA followed by Tukey’s or Bonferroni’s multiple comparison test and Kruskal Wallis test followed by a Dunn’s multiple comparison test were used for parametric and non-parametric datasets, respectively. Two-way ANOVA was used to compare time-course curves followed by Sidak’s multiple comparison test to determine the significance. P values of less than 0.05 were deemed statistically significant with *** p < 0.001, ** p <0.01 and * p < 0.05. Error bars represent standard deviation (SD) as indicated in the figure legends unless otherwise specified.
Chapter 3
The heterogeneity of alveolar macrophages \textit{in vitro}
Chapter 3 | The heterogeneity of AMφ in vitro

3.1 Introduction

Macrophages display plasticity and heterogeneity between different tissues. Within the local environment the phenotypes of macrophages can be modified by the requirements of a specific tissue during normal and pathological processes to generate subsets that are tailored to carry out specific functions. These phenotypes initially defined by in vitro studies can be broadly categorized into ‘classical’ M1 macrophages that may contribute to antimicrobial response and ‘alternative’ M2 and M2-like macrophages that may contribute to wound healing and immunoregulation respectively. However, this categorization may be an oversimplification of the situation in vivo, where a variety of macrophage inducing signals are likely to exist within the tissue environment and to vary over time. Quantitatively and qualitatively different types of signals have been identified that contribute to tissue macrophage heterogeneity. These include weak priming signals that may influence the inflammatory potential of macrophages and their response to other stimuli, or strong inflammatory signals initiated by PRR stimulation.

There has been some confusion in the comparison between classical and alternative macrophage subsets, in that M1 macrophages can be generated by both a priming and/or a stimulatory signal (IFN-γ and/or LPS respectively), whereas M2 macrophages can be generated with a priming signal only (IL-4 or IL-13). Given that both priming and stimulatory signals can activate their own gene-regulatory programme, this broad definition may likely obscure differences between different priming signals and also the stimulatory signal. The substantial differences observed between the two subsets may actually be solely dependent on the presence or absence of an antimicrobial stimulus.
The effects of priming and/or stimulatory signals on macrophage phenotype have been investigated in *in vitro* studies. BMMφ were primed with IFN-γ, IL-4 or media control, and then cultured with or without LPS. Although these two cytokines often induce opposing effects on gene transcription, the subsequent activation of BMMφ by LPS produced a strong, priming-dependent pro-inflammatory response in both macrophage phenotypes. For example, the gene expression of several key pro-inflammatory cytokines, including IL-6 and IL-12α (gene encoding the IL-12 p35 subunit) was significantly higher in IL-4 compared to IFN-γ-primed macrophages. On the other hand, in a subset of genes (e.g. IL-12α), IFN-γ priming was actually found to suppress LPS-induced gene expression in a STAT-1 dependent manner. These data suggested that IL-4 priming is not anti-inflammatory *per se*, but generates a tissue protective ‘hybrid’ macrophage subset phenotype that is also capable of generating a pro-inflammatory response following TLR4-stimulation. In the context of tissue repair and fibrosis, endogenous alarmins are often found at sites of tissue injury. These may act as stimulatory signals to various macrophage PRRs, in addition to the M2-priming cytokines IL-4 and IL-13, to influence macrophage phenotypes. The hybrid subset may therefore better represent the macrophage phenotype in tissue repair *in vivo*. There have been no studies directly addressing the heterogeneity in AMφ; all attempts to subdivide AMφ into subsets have been based on the basis of experiments that examined these cells during pulmonary diseases using subset-associated markers pre-defined in BMMφ.

<table>
<thead>
<tr>
<th>Priming</th>
<th>Stimulation</th>
<th>Subset Associated markers</th>
<th>Function</th>
</tr>
</thead>
<tbody>
<tr>
<td>M1</td>
<td>IFN-γ&lt;sup&gt;340&lt;/sup&gt;</td>
<td>TNF-α, LPS&lt;sup&gt;340&lt;/sup&gt;</td>
<td>NOS2, MHC-II, TNF-α, IL-1β, IL-6, IL-12&lt;sup&gt;739&lt;/sup&gt;</td>
</tr>
<tr>
<td>M2</td>
<td>IL-4/IL-13&lt;sup&gt;282,305&lt;/sup&gt;</td>
<td>N/A</td>
<td>Arg-1, Chi3I3, MRC, Fibronectin, PDGF, IGF-1&lt;sup&gt;739&lt;/sup&gt;</td>
</tr>
<tr>
<td>M2-like</td>
<td>IL-10/TGF-β/PGE&lt;sup&gt;306,740&lt;/sup&gt;</td>
<td>+ TLR ligand&lt;sup&gt;306,740&lt;/sup&gt;</td>
<td>IL-10, TGF-β&lt;sup&gt;740&lt;/sup&gt;</td>
</tr>
</tbody>
</table>

Table 3.1 Summary of macrophage subset inducing priming and stimulation signals, and subset associated markers. Arg-1, Arginase-1; Chi3I3, Chitinase 3 like 3; IFN-Y, Interferon-Y; IGF-1, insulin-like growth factor-1; IL, interleukin; LPS, Lipopolysaccharide; MHC, Major Histocompatibility Complex; MRC1, Mannose Receptor C, type 1; N/A, not applicable; NOS2, Nitric Oxide Synthase 2; PDGF, platelet derived growth factor; PGE, prostaglandin E; TGF-β, transforming growth factor-β; TNF-α, tumour necrosis factor-α.
In healthy humans, there is no general consensus about whether AMφ are M1- or M2-like in nature. There has been great variation in the number of MRC1-expressing M2 AMφ reported in humans, ranging from 8% in one study\textsuperscript{741}, to 50% in another study\textsuperscript{573}. Around 20% of human AMφ have been reported to express stabilin-1 (STAB-1), another protein that is associated with M2 in humans\textsuperscript{741}. These conflicting reports highlight the fact that AMφ in healthy individuals do not neatly fit into either a strict M1 or M2 classification, and it may be that a resting lung-resident macrophages cannot necessarily be driven to become one of these defined cell subtypes.

There are many potential signals that may potentially alter macrophage phenotypes within the lung of IPF patients. As mentioned above, the M2-inducing signal IL-13 has been reported to be upregulated in the lungs of IPF patients\textsuperscript{580}. Similarly the M2-like inducing signal TGF-β1 also is more abundantly expressed in the lungs of rodent models of pulmonary fibrosis and IPF patients\textsuperscript{554,561,742–745}. Hypoxic conditions are also commonly found in fibrotic lungs due to loss of alveolar architecture as a result of prolonged lung injury and dysregulated wound healing. The extreme local hypoxia is a consequence of decreased perfusion, which is secondary to microvascular injury, thrombosis or increased interstitial pressure, coupled with the metabolic activities of the recruited inflammatory cells\textsuperscript{746}. Hypoxic conditions in wounds or necrotic tissue sites have been described as less than 1% oxygen in comparison to 2.5% to 9% found in healthy tissues\textsuperscript{747–749}.

It was discovered that hypoxia share the TGF-β1 signalling pathway to promote a pro-fibrotic response in MH-S cells (an AMφ derived cell line) by protecting the transcription factor HIF-1α from degradation and thereby allowing increased secretion of pro-fibrotic mediators including PAI-1 and PDGF-AA. Silencing of HIF-1α by siRNA abolishes TGF-β induction of PAI-1 and PDGF-AA upregulation\textsuperscript{261}.

3.2 Hypothesis

To emphasize my comments above although a number of studies have been carried out to characterise the functionality of different subsets of BMMφ, few investigations have explored the phenotype of primary lung macrophages induced by different signalling pathways. Therefore reports of macrophage subsets identified in pulmonary diseases have largely been based on subset-associated markers characterised in BMMφ.

While BMMφ subsets may provide a useful in vitro model, it should be highlighted that these cells are artificially generated from bone marrow cells by application of a single inducing mediator [Granulocyte Macrophage Colony Stimulating Factor (GM-CSF) in humans; Macrophage Colony Stimulating Factor (M-
CSF) in mice, and may not truly represent a terminally-differentiated tissue resident lung macrophage that are influenced by multiple signals within its immediate environment. It is hypothesised that terminally differentiated AMφ display similar plasticity to external stimuli that has been observed in BMMφ in vitro, and may be induced into distinct subsets through priming signals ex vivo. Furthermore, stimulatory signals, such as LPS, will be able to alter the phenotype of AMφ subsets.

### 3.3 Aims

The overall aim of this chapter was to explore the heterogeneity of AMφ with respect to mRNA and protein levels in response to priming (media control, IFN-Υ, IL-4 and/or IL-13, hypoxia, and TGF-β) signals to generate distinct subsets that were observed in BMMφ, and whether or not the gene and/or protein expression of these subsets could be modified by stimulatory signals (LPS). More specifically, this chapter aims to:

1. Validate macrophage polarisation protocols obtained from literature by priming BMMφ with the M1- or M2-inducing cytokines, IFN-Y or IL-4/ IL-13 respectively.
2. Investigate the response of AMφ to the M1- or M2-inducing cytokines, IFN-Y or IL-4/ IL-13 respectively. IL-13 is a TH2 cytokine with similar properties to IL-4; both cytokines signal through a common Type II IL-4R dimer, resulting in the activation of the STAT6 pathway. IL-13 was used instead of IL-4 for the priming of AMφ, as it is considered more disease relevant in IPF, as IL-13 has been found to be upregulated in IPF patients.
3. Investigate the response of AMφ to hypoxic conditions or TGF-β1, which are commonly found in the fibrotic lung.
4. The modulatory effects of LPS on the gene and/or protein expression of these BMMφ or AMφ subsets were also reviewed.

The studies described here are the first of their kind to systematically investigate various signals affecting macrophage phenotypes in terminally differentiated AMφ, and provide a useful insight into AMφ heterogeneity for future studies in various pulmonary diseases.
3.4 Methods

3.4.1 Generation of BMMφ and AMφ

BMMφ were generated and AMφ were isolated as described in chapter 2 (sections 2.2.7 and 2.2.8, and sections 2.2.4, 2.2.5 and 2.2.6 respectively). BMMφ and AMφ were primed for the polarization of macrophages into distinct subsets.

3.4.2 Priming and stimulation of BMMφ and/or AMφ

For the generation of M1- or M2-like macrophage subsets, 1 x 10^5 BMMφ or AMφ/ well of a 48-well tissue culture plate were primed with either macrophage complete media control (RPMI-1640 + 10% fetal bovine serum + 1.5 mM sodium pyruvate + 0.05 mM β-mercaptoethanol + 1 mM penicillin/streptomycin + 50 μg/ml geneticin), 20ng/ml IFN-γ (R&D Systems), 10 ng/ml IL-4 (R&D Systems) or 10 ng/ml IL-13 (R&D Systems) and incubated for 18 hours at 37°C, 5% CO2, 20% O2. To evaluate the alterations of macrophage mRNA and/or protein levels to PRR stimulation, cytokine-primed BMMφ/AMφ were further cultured with or without 10 ng/ml LPS-EB (Invivogen), for another 4 hours for mRNA level analysis, or for 24 hours for measurements of protein levels in the AMφ supernatant.

For the priming of AMφ with lung-fibrosis associated signals, 1 x 10^5 AMφ/ well a 48-well plate were serum-starved for 24 hours, and then primed with either macrophage complete media control (RPMI-1640 + 10% fetal bovine serum + 1.5 mM sodium pyruvate + 0.05 mM β-mercaptoethanol + 1 mM penicillin/streptomycin + 50 μg/ml geneticin), 10 ng/ml TGF-β1 (R&D Systems) or subject to hypoxia (1% O2, 5% CO2) for 18 hours. AMφ were serum starved prior to hypoxia or TGF-β1 priming as fetal bovine serum (FBS) contains TGF-β that may activate the TGF-β induced HIF-1α pathway that is under investigation (section 3.1). To evaluate the alterations of macrophage mRNA and/or protein levels to PRR stimulation, hypoxia or TGF-β1-primed AMφ were then cultured with or without 10 ng/ml LPS-EB (Invivogen), for another 4 hours for gene transcript level analysis, or for 24 hours for measurements of protein levels in the AMφ supernatant.

3.4.3 Assessment of mRNA and protein levels

Total RNA extraction, reverse transcription, and qRT-PCR for mRNA level analysis were carried out as described at sections 2.2.11, 2.2.12 and 2.2.13 respectively. Measurements of proteins of interest in culture supernatant was measured as mentioned in sections 2.2.14 (fibronectin enzyme linked immunosorbent assay (ELISA) and 2.2.15 [Meso-Scale Discovery (MSD)].
3.4.4 Flow cytometry experiments for differentially primed AMφ

AMφ were cultured at 3 x 10^5 cells/well in a Corning Costar® Ultra-Low attachment culture plate (Sigma-Aldrich) with macrophage complete media control (RPMI-1640 + 10% fetal bovine serum + 1.5 mM sodium pyruvate + 0.05 mM β-mercaptoethanol + 1 mM penicillin/streptomycin + 50 μg/ml geneticin), 20ng/ml IFN-γ (R&D Systems), or 10 ng/ml IL-13 (R&D Systems) for 24 or 72 hours. Corning Costar® Ultra-Low attachment culture plate were used to avoid AMφ adherence, as mechanical (gentle scraping) or enzymatical (trypsin digestion) of cells may damage cell surface proteins under investigation. There was expected to be little contamination from other cell types as AMφ was found to constituted over 90% of BAL cells in naïve mice from differential cell count (section 2.2.5).

After 24 or 72 hours of incubation, each sample of 3 x 10^5 cells/well were separated into 3 tubes (i.e. 1 x 10^5 cells/tube), one for positive staining for surface proteins of interest (CD11c, MHC-II and MRC1), one for unstained negative control, and one for isotype control of nonspecific antibody binding. For each tube, cells were resuspended at 1x10^5 cells per 100 μl Fluorescence Activated Cell Sorting (FACS) analysis buffer (DPBS + 0.1% FCS), 1% of Fc Receptor (FcR) block (Miltenyi Biotech) and 5% mouse serum per 100μl reaction. FcR block was added to prevent non-specific binding of the Fc (constant) region of detection antibodies/ isotype controls to cell surface FcR that may generate a false positive signal. Mouse serum was added to prevent non-specific binding of the variable region of detection antibodies/ isotype controls.

For the positive staining tube, cells were incubated with FACS analysis buffer, 0.25 μg/100 μl CD11c-APC, 0.25 μg/100 μl MHC-II-FITC and 0.5 μg/100 μl MRC1-PerCP/Cy5.5. For the isotype control tube, cells were stained with the isotype controls 0.25 μg/100 μl Armenian hamster IgG-APC, 0.25 μg/100 μl Rat IgG2b-FITC and 0.5 μg/100 μl Rat IgG2a-PerCP/Cy5.5 for 30 minutes in the dark at 4°C. For the unstained control, cells were incubated with FACS analysis buffer only.

BAL cells were then washed in 2 ml DPBS and centrifuged at 500 g for 10 minutes at 4°C. The supernatant was then discarded and BAL cells were fixed and permeabilised with 300 μl BD Cytofix/Cytoperm™ (BD Biosciences) for 20 minutes in the dark at 4°C. BAL cells were then washed with 2 ml BD Perm/Wash™ (BD Biosciences). Cells were then resuspended in 500 μl 1 x Cell Fix (BD Biosciences) (diluted 1:10 from 10x stock solution), and kept in the dark at 4°C until further analysis.

Anti-Rat/ anti-Hamster IgK compensation kit (BD Biosciences) was used on the day of flow cytometric analysis to compensate for fluorophore spectral overlap in multi-colour staining. The set consists of two populations of microparticles, including the BD CompBeads Anti-Rat/Hamster Ig, κ particles (positive...
control), which bind any rat or hamster $\kappa$ light chain-bearing immunoglobulin, and the BD CompBeads Negative Control (FBS) Particles (negative control), which has no binding capacity. When mixed together with a fluorochrome-conjugated rat or hamster antibody, the BD-CompBeads provide distinct positive and negative (background fluorescence)-stained populations that can be used to set compensation levels manually. Anti-Rat/ anti-Hamster IgK compensation kit (BD Biosciences) was prepared according to manufacturer’s instructions. In brief, 60 $\mu$l of BD CompBeads Anti-Rat/Hamster Ig, $\kappa$ particles and 60 $\mu$l of BD CompBeads Negative Control (FBS) particles were added to 100 $\mu$l FACS buffer (+0.1 % FBS), and 20 $\mu$l of pre-diluted antibody stock (where final concentration in 220 $\mu$l is equivalent to that used in experiments) was subsequently added. This process was carried out separately for every different type of fluorophore that was used in multicolour experiments. The BD CompBeads Anti-Rat/Hamster Ig, $\kappa$ particles/ BD CompBeads Negative Control (FBS) particles/ antibody mixture was then incubated in the dark at 4°C for 30 minutes. The mixture was then washed with 2 ml FACS buffer and centrifuged at 500 g for 10 minutes. The supernatant containing unbound antibodies was discarded, and the pellet containing Compbeads was resuspended in 500 $\mu$l FACS buffer.

FACS data was read by FACS CANTO II (BD Biosciences). Prior to recording data for samples, compensation for spectral overlap was determined from the pre-stained BD CompBeads Anti-Rat/Hamster Ig, $\kappa$ particles/ BD CompBeads Negative Control (FBS) particles using the compensation setup on the FACS CANTO II software (BD Biosciences). FACS data was analyzed by Flowjo (Tree Star Inc). AMφ were defined as SSC$^+$ FSC$^+$ CD11c$, and the expression of the protein of interest was gated with respect to their respective isotype controls. Data was presented as median fluorescence intensity (MFI) and % positive with respect to isotype controls.

3.4.5 Statistical analysis
All statistical analysis was carried out using GraphPad Prism 6.0 software (GraphPad software, CA, USA). Normal distribution was evaluated by visual inspection of data due to insufficient numbers for normality tests. Data appeared to be parametric, and one-way ANOVA followed by Tukey’s test were used for statistical analysis for comparison of three or more sets of data over one parameter. P values of less than 0.05 were deemed statistically significant with *** $p < 0.001$, ** $p <0.01$ and * $p < 0.05$. Error bars represent standard deviation (SD) as indicated in the figure legends.
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3.5 Results

3.5.1 Preliminary experiments

In the beginning of this PhD, several preliminary experiments (n=1) were carried out to validate macrophage priming (media control, IFN-Υ or IL-4) and/or stimulation (LPS) protocols in BMMφ, and to explore AMφ heterogeneity in response to these and other pro-fibrotic signals (e.g. IL-13, TGF-β and hypoxia).

The change in mRNA and/or protein levels of AMφ in response to macrophage subset-inducing cytokines, including M1-inducing IFN-Υ and M2-inducing IL-4/IL-13, and also hypoxic conditions (1% O2, 5% CO2) or TGF-β1 that are commonly found in fibrotic tissues, were evaluated. The modulatory effect of PRR stimulation (LPS) on primed AMφ mRNA and protein levels were also assessed. The change in levels of a number of markers that have been characterised to distinct macrophage subsets [M1 (NOS2, IL-1β, MHC-II); M2 (Arg-1, fibronectin 1); TGF-β1 or hypoxia-primed macrophages (PAI-1)] mediators were investigated in these studies. The secreted protein levels of macrophage-associated mediators that may play a role in fibrosis, including IL-6, CCL2, CCL3, CCL4, CCL5, CXCL2, mMMP-9, IL-1β, were also evaluated for differentially primed AMφ to assess whether or not they are associated with a particular macrophage subset. Finally, the effect of LPS stimulation on differentially primed AMφ mRNA and protein levels were determined.

Due to the large amount of parameters and mediators that were under preliminary investigation, further repeats were carried out only for AMφ primed with IFN-Υ or IL-13 (n=3), but not the BMMφ and TGF-β1 or hypoxia-primed macrophage studies. These studies were selected for further investigation due to the novelty in investigating AMφ (vs. BMMφ) polarisation, and because IFN-Υ or IL-13-primed macrophages (versus TGF-β1 or hypoxia-primed macrophages) generated M1- or M2-like AMφ respectively that represented opposite ends of the macrophage polarisation spectrum. Characterisation of these subsets can therefore provide a greater scope of the various changes in mRNA and protein levels in response to diverse signals.

In the following section, results from preliminary studies (n=1), including BMMφ priming and/or stimulatory studies, and also TGF-β1 or hypoxia-primed AMφ are reported first (section 3.5.1). These preliminary results (n=1) are not statistically significant without further repeats; but may provide clues on how BMMφ/ AMφ may behave in response to the inducing signals under investigation. Results from preliminary experiments are then followed by those from repeated studies, including mRNA, surface protein, and secreted protein level analyses studies in IFN-Υ or IL-13-primed AMφ (section 3.5.2).
3.5.1.1 Differentially primed BMMφ express macrophage subset associated markers

3.5.1.2 Investigation of the potential modulatory effects of cytokine priming and or LPS stimulation on AMφ mRNA and protein levels

Previous publications have reported a change in BMMφ phenotype in response to priming in vitro; these changes were correlated with the priming signal used. In the experiments reported here similar conditions are used to determine whether or not disease relevant primary cells which contribute to the pathophysiological host response in a variety of respiratory diseases, namely AMφ, have the potential to be influenced in a comparable manner. Priming conditions for the generation of the macrophage subsets are first to be validated in BMMφ, and this would then provide a basis for investigating and characterizing the phenotype of AMφ primed under similar conditions.

3.5.1.2.1 Differentially primed BMMφ express macrophage subset associated markers

In order to validate the priming conditions for macrophage subsets described in previous publications, BMMφ were cultured under the same conditions and analysed for the mRNA levels of type I (M1) (NOS2) or type 2 (M2)- (Arg-1, Chi3l3 and MRC1) associated mediators following priming by the M1-inducing cytokine, IFN-Υ, or M2-inducing cytokine, IL-4 respectively. The increased levels of these mediators in response to their corresponding inducing cytokines would serve as a positive control for further cytokine-priming experiments in AMφ polarisation, which is the primary objective of these experiments. However, it should be noted that the mRNA levels of M2-associated mediators were not evaluated in M1, nor M1-associated mediators in M2 BMMφ. In other words, these experiments only reveal whether or not priming signals can induce a change in mediator mRNA levels in BMMφ, but cannot fully characterise BMMφ polarisation (whether or not M1 or M2 BMMφ have been generated).
In the BMMϕ-priming experiments, IFN-Υ-primed BMMϕ displayed increased NOS2 mRNA levels, whereas Arg-1, Chi3l3, and MRC1 mRNA levels was elevated in IL-4 primed BMMϕ (Figure 3.1). These results demonstrate that as reported in the literature, BMMϕ increase different gene transcripts in response to different cytokine priming.

Figure 3.1 Effect of cytokine priming on mRNA levels of macrophage subset markers in Bone Marrow Derived Macrophages (BMMϕ). 1 x 10^5 BMMϕ were seeded per well of a 48-well culture plates, and primed with macrophage complete media control (RPMI-1640 + 10% fetal bovine serum + 1.5 mM sodium pyruvate + 0.05 mM β-mercaptoethanol + 1 mM penicillin/streptomycin + 50 μg/ml geneticin) alone or together with 20 ng/ml Interferon-Υ (IFN-Υ) or 10 ng/ml Interluekin-4 (IL-4) for 22 hours. Nitric Oxide Synthase (NOS) 2, Arginase-1 (Arg-1), Mannose Receptor C, type 1 (MRC1), and Chitinase 3 like 3 (Chi3l3) mRNA levels were measured by Quantitative Reverse Transcription Polymerase Chain Reaction (qRT-PCR). Data are from one pilot study collected from pooled samples run in triplicates and the average was presented as relative % to the housekeeping gene Hypoxanthine Phosphoribosyltransferase 1 (HPRT-1). ND: Not detected.
3.5.1.2.2 Differentially primed BMMφ have distinct responses to LPS stimulation

TLR activation is generally ascribed to modifying macrophages to promote a pro-inflammatory phenotype. However this phenotype may be differentially regulated in various macrophage subsets. M1 macrophages have been described to promote inflammation through the release of pro-inflammatory mediators, ROS, and RNS, and augment the pro-inflammatory response following TLR stimulation. M2 macrophages have a wound healing phenotype that is characterised by Arg-1 expression\textsuperscript{581,582} and expression of various wound healing mediators (e.g. PDGF and fibronectin)\textsuperscript{120,520}, but have also been reported to have increased expression of pro-inflammatory mediators in response to LPS stimulation\textsuperscript{738}.

In order to validate these observations, a pilot study was carried out. M1 and M2 macrophages were generated as before by priming BMMφ with IFN-Υ or IL-4; these were then further stimulated with LPS for TLR4 activation.
3.5.1.2.1 IFN-Y primed macrophages have an exaggerated pro-inflammatory response to LPS stimulation

As has been reported by others\textsuperscript{282,340,738,739}, LPS stimulation promoted a pro-inflammatory response and increased both NOS2 and IL-1\(\beta\) gene transcript levels. IFN-Y-primed macrophages have an exaggerated response to LPS stimulation, which strongly augmented increased transcript levels of NOS2 by around 4-fold and to a lesser extent IL-1\(\beta\). On the other hand, IL-4-primed macrophages have a dampened pro-inflammatory response to LPS stimulation and reduced LPS-induced increase in IL-1\(\beta\) mRNA levels (Figure 3.2).

As has been reported by others\textsuperscript{283,341,738,739}, LPS stimulation promoted a pro-inflammatory response and increased both NOS2 and IL-1\(\beta\) gene transcript levels. IFN-Y-primed macrophages have an exaggerated response to LPS stimulation, which strongly augmented increased transcript levels of NOS2 by around 4-fold and to a lesser extent IL-1\(\beta\). On the other hand, IL-4-primed macrophages have a dampened pro-inflammatory response to LPS stimulation and reduced IL-1\(\beta\) upregulation (Figure 3.2).

![Figure 3.2 The effects of Lipopolysaccharide (LPS) stimulation on mRNA levels of M1-associated markers in differentially primed BMMΦ.](image)

Figure 3.2 The effects of Lipopolysaccharide (LPS) stimulation on mRNA levels of M1-associated markers in differentially primed BMMΦ. \(1 \times 10^5\) BMMΦ were seeded per well of a 48-well culture plates, and primed with macrophage complete media control (RPMI-1640 + 10% fetal bovine serum + 1.5 mM sodium pyruvate + 0.05 mM \(\beta\)-mercaptoethanol + 1 mM penicillin/streptomycin + 50 \(\mu\)g/ml geneticin) alone or together with 20 ng/ml Interferon-Y (IFN-Y) or 10 ng/ml Interleukin-4 (IL-4) for 18 hours. Primed BMMΦ were then further cultured with macrophage complete media control or 10 ng/ml LPS from E. coli 0111:B4 for 4 hours. NOS2 and IL-1\(\beta\) mRNA levels were measured by qRT-PCR. Data are from one pilot study collected from pooled samples run in triplicates and the average was presented as relative % to the housekeeping gene HPRT-1. ND: Not detected.
3.5.1.2.2 LPS stimulation shifts macrophage subset bias away from M2 phenotype

LPS stimulation promoted a pro-inflammatory phenotype, and was synergistic to increased levels of NOS2 and IL-1β gene transcripts in BMMφ induced by IFN-Υ priming (Figure 3.2). In contrast, LPS stimulation shifts the macrophage subset bias away from an IL-4 primed phenotype, as observed by the decline in Arg-1 and Chi3l3 transcript levels in IL-4-induced BMMφ by approximately 25% and 50% respectively (Figure 3.3). These results demonstrate that LPS stimulation of TLR4 may alter macrophage phenotype driven by cytokine priming.

Figure 3.3 The effects of Lipopolysaccharide (LPS) stimulation on mRNA levels of M2-associated markers in differentially primed BMMφ. 1 x 10^5 BMMφ were seeded per well of a 48-well culture plates, and primed with macrophage complete media control (RPMI-1640 + 10% fetal bovine serum + 1.5 mM sodium pyruvate + 0.05 mM β-mercaptoethanol + 1 mM penicillin/streptomycin + 50 μg/ml geneticin) alone or together with 20 ng/ml IFN-Υ or 10 ng/ml IL-4 for 18 hours. Primed BMMφ were then further cultured with macrophage complete media control or 10 ng/ml LPS from E. coli 0111:B4 for 4 hours. Arg-1 and Chi3l3 mRNA levels were measured by qRT-PCR. Data are from one pilot study collected from pooled samples run in triplicates and the average was presented as relative % to the housekeeping gene HPRT-1.
3.5.1.3 Investigation of the potential modulatory effects of pro-fibrotic conditions on AMφ phenotypes

Having shown that the mRNA levels of various macrophage subset associated genes can be differentially altered by different cytokine priming in BMMφ, I then investigated whether or not the cytokines IFN-Y or IL-13, or fibrotic conditions including TGF-β or hypoxic conditions can also alter macrophage mRNA and protein levels. Studies regarding IFN-Y and IL-13 were repeated and will be further discussed in section 3.5.2; in this section I will focus on the preliminary results from TGF-β or hypoxia-induced AMφ.

TGF-β is elevated in the BALF and lung tissue of IPF patients, and is able to induce fibrosis in vivo. TGF-β priming of BMMφ has been reported to promote a M2-like phenotype that is anti-inflammatory. It may also promote fibrosis through increased fibrin deposition by stabilisation of the transcription factor HIF-1α. Furthermore, it has been documented that TGF-β can reduce expression of the HIF-1α inhibitor Propyl Hydroxylase Domain (PHD)-2 in MH-S cells (a murine AMφ derived cell line), thereby promoting the downstream transcription of PAI-1, leading to inhibition of the fibrinolytic enzyme plasminogen activation.

Hypoxic conditions arise in lungs of IPF patients due to an injury to alveolar capillary membrane and impaired gaseous exchange, which cause cell stress and an increase in HIF-1α that can regulate expression of a number of genes that drive remodelling and fibrosis. Hypoxia can promote fibrosis through reducing PHD-2 in MH-S cells, thereby promoting HIF-1α activation, PAI-1 transcription and plasminogen inhibition, resulting in the activation of plasmin and accumulation of fibrin. Plasmin can promote fibrosis through the proteolysis and activation of its pro-fibrotic substrates (e.g. MMPs) and also promote cellular infiltration via the fibrin clot.

AMφ cultured with TGF-β1 or hypoxic conditions were analysed for changes in mRNA levels for two L-Arginine catalytic enzymes, NOS2 and Arg-1, which are associated with M1- or M2-BMMφ (and AMφ – see section 3.5.2.1) respectively, and pro-fibrotic mediators, including PAI-1, TGF-β and PDGF-β.

3.5.1.3.1 Differentially primed AMφ express macrophage subset associated markers

The results of TGF-β1 priming and hypoxia induction suggested that there was a trend in the increase of PAI-1 mRNA levels in AMφ by 5-fold and 7-fold respectively (Figure 3.4). In TGF-β1 primed AMφ there appeared to be an increase in the pro-fibrotic mediators TGF-β1 and PDGF-β, but no such elevation was observed for hypoxia induced AMφ (Figure 3.5). There was an enhancement in both M1 associated NOS2 and M2 associated Arg-1 gene transcript levels in hypoxia-induced AMφ, although no change was observed for TGF-β1 primed AMφ (Figure 3.6).
These results suggested that hypoxia and TGF-β1 are both potent signals to alter macrophage phenotypes. Hypoxia appears to promote a mixed M1-like and M2-like phenotype, increasing the transcript levels of both the M1-associated gene NOS2 and the M2-associated gene Arg-1. Hypoxia induction also triggered an increase in PAI-1 transcript levels that may promote fibrin accumulation and fibrosis. On the other hand, TGF-β favoured a pro-fibrotic phenotype, inducing elevated transcript levels for the pro-fibrotic mediators PAI-1, TGF-β and PDGF-β. Hypoxia and TGF-β have been reported to share a common HIF-1α signalling pathway to upregulate pro-fibrotic mediators such as PAI-1\textsuperscript{261}. However, other hypoxia induced HIF-1α independent pathways (e.g. Hif-2α) may also contribute to the fibrotic process, as the results presented here reveal a difference in induction of various pro-fibrotic mediators and macrophage-subset associated proteins by these two signals.
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Figure 3.4 Effect of TGF-β priming or hypoxia induction on mRNA levels of Plasminogen Activator Inhibitor (PAI-1) in AMφ. 1 x 10^5 AMΦ were seeded per well of 48-well culture plates, and primed with macrophage complete media control (RPMI-1640 + 10% fetal bovine serum + 1.5 mM sodium pyruvate + 0.05 mM β-mercaptoethanol + 1 mM penicillin/streptomycin + 50 μg/ml geneticin), 5 ng/ml TGF-β1 or hypoxia (<1% O2, 5% CO2) for 22 hours. PAI-1 mRNA levels was measured by qRT-PCR. Data are from one pilot study collected from pooled samples run in triplicates and the average was presented as relative % to the housekeeping gene HPRT-1.

Figure 3.5 Effect of TGF-β priming or hypoxia induction on mRNA levels of pro-fibrotic mediators in AMφ. 1 x 10^5 AMΦ were seeded per well of 48-well culture plates, and primed with macrophage complete media control (RPMI-1640 + 10% fetal bovine serum + 1.5 mM sodium pyruvate + 0.05 mM β-mercaptoethanol + 1 mM penicillin/streptomycin + 50 μg/ml geneticin), 5 ng/ml TGF-β1 or hypoxia (<1% O2, 5% CO2) for 22 hours. TGFβ1 and Platelet Derived Growth Factor (PDGF)-β mRNA levels were measured by qRT-PCR. Data are from one pilot study collected from pooled samples run in triplicates and the average was presented as relative % to the housekeeping gene HPRT-1.

Figure 3.6 Effect of TGF-β priming or hypoxia induction on mRNA levels of macrophage subset associated markers in AMφ. 1 x 10^5 AMΦ were seeded per well of 48-well culture plates, and primed with macrophage complete media control (RPMI-1640 + 10% fetal bovine serum + 1.5 mM sodium pyruvate + 0.05 mM β-mercaptoethanol + 1 mM penicillin/streptomycin + 50 μg/ml geneticin), 5 ng/ml TGF-β1 or hypoxia (<1% O2, 5% CO2) for 22 hours. NOS2 and Arg-1 mRNA levels were determined by qRT-PCR. Data are from one pilot study collected from pooled samples run in triplicates and the average was presented as relative % to the housekeeping gene HPRT-1. ND: Not detected.
3.5.1.3.2 Differentially primed AMΦ have distinct responses to LPS stimulation

Having identified that TGF-β1 or hypoxia that promote fibrotic responses can alter mRNA levels in AMΦ, it was next investigated whether or not such priming conditions will have affect AMΦ responses to TLR stimulation. The analysis of mRNA levels revealed that LPS stimulation induced a trend implying that PAI-1 is increased in hypoxia but not TGF-β1 primed AMΦ (Figure 3.7). It was also observed that LPS stimulation increased mRNA levels of the profibrotic mediators TGF-β and PDGF-β (Figures 3.8). This trend was accumulative for PDGF-β that had been increased following TGF-β priming (Figure 3.8). In addition, LPS stimulation was synergistic to the increased NOS2 gene transcript levels by 9-fold observed for hypoxia primed AMΦ (Figure 3.9), but on the other hand suppressed Arg-1 by about 30% (Figure 3.9).

![Figure 3.7 The effects of LPS stimulation on PAI-1 mRNA levels in differentially primed AMΦ.](image)

Figure 3.7 The effects of LPS stimulation on PAI-1 mRNA levels in differentially primed AMΦ. 1 x 10^5 AMΦ were seeded per well on 48-well culture plates, and primed with macrophage complete media control (RPMI-1640 + 10% fetal bovine serum + 1.5 mM sodium pyruvate + 0.05 mM β-mercaptoethanol + 1 mM penicillin/streptomycin + 50 μg/ml geneticin) or with 5 ng/ml TGF-β1 or hypoxia (<1% O2, 5% CO2) for 18 hours. Primed AMΦ were then further cultured with media control or 10 ng LPS-EB for 4 hours. PAI-1 mRNA level was determined by qRT-PCR. Data are from one pilot study collected from pooled samples run in triplicates and the average was presented as relative % to the housekeeping gene HPRT-1.

![Figure 3.8 The effects of LPS stimulation on pro-fibrotic mediator mRNA levels in differentially primed AMΦ.](image)

Figure 3.8 The effects of LPS stimulation on pro-fibrotic mediator mRNA levels in differentially primed AMΦ. 1 x 10^5 AMΦ were seeded per well on 48-well culture plates, and primed with macrophage complete media control (RPMI-1640 + 10% fetal bovine serum + 1.5 mM sodium pyruvate + 0.05 mM β-mercaptoethanol + 1 mM penicillin/streptomycin + 50 μg/ml geneticin), alone or with 5 ng/ml TGF-β1 or hypoxia (<1% O2, 5% CO2) for 18 hours. Primed AMΦ were then further cultured with media control or 10 ng LPS-EB for 4 hours. TGF-β and PDGF-β mRNA levels were determined by qRT-PCR. Data are from one pilot study collected from pooled samples run in triplicates and the average was presented as relative % to the housekeeping gene HPRT-1.
Figure 3.9 The effects of LPS stimulation on mRNA levels of macrophage subset associated markers in various differentially primed AMΦ. 1 x 10^5 AMΦ were seeded per well onto 48-well culture plates, and primed with macrophage complete media control (RPMI-1640 + 10% fetal bovine serum + 1.5 mM sodium pyruvate + 0.05 mM β-mercaptoethanol + 1 mM penicillin/streptomycin + 50 μg/ml geneticin), with 5 ng/ml TGF-β1 or hypoxia (<1% O2, 5% CO2) for 18 hours. Primed AMΦ were then further cultured control with media or 10 ng/ml LPS for 4 hours. NOS2 and Arg-1 mRNA levels were determined by qRT-PCR. Data are from one pilot study collected from pooled samples run in triplicates and the average was presented as relative % to the housekeeping gene HPRT-1. ND: Not detected.

3.5.1.3.3 Analysis of fibrosis-associated protein levels of differentially primed AMΦ subsets

Next the effect of AMΦ priming on protein levels of selected mediators was investigated. AMΦ primed with either IFN-Υ, IL-4, hypoxia or cultured in media alone (control cultures) were analysed for the production of various cytokines and chemokines that have been reported to play a role in IPF, including the pro-inflammatory cytokine IL-6, and the chemokines CCL2, CCL3, CCL4, CCL5, CXCL2, CXCL1, CXCL7, and MMP-9. The levels of another chemoattractant, CCL20 was also assessed.

CCL20 is predominantly expressed in lymph nodes, appendix, peripheral blood lymphocytes (PBL), liver, lung, AMΦ in the lungs of sarcoidosis patients and epithelial cells of the intestinal tissues. CCL20 exerts its chemotactic effects through binding to CCR6 that is expressed in DCs and lymphocytes. Synthetic or recombinant CCL20 is chemotactic for lymphocytes and DCs, and inhibits proliferation of myeloid progenitors in colony formation assays. The pro-inflammatory cytokine IL-6 may play a role in tissue injury while these chemokines listed above may contribute to the recruitment of pro-inflammatory myeloid cells including circulating monocytes (progenitors of terminally differentiated alveolar macrophages), to the lung in response to injury or infection. ELR+ CXC chemokines may also contribute to the pathogenesis of fibrosis through promoting angiogenesis (Section 1.2.3.1).
Results showed that CCL20 and MMP-9 protein levels were not detectable under various priming signals or LPS stimulation, suggesting that these mediators are not secreted by AMφ (data not shown). In the absence of TLR stimulation, IFN-Υ increased CCL2 protein levels; however, all the other mediators of interest were undetectable (data not shown). LPS stimulation alone increased CCL5 protein levels in AMφ culture supernatant, but had no effect on other mediators (data not shown). On the other hand, LPS stimulation of IFN-Υ primed AMφ increased IL-6 and CCL5 protein levels, which was not observed in IFN-Υ primed cells alone. CCL2 was also enhanced in IFN-Υ primed AMφ stimulated with LPS, although this increment appeared to be of no difference to that of AMφ primed with IFN-Υ alone. IL-4 primed AMφ also promoted the increased levels of CCL5 and IL-6 following LPS stimulation. However, hypoxia-primed AMφ strongly exaggerated the levels of various chemokines, including CCL2, CCL3, CCL4, CCL5, and CXCL2. These results indicate that hypoxia may be an important signal for induction of chemoattraction in AMφ (Figure 3.10).
Figure 3.10 The effects of differential cytokine priming and/or LPS stimulation on macrophage-associated protein levels in AMφ culture supernatant. 1 x 10^5 AMΦ were seeded per well of a 48-well culture plate, and primed with macrophage complete media control (RPMI-1640 + 10% fetal bovine serum + 1.5 mM sodium pyruvate + 0.05 mM β-mercaptoethanol + 1 mM penicillin/streptomycin + 50 μg/ml geneticin), 20 ng/ml IFN-γ, 10 ng/ml IL-4, or hypoxia (<1% O2, 5% CO2) for 18 hours. Culture media was changed (in the presence of priming cytokines) and AMφ were further cultured, with macrophage complete media control or with 10 ng/ml LPS-EB (Invivogen) for 24 hours. AMφ supernatant was collected and stored at -80°C prior to analysis. IL-6, CCL2, CCL3, CCL4, CCL5, and CXCL2 protein levels were detected by MSD. Data from pooled samples were ran in duplicates and presented as mean values. Data is from one pilot study. Dotted line refers to lower limit of detection. ND: Not detected. Standard curves for the MSDs are attached to Appendix 1.
3.5.2 Alveolar Macrophages (AMφ)

3.5.2.1 Differentially primed AMφ express macrophage subset associated markers

Having demonstrated that in vitro generated BMMφ differentiate into various subsets following differential cytokine priming earlier (section 3.5.1.1), the effect of priming tissue specific AMφ under conditions known to promote an M1 or M2 ‘like’ phenotype in BMMφ, were investigated next. AMφ are resident in the alveolar space and may facilitate the immune response to respiratory pathogens including bacteria and viruses, or endogenous alarmins that are released in response to tissue injury. There is evidence for increases in alarmins such as HA fragments and HMGB1 in the lungs or BALF or patients with pulmonary fibrosis.

In contrast to BMMφ, AMφ are considered terminally differentiated, and may be less responsive to exogenous priming signals. In order to investigate whether or not AMφ can also be induced to differentiate into M1-like or M2-like macrophages, primary AMφ isolated from mice were primed for 18 hours with either IFN-Υ or IL-13 respectively. Primed AMφ were then further cultured for 4 and 24 hours for mRNA and protein level analysis.

The analyses of mRNA levels of various macrophage subset associated mediators revealed that similar to BMMφ, AMφ can be induced to differentiate into distinct subsets. As with BMMφ, the level of transcript for the M1-associated gene NOS2 was elevated following IFN-Υ priming (p<0.05) whereas the level of transcript for the M2-associated gene Arg-1 was increased following IL-13 priming (p<0.01). Further mRNA level analysis showed that in IL-13 primed AMφ the gene transcript levels of the profibrotic mediators, including IGF-1 and fibronectin are enhanced by 10-fold (p<0.05) and 6-fold (p<0.01) respectively, suggesting a potential pro-fibrotic role for M2 macrophages (Figure 3.11).
Figure 3.11 Effect of cytokine priming on macrophage-subset associated mRNA levels of macrophage subset markers in AMφ. 1 x 10^5 AMφ were seeded per well of 48-well culture plates, and primed with macrophage complete media control (RPMI-1640 + 10% fetal bovine serum + 1.5 mM sodium pyruvate + 0.05 mM β-mercaptoethanol + 1 mM penicillin/streptomycin + 50 μg/ml geneticin) alone or together with 20 ng/ml IFN-Υ or 10 ng/ml IL-13 for 18 hours. Primed AMφ were then further cultured for 4 hours. NOS2, Arg-1, IGF-1, and fibronectin mRNA levels were measured by qRT-PCR. Pooled samples (per condition) were run in triplicates and the average was presented as relative % to the housekeeping gene HPRT-1. Three separate studies were carried out (n=3) and data are represented as mean ± SD. One way ANOVA with Tukey post-test was carried out; *: p<0.05, **: p<0.01, ***: p<0.005. ND, Not Detected.
Using ELISA and MSD analyses it was observed that differentially primed AMφ have distinct changes in protein levels. IFN-Υ primed AMφ produced significantly higher levels of pro-inflammatory cytokines, including IL-1β (p<0.05) and IL-12 p40 (total) (3.10-fold; p<0.05) than the untreated controls, whereas IL-13 primed AMφ produced higher levels of the ECM component fibronectin (2-fold; p<0.05) (Figure 3.12).

Figure 3.12 Effect of cytokine priming on protein levels of macrophage subset markers in AMφ. AMΦ were seeded onto culture plates, and primed with macrophage complete media control (RPMI-1640 + 10% fetal bovine serum + 1.5 mM sodium pyruvate + 0.05 mM β-mercaptoethanol + 1 mM penicillin/streptomycin + 50 μg/ml geneticin) alone or together with 20 ng/ml IFN-Υ or 10 ng/ml IL-13 for 18 hours. Primed AMΦ were then further cultured for 24 hours. IL-1β, IL-12, and fibronectin protein expression by Meso Scale Discovery (MSD) or Enzyme Linked Immunosorbent Assay (ELISA) respectively. Data are represented as mean ± SD; n=3 from three separate studies. One way ANOVA with Tukey post-test was carried out; *: p<0.05, **: p<0.01, ***: p<0.005, ****: p<0.001. NS: Not Significant. IL-12 p40 (total) includes free IL-12 p40, IL-12 p40 homodimers and IL-12 p40 heterodimers (IL-12 p70 and IL-23). See Appendix 2 for standard curves of individual ELISA/ MSD.
Flow cytometric analyses demonstrated that differentially primed AMφ have distinct cell surface marker expression. IFN-γ priming strongly promoted the surface expression of MHC-II at 24 hours but suppressed that of MRC1, and therefore can be classified as MHC-II\textsuperscript{hi} MRC1\textsuperscript{-} (Figure 3.13). Three peaks were observed in MHC-II staining for IFN-γ-primed AMφ, including a MHC-II\textsuperscript{-} MHC-II\textsuperscript{mid} and a MHC-II\textsuperscript{hi} subsets at 24 hours (Figure 3.13). These three peaks may represent heterogeneous AMφ populations within the lung that have different levels of responses to IFN-γ, perhaps due to inherent immunosuppression by endogenous anti-inflammatory mediators \textit{in vivo} that act as a homeostasis mechanism against frequently encountered airborne pathogens within the lung.

IL-13 priming also weakly increased the number of AMφ expressing surface MHC-II at 72 hours, but has no effect on MRC1 levels, and as such can be described as MHC-II\textsuperscript{-}/MRC1\textsuperscript{-} (Figure 3.14).

These results indicate that similar to BMMφ, AMφ can be driven into distinct macrophage subsets with different gene, secreted protein and cell surface protein expression. IFN-γ priming of AMφ generated M1-like macrophages that had a pro-inflammatory phenotype; IL-13-priming generated a wound healing phenotype that resembles M2 macrophages with the exception of increased surface expression of MRC1.
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24 hours

Figure 3.13 Effect of cytokine priming on cell surface protein expression of macrophage subset markers AMφ at 24 hours. 3 x 10^5 BAL cells were cultured in suspension in a 6-well Corning Costar® Ultra-Low attachment culture plate (Sigma-Aldrich) and primed with macrophage complete media control (RPMI-1640 + 10% fetal bovine serum + 1.5 mM sodium pyruvate + 0.05 mM β-mercaptoethanol + 1 mM penicillin/streptomycin + 50 μg/ml geneticin) alone or together with 20 ng/ml IFN-γ or 10 ng/ml IL-13 for 24 hours. BAL cells were then harvested and stained with cluster of differentiation (CD) 11c-APC/Cy7 (2 μg/ml), Major Histocompatibility (MHC)-II (IA/IE)-Pacific Blue (5 μg/ml) and Mannose Receptor C Type 1 (MRC1)-PerCP/Cy5.5 (2 μg/ml) antibodies. AMφ were identified as SSChi FSChi CD11c+ and autofluorescent in the FL1 (Green) channel, and analysed for changes in surface protein expression in MHC-II and MRC1 by flow cytometry as shown above. Difference in cell surface protein expression was evaluated by changes in % Population Positive and Median Fluorescence Intensity (MFI) of MHC-II (IA/IE) or MRC1 staining in the AMφ population following cytokine priming. Data is represented as mean ± SD; n=3 from three separate studies. A one way ANOVA with Tukey post-test was carried out; *: p<0.05, **: p<0.01, ***: p<0.005, ****: p<0.001. NS, Not significant. % positive population expressed as an average of n=3 studies. Dotted lines represent MFI for isotype controls; MFI = 289 for Pacific Blue™ Rat IgG2b, κ isotype control antibody (isotype control for anti-MHC-II (IA/IE)-Pacific Blue™) and MFI = 155 for Rat IgG2a κ-PerCP/Cy5.5 (isotype control for anti- MRC1-PerCP/Cy5.5). See Appendix 3 for histograms of individual repeats.
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72 hours

Figure 3.14 Effect of cytokine priming on cell surface protein expression of macrophage subset markers in AMφ at 72 hours. 3 x 10^5 BAL cells were cultured in suspension in a 6-well Corning Costar® Ultra-Low attachment culture plate (Sigma-Aldrich) and primed with macrophage complete media control (RPMI-1640 + 10% fetal bovine serum + 1.5 mM sodium pyruvate + 0.05 mM β-mercaptoethanol + 1 mM penicillin/streptomycin + 50 μg/ml geneticin) alone or together with 20 ng/ml IFN-Υ or 10 ng/ml IL-13 for 72 hours. BAL cells were then harvested and stained with CD11c-APC/Cy7 (2 μg/ml), MHC-II (IA/IE)-Pacific Blue (5 μg/ml) and MRC1-PerCP/Cy5.5 (2 μg/ml) antibodies. AMφ were identified as SSC^hi FSC^hi CD11c^+ and autofluorescent in the FL1 (Green) channel, and analysed for changes in surface protein expression in MHC-II and MRC1 by flow cytometry by flow cytometry as shown above. Difference in cell surface protein levels was evaluated by changes in % Population Positive and MFI of MHC-II (IA/IE) or MRC1 staining in the AMφ population following cytokine priming. Data is represented as mean ± SD; n=3 from three separate studies. A one way ANOVA with Tukey post-test was carried out; *: p<0.05, **: p<0.01, ***: p<0.005, ****: p<0.001. NS, Not significant. % positive population expressed as an average of n=3 studies. Dotted lines represent MFI for isotype controls; MFI = 319 for Pacific Blue™ Rat IgG2b, κ isotype control antibody (isotype control for anti-MHC-II (IA/IE)-Pacific Blue™) and MFI = 151 for Rat IgG2a κ-PerCP/Cy5.5 (isotype control for anti-MRC1-PerCP/Cy5.5). See Appendix 3 for histograms of individual repeats.
3.5.2.2 Differentially primed AMφ have distinct responses to LPS stimulation

Many receptors act synergistically generating cross-talk between different signalling pathways within the cell. There have been reports that TLR signalling can act in synergy with cytokine receptors, resulting in enhanced pro-inflammatory responses. TLRs on AMφ may have a particularly important role, as the respiratory tract in which they are located is not a sterile environment and constantly exposed to environmental pathogens and irritants. Therefore AMφ responses to cytokine priming in conjunction with TLR activation may result in the differentiation of functionally distinct subsets.

In the following studies, M1-like and M2-like AMφ were generated as before by priming AMφ with IFN-Υ or IL-13; these were then further stimulated with LPS for TLR4 activation.

3.5.2.2.1 M1 macrophages do not exhibit an exaggerated pro-inflammatory response upon LPS stimulation

In contrast to BMMφ, M1-like AMφ did not appear to have an exaggerated pro-inflammatory response upon LPS stimulation. LPS alone did not increase NOS2 mRNA levels, and also failed to augment the IFN-Υ induced increase in NOS2 transcripts. LPS alone enhanced TNF-α gene transcript levels (p<0.01), but this increase was not amplified in IFN-Υ primed cells (p<0.01). IFN-Υ priming significantly enhanced IL-1β protein levels (p<0.01) and also a trend towards increased IL-12 p40 (total) protein levels, but LPS stimulation failed to amplify the IFN-Υ induced increase in IL-1β and IL-12 p40 (total) protein levels (Figure 3.15).
Figure 3.15 The effects of Lipopolysaccharide (LPS) stimulation on mRNA and protein levels of M1-associated markers in differentially primed AMφ. 1 x 10⁵ AMΦ were seeded per well of 48-well culture plates, and primed with media control alone or together with 20 ng/ml IFN-Y or 10 ng/ml IL-13 for 18 hours. Primed AMΦ were then further cultured with macrophage complete media control (RPMI-1640 + 10% fetal bovine serum + 1.5 mM sodium pyruvate + 0.05 mM β-mercaptoethanol + 1 mM penicillin/streptomycin + 50 μg/ml geneticin) or 10 ng/ml LPS from E. coli 0111:B4, 4 hours for mRNA analysis and 24 hours for protein level analysis. NOS2 and TNF-α mRNA levels were determined by qRT-PCR. Pooled samples (per condition) were run in triplicates and the average was presented as relative % to the housekeeping gene HPRT-1. IL-1β and IL-12 p40 (total) protein levels in the culture supernatant were determined by MSD. Three separate studies were carried out (n=3) and data are represented as mean ± SD. A one way ANOVA with Tukey post-test was carried out; *: p<0.05, **: p< 0.01, ***: p<0.005. ND, Not Detected; NS, Not significant. IL-12 p40 (total) includes free IL-12 p40, IL-12 p40 homodimers and IL-12 p40 heterodimers (IL-12 p70 and IL-23). See Appendix 4 for standard curves for MSD of IL-1β and IL-12 p40 (total).
3.5.2.2.2 LPS stimulation has mixed effects on M2 phenotype

LPS stimulation had mixed effects on different M2 associated genes. Similar to BMMφ, LPS stimulation reduced transcript levels of Arg-1 that had been increased following treatment with IL-13 by 5-fold in IL-13 induced AMφ (p<0.01). However, there was no effect on either the pro-fibrotic mediator IGF-1 or the protein levels of fibronectin (Figure 3.16).

These results suggest that LPS stimulation of TLR4 appear to influence the AMφ phenotypes generated by cytokine priming. These effects however appear to differ to that observed in BMMφ in that LPS stimulation had no augmenting effects on M1-associated gene expression, and had mixed effects on M2-associated genes, increasing the transcript levels of Arg-1 while having no effects on others (IGF-1 and fibronectin). Further experiments using alternate TLR ligands (e.g. Pam2CSK4) will have to be carried out to investigate the effects of TLR stimulation on mRNA and protein levels of AMφ.

![Graphs showing effects of LPS stimulation on mRNA and protein levels of M2-associated markers](image)

Figure 3.16 The effects of Lipopolysaccharide (LPS) stimulation on mRNA and protein levels of M2-associated markers in differentially primed AMφ. 1 x 10⁶ AMφ were seeded per well of 48-well culture plates, and primed with media control alone or together with 20 ng/ml IFN-Y or 10 ng/ml IL-13 for 18 hours. Primed AMφ were then further cultured with media control or 10 ng/ml LPS from E. coli 0111:B4, 4 hours for mRNA level analysis and 24 hours for protein level analysis respectively. Arg-1 and IGF-1 mRNA levels were determined by qRT-PCR. Pooled samples (per condition) were run in triplicates and the average was presented as relative % to the housekeeping gene HPRT-1. Fibronectin protein levels in the culture supernatant were determined by M SD. Three separate studies were carried out (n=3) and data are represented as mean ± SD. One way ANOVA with Tukey post-test was carried out; *: p<0.05, **: p<0.01, ***: p<0.005, ****: p<0.001. ND, Not Detected; NS, Not significant. See Appendix 4 for standard curves for fibronectin ELISA.
In conclusion, AMφ displayed heterogeneity in phenotype similar to that observed in BMMφ, and can be induced into distinct subsets by external priming signals. IFN-Υ induced M1 macrophages have a pro-inflammatory phenotype, whereas IL-13-induced M2 macrophages have a pro-wound healing phenotype. Hypoxia and TGF-β, two conditions that have been reported to be prevalent in pulmonary fibrosis, are also potent priming signals to drive a wound healing macrophage phenotype.

LPS stimulation may also alter macrophage phenotype to generate a pro-inflammatory response, although this appears to be independent of the IFN-Υ-induced M1-like phenotype as distinctly different pro-inflammatory mediators were upregulated. LPS stimulation also altered macrophage subset phenotypes induced by different priming signals. In AMφ, LPS stimulation resulted in mixed response in subset associated changes in gene and protein levels, and did not bias towards or away from a particular subset associated phenotype. This highlights the fact that stimulatory signals possess their own gene expression programme, and should not be associated solely with a particular macrophage subset.
3.6 Discussion

In this chapter the focus was to investigate the effects of various mediators, hypoxia, and PRR stimulation on the phenotypes of BMMφ and/or AMφ ex vivo. In brief the results demonstrated that BMMφ and AMφ can be induced by different cytokines to differentiate into distinct phenotypes. TGF-β and hypoxic conditions, which are commonly found in the lungs of IPF patients, were also potent signals in altering AMφ phenotypes. LPS stimulation can modulate the mRNA and protein levels of BMMφ and AMφ that have been primed by various mediators (cytokines, growth factors or hypoxia). In the following section, I will first discuss the experimental limitations to these studies and their implications to results analyses (section 3.6.1). The implications of the experimental results will then be evaluated (sections 3.6.2-3.6.3), future directions will be discussed in section 3.7 and chapter concluded at section 3.8.

3.6.1 Experimental limitations

There are several limitations to the above studied here that have been identified and requires to be rectified. Preliminary studies (n=1) were carried out for the BMMφ studies, the TGF-β and hypoxia-induced AMφ studies, and also the protein level analysis of fibrosis-associated mediators in AMφ primed with IFN-γ, IL-4, or hypoxia. These studies provide an indication of how BMMφ and/or AMφ might behave under these different priming and/or stimulatory conditions, although further repeats will have to be carried out to verify these observations.

Macrophage subset mediators relating to M1- (NOS2) or M2- (Arg-1) were assessed in IFN-γ or IL-4-induced BMMφ respectively. While these studies were sufficient to indicate whether or not BMMφ were responding to the said cytokines, and thereby serve as a positive control for the use of these cytokines and priming procedure for later AMφ priming studies, it was unable to fully demonstrate whether or not M1 or M2 BMMφ (rather than a hybrid phenotype per se) has been generated. Further experiments assessing the mRNA levels for M2- or M1-associated proteins for IFN-γ or IL-4-primed BMMφ is therefore required to complete the BMMφ polarisation assessment.

There were also no controls put in place to verify the efficiency of generating M1- or M2-like BMMφ by IFN-γ or IL-4 priming respectively, M1- or M2-like AMφ by IFN- or IL-13 respectively, and pro-fibrotic macrophages by TGF-β or hypoxia. The results for changes in mRNA and protein levels in BMMφ and/or AMφ may therefore be a net effect of the responders and potential non-responders to such priming signals. Further studies involving flow cytometry and/or mass cytometry that assesses the protein levels
of M1-(NOS2, MHC-II) or M2-(Arg-1, fibronectin) associated mediators at a cellular level will have to be carried out to achieve this.

Moreover, preliminary experiments (n=1) have been carried out for BMMϕ experiments and also TGF-β and hypoxia-induced AMϕ experiments discussed below (sections 3.6.2); as no repeats were carried out the results from these studies should be treated as initial observations rather than conclusive results. The repeated experiments (n=3) in IFN-Y or IL-13 primed AMϕ (sections 3.6.3) may provide an indication of the overall trend in gene transcript and protein levels of various mediators in AMϕ subsets, although these are more likely than not to be an underestimate, as a result of the lack of control for the efficiency for macrophage priming by various mediators.

Despite the experimental limitations, these studies are able to provide an initial overview of AMϕ heterogeneity in response to various priming and stimulatory signals, which will be discussed further below. Observations from preliminary experiments of mRNA and protein level analyses of TGF-β and hypoxia-primed AMϕ will first be evaluated, followed by conclusions drawn from the repeated studies of IFN-γr IL-13 primed AMϕ.

3.6.2 Preliminary experiments

3.6.2.1 Hypoxia and TGF-β priming promotes a pro-fibrotic response

Hypoxia and TGF-β are two common conditions found in the fibrotic lung, and previous literature has reported that both of these conditions altered the phenotype of MH-S cells260 and promoted a pro-fibrotic response261. The hypoxic response is predominantly controlled by HIF, a heterodimeric transcription factor that in turn is regulated by both oxygen and iron746. The HIF DNA-binding complex is comprised of the constitutively expressed HIF-1β subunit, and one of two α- subunit isoforms (HIF-1α or HIF-1β)762. Under hypoxic conditions, HIF α-subunits accumulate and translocate to the nucleus where they bind HIF-1β, and the heterodimeric HIF complex binds to hypoxia-response elements (HREs) in the promoter of target genes. The TGF-β responses are mediated by either the HIF-1α signalling pathway that is shared by hypoxia induction, or the mothers against decapentaplegic homolog (Smad) signalling pathway763. In the studies presented here it was evaluated whether or not primary AMϕ were prone to alter their phenotype in response to signalling via hypoxia and TGF-β as well. Several parameters were assessed, including the serine protease PAI-1 (Figure 3.4), the macrophage subset-associated mediators NOS2 (M1) and Arg-1 (M2) (Figure 3.6), and the pro-fibrotic mediators TGF-β and PDGF-β (Figure 3.5).
The findings revealed that both hypoxia and TGF-β priming resulted in enhanced PAI-1 gene transcript levels; however hypoxia was the more potent inducer (Figure 3.4). PAI-1 is the primary inhibitor of plasminogen activators tissue plasminogen activator (tPA) and urokinase (uPA), which are serine proteases that catalyse the activation of plasminogen into plasmin. Plasmin in turn acts to cleave the ECM component fibrin. By the increased expression of PAI-1, fibrin is able to accumulate and form the fibrin clot that aids the normal wound healing process. On the other hand, PAI-1 may play a role in fibrosis upon persistent tissue injury and dysregulated wound healing. PAI-1 is consistently and markedly upregulated in pulmonary fibrosis\textsuperscript{262}, and plays a key role in BLM-induced pulmonary fibrosis\textsuperscript{263,264}. This fibrosis was more severe in transgenic mice overexpressing PAI-1 gene than WT mice\textsuperscript{264}, whereas PAI-1 knockout mice were protected from fibrosis\textsuperscript{263,264}. Several hypotheses have been proposed to explain how PAI-1 deficiency protects from fibrosis; it was thought that the lack of PAI-1 may result in increased plasmin-mediated proteolysis of fibrin, and/or enhanced proteolysis of growth factors or MMPs that degrade matrix glycoproteins\textsuperscript{265}.

The gene transcript levels for two different L-Arginine metabolizing enzymes, namely the M1-associated NOS2 and also the M2-associated Arg-1 were elevated in hypoxia-primed AM\(\Phi\) but not TGF-β-primed AM\(\Phi\) (Figure 3.6). The transcription of the genes for these two enzymes of divergent roles is reported to be catalysed by different HIF\(\alpha\)-subunits of the HIF transcription factor. NOS2 is induced by HIF-1\(\alpha\), whereas Arg-1 is induced by HIF-2\(\alpha\) containing HIF heterodimer\textsuperscript{764}. Interestingly, it was also reported that IFN-\(\Upsilon\) induced BMM\(\Phi\) (M1) and IL-4 or IL-13 induced BMM\(\Phi\) (M2) increased the transcript levels of HIF-1\(\alpha\) or HIF-2\(\alpha\) respectively\textsuperscript{764}, suggesting that hypoxia priming is synergistic with both macrophage subset responses. This allows macrophages to launch an effective and targeted response against different types of pathogens/stimuli at sites of tissue injury where extreme local hypoxia is prominent. Further studies will have to be carried out for AM\(\Phi\) isolated from mice challenged with either T\(\text{h}1\)-or T\(\text{h}2\)-inducing pathogens (e.g. viruses vs. parasites), cultured under hypoxic conditions \textit{ex vivo}, to investigate whether or not hypoxia amplifies NOS2 and Arg-1 mRNA levels respectively as compared to normoxic controls (The T\(\text{h}1\) or T\(\text{h}2\) cytokines, IFN-\(\Upsilon\) or IL-13, induced M1 or M2 respectively). In addition, the effect of hypoxia on the mRNA and protein levels of other M1- or M2-like mediators on differentially primed AM\(\Phi\) can also be analysed.

The gene transcript levels of the pro-fibrotic mediators TGF-β and PDGF-β were increased in primary AM\(\Phi\) induced with TGF-β (Figure 3.5), suggesting a positive feedback loop in the wound healing response. In the context of pulmonary fibrosis, the elevation of TGF-β levels within the lung as a result of
dysregulated wound healing may therefore further propagate and amplify the fibrotic response by the induction of AMφ. TGF-β promotes multiple features associated with fibrosis, including fibroblast migration\textsuperscript{561}, proliferation\textsuperscript{561}, myofibroblast differentiation\textsuperscript{562}, EMT\textsuperscript{131}, excessive production of ECM components in myofibroblasts\textsuperscript{563} and AEC apoptosis\textsuperscript{564}. PDGF may contribute to the proliferation of fibroblasts and their transformation to ECM-producing myofibroblasts\textsuperscript{120}.

3.6.2.2 LPS stimulation biases towards M1-like phenotype in hypoxia-primed AMφ

LPS stimulation altered the transcript level of genes that were increased by hypoxia or TGF-β priming in AMφ (Figures 3.7 – 3.9). In hypoxia-primed AMφ, LPS stimulation amplified the increased transcript levels of the HIF-1α mediated genes, NOS2 (Figure 3.9) and PAI-1 (Figure 3.7), but suppressed that of the HIF-2α mediated gene Arg-1 (Figure 3.9). The synergy between the HIF-1α mediated genes and LPS stimulation is consistent with previous reports in the literature, which has demonstrated that NFκB, the main transcription factor facilitating TLR responses, mediated increase of HIF-1α mRNA expression\textsuperscript{765,766}. However there have been no reported studies investigating the role of the HIF-2α subunit in the HIF-NFκB pathway\textsuperscript{746}, although in the context of Arg-1 from these studies a modulatory role is expected. Indeed, it has been reported that HIF-1α, but not HIF-2α, was responsible for the synergistic induction of NOS2 expression and other transcriptional activities in macrophages following exposure to LPS and hypoxia\textsuperscript{767}.

3.6.2.3 LPS stimulation promotes the increase in mRNA levels of pro-wound healing mediators in TGF-β primed AMφ

Along with a pro-inflammatory response as observed before (Figure 3.2), the results showed that LPS stimulation alone in unprimed AMφ increased the transcript levels of several anti-inflammatory or pro-wound healing mediators, including TGF-β and PDGF-β (Figure 3.8). These results may reflect the presence of a negative feedback loop for the regulation of inflammation following LPS stimulation\textsuperscript{768}. In TGF-β induced AMφ, LPS stimulation amplified the increased transcript levels of PDGF-β (Figure 3.8). Collectively these results suggest that TGF-β biases the LPS stimulatory response towards a wound healing phenotype in AMφ.

It was observed that LPS stimulation did not affect PAI-1 transcript levels in TGF-β induced AMφ (Figure 3.7) as was the case in hypoxia-induced AMφ. Although TGF-β and hypoxia induction share a common downstream HIF-1α pathway that regulates PAI-1 transcription\textsuperscript{261}, other pathways, such as the SMAD pathway is also used downstream of TGF-β signalling\textsuperscript{261}. Perhaps PAI-1 transcription in TGF-β induced AMφ is more dependent on SMAD as opposed to HIF-1α signalling. It is possible to assess SMAD signalling...
pathway activation through measuring the change in levels of activated signalling intermediates (e.g. phosphorylated SMAD) by western blot or MSD in BLM- versus saline-challenged mice, or by inhibiting the signalling intermediates and assessing the change in pathology in BLM-induced pulmonary fibrosis.

3.6.2.4 IFN-Υ and IL-4 amplified LPS-induced IL-6 and CCL5 expression
Finally the levels of several other proteins that play a role in pulmonary fibrosis were assessed for AMφ that had been primed with various cytokines, in the presence or absence of TLR stimulation (Figure 3.10). It was proposed that differential cytokine priming and PRR stimulation might also contribute to the increased expression of these proteins. CCL2 appeared to be a M1-associated mediator, as its protein levels are increased in IFN-Υ induced AMφ (Figure 3.10). LPS stimulation promoted a pro-inflammatory response, increasing protein levels of various pro-inflammatory cytokines and chemokines. Similar to that observed in BMMφ, AMφ induced with IFN-Υ or IL-4 amplified LPS stimulation in terms of IL-6 and CCL5 levels (Figure 3.10).

3.6.2.5 Hypoxia priming amplified LPS-induced increased expression of pro-inflammatory mediators
Perhaps the most notable results in these studies is that hypoxia-induced AMφ have highly elevated levels of pro-inflammatory chemokines, including CCL2, CCL3, CCL4, CCL5 and CXCL2 following LPS stimulation (Figure 3.10). As mentioned above LPS stimulation can promote hypoxic responses through the NFκB mediated increase of HIF-1α mRNA expression. Conversely, hypoxia can also amplify pro-inflammatory responses by stimulating NFκB activation by inhibiting prolyl hydroxylases that negatively modulate inhibitor of IKKB catalytic activities. These results reinforce the hypothesis that extreme local hypoxia may serve as an amplification signal to maximise the immune response at the site of tissue injury.

3.6.3 Repeated experiments on AMφ heterogeneity
3.6.3.1 AMφ can be induced into distinct subsets following cytokine priming
Studies exploring the role of lung macrophage subsets in IPF in humans and murine models of pulmonary fibrosis have depended on macrophage subset associated markers derived largely from the in vitro analysis of BMMφ in mice and blood monocyte derived macrophages (BMDM) in humans. However, there may be differences in macrophage subset phenotypes between those generated in vitro by the stimulation of BMMφ under selected conditions compared to terminally differentiated pulmonary macrophages exposed to the environment of the lungs that may behave differently. With this in mind experiments were carried out to characterise macrophage subsets derived from freshly isolated AMφ by systematically stimulating these cells with various mediators that have
been reported to generate functionally distinct BMMϕ subsets in vitro. The ability of TLR stimulation to modulate AMϕ mRNA and protein levels following cytokine priming was also assessed and discussed further in section 3.6.3.3.

Here it is demonstrated that AMϕ, under the same cytokine priming signals as BMMϕ, can be induced to differentiate into distinct subsets in vitro. AMϕ primed by IFN-γ displayed a M1-like phenotype, and is characterised by increased transcript levels of NOS2 (Figure 3.11), and protein levels of IL-1β, IL-12 p40 (total) (Figure 3.12). Furthermore flow cytometric analysis revealed that the surface phenotype of these cells was MHC-IImid/hi MRC1−/lo (Figure 3.13). The increased production of RNS and pro-inflammatory mediators would promote microbial killing and the high expression of MHC-II would facilitate efficient antigen-presentation, suggesting an antimicrobial response for M1-like AMϕ.

IL-12 p40 (total) measures free, homodimeric and heterodimeric forms of IL-12 p40, including IL-12 p70 (p40 + p35) and IL-23 (p40 + p19). Therefore it is unclear from these experiments which specific forms of IL-12 p40 are found in IFN-γ primed AMϕ. IFN-γ has been reported to increase the protein expression of both IL-12 p70 and IL-23 in BMMϕ. IL-12 p70 was produced in murine BMMϕ following IFN-γ priming with or without Mycobacterium bovis Bacillus Calmette-Guérin (BCG) or LPS stimulation, but production was attenuated in mutant mice lacking the IFN-γ receptor. In Sendai virus stimulated monocyte derived macrophages, IFN-γ priming further amplified the production of IL-12 p40, IL-12 p35 and IL-23 p19 gene expression. Further protein analyses experiments that specifically detect IL-12 p40, IL-12 p70, and IL-23 will have to be carried out to verify whether or not IFN-γ induces IL-12 p40, IL-12 p70 and/or IL-23 production in AMϕ.

The phenotype of AMϕ primed by IL-13 is M2-like, with elevated transcripts for Arg-1, IGF-1 and fibronectin (Figure 3.11), and higher levels of fibronectin protein (Figure 3.12). These cells also weakly increased the number of AMϕ with surface expression of MHC-II proteins (Figure 3.14). These results suggest that M2-like AMϕ may play a role in wound healing, but in the presence of persistent stimulation may drive fibrosis. Together these results demonstrate that AMϕ can adjust their phenotype to their environment and polarise into distinct subsets.

3.6.3.2 There is no increased surface protein levels of MRC1 on M2-like AMϕ

The use of MRC1 as a M2 marker originates from a study in which an increase of both intracellular and extracellular MRC1 in mouse peritoneal macrophages was observed following induction by IL-4 or IL-10, which are cytokines associated with alternative activation of macrophages. Surface expression of
MRC1 has since been widely used as a marker for M2-like macrophages in flow cytometry analyses in various diseases, including adipose tissue macrophages in diabetes, splenic macrophages in experimental autoimmune encephalomyelitis and TAMs in breast cancer. However in this report an increase of MRC1 surface expression was not observed on M2-like AMφ generated by IL-13 priming at 24 and 72 hours (Figures 3.13 and 3.14). Several factors may give rise to this difference. IL-13 has lower potency compared to IL-4 in inducing MRC1 expression and activity as reported in mouse peritoneal macrophages. Conversely, there may be different response between peritoneal macrophages and AMφ to IL-13 priming such that MRC1 is induced in the former but not the latter. Another possibility is that MRC1 protein expression is induced in IL-13 primed AMφ, but remains in an intracellular location. Regardless these results propose reconsidering using cell surface MRC1 as a M2-associated marker and particularly for AMφ. On the other hand there was a non-significant trend suggesting decrease in MRC1 surface expression in IFN-Υ-induced AMφ (Figure 3.13) compared to media control, suggesting that these cells are MRC1⁻.

Together these results demonstrate that terminally differentiated AMφ are able to adjust their phenotypes, in a similar manner to that reported for BMMφ, in response to cytokine priming. Similar to BMMφ, IFN-Υ induces a M1-like antimicrobial response in AMφ, whereas IL-13 induces a M2-like wound-healing phenotype. Several commonly used BMMφ subset associated markers have been shown here to also be increased in corresponding AMφ subsets: IFN-Υ primed M1-like AMφ can be described as NOS2hi TNFhi IL-1βhi IL-12 p40 (total)hi MHC-IIhi MRC1⁺, and M2-like AMφ can be characterised as Arg-1hi IGF-1hi fibronectinh MRC1⁻/lo, for IL-13 generated. These results have validated the use of several BMMφ-derived macrophage subset markers in characterising AMφ populations with different functional profiles and provide a useful guide for future studies of AMφ heterogeneity in other murine pulmonary studies. Further gene microarray studies can expand these studies to detect differential expression of MMPs, chemokines, and cytokines, between the two different AMφ subsets to better understand their role in host defence, inflammation, repair, tissue remodelling and/or fibrosis within the lung.

3.6.3.3 LPS stimulation alters AMφ phenotype induced by cytokine priming

LPS stimulation induces a pro-inflammatory response in macrophages, and differential cytokine priming can promote or repress these responses. The M1-inducing cytokine IFN-Υ can synergize with or have additive effects to LPS stimulation, which may be due to the coexistence of binding sites for their main signalling mediators, IRF and NFκB respectively, in proximal promoters of regulated genes. The M2-inducing cytokine IL-4 can also synergistically upregulate some pro-inflammatory mediators upon LPS
stimulation of BMMφ. This prompted an investigation as to whether or not LPS stimulation would have an effect on the phenotype of AMφ subsets.

The results showed that LPS stimulation alone increases TNF-α gene transcript levels, but neither IFN-Υ nor IL-13-priming elicited any additive effects to LPS stimulation of TNF-α. LPS stimulation also had no effect on NOS2 transcript, or IL-1β protein levels (Figure 3.15). On the other hand, IL-12 p40 (total) levels were amplified in IFN-Υ induced mice following LPS stimulation (Figure 3.15). IL-12 p40 can form the bioactive IL-12 p70 following LPS stimulation of IFN-Υ primed monocytes.

LPS stimulation may also suppress IL-4/IL-13 signalling through the inhibition of STAT6 by upregulating its inhibitor SOCS1, and also through the PI3K pathway by SOCS3. In these studies the effect of LPS stimulation on the levels of IL-13-induced, M2-associated mediators was evaluated. The results presented here revealed that LPS stimulation reduced IL-13-induced increase in Arg-1 gene transcript levels, but had no effect on IGF-1 gene transcript levels, or fibronectin protein levels (Figure 3.16). These results indicate that an alternative IL-13 signalling pathway independent of STAT6 or PI3K signalling responsible for the elevated levels of the pro-fibrotic mediators, IGF-1 and fibronectin, may exist. Combined with the results above, it appears that LPS stimulation favours a pro-inflammatory phenotype, but does not necessarily suppress a wound healing phenotype. AMφ display a high degree of flexibility and are able to respond to two divergent signals simultaneously by forming hybrid phenotypes. This is best illustrated by AMφ primed with IL-13 and then stimulated with LPS, where transcript levels of the pro-inflammatory cytokine TNF-α (Figure 3.15), the pro-fibrotic mediator IGF-1 (Figure 3.16), and protein levels of the ECM component fibronectin (Figure 3.16) were all elevated. Such hybrid phenotypes may be more relevant to the disease phenotype observed in fibrotic lungs, where levels of both IL-13 and PRR-stimulating alarmins are elevated.

### 3.7 Future directions

As aforementioned in section 3.6.1, there are several experimental limitations to the studies above that requires to be amended, including repeats of preliminary experiments, investigation of the gene transcript and protein levels of both M1- and M2-associated mediators in IFN-Υ or IL-4-primed BMMφ to ascertain that distinct subsets, rather than hybrid macrophages are generated, and flow or mass cytometry to evaluate the efficiency of macrophage priming in generating distinct subsets. With respect to IL-12 protein level analysis, the IL-12 p40 (total) MSD measured all oligomers containing IL-12 p40, including IL-12 p40 monomer, IL-12 p40 homodimer, IL-12 p70 (p40 + p35) and IL-23 (p40 + p19).
Further studies measuring the specific oligomeric forms of IL-12p40 will have to be carried out to resolve this.

Future experiments can be carried out to further explore macrophage heterogeneity. These current studies reflect only a certain time point following cytokine/growth factor/hypoxia priming and LPS stimulation. Different genes/proteins may be released at different time points following AMϕ activation. For example, acute LPS stimulation induces a M1 associated pro-inflammatory response, but chronic LPS stimulation promotes the increased expression of M2-like associated mediators, including IL-10, TGF-β and Arg-1 in peritoneal macrophages. Kinetic studies for the aforementioned proteins of interest will therefore have to be carried out for AMϕ induced by various priming and/or stimulatory mediators. The changes in gene transcript or protein levels of these mediators produced by *ex vivo* generated AMϕ subsets will then be compared to those that are generated in the BLM-induced model of pulmonary fibrosis *in vivo*. A huge body of work will be involved in assessing a wide range of mediators over multiple time points. Therefore, a more efficient screening method in the form of microarrays could be used to allow the simultaneous assessment of the transcript levels of a large number of pre-selected genes. The quantity and quality of RNA required for microarray analyses is also pivotal for its result. As there are small numbers of AMϕ that can be isolated per mouse, AMϕ pooled from different BLM-challenged mice will have to be assessed. Alternatively, mRNA from AMϕ can be pre-amplified prior to gene transcript analyses using commercially available kits (Life Technologies). These microarray analyses will provide a useful reference for future studies of lung macrophage subsets in pulmonary diseases. The use of microarrays will be discussed in greater detail in the general discussion (section 6.4.1).

In these studies, LPS was chosen as the stimulatory signal for TLR4 on AMϕ as it is able to induce pronounced inflammation, which allows a greater window of opportunity to study the changes in expression of pro-inflammatory mediators in response to cytokine priming. In addition to microbial derived proteins alarmins would also relevant ligands for PRRs. Alarmins may simultaneously activate a number of PRRs that produces a different cytokine profile as compared to LPS. For example, HMGB1 activates both TLR2 and TLR4, and is able to act as a molecular chaperone to the ligands of TLR2, TLR4 and TLR9. TLR4 and TLR9 ligation induces a predominantly Th1 response, and the latter also promotes the production of type 1 IFNs. TLR2 is also able to induce a mixed Th1 and Th2 response. It would therefore be interesting to carry out further *ex vivo* studies to evaluate the effects of various alarmins on differentially primed AMϕ subsets to better understand macrophage polarization in tissue injury.
Pulmonary macrophages exposed in the lung environment are subject to a multitude of signals that may alter their gene transcript and protein levels, as observed in the change in the studies above. Since the lung environment is ever changing, long-lived pulmonary macrophages would likely have to display plasticity and adapt to these alterations in environmental signals. In other words, AM\(\phi\) that are polarised towards a certain direction can be reversed and polarised towards an opposite direction (e.g. M1 \(\rightarrow\) M2 or vice versa). In order to test for the plasticity of AM\(\phi\), experiments can be carried out to reverse AM\(\phi\) that are polarised either ex vivo (by cytokine priming) or in vivo (in Th1-or Th2 driven disease models, e.g. endotoxin-induced lung inflammation or helminth infection respectively).

3.8 Conclusion

Collectively, these results demonstrate that terminally differentiated AM\(\phi\) freshly isolated from the lung can alter their mRNA and protein levels in response to various external signals, including cytokines, growth factors and hypoxia. There are a high number of signals contributing to the diversity of macrophage function, including the synergistic or antagonistic effects of different cytokines and related signals on their differential expression. For example, IL-10 promotes IL-4 induced M2 phenotype in BMM\(\phi\), and increased the gene expression of Arg-1, Retn1\(\alpha\) and MRC1\(^{784}\). In addition, LPS stimulation can antagonise Arg-1 expression in IL-13 induced AM\(\phi\) (Figure 3.16) but increase PDGF-\(\beta\) gene expression by TGF-\(\beta\) induced AM\(\phi\) (Figure 3.8). It is highly likely that multiple priming and stimulatory signals will exist within the lung environment, such that AM\(\phi\) phenotype will not be strictly M1-like or M2-like in vivo, but will exhibit characteristics of both polarities. Also the response in the lungs will be dynamic and therefore AM\(\phi\) phenotypes have the capacity to alter as pulmonary inflammation and fibrosis develop. This suggests that AM\(\phi\) participating in various pulmonary diseases should not simply be defined as being M1-like or M2-like, but rather characterised as a separate phenotype ex vivo. The phenotype of AM\(\phi\) in BLM-induced pulmonary fibrosis will be explored in the next chapter (chapter 4).
Chapter 4
Alveolar macrophage heterogeneity in Bleomycin-induced pulmonary fibrosis
Chapter 4 | AMφ heterogeneity in BLM-induced pulmonary fibrosis

4.1 Introduction

4.1.1 Macrophage subsets in IPF

In the previous chapter, it was demonstrated that terminally differentiated AMφ were able to respond to external conditions and alter their phenotypes to generate distinct macrophage subsets. These macrophage subsets potentially could play a role in IPF.

Macrophage subset-associated markers have been identified in IPF: M1-like pro-inflammatory FRβ-positive macrophages are present in patients with this disease; M2-associated markers, including galactin (Gal)3, CCL18, chitinase 3 like 3 (Chi3l3), CD163, insulin like growth factor (IGF)-1, and platelet derived growth factor (PDGF) are also reported to be elevated in AMφ. In addition, there is an increase in the M2-inducing cytokine IL-13 in IPF patients compared to controls, and AMφ isolated from these patients produced more IL-13 than that detected in control lungs. M2 markers, including Arg-1, found in inflammatory zone (FIZZ)1 and MRC1 have also been found to be elevated in murine models of pulmonary fibrosis.

Distinct macrophage subsets are reported to play different roles in the pathology of IPF. In a BLM-induced pulmonary fibrosis mouse model, M2 and/or M2-like macrophages contribute to fibrosis and tissue remodelling. Depletion of lung macrophages using liposomal chondrate or DTR during the progressive fibrotic phase abolished collagen deposition and fibrosis. This was accompanied by a decline in M2-associated markers, including Arg-1 or Chi3l3 but not the M1-associated marker NOS2 in the lung. M2 or M2-like macrophages are associated with wound healing and if this process is dysregulated, as could occur in IPF, the overexpression of a number of pro-fibrotic mediators may drive pulmonary fibrosis (Table 4.1).
In contrast M1 macrophages have a less well established role in pulmonary fibrosis. They have a pro-inflammatory phenotype, and are therefore likely to contribute to acute lung injury in BLM-induced pulmonary fibrosis (Table 4.2), although further studies are required to confirm this. However, depletion of lung macrophages during the inflammatory phase had no effect on early stage or peak fibrosis. This suggests that either macrophages in the inflammatory phase are not essential, or that inflammation itself is non-essential for the development of fibrosis.
4.1.2 Multiple oropharyngeal dose of BLM-induced pulmonary fibrosis

Despite published reports of increased expression of distinct macrophage subset-associated mediators at various stages of pulmonary fibrosis, it should be noted that macrophages found in vivo are unlikely to fall into separate in vitro defined subsets, but rather form a gradient of phenotypes, for as discussed above there will be a variety of diverse signals within the lungs that may influence macrophage gene and protein expression.

In the following studies, a comprehensive analysis of macrophage heterogeneity was carried out in a BLM-induced pulmonary fibrosis mouse model using a novel repeated oropharyngeal dosing regimen, developed by Dr. Elizabeth Jarman at Novartis Institute of Biomedical Research (NIBR), Horsham. Three doses of BLM at lower concentrations (0.13 IU) were applied at 72 hour intervals, rather than a single dose at a higher concentration, as commonly described in the literature, via an oropharyngeal route. An oropharyngeal delivery route was chosen to mimic direct aggravation of environmental irritants to the lung, and multiple doses were applied to mimic the persistent irritation that is thought to give rise to pulmonary fibrosis. This model has the advantage over single dose regimens previously used in the

<table>
<thead>
<tr>
<th>Mediator</th>
<th>Function</th>
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<tbody>
<tr>
<td><strong>TNF-α</strong></td>
<td>Pro-inflammatory cytokine contributing to tissue injury; potential anti-fibrotic role in inhibiting collagen synthesis in myofibroblasts&lt;sup&gt;313,367,739&lt;/sup&gt;</td>
</tr>
<tr>
<td><strong>IL-1β</strong></td>
<td>Pro-inflammatory cytokine contributing to tissue injury; also induces the production of pro-fibrotic mediators TGF-β1 and PDGF&lt;sup&gt;376,567,739&lt;/sup&gt;</td>
</tr>
<tr>
<td><strong>IL-6</strong></td>
<td>Pro-inflammatory cytokine contributing to tissue injury&lt;sup&gt;739&lt;/sup&gt;</td>
</tr>
<tr>
<td><strong>IL-12</strong></td>
<td>Pro-inflammatory cytokine contributing to tissue injury; facilitates IL-17A dependent pulmonary fibrosis&lt;sup&gt;124,739&lt;/sup&gt;</td>
</tr>
<tr>
<td><strong>ROS</strong></td>
<td>Tissue injury&lt;sup&gt;739&lt;/sup&gt;</td>
</tr>
<tr>
<td><strong>NO</strong></td>
<td>Tissue injury&lt;sup&gt;739&lt;/sup&gt;</td>
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Table 4.2 M1-associated proteins and their potential role in Acute Lung Injury (ALI). IL, Interleukin; NO, nitric oxide; PDGF, platelet derived growth factor; ROS, reactive oxygen species; TGF-β, transforming growth factor-β; TNF-α, tumour necrosis factor-α.
literature where no such repeated irritation was induced\textsuperscript{175}. There is also reduced mortality in mice presumably due to better tolerance to lower concentration of BLM applied at each dose.

![Figure 4.1 Diagram illustrating time course of pathogenesis in multiple oropharyngeal dose BLM-induced pulmonary fibrosis. Mice were dosed via an oropharyngeal route at 72 hour intervals at days -7, -4 and -1. Mice were culled at days 7, 14 and 21 for analysis at the late inflammatory, active fibrotic and established fibrotic phases respectively. o.p., oropharyngeal; U, international units.](image)

BLM-induced pulmonary fibrosis consists of three phases, including inflammation-induced ALI, active fibrosis and established fibrosis\textsuperscript{595,620}. For the current model of multiple oropharyngeal dose of BLM induced pulmonary fibrosis, ALI occurs within the first week after the last dose of drug delivery, characterized by an intense inflammation and oedema with an elevation of cytokines (e.g. TNF-\(\alpha\) and IL-1\(\beta\)). By the second week (approximately day 14), the expression of the pro-fibrotic mediator TGF-\(\beta\) is upregulated as well. By the second to third week post-challenge, there is development of patchy isolated collagen deposits representative of fibrosis, with conspicuous deposition of ECM components, such as fibronectin and collagen (Figure 4.1). In-house data also revealed that BLM-challenged mice had a decline in lung function, including total lung capacity (TLC) and tissue elastance as measured by Flexivent\textsuperscript{\textregistered}, and a decline in oxygen saturation of arterial blood as determined by pulse oximetry (data not shown).

### 4.1.3 Investigation of the potential role of foamy macrophages in Hermansky Pudlak Syndrome (HPS1) mice

Foamy macrophages are macrophages that have accumulated cholesterol crystals within their lysosomes, either through excessive uptake of modified LDL, or by defective cholesterol efflux. Accumulation of cholesterol crystals have been reported to modify macrophage gene and protein expression and bias it towards a pro-inflammatory phenotype, potentially via the activation of the NLRP3 inflammasome. However, one study has found that oxidised LDL (oxLDL) induced a M2-like phenotype \textit{in vitro}, and the author speculated that whether or not a M1- or M2- profile was induced
was dependent on the level of oxidation (or modification of LDL). Increased number and size of lysosomes due to the accumulation of cholesterol crystals may also lead to enhanced fragility of lysosomes leading to more severe lung abnormalities including lung cell apoptosis that may generate persistent irritation that leads to pulmonary fibrosis. Foamy macrophages are found to accumulate in IPF patients, and may contribute to pathology through the release of pro-inflammatory and/or pro-fibrotic mediators. It would therefore be interesting to investigate the role of foamy macrophages in pulmonary fibrosis. Foamy macrophages have been reported to accumulate in the lung tissue of BLM-induced pulmonary fibrosis, and it is possible to enrich for foamy macrophages by FACS sorting of macrophages that are larger in size and have greater autofluorescence (as a result of increased cholesterol accumulation). However, FACS sorting is technically challenging and may be disruptive to cells, and therefore a simpler, less intrusive alternative method was explored. Rather than attempting to enrich for foamy macrophages isolated from BLM-challenged mice by FACS sorting \textit{ex vivo}, it was thought that enrichment \textit{in vivo}, where there is increased intrinsic accumulation of foamy macrophages within the lung, in the HPSPF mouse models, can provide a simpler method reveal the phenotype of these cells.

As aforementioned in the general introduction, HPS is a collection of autosomal recessive genetic disorders that is characterized by abnormal biogenesis, trafficking and/or function of LROs found in specialized secretory cells, such as AECs and/or AMΦ. HPS-1, HPS-2 and HPS-4 patients have been associated with the development of interstitial pulmonary fibrosis (HPSPF) that resembles IPF in terms of disease progression and its UIP histopathology. However, HPSPF is characterized by giant lamellated body formation in AEC2, resulting in AEC2 swelling. An accumulation of foamy macrophages has also been observed in the lungs of HPSPF patients, and may arise as a result of accumulation of ceroid deposition due to lysosomal defects. Defective lysosome related organelle (LRO) trafficking and biogenesis have been suggested to promote pulmonary fibrosis in several ways. Increased number and size of lysosomes may lead to enhanced fragility of lysosomes leading to more severe lung abnormalities including lung cell apoptosis that may generate persistent irritation that leads to pulmonary fibrosis. Accumulation of lysosomes may also lead to ligation of cytosolic NLRP3 inflammasome and result in AMΦ activation and subsequent release of pro-inflammatory mediators. Reduced secretion of lysosome contents, such as pulmonary surfactant from AEC2s, may also disrupt normal lung physiology.

Pulmonary surfactants are important lubricants that prevent alveolar collapse; indeed mutations in surfactant folding and secretion, including surfactants A and C, are responsible for the onset of certain subsets of familial pulmonary fibrosis.
Three HPS mouse models have mainly been used to study HPSPF, including HPS1/ep mice carrying a homozygous recessive mutation in the HPS1 gene, HPS2/pe mice carrying a homozygous recessive mutation in the HPS2 gene, and HPS1/HPS2 mice with homozygous recessive mutations in both HPS1 and HPS2 genes.

HPS1 and HPS2 mice have been shown to have structural abnormalities in the alveolar compartments that are similar to those observed in humans with HPS, including foamy AMφ and enlarged AEC2 containing irregular dense inclusions\(^790\). HPS1 and HPS2 mice also have basal inflammatory dysregulation and constitutive AMφ activation that parallels the abnormalities observed in HPS patients\(^695,709\). Silica challenged HPS1 mice also develop a persistent accumulation of AMφ and increased collagen fibres in alveolar tissue\(^790\). HPS1 and HPS2 mice have an increased susceptibility to BLM-induced pulmonary fibrosis with an increase in collagen deposition and elevated levels of TGF-β and IL-12/23 p40 in the lungs and secreted by AMφ as compared to saline controls\(^696\). However, unchallenged HPS1 and HPS2 mice do not spontaneously develop histologically significant pulmonary fibrosis by 1 year of age, but instead exhibit progressive airspace enlargement\(^697\). It is unclear for the cause for increased susceptibility to BLM-induced pulmonary fibrosis in HPS1 and HPS2 mice, although it is speculated to be due to defective lysosomal transport in pulmonary cells as is the case for HPSPF patients as mentioned above. HPS1 and HPS2 mice do not spontaneously develop pulmonary fibrosis, possibly due to some degree of redundancy in gene function between mouse HPS genes, although this remains to be tested.

HPS1/HPS2 mice carrying double homozygous recessive mutation in both HPS1 and HPS2 have impaired lamellar body secretion from AEC2\(^791\) and their lung hydroxyproline content is significantly increased compared to controls\(^717\). These mice spontaneously develop pulmonary fibrosis after 3 months of age, patchy and subpleural at first, which then progresses to established fibrosis by the age of 9 months with cellular infiltration, collagen accumulation and increased hydroxyproline content within the lung\(^719\). HPS1/HPS2 mice has been proposed as a model of spontaneous onset pulmonary fibrosis that may be used to increase our understanding of mechanisms underlying disease pathogenesis in familial interstitial pulmonary fibrosis linked to mutations in surfactant proteins, as well as in IPF.

The role of foamy AMφ in pulmonary fibrosis is unclear, although it was thought that they may contribute to pathology through the release of a number of pro-inflammatory and pro-fibrotic mediators. Initially it was planned to use HPS1/HPS2 double homozygous recessive mice as a model of HPSPF in which to study foamy AMφ in terms of cholesterol accumulation, and changes in mRNA and protein levels of various pro-inflammatory and pro-fibrotic mediators in association with pulmonary
Breeding pairs of HPS1 and HPS2 mice backcrossed to a C57BL/6 background were purchased from the Jackson laboratory (Maine, USA) and the resulting colonies were maintained at Novartis Institute of Biomedical Research (Horsham, UK). These two strains of mice were then bred together in an attempt to generate the HPS1/HPS2 double mutant mouse. Several breeding attempts have generated a few mice with albino coat colour and hypopigmented eyes matching the phenotype of the HPS1/HPS2 double mutant. However, these did not survive infancy (2-3 weeks old).

As HPS1/HPS2 mice could not be generated after several attempts, an alternative plan to study the effects of foamy AMφ on interstitial pulmonary fibrosis was devised. To this end, AMφ isolated from BLM-challenged HPS1 mice was to be compared to that from WT controls. As mentioned above, HPS1 mice do not spontaneously develop histologically significant pulmonary fibrosis, but have an increased susceptibility to BLM-induced pulmonary fibrosis. This may be caused by the increased accumulation of foamy macrophages and/or AEC2 that results from the defective intracellular organelle trafficking in pulmonary cells as a consequence of the HPS1 mutation. These foam cells may promote pulmonary fibrosis through the increased release of pro-inflammatory and/or pro-fibrotic mediators. Indeed, foamy macrophages that are constitutively activated are found to accumulate in naive HPS1 mice. The comparison of total AMφ isolated from BLM-challenged HPS1 mice, where there is thought to be increased foamy AMφ accumulation, as compared to that from WT controls, where less foamy AMφ are present, should therefore provide clues on the phenotype of foamy AMφ.

4.2 Hypothesis

The hypothesis to be tested here is that AMφ are able to adapt to the changing lung environment in vivo following persistent lung irritation by altering their phenotype and thereby contribute to pulmonary fibrosis. The reported accumulation of foamy macrophages in HPS1 mice, potentially as a result of defective lysosomal trafficking, may lead to the observed increased susceptibility to BLM-induced pulmonary fibrosis (day 7).

4.3 Aims

The first part of this chapter aims to assess the phenotype of AMφ isolated from WT C57BL/6 mice at various stages of BLM-induced pulmonary fibrosis, including the late inflammatory phase (day 7), the early fibrotic phase (day 14) and the established fibrotic phase (day 21). AMφ will be characterised based on gene transcript and secreted protein levels of mediators that are associated with inflammation and
pulmonary fibrosis and surface protein expression of M1- or M2-like surface proteins. The first part of chapter 4 aims to:

1. Characterise AMϕ isolated from BLM-challenged mice or saline controls at days 7, 14 and 21 based on their mRNA levels of the M1-associated gene NOS2, or the M2-associated gene Arg1.
2. Characterise AMϕ isolated from BLM-challenged mice or saline controls at days 7, 14 and 21 based on their surface protein expression levels of M1-associated markers (MHC-II^mid/hi^ MRC1^+^) and M2-associated markers (MHC-II^low^ MRC1^-^).
3. Characterise AMϕ isolated from BLM-challenged mice or saline controls at days 7, 14 and 21 based on the protein levels of M1-associated mediators, including IL-1β and IL-12 p40 (total), or M2-associated mediator, fibronectin, in culture supernatant. The protein levels of other mediators, including TNF-α, IL-6, IL-10, CCL2, CCL3, CCL4, CCL5, CCL20, and CXCL1, were also assessed in AMϕ culture supernatant.
4. Evaluate the pro-fibrotic role of AMϕ isolated from BLM-challenged mice at days 7, 14 and 21, as compared to saline controls, in inducing increased Col1α1 mRNA levels in mouse pulmonary fibroblasts ex vivo.

This is the first time macrophage heterogeneity is investigated in a novel repeated oropharyngeal dosing regimen of BLM induced pulmonary fibrosis, and the results may provide further information on the biology of the macrophages involved in the disease pathophysiology.

The second part of this chapter aims to compare the phenotype of AMϕ isolated from HPS1 mice with WT C57BL/6 mice at various stages of BLM-induced pulmonary fibrosis, including the late inflammatory phase (day 7), the early fibrotic phase (day 14) and the established fibrotic phase (day 21). HPS1 mice are reported to have an increased susceptibility to BLM-induced pulmonary fibrosis that may be caused by their intrinsic accumulation of foamy macrophages. Comparison of total AMϕ from BLM-challenged HPS1 mice, where there is likely to be a higher proportion of foamy AMϕ, with WT C57BL/6 controls, may provide clues to the phenotype and contributions of these cells to pathology. Comparisons between naïve HPS1 mice and WT C57BL/6 controls were first made to identify constitutive differences between the two mouse strains, which is followed by the comparison of differences in susceptibility to BLM-induced pulmonary fibrosis. More specifically, part 2 of chapter 4 aims to:

1. Examine and detect for lung inflammation and fibrosis by histology and gene expression analysis in naïve HPS1 mice backcrossed to a C57BL/6 background to validate observations from previous
reports and establish whether or not HPS1 mice spontaneously develop pulmonary fibrosis. Differential cell count and the number of foamy macrophages in BAL will also be quantified as will the basal expression of various pro-inflammatory and pro-fibrotic mediators in HPS1 and WT C57BL/6 control mice.

2. Evaluate the tolerance of HPS1 mice to BLM challenge with respect to percentage weight loss, and also susceptibility to BLM-induced pulmonary fibrosis by histological analysis of lung inflammation and fibrosis, and the gene expression profiles of inflammatory and fibrotic mediators within the lung at days 7, 14 and 21.

3. Investigate the potential contribution of foamy AMφ to increased susceptibility to BLM-induced pulmonary fibrosis in HPS1 mice will be investigated. The number of foamy AMφ in saline and BLM-challenged HPS1 and WT C57BL/6 control mice and their protein expression of the pro-inflammatory mediator IL-12 p40 (total) and the ECM component fibronectin will be determined.

4.4 Methods

4.4.1 Multiple oropharyngeal dose protocol for BLM-induced pulmonary fibrosis

BLM-induced pulmonary fibrosis in mice were carried out as described in section 2.2.2. In brief, WT C57BL/6 male mice or male or female HPS1 mice were challenged three times by oropharyngeal instillations of 50μl of 0.13U BLM sulfate (Kyowa Nippon, Kayakin Co Ltd.) or saline controls at 72 hour intervals on Days -7, -4 and -1. Mice were culled on days 7, 14 and 21 post-challenge, which include time points in the ALI phase, early fibrotic phase and the progressive fibrotic stage respectively (Figure 2.1).

4.4.2 Histology

Preparation of histological slides and staining procedures for H&E, PSR and CD68 stains are described in section 2.2.3.

4.4.3 AMφ enrichment and culture

AMφ were isolated from BAL of BLM-challenged mice or saline controls at days 7, 14 and 21 following two PBS washes in mice, and enriched by plastic adherence to tissue culture plates, the details of which are described in detail in sections 2.2.4, 2.2.5 and 2.2.6. 1 x 10^5 AMφ that were enriched by plastic adherence per well of a 48-well plate were further cultured in macrophage complete media for 4 or 24 hours for mRNA and protein level analysis respectively.
4.4.4 AMφ mRNA and protein level determination
Total RNA extraction, reverse transcription, and qRT-PCR for gene expression analysis were carried out as described at sections 2.2.11, 2.2.12 and 2.2.13 respectively. Measurements of proteins of interest in culture supernatant was measured as mentioned in sections 2.2.14 (fibronectin enzyme linked immunosorbent assay (ELISA) and 2.2.15 [Meso-Scale Discovery (MSD)].

4.4.3 Flow cytometry
3 x 10^5 cells/ml of BAL cells were separated into 3 tubes (i.e. 1 x 10^5 cells/tube), one for positive staining for surface proteins of interest (CD11c, MHC-II and MRC1), one for unstained negative control, and one for isotype control of nonspecific antibody binding. For each tube, cells were resuspended at 1x10^5 cells per 100 μl Fluorescence Activated Cell Sorting (FACS) analysis buffer (DPBS + 0.1% FCS), 1% of Fc Receptor (FcR) block (Miltenyi Biotech) and 5% mouse serum per 100μl reaction. FcR block was added to prevent non-specific binding of the Fc (constant) region of detection antibodies/ isotype controls to cell surface FcR that may generate a false positive signal. Mouse serum was added to prevent non-specific binding of the variable region of detection antibodies/ isotype controls.

For the tube stained for proteins of interest, cells were incubated with FACS analysis buffer, 0.25 μg/100 μl CD11c-APC, 0.25 μg/100 μl MHC-II-FITC and 0.5 μg/100 μl MRC1-PerCP/Cy5.5 for 30 minutes in the dark at 4°C. For the isotype control tube, cells were incubated with 0.25 μg/100 μl Armenian hamster IgG-APC, 0.25 μg/100 μl Rat IgG2b-FITC and 0.5 μg/100 μl Rat IgG2a-PerCP/Cy5.5 for 30 minutes in the dark at 4°C. For unstained negative control, cells were incubated in FACS analysis buffer only for 30 minutes in the dark at 4°C.

BAL cells were then washed in 2 ml DPBS and centrifuged at 500 g for 10 minutes at 4°C. The supernatant was then discarded and BAL cells were fixed and permeabilised with 300 μl BD Cytofix/Cytoperm™ (BD Biosciences) for 20 minutes in the dark at 4°C. BAL cells were then washed with 2 ml BD Perm/Wash™ (BD Biosciences). Cells were then resuspended in 500 μl 1 x Cell Fix (BD Biosciences) (diluted 1:10 from 10x stock solution), and kept in the dark at 4°C until further analysis.

Anti-Rat/anti-Hamster IgK compensation kit (BD Biosciences) was used on the day of flow cytometric analysis to compensate for fluorophore spectral overlap in multi-colour staining. The set consists of two populations of microparticles, including the BD CompBeads Anti-Rat/Hamster Igκ particles (positive control), which bind any rat or hamster κ light chain-bearing immunoglobulin, and the BD CompBeads Negative Control (FBS) Particles (negative control), which has no binding capacity. When mixed together
with a fluorochrome-conjugated rat or hamster antibody, the BD-CompBeads provide distinct positive and negative (background fluorescence)-stained populations that can be used to set compensation levels manually. Anti-Rat/anti-Hamster Igκ compensation kit (BD Biosciences) was prepared according to manufacturer’s instructions. In brief, 60 μl of BD CompBeads Anti-Rat/Hamster Igκ particles and 60 μl of BD CompBeads Negative Control (FBS) particles were added to 100 μl FACS buffer (+0.1 % FBS), and 20 μl of pre-diluted antibody stock (where final concentration in 220 μl is equivalent to that used in experiments) was subsequently added. This process was carried out separately for every different type of fluorophore that was used in multicolour experiments. The BD CompBeads Anti-Rat/Hamster Igκ particles/BD CompBeads Negative Control (FBS) particles/antibody mixture was then incubated in the dark at 4°C for 30 minutes. The mixture was then washed with 2 ml FACS buffer and centrifuged at 500 g for 10 minutes. The supernatant containing unbound antibodies was discarded, and the pellet containing Compbeads was resuspended in 500 μl FACS buffer.

FACS data was read by FACS CANTO II (BD Biosciences). Prior to recording data for samples, compensation for spectral overlap was determined from the pre-stained BD CompBeads Anti-Rat/Hamster Igκ particles/BD CompBeads Negative Control (FBS) particles using the compensation setup on the FACS CANTO II software (BD Biosciences). FACS data was analyzed by Flowjo (Tree Star Inc). AMφ were defined as SSChi FSChi CD11c+, and the expression of the protein of interest was gated with respect to their respective isotype controls. Data was presented as median fluorescence intensity (MFI) and % positive with respect to isotype controls.

4.4.4 Statistical analyses
All statistical analysis was carried out using GraphPad Prism 6.0 software (GraphPad software, CA, USA). Normal distribution was evaluated by visual inspection of data due to insufficient numbers for normality tests.

For the studies evaluating AMφ phenotype in C57BL/6 mice only, two-way ANOVA was used to compare time-course curves followed by Sidak’s multiple comparison test to determine the significance. P values of less than 0.05 were deemed statistically significant with *** p < 0.001, ** p < 0.01 and * p < 0.05. Error bars represent standard deviation (SD) as indicated in the figure legends.

For the studies comparing HPS1 mice with WT C57BL/6 mice, when comparing two groups unpaired two-tailed t-tests (followed by Welch’s correction test for non-equal standard deviations) and Mann-Whitney tests were used for parametric and non-parametric datasets, respectively.
4.5 Results

4.5.1 AMφ heterogeneity in BLM-induced pulmonary fibrosis in WT C57BL/6 mice

4.5.1.1 Histological analysis of BLM-induced pulmonary fibrosis in C57BL/6 mice

Haematoxylin and eosin (H&E) were used to evaluate the cellular influx into the lungs following challenge with BLM. Picrosirius Red (PSR) staining which detects collagen deposition was employed as an indicator of fibrotic pathology and tissue remodelling. Intracellular staining with CD68 was carried out to measure the influx of macrophages into the lung following exposure to BLM in order to validate their presence during fibrosis.

4.5.1.1.1 H&E staining

H&E staining showed that there was a cellular influx within the lung post-BLM challenge, with cells accumulating from day 7 during the late inflammatory phase, and the cellular infiltration was sustained through the early and late fibrotic phase at days 14 and 21. The loss of alveolar architecture in the interstitium of the BLM-challenged lung can also be observed (Figure 4.2). There was a loss of the regular mesh like alveoli structure and the infiltration of a large number of cells. It was also noted that there is spatial heterogeneity in cellular infiltration across the entire lung slice used for histology (data not shown), in which areas of normal tissue were often found adjacent to affected tissue. This may reflect the nature of BLM-induced injury, in which only regions within the lung that comes into direct contact with BLM is affected.

4.5.1.1.2 PSR staining

PSR staining revealed an increase in collagen deposition in BLM-challenged lungs. Collagen deposition was observed from day 7 post challenge, and was greatest at around day 14 and sustained until at least day 21 occurring within the interstitium and around the small airways (Figure 4.3).

4.5.1.1.3 CD68+ Staining for Macrophages

CD68 is an intracellular glycoprotein that binds low density lipoproteins, which are found exclusively in monocytes and macrophages. CD68 staining indicated that there was an influx of macrophages within the lungs in BLM-challenged mice (Figure 4.4). Positive pixel count of CD68+ cells showed that macrophage numbers were increased from day 7 by around 15-fold (p<0.001), and remained elevated by 15-fold at day 14 (p<0.005), and 10-fold to until at least day 21 (p<0.05) (Figure 4.5). There is great variation in CD68+ cell number at day 21 of BLM-challenged mice (Figure 4.5), suggesting that these cells are not essential at the established fibrotic phase.
Together these results demonstrate that BLM induces pulmonary fibrosis in mice characterised by the deposition of collagen within the lung. This was accompanied by a cellular influx from at least the late inflammatory phase (day 7) until the late fibrotic phase (day 21) and CD68+ macrophages constitute a component of the infiltrate.

Figure 4.2 Haematoxylin and Eosin (H&E) staining of lung from either saline or bleomycin (BLM)-challenged C57BL/6 mice. 8-10 week old male mice were challenged via an oropharyngeal route three times at 72 hour intervals with either saline, or 0.13 International Units (IU) BLM, and were harvested at days 7, 14 and 21 after the last dose. Paraffin-embedded sections of the left lung were stained for cellular influx using the H&E stain; n=5 for saline or n=4 for BLM-challenged mice from a single representative study.
Figure 4.3 Picrosirius Red (PSR) staining of lung from either saline or BLM-challenged C57BL/6 mice. 8-10 week old male mice were challenged three times via an oropharyngeal route at 72 hour intervals with either saline, or 0.13 IU BLM, and were harvested at days 7, 14 and 21 after the last dose. Paraffin-embedded sections of the left lung were stained for collagen using PSR stain; n=5 for saline or n=4 for BLM-challenged mice from a single representative study.
Figure 4.4 CD68+ staining of lung from either saline or BLM-challenged C57BL/6 mice. 8-10 week old male mice were challenged via an oropharyngeal route three times at 72 hour intervals with either saline, or 0.13 IU BLM, and were harvested at days 7, 14 and 21 after the last dose. Paraffin-embedded sections of the left lung were stained for CD68+ cells; n=5 for saline or n=4 for BLM-challenged mice from a single representative study.

Figure 4.5 Positive Pixel Count of CD68+ cells in lung of either saline or BLM-challenged C57BL/6 mice. 8-10 week old male mice were challenged three times via an oropharyngeal route at 72 hour intervals with either saline, or 0.13 IU BLM, and were harvested at days 7, 14 and 21 after the last dose. n=5 for saline or n=4 for BLM-challenged mice from a single representative study. Two way ANOVA with Tukey post-test was carried out for days 7 and 14 and data was represented as mean ± SD; for day 21, data appeared non-parametric and non-parametric student t-test was carried out for saline vs. BLM foam cell count; data was represented as mean ± interquartile range. *: p<0.05, **: p<0.005, ***: p<0.001. BLM, Bleomycin.
4.5.1.2 BAL differential cell count
In order to analyse cellular composition within the alveolar space during BLM-induced pulmonary fibrosis, differential cell counts were carried out on BAL. The results revealed an influx of macrophages in the alveolar space, with numbers increasing by about 8-fold (p<0.005) at day 7, and they remained elevated (~3-fold) at day 14 (p<0.01) and day 21 (~3-fold; p<0.05). Granulocyte levels in BAL were not significantly increased from day 7 onwards, indicating that this time point may represent the end of the inflammatory phase or a shift in the cellular components of the inflammatory response. There is also some lymphocytic influx at day 7 (p<0.005), but this had mostly subsided by day 14 (Figure 4.6).

4.5.1.3 BAL foam cell count
Foam cells are lipid-laden cells generated through the excessive phagocytosis of cholesterol via its carrier modified Low Density Lipoprotein (LDL), or the defective efflux of cholesterol. Foamy macrophages generated \textit{in vitro} or isolated \textit{in vivo} from atherosclerosis patients have been reported to have a pro-inflammatory phenotype, although one \textit{in vitro} study reported a pro-fibrotic phenotype. Foamy macrophages have been found to accumulate in BALF of IPF patients and HPS patients with interstitial pulmonary fibrosis. The cause of the formation of the foam cells is unknown in these patients, although a candidate for this is defective cholesterol efflux of macrophages.

Apolipoprotein-AI (Apo-AI) is a major component of high density lipoprotein (HDL) that facilitates cholesterol efflux from cells. In one report, Apo-AI is reported to be downregulated in BALF of IPF patients, and this is inversely correlated with the number of foamy macrophages. Treatment of BLM-challenged mice with dosage of Apo-AI via an intranasal route reduced collagen deposition and inflammatory cell accumulation. Foamy macrophages may play a role in pulmonary fibrosis through promoting tissue injury and/or fibrosis.

Oil Red O (ORO) is a lysochrome (fat-soluble) diazo dye that stains for neutral triglycerides and lipids, and can be used to stain for cholesterol crystals that are characteristic of foam cells. Application of Oil Red O (ORO) lipid staining showed that there is an accumulation of lipid-laden foam cells, with an increase from below 2% of cells in BALF in the saline controls to an average of around 8% at day 7 (p<0.005), 8% at day 14 (p<0.01) and a non-significant trend suggesting an increase of 4.4% at day 21 (Figures 4.7 & 4.8).

Collectively these results demonstrate that there is an accumulation of AMφ, including foamy macrophages, during BLM-induced pulmonary fibrosis, which may suggest a role for these cells in lung pathology that leads to fibrosis.
Figure 4.6 Differential Cell Count for BAL cells in saline or BLM-challenged C57BL/6 mice. 8-10 week old male mice were challenged via an oropharyngeal route three times at 72 hour intervals at days -7, -4 and -1, with either saline, or 0.13 IU BLM, and were harvested at days 7, 14 and 21 after the last dose. BAL was collected by 2 x 0.5 ml DPBS washes of the lung; 100 μl aliquot was prepared for differential cell count by cytospin followed by Wright-Giemsa stain. Differential cell count was carried out by counting at least 300 stained cells across 3 different fields. Total cell count was carried out by Trypan blue exclusion of dead cells. Data are represented as mean ± SD; n=6 for saline or n=8 for BLM-challenged mice from a single representative study. Two way ANOVA with Sidak post-test was carried out; *: p<0.05, **: p< 0.01, ***: p<0.005, ****: p<0.001. BLM, Bleomycin; NS, Not Significant.
Figure 4.7 Foam cell staining in BAL of saline or BLM-challenged C57BL/6 mice. 8-10 week old male mice were challenged via an oropharyngeal route three times at 72 hour intervals with either saline, or 0.13 IU BLM, and were culled at days 7, 14 and 21 after the last dose. BAL was collected by 2 x 0.5 ml DPBS washes of the lung; 100 μl aliquot was prepared for foam cell count by cytopsin followed by Oil Red O (ORO) stain. Differential cell count was carried out by counting at least 300 stained cells across 3 different fields. Results are from 2 studies; n=6 for saline or n=4 for BLM-challenged mice from a single representative study. Representative images are shown.
Chapter 4 | AMφ heterogeneity in BLM-induced pulmonary fibrosis

4.5.1.4 Gene and phenotypic changes in AMφ following BLM challenge

It was previously shown that AMφ isolated from naïve mice can be induced by selective conditions of cytokine priming into distinct subsets \textit{in vitro} (section 3.5.1.3), indicating that these terminally differentiated tissue macrophages display plasticity and are able to respond to their immediate environment by altering their phenotype.

In the current chapter, it is validated that BLM induces pulmonary fibrosis in mice (section 4.5.1.1), and AMφ were observed to accumulate in the lung (section 4.5.1.2). The next set of experiments were designed to investigate whether or not these AMφ from a disease model altered their phenotype to adapt to the change in pulmonary environment. It has been proposed that M1 macrophages play a more important role in inflammation-induced tissue injury, whereas M2 or M2-like macrophages have a greater role in active fibrosis\textsuperscript{278}. In the following studies, AMφ were isolated from saline or BLM-challenged mice at various time points and analysed \textit{ex vivo} for their gene, protein and surface protein expression of several key macrophage subset-associated markers.

The mRNA levels of the M2-associated mediator Arg-1 was increased in BLM-challenged mice. Arg-1 mRNA levels was significantly elevated from day 7 (51.3-fold; p<0.005); this trend appeared to sustain until at least day 21, although this was non-significant from day 14 (Figure 4.9). The mRNA levels of the M1-associated marker NOS2 was marginally enhanced at day 7 but this increase was not significant and was undetectable from day 14 onwards (Figure 4.9). A trend suggesting reduction of the gene transcript levels of the TGF-β/ hypoxia-associated PAI-1 was observed in AMφ isolated from BLM challenged mice as compared to saline controls (Figure 4.10).

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Figure 4.8 Foam cell count in BAL of saline or BLM-challenged C57BL/6 mice. 8-10 week old male mice were challenged via an oropharyngeal route three times at 72 hour intervals with either saline, or 0.13 IU BLM, and were culled at days 7, 14 and 21 after the last dose. BAL was collected by 2 x 0.5 ml DPBS washes of the lung; 100 μl aliquot was prepared for foam cell count by cytopin followed by ORO stain. Differential cell count was carried out by counting at least 300 stained cells across 3 different fields. Results are from 2 studies, n=6 for saline or n=6 for BLM-challenged mice from a single representative study. Two way ANOVA with Sidak post-test was carried out; ****: p<0.001. BLM, Bleomycin; NS, Not Significant.
These results were surprising as increased PAI-1 levels in AMφ have been associated with TGF-β or hypoxia induction in the previous chapter (section 3.5.1.3.1), two signals that have been reported to be prominent in the fibrotic environment118,261. Perhaps a negative feedback loop exists in which PAI-1 in AMφ is downregulated followed prolonged induction.

Figure 4.9 mRNA level analysis of macrophage subset-associated markers in AMφ isolated from C57BL/6 mice at various stages of BLM-induced pulmonary fibrosis. 8-10 week old male mice were challenged via an oropharyngeal route three times at 72 hour intervals with either saline, or 0.13 IU BLM, and were culled at days 7, 14 and 21 after the last dose. 1x10⁵ AMφ were seeded per well of 48-well culture plates for 90 minutes, and plastic adherence enriched AMφ were then analysed for change in mRNA levels of macrophage subset associated genes. The mRNA levels of NOS2 and Arg-1 were determined by qRT-PCR. Each sample was run in triplicates in qRT-PCR and the average was used to calculate the mean of sample repeats. Data is represented as mean ± SD; n=3 (days 14 and 21) for saline or n=7 (NOS2), n=9 (Arg-1) from three separate studies. Two way ANOVA with Sidak post-test was carried out; ***: p<0.005. BLM, Bleomycin; ND, Not Detected; NS, Not significant.

Figure 4.10 mRNA level analysis of PAI-1 in AMφ isolated from C57BL/6 mice at various stages of BLM-induced pulmonary fibrosis. 8-10 week old male mice were challenged via an oropharyngeal route three times at 72 hour intervals with either saline, or 0.13 IU BLM, and were culled at days 7, 14 and 21 after the last dose. 1x10⁵ AMφ were seeded per well of 48-well culture plates for 90 minutes, and plastic adherence enriched AMφ were then analysed for change in mRNA levels of macrophage subset associated genes. The mRNA levels of PAI-1 were determined by qRT-PCR. Each sample was run in triplicates in qRT-PCR and the average was used to calculate the mean of sample repeats. Data is represented as mean ± SD; n=3 from three separate studies. Two way ANOVA with Sidak post-test was carried out. BLM, Bleomycin; ND, Not Detected; NS, Not significant.
No increase in the levels of the M1-associated protein IL-1β was detectable in BLM-challenged mice beyond the late inflammatory phase at day 7 as compared to saline controls. On the other hand, the expression of another M1-associated protein IL-12 p40 (total), including free IL-12 p40, IL-12 p40 homodimers, or IL-12 p40 heterodimers (IL-12 p70 or IL-23) was elevated from day 7 onwards, from below 200 pg/ml in saline controls to ~600 pg/ml on day 7, ~800 pg/ml on day 14 and persists to ~1000 pg/ml at day 21 in BLM-challenged mice (p<0.01). However, the bioactive IL-12 p70 was undetectable (data not shown), indicating that IL-12 p40 (or IL-23), rather than IL-12 p70 participates in pulmonary fibrosis. Fibronectin, which is associated with a M2-phenotype, was increased by 6-fold during BLM-induced pulmonary fibrosis, from day 7 (p<0.01) through day 14 (p<0.005) to day 21 (p<0.01) (Figure 4.11).

**Figure 4.11** Protein level analysis of macrophage-associated markers in AMφ isolated from C57BL/6 mice at various stages of BLM-induced pulmonary fibrosis. 8-10 week old male mice were challenged via an oropharyngeal route three times at 72 hour intervals with either saline, or 0.13 IU BLM, and were culled at days 7, 14 and 21 after the last dose. 1x10⁵ AMφ were seeded per well of 48-well culture plates for 90 minutes, and plastic adherence enriched AMφ were then further cultured for 24 hours in fresh media. Isolated AMφ were then analysed for change in levels of macrophage subset associated proteins. The protein levels of IL-1β and IL-12 p40 (total), and fibronectin, were determined by ELISA and MSD respectively. Each sample was run in duplicates in MSD/ELISA and the average was used to calculate the mean of sample repeats. Data is represented as mean ± SD; IL-1β (saline): n=6 (day 7), n=7 (days 14 and 21), IL-1β (BLM): n=9 (days 7, 14 and 21); IL-12 p40 (total) (saline): n=6 (day 7), n=7 (days 14 and 21), IL-12 p40 (total) (BLM): n=8 (days 7 and 14), n=7 (day 21), fibronectin (saline): n=3 (days 7 and 21), n=5 (day 14), fibronectin (BLM): n=4 (day 7), n=6 (days 14 and 21) from two separate studies. Two way ANOVA with Sidak post-test was carried out; **: p<0.01, ***: p<0.005, ****: p<0.001. BD, Below Detection; BLM, Bleomycin; NS, Not significant. IL-12 p40 (total) incudes free IL-12 p40, IL-12 p40 homodimers and IL-12 p40 heterodimers (IL-12 p70 and IL-23). Standard curves for ELISA/MSD of these proteins of interest can be found in Appendix 4.
AMφ from saline or BLM-challenged mice were co-stained for the cell surface proteins CD11c, MHC-II and MRC1. As predefined in the previous chapter (see Chapter 3, section 3.5.2.1), M1 macrophages are CD11c+ MHC-IIhi MRC1-, whereas M2 macrophages are CD11c+ MHC-II-/lo MRC1-/lo. In the flow cytometry analysis below, AMφ were defined as CD11c+ FSC hi SSC hi, and quadrants for MHC-II and MRC1 were set with respect to their respective isotype controls. The results revealed that M1-like macrophages accumulated at day 7, but they are gradually replaced by M2-like macrophages to at least day 21 post BLM challenge (Figures 4.12 and 4.13). Collectively these results demonstrate that in BLM-induced pulmonary fibrosis that M1-like AMφ are present by day 7 and thus may contribute to the late inflammatory response, whereas M2-like AMφ appear later and therefore may play a role in the fibrotic phase.

**Saline**

![Figure 4.12](image)

**Day 7**

**Day 14**

**Day 21**

Figure 4.12 Cell surface protein level analysis of macrophage subset-associated markers in AMφ isolated from saline-challenged C57BL/6 mice at various stages of BLM-induced pulmonary fibrosis. 8-10 week old male mice were challenged via an oropharyngeal route, with either saline or 0.13 IU bleomycin, for 3 times at 72 hour intervals. Mice were culled at 7, 14 and 21 days after the last dose. BAL cells were then harvested and stained with CD11c-AP/Cy7 (2 μg/ml), MHC-II-FITC (2 μg/ml) and MRC1-PerCP/Cy5.5 (2 μg/ml) antibodies by flow cytometry. AMφ were identified as Side Scatter (SSC) hi Forward Scatter (FSC) hi CD11c+ and autofluorescent in the FL1 channel, and analysed for changes in surface protein expression in MHC-II and MRC1 by flow cytometry as shown above. Quadrants were set based on the MFI of their respective isotype controls. Representative images are shown; n=8 for saline or n=5 for BLM-challenged mice from two separate studies. Graphs for repeats of this study can be found in Appendix 5.
Figure 4.13 Cell surface protein level analysis of macrophage subset-associated markers in AMφ isolated from BLM-challenged C57BL/6 mice at various stages of BLM-induced pulmonary fibrosis. 8-10 week old male mice were challenged via an oropharyngeal route, with either saline or 0.13 IU bleomycin, for 3 times at 72 hour intervals. Mice were culled at 7, 14 and 21 days after the last dose. BAL cells were then harvested and stained with CD11c-AP/Cy7 (2 μg/ml), MHC-II-FITC (2 μg/ml) and MRC1-PerCP/Cy5.5 (2μg/ml) antibodies by flow cytometry. AMΦ were identified as Side Scatter (SSC)\textsuperscript{hi} Forward Scatter (FSC)\textsuperscript{hi} and autofluorescent in the FL1 channel, and analysed for changes in surface protein expression in MHC-II and MRC1 by flow cytometry as shown above. Quadrants were set based on the MFI of their respective isotype controls. Representative images are shown; n=8 for saline or n=5 for BLM-challenged mice from two separate studies. Graphs for repeats of this study can be found in Appendix 5.
These results also demonstrate that AMφ are capable of altering their phenotype to adapt to the immediate environment in BLM challenged mice, and have different gene and protein expression at different stages of pulmonary fibrosis. The AMφ phenotypes do not necessarily fall within a predefined subset that has been characterised in vitro, but rather express a mixture of M1- and M2-associated mediators when analysed ex vivo. These results suggested that there was either the presence of two independent macrophage subset populations, or a hybrid population expressing mediators of both subsets.

At the late inflammatory phase (day 7), AMφ displayed a trend suggesting increase of the M1-associated markers, including NOS2 (Figure 4.9) and IL-1β (Figure 4.11). It was also observed that AMφ had a significant increase in another M1-associated mediator IL-12 p40 (total) (Figure 4.11), and also the M2-associated products Arg-1 (Figure 4.9) and fibronectin (Figure 4.11). Flow cytometry analysis of M1- or M2-associated surface protein expression has revealed two separate populations, including a MHC-II^hi MRC1^- and a MHC-II^-/lo MRC1^- population (Figures 4.12 and 4.13) that may contribute to the mixture of M1- and M2-associated phenotypes.

The M1-associated mediator IL-12 p40 (total), and also the M2-associated mediator fibronectin both persist throughout the early and established fibrotic phases (days 14 and 21) (Figure 4.11); analysis of the changes in mRNA levels of the M2-associated mediator Arg-1 also revealed a trend suggesting its increase in the fibrotic phases (Figure 4.9). However, flow cytometric analysis of M1- or M2-associated surface protein levels has revealed one predominant population, MHC-II^-/lo MRC1^-/lo (Figures 4.12 and 4.13), suggesting that a hybrid macrophage population may participate in active fibrosis. These results may reflect the presence of a complex variety of signals within the lung environment that may influence AMφ phenotype during pulmonary fibrosis, such that they cannot be neatly classified into pre-defined subsets that have been characterised in vitro.

### 4.5.1.5 Other phenotypic changes in AMφ isolated from BLM-challenged mice

Having identified that there is a shift in AMφ phenotype from M1-like to M2-like from the late inflammatory phase to the fibrotic phases, the next set of experiments were designed to investigate whether or not this phenotypic reprogramming was associated with changes in other macrophage derived proteins that have been reported to play a role in pulmonary fibrosis. AMφ were isolated from saline or BLM-challenged mice at days 7, 14 and 21, cultured in media ex vivo for 24 hours, and the culture supernatant was measured for protein levels of pulmonary fibrosis-associated mediators, including IL-6, CCL2 (MCP-1), CCL3 (MIP-1α), CCL4 (MIP-1β), CCL5 (RANTES), CXCL2 (MIP-2), CXCL20 (MIP-3α), and mMMP-9. Surprisingly, CCL3, CCL4, CCL20 and mMMP-9 levels were all below detectable level in both saline and BLM
challenged mice. There was a small increase in CCL5 in AMϕ BLM challenged mice at day 7, but this change in expression disappeared by day 14. There was also a trend indicating an elevation in IL-6 levels and CCL2 that mirrored that of CCL5, but significance was not reached possibly as the increase in production was limited. No enhancement was observed for CXCL2 (Figure 4.14). This lack of significance in the increase in pro-inflammatory mediators associated with IPF may reflect the decline of inflammation by the late inflammatory phase when expression was first measured. Alternatively BLM-induced pulmonary fibrosis may have different characteristics to IPF as compared to saline controls.

Figure 4.14. Protein level analysis of pro-inflammatory mediators in AMϕ isolated from C57BL/6 mice at various stages of BLM-induced pulmonary fibrosis. 8-10 week old male mice were challenged via an oropharyngeal route three times at 72 hour intervals with either saline, or 0.13 IU bleomycin, and were culled at days 7, 14 and 21 after the last dose. 1x10^5 AMϕ were seeded per well of 48-well culture plates for 90 minutes, and plastic adherence enriched AMϕ were then further cultured for 24 hours in fresh media. Isolated AMϕ were then analysed for changes in levels of pro-inflammatory proteins. The protein levels of IL-6, CCL2, CCL5 and CXCL2 were determined by MSD. Each sample was run in duplicates in MSD/ELISA and the average was used to calculate the mean of sample repeats. Data is represented as mean ± SD; n=6 (saline), n=7 (BLM) from two separate studies. Two way ANOVA with Sidak post-test was carried out; *: p<0.05, **: p<0.01, ***: p<0.005, ****: p<0.001. BD, Below Detection; BLM, Bleomycin; NS, Not significant. Standard curves for ELISA/MSD of these proteins of interest can be found in Appendix 6.
In conclusion, the results reported here demonstrate that this novel multi-dose oropharyngeal challenge of BLM induces pulmonary fibrosis in mice, as observed by excessive collagen deposition and influx of inflammatory cells. There is an accumulation of CD68+ lung macrophages, which included a population of foamy AMφ present in the BAL. An influx of lymphocytes was also observed in BAL. The macrophage infiltrate may contribute to pulmonary fibrosis by differentiating into distinct functional phenotypes. M1-like macrophages accumulate during the late inflammatory phase (day 7), and may be a key pathogenic component of the acute response that causes lung injury. However their numbers subside by the early fibrotic phase (day 14), and are accompanied by reduced biosynthesis of pro-inflammatory mediators. A M1/M2-like hybrid phenotype was identified from the late inflammatory phase (day 7) until at least the established fibrotic phase (day 21). This may be an active cellular component that drives fibrosis through the direct release of the ECM component fibronectin and potentially other pro-fibrotic mediators, and also macrophage recruitment through the release of IL-12 p40 (total).

4.5.1.6 Co-culture of Mouse Pulmonary Fibroblasts (MPF) with supernatant from AMφ isolated from saline or BLM-challenged mice

In the above studies it was observed that the phenotype of AMφ was influenced by its surroundings following BLM challenge, and resulted in the increased expression of many pro-inflammatory or pro-fibrotic mediators. These mediators may influence the phenotype of other cells downstream to further facilitate fibrosis. In the following preliminary studies, the influence of AMφ activation on the phenotype of MPFs, one of the major contributors to ECM deposition in pulmonary fibrosis, was investigated. Supernatants were collected from AMφ isolated from BLM-challenged mice or saline controls, and then co-cultured with MPF. The change in MPF gene expression of collagen 1α1 (Col1α1), a major ECM component found in lung fibrosis was then analysed.

The results indicate that the co-culture of supernatants from AMφ from BLM-challenged mice, but not saline controls, promoted Col1α1 gene expression in MPF in the late inflammatory phase (day 7) to early fibrotic phase (day 14). However the increase in expression of Col1α1 that was observed early on in the response returned to basal levels by the late fibrotic phase (day 21) (Figure 4.15). These results suggest that in addition to directly increasing the expression of the ECM component fibronectin, AMφ may also promote fibrosis indirectly by activating pulmonary fibroblasts through the release of various pro-fibrotic mediators. Further studies will have to be carried out to confirm the identity of these mediators.
Figure 4.15 Secreted proteins produced by AMΦ isolated from BLM-challenged C57BL/6 mice promote a pro-fibrotic response in mouse pulmonary fibroblasts (MPF). 8-10 week old male mice were challenged via an oropharyngeal route, with either saline or 0.13 U BLM, for 3 times at 72 hour intervals. Mice were culled at 7, 14 and 21 days after the last dose. 1x10⁵ AMΦ were seeded per well of 48-well culture plates for 90 minutes, and then further cultured in fresh media for 24 hours; AMΦ culture supernatant was collected and then co-cultured with 1x10⁵ MPF per well of a 48-well plate from naïve mice for 24 hours. MPF were then harvested and analysed for Col1α1 mRNA levels. Secreted protein by AMΦ isolated from BLM-challenged mice promotes a pro-fibrotic response, as indicated by the increase in Col1α1 mRNA levels in MPF co-cultured with AMΦ supernatant from BLM-challenged mice as compared to saline controls. Data are represented as mean ± SD; n=3 from two separate studies. Two way ANOVA with Sidak post-test was carried out; ***: p<0.005, ****: p<0.001. NS: Not Significant.
4.5.2 Comparison between HPS1 mice and WT C57BL/6 mice

Foamy macrophages were reported to accumulate in IPF and HPSPF patients, and this was also observed in my own studies of BLM-induced pulmonary fibrosis. Foamy macrophages arise from increased modified LDL uptake by scavenger receptors on macrophages, or defective cholesterol efflux from macrophages. It was proposed that foamy macrophages may contribute to pulmonary fibrosis through increased release of pro-inflammatory and/or pro-fibrotic mediators.

As aforementioned in the introduction, HPS1 mice have defects in lysosomal transport that may result in the excessive accumulation of foamy macrophages, which may contribute to their reported increased susceptibility to BLM-induced pulmonary fibrosis. By comparing BLM-induced pulmonary fibrosis in HPS1 mice that are more likely than not to have an increased accumulation of foamy macrophages (versus non foamy counterparts) as compared to WT C57BL/6 controls, the potential phenotype of foamy macrophages can be evaluated.

4.5.2.1 Comparison between naïve HPS1 mice and WT C57BL/6 mice

The phenotypes of naïve HPS1 mice and WT C57BL/6 controls were first evaluated to identify any baseline differences that may exist between the two strains. Several parameters were assessed: first, the generation of HPS1 mice was confirmed by visual inspection of mice appearance, and also outsourced genotyping (Transnetyx (Tennessee, United States)). Second, spontaneous inflammation and/or fibrosis in HPS mice were evaluated by histological analyses of cellular infiltration and collagen accumulation, and mRNA level analyses of the pro-inflammatory mediator IL-6, and the pro-fibrotic mediators Col1α1 and fibronectin. Third, the constitutive activation state of AMφ was assessed by measuring for the pro-inflammatory mediators TNF-α and CXCL1, and the pro-fibrotic mediator fibronectin, in the culture supernatant.
4.5.2.1.1 Differences in phenotype between HPS1 and WT C57BL/6 mice

The breeding pair of HPS1 mice were purchased from Jackson Laboratory (Maine) and the colony was bred and maintained in-house. HPS1 mice were genotyped by Transnetyx (Tennessee, United States). As published HPS1 mice have hypopigmented ears and tails compared to their wild type counterparts (Figure 4.16), and often referred to as ‘pale ear’. This phenotypic difference may result from defective intracellular trafficking in melanosomes found in the ears and tail of HPS1 mice.

![WT C57BL/6 and HPS1 mice](image)

Figure 4.16 Differences in phenotype between wild type (WT) C57BL/6 and Hermansky Pudlak Syndrome 1 (HPS1) mice. The breeding pair of HPS1 mice were purchased from The Jackson Laboratory (Maine) and the colony was bred and maintained in-house. HPS1 mice generated were genotyped by Transnetyx (Tennessee, United States).
4.5.2.1.2 Histological analysis of the lungs of naïve HPS1 and WT mice

Histological analysis of the lung showed that there was no constitutive cell accumulation or fibrosis in the lungs of HPS1 mice, as indicated by H&E (Figure 4.17) and PSR staining (Figure 4.18) respectively.

**Figure 4.17** Haematoxylin and Eosin (H&E) staining of lung sections in WT C57BL/6 and HPS1 mutant mice. 8-14 week HPS1 mice and WT C57BL/6 controls were culled. Bronchoalveolar Lavage (BAL) was performed for the isolation of alveolar macrophages (AMφ). Lungs were then inflated with Optimal Cutting Temperature (OCT) and snap frozen in liquid nitrogen; 10μm sections were cut for histology analysis. The remainder of the frozen lung was crushed for mRNA level analysis. Representative images are shown. WT C57BL/6, n=3; HPS1, n=4 from one study.

**Figure 4.18** Picrosirius Red (PSR) staining of lung sections in WT C57BL/6 and HPS1 mutant mice. 8-14 week HPS1 mice and WT C57BL/6 controls were culled. BAL was performed for the isolation of AMφ. Lungs were then inflated with OCT and snap frozen in liquid nitrogen; 10μm sections were cut for histology analysis. The remainder of the frozen lung was crushed for mRNA level analysis. Representative images are shown. WT C57BL/6, n=3; HPS1, n=4 from one study.
4.5.2.1.3 Analysis of mRNA levels in lung tissue from HPS1 and WT mice

The analysis of mRNA levels in the lungs following BAL washing (i.e. in the absence of AMΦ) revealed the presence of a non-significant trend towards increased expression of the pro-inflammatory cytokines IL-6 and TNF-α in the lungs of HPS1 mice aged 8-14 weeks (Figure 4.19). There is a high level of variation in the mRNA levels of IL-6 and TNF-α in the lungs of HPS1 mice, potentially because there is variation in the degree of functional lysosomal transport caused by the HPS1 mutation between individual mice. HPS1 mice with less functional lysosomal transport may have greater intrinsic lung injury that is caused by defective surfactant proteins by AEC2, or cholesterol accumulation in foamy AMΦ, which results in increased inflammation.

This is concurrent with the lack of cellular infiltration as identified in H&E staining of histological analysis (section 4.5.2.1.2). The increased pro-inflammatory mediator transcript levels may be found in activated AECs in response to intrinsic lung injury. Further repeats and protein level analysis of these mediators will have to be carried out to assess whether or not constitutive inflammation is prevalent in HPS1 mice. There have been no previous reports of constitutive inflammation in young HPS1 mice, although histological analysis has revealed that CD45+ inflammatory cell infiltrates accumulate in aged HPS1 mice (>1.5 years old)\textsuperscript{718}.

Figure 4.19 The mRNA level analysis of the pro-inflammatory mediators in lung tissue from HPS1 mice and WT C57BL/6 controls. 8-14 week HPS1 mice and WT C57BL/6 controls were culled; BAL was performed for the isolation of AMΦ. Lungs were then inflated with OCT and snap frozen in liquid nitrogen; 10μm sections were cut for histology analysis. The remainder of the frozen lung was crushed for mRNA level analysis. The mRNA of the pro-inflammatory mediators IL-6 and TNF-α were determined by qRT-PCR. Data are from one pilot study, and each sample was run in triplicates and the average was presented as relative % to the housekeeping gene HPRT-1. Data is represented as mean ± SD; WT, n=4; HPS1, n=4. Student t-test was carried out; NS: Not significant.
There was a trend suggesting a reduction of fibronectin in HPS1 mice, although this was not significant (Figure 4.20). There appeared to be a large increase in Col1α1 mRNA levels in one of the HPS1 mice compared to controls. As with the spread of mRNA levels of pro-inflammatory mediators observed above, it is possible that different levels of defective lysosomal transport, as a result of HPS1 mutation, may lead to various degrees of tissue injury, thereby inducing different amounts of Col1α1 gene expression. Further repeats assessing gene transcript levels and assays evaluating protein levels will have to be carried out to confirm this.

Figure 4.20 The mRNA levels of ECM components in lung tissue from HPS1 mice and WT C57BL/6 controls. 8-14 week HPS1 mice and WT C57BL/6 controls were culled; BAL was performed for the isolation of AMφ. Lungs were then inflated with OCT and snap frozen in liquid nitrogen; 10μm sections were cut for histology analysis. The remainder of the frozen lung was crushed for mRNA level analysis. The mRNA levels of the ECM components collagen 1 α 1 (Col1α1) and fibronectin were determined by qRT-PCR. Data are from one pilot study, and each sample was run in triplicates and the average was presented as relative % to the housekeeping gene HPRT-1. Non-parametric Mann-Whitney post-test was carried out for Col1α1 data, and presented as median ± interquartile range. Parametric student t-test was carried out for Fibronectin data, and is represented as mean ± SD. WT C57BL/6 control, n=4; HPS1, n=4 from one study. NS: Not significant.
4.5.2.1.4 Differential cell count of BAL cells from HPS1 and WT mice

Differential cell count by Wright-Giemsa staining showed that there was no difference in cellular composition in BALF isolated from HPS1 mice compared to C57BL/6 controls. In both cases AMφ was the predominant BAL cell population (>95%), with few lymphocytes or granulocytes observed (Figure 4.21).

Figure 4.21 Differential cell count of BAL cells isolated from HPS1 mice and WT C57BL/6 controls. 8-14 week HPS1 mice and WT C57BL/6 controls were culled; BAL was performed by 2 x 1 ml DPBS washes. 100 μl of BAL was aliquoted and BAL cells were centrifuged onto microscopic slides and stained with Wright-Giemsa stain for differential cell count. Differential cell count was carried out by counting 300 cells from at least 3 fields per sample. Data is presented as number of cells/mouse [i.e. no. of cells counted x 20 (2 ml/ 100 μl)] and is represented as mean ± SD; WT, n=4; HPS1, n=4 from one pilot study. One way ANOVA with Tukey post-test was carried out; NS: Not significant.
4.5.2.1.5 Naïve HPS1 mice do not have altered numbers of foam cells in their lungs compared to WT C57BL/6 mice

As foamy macrophages have been reported to accumulate in the lungs of HPSPF patients, it was investigated whether or not such accumulation also occurred in HPS1 mice. However, as shown by the lack of ORO staining of the AMφ there were no foamy macrophages detected in either HPS1 mice or WT C57BL/6 controls (Figure 4.22).

![WT C57BL/6](image1)

![HPS1](image2)

**Figure 4.22** Oil Red O (ORO) staining of BAL cells isolated from naïve HPS1 mice or WT C57BL/6 controls. 8-14 week HPS1 mice and WT C57BL/6 controls were culled. 1 x 10^5 AMφ were seeded per well of a 48-well tissue culture plate for 90 minutes. AMφ enriched by plastic adherence were then further cultured in fresh media for 24 hours. ORO stained cells were determined by counting 300 cells from at least 3 fields per sample. Representative images are shown; n=4 (WT C57BL/6) and n=3 (HPS1) from one study.

4.5.2.1.6 Measurement of IL-12 p40 (total) and fibronectin protein levels in AMφ isolated from naïve HPS1 and WT C57BL/6 mice

AMφ isolated from naïve HPS1 or WT C57BL/6 mice were then analyzed for constitutive protein expression of pro-inflammatory mediators and fibronectin. In contrast to a previous report where AMφ isolated from HPS1 were described as constitutively activated, there appeared to be no such activation of AMφ from naïve HPS1 mice here, as observed by unaltered levels of TNF-α and CXCL1 in HPS1 mice as compared to WT C57BL/6 controls (Figure 4.23). Levels of IL-1β were also investigated, but they were below detection limit in both mouse strains (data not shown). There was also no increased levels of fibronectin in HPS1 mice compared to WT C57BL/6 controls (Figure 4.24). Overall these results suggest that AMΦ do not contribute towards increased inflammation and fibrosis in naïve HPS1 mice.
Figure 4.23 Protein level analyses of pro-inflammatory mediators in AMΦ from HPS1 mice and WT C57BL/6 controls. 8-14 week HPS1 mice and WT C57BL/6 controls were culled; BAL was performed for the isolation of AMΦ by 2 x 1 ml DPBS washes. 1 x 10^5 AMΦ was seeded per well of a 48-well tissue culture plate and isolated from other BAL cells by plastic adherence for 90 minutes. Culture media was then changed and AMΦ were further cultured for 24 hours. Culture supernatant then was collected and subject to TNF-α and CXCL1 protein level analysis. Protein levels of the pro-inflammatory mediators TNF-α and CXCL1 were determined by MSD. WT, n=3; HPS1, n=3 from one pilot study and evaluation per samples were carried out in duplicates. Parametric student t-test was carried out for TNF-α data, and is represented as mean ± SD. Non-parametric Mann-Whitney post-test was carried out for CXCL1 data, and presented as median ± interquartile range. NS: Not significant. Standard curves for MSD of TNF-α and CXCL1 are shown in appendix 7.

Figure 4.24 Protein level analysis of Fibronectin in AMΦ from HPS1 mice and WT C57BL/6 controls. 8-14 week HPS1 mice and WT C57BL/6 controls were culled; BAL was performed for the isolation of AMΦ by 2 x 1 ml DPBS washes. 1 x 10^5 AMΦ was seeded per well of a 48-well tissue culture plate and isolated from other BAL cells by plastic adherence for 90 minutes. Culture media was then changed and AMΦ were further cultured for 24 hours. Culture supernatant then was collected and subject to fibronectin protein level analysis. WT, n=4; HPS1, n=4 from one pilot study and evaluation per samples were carried out in duplicates. Protein level of fibronectin was determined by ELISA. Data is represented as median ± interquartile range. Non-parametric Mann-Whitney test was carried out; NS: Not significant. Standard curves for ELISA of Fibronectin is shown in appendix 7.
4.5.2.2 Comparison of BLM-induced pulmonary fibrosis between HPS1 mice and WT C57BL/6 mice

Having identified that there were no differences in basal levels of inflammation, collagen accumulation and fibrosis, foamy macrophage accumulation and macrophage activation between naïve HPS1 mice and WT C57BL/6 controls, next it was assessed whether or not HPS1 mice had an increased susceptibility to BLM-induced pulmonary fibrosis as reported before, and if an increased accumulation of foamy macrophages was observable when compared to WT controls.

4.5.2.2.1 Comparison of weight change between HPS1 mice and WT C57BL/6 controls following BLM-challenge

Having established that there was no lung inflammation or fibrosis in naïve HPS1 mice as compared to WT C57BL/6 controls, next it was investigated whether or not HPS1 mice have increased susceptibility to BLM-induced pulmonary fibrosis challenge.

Although Young and colleagues have investigated the susceptibility of HPS1 mice to BLM-induced pulmonary fibrosis, this study differs in two key aspects. First, in contrast to previous work HPS1 and WT C57BL/6 mice received multiple doses of BLM, namely three times via an oropharyngeal route rather than a single intratracheal dose. This protocol allows better tolerance of the mice to BLM challenge, and mimics more appropriately the persistent irritation encountered during pulmonary fibrosis. Secondly, the effects of BLM challenge on HPS1 and WT C57BL/6 mice were also studied beyond the late inflammatory phase at day 7 (as previously reported Young LR, 2007) until day 21 (established fibrotic phase).

Increased susceptibility to BLM-induced pulmonary fibrosis is associated with reduced tolerance to BLM challenge that is marked with elevated weight loss in mice. It was observed that mice that are less tolerant of BLM are more poorly and have a reduced appetite, resulting in a greater weight loss. Tolerance of mice to BLM challenge was initially determined by percentage weight loss. The results demonstrated that there was a significant increase in % weight loss in BLM-challenged HPS1 mice compared to WT C57BL/6 controls, from a loss of 0.978% in WT C57BL/6 controls to 7.36% in HPS1 mice at day 8 (7.53-fold; p<0.01), and from a weight gain of 2.31% in WT C57BL/6 mice to a weight loss of 9.45% in HPS1 mice at day 11 (5.09-fold; p<0.05), around the late inflammatory phase. However, there was no significant weight change between saline challenged HPS1 and WT C57BL/6 mice beyond day 11 (Figure 4.25). As significant weight loss occurred at days 8 and 11 of the late inflammatory phase but not later in the fibrotic phase, this would suggest that reduced tolerance to BLM challenge in HPS1 mice may be due to enhanced inflammation rather than increased pulmonary fibrosis.
Figure 4.25 Weight change in HPS1 and WT C57BL/6 control mice after BLM or saline challenge. 8-14 week old HPS1 and WT C57BL/6 mice were challenged via an oropharyngeal route, with either saline or 0.13 U BLM, for 3 times at 72 hour intervals at days -7, -4 and -1. Mice were culled at 7, 14 and 21 days after the last dose. Mice were weighed daily from the first day of oropharyngeal challenge. The average weight of each group per time point is reported, and data is represented as mean ± SD. Saline-challenged WT C57BL/6 mice (day-7, day-4, day-1, day 2, and day 5, n=12; day 8 and day 11, n=8; day 14, day 17 and day 20, n=4), BLM-challenged WT C57BL/6 mice (day-7, day-4, day-1, day 2, and day 5, n=12; day 8, day 11 and day 14, n=8; day 17 and day 20, n=4), saline-challenged HPS1 mice (day-7, day-4, day-1, day 2, and day 5, n=12; day 8 and day 11, n=8; day 14, n=6; day 17 and day 20, n=4), BLM-challenged HPS1 mice (day-7, day-4, day-1, n=11; day 2, and day 5, n=10; day 8, n=7; day 11, n=6; day 14, n=4; day 17 and day 20, n=2). Two way ANOVA with Sidak post-test was carried out; NS: Not significant, *: p<0.05, **: p< 0.01, ***: p<0.005, ****: p<0.001.

4.5.2.2.2 Histological analysis of lung tissue from saline or BLM-challenged HPS1 and WT C57BL/6 mice

The reduced tolerance of HPS1 mice to BLM challenge may be due to increased inflammation and/or fibrosis in HPS1 mice compared to the WT C57BL/6 controls. In order to investigate this hypothesis, histological analysis by H&E and PSR staining was first carried out to confirm if cellular infiltration and collagen deposition has occurred. Active inflammation and fibrosis in each mouse strain was then quantified by mRNA level analysis of lung tissue. Results from the H&E and PSR histological analyses show that BLM-challenge induced cellular influx (Figure 4.26) and collagen deposition (Figure 4.27) respectively in the interstitium of the lungs of both HPS1 mice and WT C57BL/6 controls. The area of cellular infiltration and collagen accumulation in BLM-challenged HPS1 mice appears to be less than that in WT C57BL/6 controls (Figures 4.26 and 4.27). The potential reduction in cellular infiltration in BLM-challenged HPS1 mice can be quantified using positive pixel count of hematoxylin stained cells.
Figure 4.26 H&E staining of HPS1 and WT C57BL/6 control mice following saline or BLM challenge. 8-14 week old HPS1 and WT C57BL/6 mice were challenged via an oropharyngeal route, with either saline or 0.13 U BLM, for 3 times at 72 hour intervals. Mice were culled at 7, 14 and 21 days after the last dose. BAL was performed for the isolation of AMφ; lungs were then inflated with OCT and snap frozen in liquid nitrogen. 10μm frozen lung sections were sliced for histology analysis; the remainder was crushed for RNA analysis. Representative images are shown from one study.
Figure 4.27 PSR staining of HPS1 mice or WT C57BL/6 controls following saline or BLM challenge. 8-14 week old HPS1 and WT C57BL/6 mice were challenged via an oropharyngeal route, with either saline or 0.13 U BLM, for 3 times at 72 hour intervals. Mice were culled at 7, 14 and 21 days after the last dose. BAL was performed for the isolation of AMφ; lungs were then inflated with OCT and snap frozen in liquid nitrogen. 10μm frozen lung sections were sliced for histology analysis; the remainder was crushed for RNA analysis. Representative images are shown from one study.
4.5.2.2.3 Analysis of mRNA levels of lung tissue from saline or BLM-challenged HPS1 mice or WT C57BL/6 mice

Having confirmed that BLM challenge induces both cellular infiltration in HPS1 mice as well as WT C57BL/6 mice, active inflammation and fibrosis was then evaluated by mRNA level analysis. In the following studies, lung tissues were first washed with DPBS (BAL), and AMΦ in BAL was isolated by plastic adherence on tissue culture plates for later studies. Lavaged lung tissue that is depleted of AMΦ is then subject to mRNA level analysis, and it is therefore presumed that any changes in mRNA levels to the lung observed in these studies are independent of AMΦ. The role of AMΦ was investigated separately (section 4.4.2.2.6). IL-6 was chosen as this pro-inflammatory cytokine has an established role in pulmonary fibrosis. IL-6 is elevated in the lungs of mice with experimental pulmonary fibrosis407, and also in BALF of IPF patients88. Genetic or pharmacological ablation of IL-6 attenuated pulmonary fibrosis in BLM challenged mice407. IL-6 may play a role in pulmonary fibrosis by inducing the conversion of lung fibroblasts into myofibroblasts403, and thereby promote collagen synthesis. Results showed that on day 7 post-BLM challenge, there was a significant increase of 9.31-fold (p<0.05) in IL-6 gene transcript levels in the lungs of BLM-challenged HPS1 mice, and a non-significant trend towards an increase of 2.96-fold (p>0.05) in BLM-challenged WT C57BL/6 mice compared to their respective saline controls (Figure 4.28). IL-6 gene transcript levels returned to basal levels by day 14 in both BLM-challenged HPS1 and WT C57BL/6 mice. This potential early increase in the inflammatory response in HPS1 mice may contribute to their reduced tolerance and increased weight loss following exposure to BLM (Figure 4.25).

As regards to the fibrotic response it was noted that there was an increase in the mRNA levels of the ECM component Col1α1 in both WT C57BL/6 mice and HPS1 mice upon BLM challenge as compared to saline controls. Col1α1 gene transcript levels in WT C57BL/6 mice were increased by 3.34-fold at day 7 (p<0.05) and 2.89-fold at day 14 (p<0.01), but returned to basal levels at day 21 post BLM challenge. A rise in Col1α1 gene transcript levels in HPS1 mice by 3-fold was also observed at day 7 (p<0.05), but this returned to basal levels at day 14 post BLM challenge, suggesting a decreased duration of active fibrosis (Figure 4.29). However, there is no difference in the amount of increase between the two strains of mice at day 7 post BLM challenge (Figure 4.29). This difference in gene transcript levels was reflected in histology analysis using PSR staining, where reduced fibrosis was observed in BLM-challenged HPS1 mice as compared to WT C57BL/6 controls (Figure 4.27).

A similar trend was observed for another ECM component fibronectin (Figure 4.30). Fibronectin mRNA levels in WT C57BL/6 mice were elevated by 5.27-fold at day 7 (p<0.05) and 4.32-fold at day 14 (p<0.005), but returned to basal levels at day 21 post BLM challenge. There was a trend
suggesting increase in mRNA levels of fibronectin in HPS1 mice by 3.73-fold at day 7, but this was no longer observed by day 14 post BLM challenge. This suggests that enhancement of ECM gene transcript levels in response to BLM challenge is short-lived in HPS1 mice as compared to WT C57BL/6 controls. Such increase in ECM levels also does not appear to affect long-lasting accumulation, as less collagen deposition was observed across the whole lung in HPS1 mice as compared to WT C57BL/6 controls, observed from PSR staining (data not shown).

Collectively these results reveal a trend in elevated levels of inflammation but not fibrosis within the lungs of HPS1 mice compared to WT C57BL/6 controls. In contrast, there may be less ECM deposition within the lungs of HPS1 mice, as revealed by a shorter duration of increased transcript levels of the key pro-fibrotic mediators Col1α1 and fibronectin within the lungs of HPS1 mice, and also a lesser extent of PSR staining observed. These suggest that reduced tolerance in HPS1 mice to BLM challenge compared to WT C57BL/6 controls is not caused by an increased susceptibility to BLM-induced pulmonary fibrosis but perhaps an increased pro-inflammatory response.

4.5.2.2.4 Differential cell count of BAL cells from saline and BLM challenged HPS1 and WT C57BL/6 mice

The results showed that the inflammatory response in the BLM challenged HPS1 mice and WT C57BL/6 controls were comparable. However, there was a difference in the kinetics of active fibrosis, in that increase of gene transcript levels of ECM components in HPS1 mice returned to basal level at an earlier time point than that observed in the WT C57BL/6 counterparts. In the next set of experiments I then investigated whether or not the influx of immune cells into the lungs varied between the two mouse strains after BLM challenge. The differential cell count was carried out by Wright-Giemsa staining of BAL cells. AMφ are identified by their larger size (10-20 μm), round shape, with a large central nucleus and clear vacuolated cytoplasm. Lymphocytes are smaller in size (8-10 μm) with a large central nucleus and little cytoplasm. Granulocytes are also small in size (8-10 μm) but are polynucleated and are characterised by their cytoplasmic granules.

As the results indicate, there was an influx of AMφ upon BLM challenge in both HPS1 and WT C57BL/6 mice at day 7 post-challenge. The number of AMφ present significantly increased from 2.53 x 10^4 to 15.0 x 10^4 in WT C57BL/6 mice following BLM challenge (5.92-fold; p<0.05), and there was a trend suggesting an escalation in the number of AMφ, from 6.56 x 10^4 to 18.0 x 10^4, in the HPS1 mice (2.74-fold). However, this returned to basal levels by day 14 post-challenge in both mouse strains (Figure 4.31). Furthermore, there is no apparent difference in the number of AMφ infiltrating the lungs of BLM-challenged HPS1 or WT C57BL/6 mice (Figure 4.31).
An influx of lymphocytes was noted in WT C57BL/6 mice in response to BLM challenge, increasing from $0.0361 \times 10^4$ to $3.53 \times 10^4$ at day 7 (96.5-fold; $p<0.01$), and $0.0915 \times 10^4$ to $1.97 \times 10^4$ at day 14 (21.5-fold; $p<0.01$) after challenge. This enhancement returned to basal levels at day 21 (Figure 4.32). In contrast no lymphocytic infiltration was observed in HPS1 mice post BLM-challenge (Figure 4.32).

There was a trend suggesting an increased accumulation of granulocytes in BLM challenged WT C57BL/6 mice at day 7, from undetectable levels to $1.95 \times 10^3$ cells; however by day 14 post challenge this had returned to baseline levels, whereas in the HPS1 mice there was no indication of a granulocyte infiltration into the lungs (Figure 4.33). This granulocytic infiltration was observed to be that of neutrophils based on the polynucleated horseshoe-shaped nucleus observed.

These results indicate that there is a disparity both in the number and type of immune cells that infiltrate in the lungs of WT C57BL/6 and HPS1 mice upon BLM challenge. This difference may contribute to the altered patterns of gene transcript levels of inflammatory and pro-fibrotic mediators observed within the lungs of BLM challenged HPS1 and WT C57BL/6 mice (Section 4.5.2.2.3).
Figure 4.28 IL-6 mRNA levels in HPS1 mice compared to WT C57BL/6 controls following saline or BLM challenge. 8-14 week old HPS1 and WT C57BL/6 control mice were challenged via an oropharyngeal route, with either saline or 0.13 IU BLM, for 3 times at 72 hour intervals. Mice were culled at 7, 14 and 21 days after the last dose. BAL was performed for the isolation of AMφ; lungs were then inflated with OCT and snap frozen in liquid nitrogen. 10 μm frozen lung sections were sliced for histology analysis; the remainder was crushed for RNA analysis. IL-6 mRNA levels was determined by qRT-PCR. Each sample was measured by qRT-PCR in triplicates and the average was used to calculate the overall mean of different repeats. Data is represented as mean ± SD; WT C57BL/6 (Saline) (days 7, 14 and 21), n=4; WT C57BL/6 (BLM) (days 7, 14 and 21), n=4; HPS1 (Saline) (days 7, and 14), n=3; HPS1 (BLM) (day 21), n=2 from one study. Two way ANOVA with Sidak post-test was carried out; NS: Not significant, *= p<0.05.
Figure 4.29 Col1α1 mRNA levels in HPS1 mice compared to WT C57BL/6 controls following saline or BLM challenge. 8-14 week old HPS1 and WT C57BL/6 control mice were challenged via an oropharyngeal route, with either saline or 0.13 IU BLM, for 3 times at 72 hour intervals. Mice were culled at 7, 14 and 21 days after the last dose. BAL was performed for the isolation of AMφ; lungs were then inflated with OCT and snap frozen in liquid nitrogen. 10μm frozen lung sections were sliced for histology analysis; the remainder was crushed for RNA analysis. Col1α1 mRNA levels was determined by qRT-PCR. Each sample was measured by qRT-PCR in triplicates and the average was used to calculate the overall mean of sample repeats. Data is represented as mean ± SD; WT C57BL/6 (Saline) (days 7, 14 and 21), n=4; WT C57BL/6 (BLM) (days 7, 14 and 21), n=4; HPS1 (Saline) (days 7, and 14), n=4; HPS1 (Saline) (day 21), n=3; HPS1 (BLM) (days 7, and 14), n=3; HPS (BLM) (day 21), n=2 from one study. Two way ANOVA with Sidak post-test was carried out; NS: Not significant, *: p<0.05, **: p<0.01.
Figure 4.30 Fibronectin mRNA levels in HPS1 mice compared to WT C57BL/6 controls following saline or BLM challenge. 8-14 week old HPS1 and WT C57BL/6 control mice were challenged via an oropharyngeal route, with either saline or 0.13 IU BLM, for 3 times at 72 hour intervals. Mice were culled at 7, 14 and 21 days after the last dose. BAL was performed for the isolation of AMφ; lungs were then inflated with OCT and snap frozen in liquid nitrogen. 10μm frozen lung sections were sliced for histology analysis; the remainder was crushed for RNA analysis. Fibronectin mRNA levels was determined by qRT-PCR. Each sample was measured by qRT-PCR in triplicates and the average was used to calculate the overall mean of sample repeats. Data is represented as mean ± SD; WT C57BL/6 (Saline) (days 7, 14 and 21), n=4; WT C57BL/6 (BLM) (days 7, 14 and 21), n=4; HPS1 (Saline) (days 7, and 14), n=4; HPS1 (Saline) (day 21), n=3; HPS1 (BLM) (days 7, and 14), n=3; HPS (BLM) (day 21), n=2 from one study. Two way ANOVA with Sidak post-test was carried out; NS: Not significant, *: p<0.05, **: p<0.01, ***: p<0.005, ****: p<0.001.
Figure 4.31 AMφ count from BAL isolated from HPS1 and WT C57BL/6 control mice. 8-14 week HPS1 and WT C57BL/6 control mice were culled; BAL was performed and BAL cells were stained with Wright-Giemsa stain for differential cell count. Differential cell count of BAL cells was carried out by counting 300 cells from at least 3 fields per sample. Data is represented as mean ± SD; WT C57BL/6 (Saline) (days 7 and 21), n=3; WT C57BL/6 (Saline) (day 21), n=4; WT C57BL/6 (BLM) (days 7, 14 and 21), n=4; HPS1 (Saline) (days 7 and 14), n=4; HPS1 (Saline) (day 21), n=3; HPS1 (BLM) (days 7, 14 and 21), n=2 from one study. Two way ANOVA with Sidak post-test was carried out; NS: Not significant, *: p<0.05.
Figure 4.32 Lymphocyte count from BAL isolated from HPS1 and WT C57BL/6 control mice. 8-14 week HPS1 and WT C57BL/6 control mice were culled; BAL was performed and BAL cells were stained with Wright-Giemsa stain for differential cell count. Differential cell count of BAL cells was carried out by counting 300 cells from at least 3 fields per sample. Data is represented as mean ± SD; WT C57BL/6 (Saline) (days 7 and 21), n=3; WT C57BL/6 (Saline) (day 21), n=4; WT C57BL/6 (BLM) (days 7, 14 and 21), n=4; HPS1 (Saline) (days 7 and 14), n=4; HPS1 (Saline) (day 21), n=3; HPS1 (BLM) (days 7, 14 and 21), n=2 from one study. Two way ANOVA with Sidak post-test was carried out; NS: Not significant, **: p< 0.01.
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Figure 4.33 Granulocyte count from BAL isolated from HPS1 and WT C57BL/6 control mice. 8-14 week HPS1 and WT C57BL/6 control mice were culled; BAL was performed and BAL cells were stained with Wright-Giemsa stain for differential cell count. Differential cell count of BAL cells was carried out by counting 300 cells from at least 3 fields per sample. Data is represented as mean ± SD; WT C57BL/6 (Saline) (days 7 and 21), n=3; WT C57BL/6 (Saline) (day 21), n=4; WT C57BL/6 (BLM) (days 7, 14 and 21), n=4; HPS1 (Saline) (days 7 and 14), n=4; HPS1 (Saline) (day 21), n=3; HPS1 (BLM) (days 7, 14 and 21), n=2 from one study. Two way ANOVA with Sidak post-test was carried out; NS: Not significant.
4.52.2.5 The presence of foamy AM\(\phi\) in saline and BLM-challenged HPS1 and WT C57BL/6 mice

Foamy AM\(\phi\) accumulation has been observed in IPF\(^{715}\) and HPSPF patients\(^{788}\), and can adopt either a pro-inflammatory\(^{709}\) or a pro-fibrotic M2-like phenotype\(^{714}\), which may contribute to pulmonary fibrosis through tissue injury and excess ECM deposition respectively. Although no constitutive accumulation of foamy AM\(\phi\) was observed for HPS1 mice, there may be a predisposition for these cells to accumulate following BLM challenge when compared to the WT C57BL/6 controls.

ORO staining of lipid droplets demonstrated that there were no foamy AM\(\phi\) detectable in either HPS1 or C57BL/6 mice when challenged with saline. In contrast there was a marked accumulation of these cells in both strains of mice after exposure to BLM (Figure 4.34). However no difference in foamy AM\(\phi\) numbers was observed between HPS1 or WT C57BL/6 control mice (Figure 4.35). The lack of a detectable increase in the accumulation of foamy AM\(\phi\) in HPS1 mice compared to the WT C57BL/6 controls suggests that these cells may not play a role in the reduced tolerance to BLM-challenge observed in HPS1 mice.

4.52.2.6 Measurement of IL-12 p40 (total) and fibronectin protein in AM\(\phi\) isolated from saline and BLM challenged HPS1 and WT C57BL/6 mice

AM\(\phi\) from saline and BLM challenged HPS1 and WT C57BL/6 control mice were then analysed to investigate if there was differential expression of pro-inflammatory or pro-fibrotic mediators that might in part help to explain the reduced tolerance of HPS1 mice to BLM.

The results indicated that there was a trend suggesting the IL-12 p40 (total) protein levels in AM\(\phi\) from HPS1 mice and WT C57BL/6 controls was amplified following BLM challenge. Analysis of IL-12 p40 (total) in WT C57BL/6 mice following BLM challenge revealed a non-significant trend suggesting increased levels from 10.4 pg/ml to 554 pg/ml at day 7 (53.4-fold), and this was significantly elevated from 15.0 pg/ml to 390 pg/ml at day 14 (30-fold; p<0.005). IL-12 p40 (total) production in HPS1 mice post BLM-challenge was significantly enhanced from 92.7 pg/ml to 1,040 pg/ml at day 7 (11.2-fold), and had a non-significant trend of escalation from 2.95 pg/ml to 209 pg/ml at day 14 (70.8-fold). IL-12 p40 (total) levels in both strains returned to basal levels by day 21. Despite increased levels of IL-12 p40 (total) in HPS1 derived AM\(\phi\), both at the baseline and following BLM-challenge, there was no significant difference in IL-12 p40 (total) levels between HPS1 mice and WT C57BL/6 controls (Figure 4.36).

Fibronectin production increased significantly from day 7 to day 14 post BLM challenge in both HPS1 and WT C57BL/6 control mice. The levels of fibronectin underwent a significant increase in the WT
C57BL/6 mice post BLM challenge, from undetectable levels to 42.8 ng/ml at day 7 (p<0.01), and from 3.13 ng/ml to 131 ng/ml at day 14 (42-fold; p<0.005). In the HPS1 mice, fibronectin levels were similarly amplified at significant levels from 13.3 ng/ml to 106 ng/ml at day 7 (7.96-fold; p<0.001), and from 11.7 ng/ml to 135 ng/ml at day 14 (11.6-fold; p<0.005). There was a trend suggesting enhanced protein levels of fibronectin at day 21 post BLM-challenge in both strains, from 10.5 ng/ml to 121 ng/ml in WT C57BL/6 mice (11.6-fold), and from 11.6 ng/ml to 93.5 ng/ml (8.04-fold) in HPS1 mice (Figure 4.37). Together these results revealed an increased pro-fibrotic response by AMφ isolated from BLM-challenged HPS1 mice at day 7, which was matched by WT C57BL/6 controls by day 14 through to day 21. This may suggest that the fibrotic response was induced earlier in HPS 1 mice than their WT C57BL/6 counterparts.

Collectively these observations suggest that HPS1 mice may have slightly different kinetics in the development of pulmonary fibrosis in response to BLM compared to WT C57BL/6 mice. Gene transcript levels analysis of the lung have suggested increased inflammation at day 7, but shortened duration of active fibrosis in HPS1 mice. The latter observation corresponded with the overall less PSR staining of collagen observed across whole lung slices of BLM-challenged HPS1 mice compared to WT C57BL/6 controls that suggests reduced established fibrosis. Protein level analysis of AMφ supernatants has revealed an exaggerated pro-fibrotic response in AMφ from HPS1 mice at day 7 that was matched by WT C57BL/6 controls at day 14. From these results it can be proposed that AMφ in HPS1 mice have an earlier and accelerated pro-fibrotic response. Further experiments will have to be carried out at earlier time points to validate these hypotheses.
Figure 4.34 ORO stain for foamy AMφ in HPS1 mice or WT C57BL/6 controls following saline or BLM challenge. 8-14 week old HPS1 and WT C57BL/6 mice were challenged via an oropharyngeal route, with either saline or 0.13 U BLM, for 3 times at 72 hour intervals. Mice were culled at 7, 14 and 21 days after the last dose. AMφ was isolated from BAL by plastic adherence to culture plates for 90 minutes and further cultured for 24 hours. ORO stained foamy macrophages were quantified by counting at least 300 cells from 3 separate fields. Representative images are shown; WT C57BL/6 (Saline) (days 7, 14 and 21), n=4; WT C57BL/6 (BLM) (days 7, 14 and 21), n=4; HPS1 (Saline) (days 7, 14 and 21), n=4; HPS1 (BLM) (days 7, and 14), n=3; HPS (BLM) (day 21), n=2 from one study.
Figure 4.35 Count of ORO stained foamy AMφ in HPS1 mice or WT C57BL/6 controls following saline or BLM challenge. 8-14 week old HPS1 and WT C57BL/6 mice were challenged via an oropharyngeal route, with either saline or 0.13 U BLM, for 3 times at 72 hour intervals. Mice were culled at 7, 14 and 21 days after the last dose. AMφ was isolated from BAL by plastic adherence to culture plates for 90 minutes and further cultured for 24 hours. ORO stained foamy macrophages were quantified by counting at least 300 cells from 3 separate fields. Representative images are shown; WT C57BL/6 (Saline) (days 7, 14 and 21), n=4; WT C57BL/6 (BLM) (days 7, 14 and 21), n=4; HPS1 (Saline) (days 7, 14 and 21), n=4; HPS1 (BLM) (days 7 and 14), n=3; HPS (BLM) (day 21), n=2 from one study. Two way ANOVA with Sidak post-test was carried out; NS: Not significant.
**Figure 4.36** IL-12 p40 (total) protein levels in AMφ from HPS1 mice or WT C57BL/6 controls following saline or BLM challenge. 8-14 week old HPS1 and C57BL/6 mice were challenged via an oropharyngeal route, with either saline or 0.13 U BLM, for 3 times at 72 hour intervals. Mice were culled at 7, 14 and 21 days after the last dose. AMφ was isolated from BAL by plastic adherence to culture plates for 90 minutes and further cultured for 24 hours. Culture supernatant was collected and subject to protein level analysis; IL-12 p40 (total) level was determined by MSD. Each sample was measured by MSD in duplicates and the average was used to calculate the overall mean of sample repeats. Data is represented as mean ± SD; WT C57BL/6 (Saline) (days 7, 14 and 21), n=4; WT C57BL/6 (BLM) (days 7, 14 and 21), n=4; HPS1 (Saline) (days 7, 14 and 21), n=4; HPS1 (BLM) (days 7, and 14), n=3; HPS (BLM) (day 21), n=2 from one study. Two way ANOVA with Sidak post-test was carried out; NS: Not significant, **: p<0.01, ***: p<0.005. See Appendix 8 for IL-12 p40 standard curve.
Figure 4.37 Fibronectin protein levels in AMφ from HPS1 mice or WT C57BL/6 controls following saline or BLM challenge. 8-14 week old HPS1 and C57BL/6 mice were challenged via an oropharyngeal route, with either saline or 0.13 U BLM, for 3 times at 72 hour intervals. Mice were culled at 7, 14 and 21 days after the last dose. AMφ was isolated from BAL by plastic adherence to culture plates for 90 minutes and further cultured for 24 hours. Culture supernatant was collected and subject to protein level analysis; Fibronectin protein level was determined by MSD. Each sample was measured by MSD in duplicates and the average was used to calculate the overall mean of sample repeats. Data is represented as mean ± SD; WT C57BL/6 (Saline) (days 7, 14 and 21), n=4; WT C57BL/6 (BLM) (days 7, 14 and 21), n=4; HPS1 (Saline) (days 7, 14 and 21), n=4; HPS1 (BLM) (days 7, and 14), n=3; HPS (BLM) (day 21), n=2 from one study. Two way ANOVA with Sidak post-test was carried out; NS: Not significant, **: p< 0.01, ***: p<0.005, ****: p<0.001. See Appendix 8 for fibronectin standard curve.
4.6 Discussion

In the first part of chapter 4, the phenotype of AMφ isolated at various stages of BLM-induced pulmonary fibrosis, using a novel repeated oropharyngeal dosing regimen, was investigated in the results reported here. Histology analyses confirmed collagen accumulation and infiltration of CD68+ pulmonary macrophages. Assessment of the different immune cell types in BAL also demonstrated that there was an accumulation of AMφ. Using macrophage subset associated mediators that have been predefined in vitro in Chapter 1, it was revealed that AMφ of different phenotypes accumulated at various stages of BLM-induced pulmonary fibrosis.

These studies for the first time study the kinetics of the changes in mRNA and protein levels of AMφ in BLM-induced pulmonary fibrosis, and demonstrate that AMφ have the capacity to alter their phenotype in response to signals in vivo, as well as in vitro which is reported in Chapter 1. These phenotypic changes involved the increased levels of pro-inflammatory and pro-fibrotic mediators that may contribute to the pathophysiology of pulmonary fibrosis. It was also demonstrated that in a paracrine culture, supernatant from AMφ isolated from BLM challenged mice were able to stimulate an increase in Col1α1 gene transcript levels in MPF, indicating that AMφ may promote pulmonary fibrosis by the activation of MPF.

In the second part of chapter 4, the potential role of foamy macrophages in BLM-induced pulmonary fibrosis was investigated. For these studies, HPS1 mice that have been reported to have an accumulation of foam cells, including foamy macrophages and AEC2, as a result of defective lysosomal trafficking, were used. The increased number of foamy macrophages in HPS1 mice should give clues about their role when compared to WT C57BL/6 mice where such cells are less populated. HPS1 mice represent an experimental model of HPS1 patients, of whom often develop pulmonary fibrosis (HPSPF) that resembles IPF in terms of disease progression and its UIP histopathology. HPS1 mice do not spontaneously develop pulmonary fibrosis in contrast to HPS1 patients, but display increased susceptibility to BLM-induced pulmonary fibrosis at day 7 post challenge. It is possible that this increased susceptibility may be promoted by elevated foamy macrophages accumulation through elevated pro-inflammatory and pro-fibrotic mediator release.

The phenotype of naïve HPS1 mice was first compared to that of the WT C57BL/6 control. It was observed in accordance with literature these had hypopigmented ears, paws and tails but not coat (Figure 4.16). HPS1 mice did not have intrinsic cellular infiltration (Figure 4.17) or collagen accumulation (Figure 4.18), although there was a non-significant trend suggesting increase in pro-inflammatory (TNF-α, and IL-6) (Figure 4.19), and reduction of the ECM component fibronectin (Figure 4.20). HPS1 mice did not have different numbers of AMφ, lymphocytes and granulocytes as
compared to WT C57BL/6 controls (Figure 4.21), nor increased number of foamy AMφ (Figure 4.22), and AMφ had no increased levels of pro-inflammatory (TNF-α, CXCL1) (Figure 4.23) and pro-fibrotic (fibronectin) mediators (Figure 4.24).

HPS1 mice were then challenged with BLM using the multiple oropharyngeal dosing method, and subsequent pathology (e.g. cellular infiltration, collagen accumulation, mRNA levels of pro-inflammatory and pro-fibrotic mediators) in BLM-induced pulmonary fibrosis was observed for the first time, beyond day 7 of the late inflammatory phase, into days 14 and 21 of the active and established fibrotic phases. The number of foamy macrophages was also measured by ORO staining to evaluate their potential increased accumulation in HPS1 mice as compared to WT C57BL/6 controls. The protein levels of pro-inflammatory and/or pro-fibrotic mediators released by these AMφ were also assessed to determine whether or not they were involved in the reported increased susceptibility to BLM-induced pulmonary fibrosis.

HPS1 mice showed reduced tolerance to BLM challenge as observed by increased weight loss as compared to WT C57BL/6 controls (Figure 4.25), which was in accordance to the literature. However, increased weight loss occurred during the late inflammatory phase (days 8-11); this was also not accompanied by increased collagen transcript levels (Figure 4.29) and accumulation (Figure 4.27) within the lungs, suggesting that reduced BLM tolerance was due to increased inflammation but not increased susceptibility to BLM-induced pulmonary fibrosis. Indeed there was a trend suggesting increase in IL-6 in the lungs to support this hypothesis (Figure 4.28). There was no difference in AMφ (Figure 4.31) or granulocyte (Figure 4.33) infiltration, although there was a lack of accumulation of lymphocytes (Figure 4.32). There was no difference in the number of foamy AMφ (Figure 4.34 and 4.35), suggesting that accumulation of cholesterol crystals in AMφ is not prerequisite of reduced tolerance in HPS1 mice. There were increased protein levels of IL-12 p40 (total) (Figure 4.36) and fibronectin (Figure 4.37) in HPS1 mice as compared to WT C57BL/6 controls at day 7 that returns to basal levels by day 14. Increased IL-12 p40 levels may lead to the reduced BLM tolerance in HPS1 mice observed in the late inflammatory phase at day 7.

In the following sections, AMφ heterogeneity in BLM-challenged pulmonary fibrosis in WT C57BL/6 mice will first be evaluated in section 4.6.1. The comparisons between naïve, saline or BLM-challenged mice HPS1 mice and WT C57BL/6 mice, for the investigation of the role of foamy macrophages in pulmonary fibrosis, will be deliberated in section 4.6.2.

4.6.1 AMφ heterogeneity in BLM-induced pulmonary fibrosis in WT C57BL/6 mice

In this section, AMφ heterogeneity in BLM-induced pulmonary fibrosis in WT C57BL/6 mice will be evaluated. This section begins with experimental limitations and their potential implications,
followed by discussion of the characterisation of AMφ at different stages of BLM-induced pulmonary fibrosis, and their role in inducing collagen expression in mouse lung fibroblasts \textit{ex vivo}, and finally the future directions of these studies will be deliberated.

\textbf{4.6.1.1 Experimental limitations}

Several experimental limitations have been identified in these studies. First, there was no quantification for cellular infiltration and collagen accumulation for histology analyses. While drastic differences in these parameters could be observed visually between saline and BLM-challenged mice, further positive pixel count of the H&E and PSR stained slides will have to be carried out to quantify the extent of these alterations.

Second, in the flow cytometry analyses, multiple stained AMφ were gated with respect to the MFI of their isotype controls, rather than using a fluorescence minus one (FMO) regime. Isotype controls are antibodies of the same isotype of that of the primary antibody but without the specificity to the target protein, and may serve as negative controls for non-specific antibody binding. However, isotype controls may have different activities to the primary antibody (since they are different antibodies) and therefore are not the best candidates for identifying staining boundaries. Instead FMO controls, which consists of all primary antibodies but the one that is being controlled, is widely accepted as gating controls. Gating strategies employed in these studies may therefore be misplaced and further FMO controls will have to be established to properly set up gates for analysis.

Third, there was no test for normal distribution in these studies due to limited number of samples available. The Kolmogorov-Smirnov test requires 5 or more values, the Shapiro-Wilk test requires 7 or more values, and the D’Agostino test requires 8 or more values, whereas \( n<5 \) in some studies. Results were therefore classified as parametric or non-parametric based on visual inspection on the spread of data, which may be unreliable. Further repeats will have to be carried out to increase sample size for normal distribution testing and the validation of the statistical testing used.

In light of these caveats however, drastic changes were still observed in AMφ mRNA and protein levels in BLM-challenged mice with respect to saline controls. These results will be further discussed in the following sections (section 4.6.1.2 – 4.6.1.6); future directions to further these studies will be deliberated in section 4.6.1.7.

Despite these shortfalls in experimental design, observations from the current studies may provide a useful indication of changes in mRNA and protein levels of AMφ in saline versus BLM-challenged mice at the late inflammatory (day 7), early fibrotic (day 14) and late fibrotic (day 21) phases, which will be discussed further in the sections below. However, it is acknowledged here that further
experiments employing the controls mentioned above will have to be carried out for a more
definitive comparison.

### 4.6.1.2 A novel repeated oropharyngeal dosage of BLM-induced pulmonary inflammation and fibrosis

A novel repeated oropharyngeal dose protocol for BLM-induced pulmonary fibrosis in mice recently
developed by Dr. Elizabeth Jarman (Novartis, Horsham) was used in these studies. Histological
analyses of cellular infiltration and collagen accumulation were carried out in order to validate this
model. H&E staining showed that there was a large cellular influx into the lungs that is coupled with
an apparent destruction of the alveoli and emphysema (Figure 4.2). These cell infiltrates included
CD68+ macrophages (Figures 4.4 and 4.5), indicating that these cells may contribute to the
pathology. PSR staining also revealed a gradual increase in collagen accumulation, a hallmark of the
onset of fibrosis (Figure 4.3). This temporal heterogeneity of collagen deposition is indicative that
pulmonary fibrosis is a progressive disease that is caused by increased production and/or reduced
removal of ECM components. Spatial heterogeneity was also observed across the whole lung slice in
both H&E and PSR staining where areas of cellular infiltration/fibrosis were immediately adjacent to
unaffected areas. This finding suggests a localised rather than a general response to BLM-induced
injury to the lung tissue. Differential cell counts in BAL revealed that AMφ were increased following
BLM-challenge between the late inflammatory phase (day 7) to the late fibrotic phase (day 21),
suggesting a role for these cells in the initiation and progression of pulmonary fibrosis (Figure 4.6).
Lymphocytes were also increased on day 7, indicating a role for these cells in the initiation of
pulmonary fibrosis (Figure 4.6). However there was no evidence of elevated numbers of
granulocytes (Figure 4.6), which reflect the lungs of IPF patients in that the progression of fibrosis is
granulocyte-independent.

### 4.6.1.3 M1-like AMφ accumulated in the alveolar space at day 7, whereas M1/M2-like hybrid AMφ were
found from days 7 to 21 of BLM-induced pulmonary fibrosis

The phenotype of AMφ within the fibrotic lung was assessed to study macrophage heterogeneity in
a disease model. These studies are the first of its kind to investigate the change in AMφ phenotype
at different stages of BLM-induced pulmonary fibrosis. Flow cytometric analyses revealed that there
were two populations expressing M1-like ($\text{MHCI}^{\text{hi}} \cdot \text{MRC1}^{\text{-}}$) and M2-like ($\text{MHCI}^{\text{-}/\text{lo}} \cdot \text{MRC1}^{\text{-}/\text{lo}}$) surface
proteins at day 7, but by day 14 and then day 21 only the latter group ($\text{MHCI}^{\text{-}/\text{lo}} \cdot \text{MRC1}^{\text{-}/\text{lo}}$) remained
(Figure 4.12 and 4.13). The mRNA and protein level analyses of select macrophage subset associated
mediators have largely supported this observation, with a trend suggesting an increase in the M1-
associated mediators NOS2 (Figure 4.9) and IL-1β (Figure 4.11) at day 7, and an elevation of the M2-
associated mediators Arg-1 (Figure 4.9) and fibronectin (Figure 4.11) between days 7 to 21.
Conversely it was observed that the M1-associated mediator IL-12 p40 (total), including monomeric
and/or homodimeric IL-12 p40, heterodimeric IL-12 p70 (p40 + p35) and/or IL-23 (p40 + p19) (Figure 4.11), was also elevated at day 7 and persisted until at least day 21. It is therefore possible that a M1-/M2-like hybrid population of AMφ may contribute to the release of IL-12 p40 (total) from at least day 14 to day 21 when M1-like AMφ were absent.

IL-12 p40 (total) was increased in the supernatants of AMφ from BLM-challenged mice from days 7 to 21 (Figure 4.11). Follow-up studies have shown that despite the increased levels of IL-12 p40 (total), levels of the bioactive IL-12 p70 were undetectable (data not shown). This suggests that the increase of IL-12 p40 (total) is not contributed by the elevation of IL-12 p70, but of IL-12 p40 monomers, homodimers and/or IL-23. IL-12 p40 may play a role in macrophage recruitment, as was observed in a mouse model of silica-induced pulmonary fibrosis. Alternatively IL-12 p40 may combine with the p19 subunit to form IL-23 to promote pulmonary fibrosis. IL-23 can play a significant role in BLM-induced pulmonary fibrosis. IL-23 may promote the differentiation of IL-17 producing T\(_\text{H}17\) cells, and IL-17A can mediate BLM or IL-1β induced pulmonary fibrosis. Further studies will have to be carried out to establish whether IL-12 p40 and/or IL-23 is responsible for the progression of pulmonary fibrosis. The potential role of IL-12 p40 monomers/homodimers in monocyte recruitment and the pathology of pulmonary fibrosis can be evaluated by administration into the lungs of BLM-challenged mice and assessing the change in collagen accumulation and pulmonary fibrosis. The potential role of IL-23 can be determined by measuring its protein levels in the supernatants of AMφ, BAL and lung of BLM-challenged mice. The effect of lung-specific instillation of IL-23, or retrospectively removal of IL-23 by specific antibodies, to BLM-induced pulmonary fibrosis can also be analysed to determine its role in pathology.

This is the first time that a hybrid phenotype, rather than a strictly M2-like phenotype, has been reported to play a role in pulmonary fibrosis. The hybrid macrophage may be generated by diverse signals promoting dysregulated wound healing (e.g. IL-4, IL-13, TGF-β, and IL-10) and persistent irritation (e.g. IFN-Υ, and TNF-α) within the fibrotic lung, which induces a M2-like and M1-like phenotype respectively. However prolonged TLR stimulation can induce tolerance in peritoneal macrophages leading to diminished M1-like responses and elevated M2-like responses. The accumulation of oxidised phosphatidylcholine (oxPc) can also induce a M2-like phenotype and a foamy appearance in human and mice AMφ. Indeed, ORO staining revealed an accumulation of foam cells in BAL (Figures 4.7 and 4.8). These results therefore suggest the presence of a M1-like phenotype during the late inflammatory phase (day 7) that is gradually replaced by a M1-/M2-like hybrid phenotype (days 7 to 21) as pathology progress.
4.6.1.4 PAI-1 gene transcript levels were decreased in BLM-challenged mice

In Chapter 3, PAI-1 transcript levels were found to be increased in AMφ following TGF-β or hypoxia induction (Chapter 3, section 3.5.1.3.1), and it is well documented that these two signals are increased in the fibrotic lung. PAI-1 was also reported to be elevated in the lung tissue of fibrotic lungs and essential for BLM-induced pulmonary fibrosis. It was therefore surprising that a trend gene suggesting decreased transcript levels of PAI-1 was observed in AMφ isolated from BLM-challenged mice compared to saline controls (Figure 4.10). It is unclear why this was the case, although several potential reasons can be considered. First, there may be a difference in pathology mechanism between the repeated lower doses regimens used in these studies versus the single higher doses regimens applied in the literature. Perhaps multiple lower doses allow AMφ to tolerate or even overcompensate for BLM-induced PAI-1 gene expression. This may call into question the representativeness of the multiple dose BLM-induced pulmonary fibrosis mouse model to IPF, with particular respect to hypoxia-related studies, and further experiments, as discussed below, will have to be carried out to evaluate this. Second, PAI-1 gene transcript levels by disease-relevant AMφ may be masked by that of bystander AMφ due to spatial heterogeneity of fibrosis (neighbouring regions of normal and fibrotic tissue) within the lung. Third, there may be increased degradation of PAI-1 mRNA in AMφ within the fibrotic lung. Indeed, PAI-1 mRNA are regulated at a post-transcriptional level by a variety of mediators, including phorbol myristate acetate (PMA), insulin, IGF-1, and cyclic nucleotide analogues. It may therefore be more informative to investigate PAI-1 levels at a protein levels for a more accurate analysis of their levels in pathology. Further studies could be carried out by in situ hybridisation or immunohistochemistry to determine the cellular localisation of PAI-1 RNA or protein respectively, and the location within the lung where such cells are present.

4.6.1.5 AMφ have increased CCL5 and increased trends of IL-6 and CCL2 protein levels at day 7

Levels of macrophage-associated proteins associated with pulmonary fibrosis have been assessed in the supernatants of AMφ isolated from different stages of BLM exposed and control mice ex vivo (Figure 4.14). The results revealed that CCL3, CCL4, CCL20 and MMP-9 were undetectable, while CCL5 was significantly increased at day 7, and a trend suggesting an increase in IL-6 and CCL2 levels in AMφ from BLM-challenged mice was observed. The protein levels of CXCL2 remained unchanged. It was revealed that AMφ do not produce detectable levels of CCL20 and mMMP-9 (Chapter 3, Section 3.5.1.3.3). Preliminary studies have shown that hypoxia induced CCL3, CCL4 and CXCL2 production in AMφ (Chapter 3, Section 3.5.1.3.3). The failure to generate measurable levels of CCL3, CCL4, and the unchanged amounts of CXCL2 suggests that the hypoxia induced AMφ phenotype was not prevalent in BLM-induced pulmonary fibrosis, a feature that was mirrored by the lack of PAI-1 gene transcript increase (Figure 4.10). This calls into question whether or not hypoxia-inducible mediators involved in pathology are directly translatable from mouse model to...
human patients. The role of hypoxia in this current model of BLM-induced pulmonary fibrosis has to be further evaluated by measuring the protein levels of the hypoxia induced transcription factors HIF-1α and HIF-2α within cells. The representativeness of this model in the study of pulmonary fibrosis-associated hypoxia will also have to be evaluated using by measurement of protein levels of hypoxia-associated proteins (e.g. PAI-1).

The role of CCL5 in pulmonary fibrosis has not been extensively investigated, although it is a chemoattractant for mast cells. It is known that mast cells accumulate in pulmonary fibrosis, and clinically correlations between mast cells and fibrosis have been observed. Mast cells may drive fibrosis through the stimulation of fibroblasts resident in the lung. Antagonism of CCL5 also protected from liver fibrosis, possibly through the modulation of monocyte subpopulations. The role of AMφ-derived CCL5 in the recruitment of mast cells can be assessed by chemoattractant assays ex vivo. Its contribution to BLM-induced pulmonary fibrosis can be evaluated by genetic or pharmacological inhibition, or retrospectively by lung-specific CCL5 overexpression studies, with the corresponding changes in fibrosis represented by collagen and mast cell accumulation.

The roles of CCL2 and IL-6 in pulmonary fibrosis are well established. Monocytes and fibrocytes are attracted into the airways following lung injury by CCL2, and it can also stimulate fibroblast collagen production via upregulation of TGF-β expression. IL-6 participates in airway remodelling in asthma, and induces differentiation of fibroblasts into myofibroblasts. It was therefore unexpected that these two essential AMφ-derived pro-fibrotic mediators were only weakly increased in BLM-challenged mice. Perhaps these mediators are only increased in pro-fibrotic AMφ that exerts its effects locally in a paracrine manner. Immunohistochemical investigations of these mediators would allow the identification of their location within the fibrotic lung.

4.6.1.6 Supernatants of AMφ isolated from BLM-challenged mice promoted collagen gene transcript levels in MPFs

Supernatants from AMφ isolated from BLM-challenged mice, but not saline controls, increased Col1α1 gene transcript levels in MPFs (Figure 4.15). These novel results demonstrate that AMφ derived from BLM-induced pulmonary fibrosis have a pro-fibrotic role ex vivo in a paracrine manner. While it has not been determined here which mediator(s) is/are responsible for such induction, potential candidates include IGF-1, TGF-β, and PDGF. Further genetic ablation or pharmacological inhibition studies will have to be carried out to determine the key mediator(s) responsible for this process.

4.6.1.7 Future directions

4.6.1.7.1 Spatial heterogeneity of BLM-induced pulmonary fibrosis

In these studies, AMφ from the whole lung were analysed. Since the AMφ from BLM-challenged mice in this study are a mixture of macrophages from both fibrotic areas and unaffected areas the
functional phenotype and plasticity of the disease associated cells may be underrepresented. Future studies using laser capture microdissection (LCM) can isolate diseased areas from the fibrotic lung and allow for a more representative comparison. Alternatively, single lung dosing of mice can be carried out, where a high dose of BLM is applied intratracheally. The high dose of BLM can induce more homogenous fibrosis in the challenged lung, whereas the unchallenged lung can maintain survival of the mice and serve as the direct negative control for BLM challenge.

4.6.1.7.2 AMφ heterogeneity in BLM-induced pulmonary fibrosis

A particular difficulty in studying disease-associated AMφ mRNA and protein levels ex vivo is to separate the heterogeneous subpopulations, such that the same functional subset, rather than a pooled sample of different subsets are analysed. As demonstrated above, macrophage subsets predefined in vitro, namely M1-like and M2-like macrophages may be insufficient to fully represent the complexity of macrophage genotype in vivo. However, these may serve as a good starting point of which to pre-sort AMφ for further analyses. The transcriptome and proteome of AMφ in pulmonary fibrosis can be analysed by DNA microarray and next generation sequencing (NGS), or protein microarrays.

4.6.1.7.3 IL-12 p40 (total) MSD measures different forms of IL-12 p40 oligomers

As mentioned above IL-12 p40 (total) measures different forms of IL-12 p40m including monomeric and homodimeric IL-12 p40, and also heterodimeric IL-12 p70 (p40 + p35) and IL-23 (p40 + p19). IL-12 p40 (total) was found to be increased in AMφ of BLM-challenged mice, although the exact oligomer form it is increased in is unknown. As different IL-12p40 isoforms have different functions, it is important to determine the specific isoform that is increased to determine its function.

4.6.1.8 Summary

Collectively it is shown in these studies that AMφ display heterogeneity during the fibrotic process to release a variety of pro-inflammatory and pro-fibrotic mediators at various stages of BLM-induced pulmonary fibrosis. AMφ-derived pro-fibrotic mediators can directly increase collagen transcript levels in MPF in a paracrine manner, and AMφ therefore has the capacity to promote fibrosis.

4.6.2 The comparison of HPS1 mice and WT C57BL/6 controls in BLM-induced pulmonary fibrosis

In this section, the susceptibility of HPS1 mice to BLM-induced pulmonary fibrosis was assessed. HPS1 mice were used here as they represent an experimental model of HPS1 patients. HPS1 patients often develop pulmonary fibrosis (HPSPF) that resembles IPF in terms of disease progression1,693 and its UIP histopathology7. HPSPF patients also have increased numbers of foamy macrophages, potentially due to defective cholesterol efflux in these cells788.
Unlike their human counterparts, HPS1 mice do not spontaneously develop pulmonary fibrosis, but display increased susceptibility to BLM-induced pulmonary fibrosis at day 7 post challenge. As foamy macrophages accumulate in IPF patients and also in the lungs of mice with BLM-induced pulmonary fibrosis (Figure 4.7 and 4.8), and foamy macrophages have been described to have a pro-inflammatory and pro-fibrotic phenotype, it is possible that these cells may contribute to pulmonary fibrosis through promoting tissue injury and fibrosis. The increased susceptibility of HPS1 mice to BLM-induced pulmonary fibrosis may therefore be resulting from the increased accumulation of these foamy AMφ. The susceptibility of HPS1 mice to a novel multiple oropharyngeal BLM-induced pulmonary fibrosis, and the phenotype of AMφ were investigated in the above studies.

In the studies here the phenotype of naïve HPS1 mice was first reaffirmed. It was observed in accordance with literature these had hypopigmented ears, paws and tails but not coat (Figure 4.16). HPS1 mice did not have intrinsic cellular infiltration (Figure 4.17) or collagen accumulation (Figure 4.18), although there was a non-significant trend suggesting increase in pro-inflammatory (TNF-α and IL-6) (Figure 4.19), and reduction in pro-fibrotic (Col1α1 and fibronectin) (Figure 4.20) mediators. HPS1 mice did not have different numbers of AMφ, lymphocytes and granulocytes as compared to WT C57BL/6 controls (Figure 4.21), nor increased number of foamy AMφ (Figure 4.22), and AMφ had no increased levels of pro-inflammatory (TNF-α, CXCL1) (Figure 4.23) and pro-fibrotic (fibronectin) mediators (Figure 4.24).

HPS1 mice showed reduced tolerance to BLM challenge as observed by increased weight loss as compared to WT C57BL/6 controls (Figure 4.25), which was in accordance to the literature. However, increased weight loss occurred during the late inflammatory phase (days 8-11); this was also not accompanied by increased collagen transcript levels (Figure 4.29) and accumulation (Figure 4.27) within the lungs, suggesting that reduced BLM tolerance was due to increased inflammation but not increased susceptibility to BLM-induced pulmonary fibrosis. Indeed there was a trend suggesting increase in IL-6 in the lungs to support this hypothesis (Figure 4.28). There was no difference in AMφ (Figure 4.31) or granulocyte (Figure 4.33) infiltration, although there was a lack of accumulation of lymphocytes (Figure 4.32). There was no difference in the number of foamy AMφ (Figure 4.34 and 4.35), suggesting that accumulation of cholesterol crystals in AMφ is not prerequisite of reduced tolerance in HPS1 mice. There were increased protein levels of IL-12 p40 (total) (Figure 4.36) and fibronectin (Figure 4.37) in HPS1 mice as compared to WT C57BL/6 controls at day 7 that returns to basal levels by day 14. Increased IL-12 p40 levels may lead to the reduced BLM tolerance in HPS1 mice observed in the late inflammatory phase at day 7.
In the following sections, the limitations of the HPS1 mice versus WT C57BL/6 control studies and their potential implications will first be assessed in section 4.6.2.1. The experimental results will then be discussed in sections 4.6.2.2 – 4.6.2.5, and the future directions will be evaluated in section 4.6.2.6.

4.6.2.1 Experimental limitations

The primary experimental limitations in the comparison studies between HPS1 and WT C57BL/6 mice, in both the experiments with naïve mice and also BLM-induced pulmonary fibrosis, were that due to time constraints, only one study was carried out to a limited sample size. The data from HPS1 mice with respect to pro-inflammatory and pro-fibrotic mediators and also cellular infiltration was very diverse, perhaps because the increased levels of these parameters are secondary to tissue injury resulting from defective lysosomal transport. In other words, there may be other factors aside from HPS1 mutation associated defective lysosomal transport that may contribute to lung injury in HPS1 mice. A greater number of mice is therefore required to test for significance, and the number of mice needed can be determined using online power calculators. This will be discussed further in the discussion of results later.

Similar to the BLM-induced pulmonary fibrosis in WT C57BL/6 mice only studies (section 4.6.1), histological analyses for cellular infiltration (H&E staining) and collagen accumulation (PSR staining) have been described qualitatively but not measured quantitatively. Further positive pixel counts of the histological slides will be required to assess the level of staining.

In the following sections, experimental results from the comparison of HPS1 mice with WT C57BL/6 controls in unchallenged (naïve), or saline or BLM-challenged conditions are discussed with respect to the limitations mentioned above (sections 4.6.2.2 to 4.6.2.5). Section 4.6.2.6 explores future directions to the HPS1 studies, and section 4.6.2.7 concludes the findings of section 4.6.2.

4.6.2.2 HPS1 mice have hypopigmentation in their ears, paws and tails

In initial studies, the phenotype of HPS1 mice was assessed with respect to appearance and histological findings to validate findings from others, and to establish a baseline reference for further studies in BLM-induced pulmonary fibrosis. HPS1 mice that have been generated in-house had hypopigmented ears, paws, and tail but normal (black) coat colour compared to WT C57BL/6 controls (Figure 4.16), which was in accordance to the literature. Melanocytes only reside in follicles on the adult mouse back skin, whereas melanocytes are found in interfollicular epidermis and dermis as well as follicles of tail and skin813. Hypopigmentation may be caused by faulty intracellular trafficking in melanocytes as a result of defective HPS1 protein. Hypopigmentation in the ears and
tails but not coat colour can be explained by differential regulation of different types of melanocytes found at these sites.

4.6.2.3 HPS1 mice do not spontaneously develop pulmonary fibrosis
Observations from histological slides revealed that in accordance with previous literature there appeared to be no alterations in alveolar architecture, cellular infiltration (Figure 4.17) and excess collagen accumulation (Figure 4.18) in naïve HPS1 mice compared to WT C57BL/6 controls. Differential cell count also confirmed that there was no difference in cellular composition within the alveolar space between naïve HPS1 mice and WT C57BL/6 controls (Figures 4.21). This was in line with a previous report in that HPS1 mice, unlike their human counterparts, do not spontaneously develop pulmonary fibrosis. These results suggest that naïve HPS1 mice have neither intrinsic inflammation nor pulmonary fibrosis. However, as these observations have been made qualitatively, further measurements involving positive pixel count of hematoxylin- or PSR-stained lungs will have to be carried out to quantify cellular infiltration and collagen accumulation respectively.

Having verified from histological analyses that HPS1 mice do not have increased cellular infiltration nor fibrosis, novel studies were carried out here to evaluate whether or not HPS1 lungs had low level underlying active inflammation and/or fibrosis that may be amplified rapidly in response to an insult (e.g. BLM-challenge). Gene transcript analyses showed a trend suggesting increase in inflammation (IL-6 and TNF-α) (Figure 4.23), but a trend suggesting reduction for fibronectin production (Figure 4.24). It is possible that IL-6 and TNF-α transcripts may be increased as a response to mild tissue injury resulting from defective release by AECs of homeostatic proteins, such as pulmonary surfactants and mucin. It may also be possible that HPS1 mice are more susceptible to infection by airborne pathogens due to lysosomal defects, although this has not been reported. The increased gene transcripts may be contributed by resident AEC2s. IL-6 is an inducer of Col1α1 and fibronectin expression via promoting TGF-β expression; it was therefore surprising that a non-significant trend suggesting reduced levels of these ECM components were observed in HPS1 mice. Perhaps other low level of pro-inflammatory mediators present, such as TNF-α, is present to inhibit Col1α1 gene expression. The enhanced inflammation in response to BLM challenge, rather than any pro-fibrotic response, are more likely to be associated with the weight loss observed. AMφ did not appear to contribute to neither inflammation (Figure 4.23) nor fibrosis (Figure 4.24) in naïve HPS1 mice. Further repeats will have to be carried out to test the significance of these observations.

4.6.2.4 Reduced tolerance to BLM challenge is not correlated with more severe pulmonary fibrosis in HPS1 mice
Following the characterisation of naïve HPS1 mice, their susceptibility to BLM-induced pulmonary fibrosis was assessed. In these studies the novel multiple low dose oropharyngeal protocol was used...
to challenge HPS1 mice and WT C57BL/6 controls with BLM. In addition to mimicking repeated challenge to the lung by a persistent irritant, lower dosage per challenge allows better BLM tolerance in mice. This has enabled for the first time evaluation of BLM-induced pulmonary fibrosis in HPS1 mice beyond the late inflammatory phase (day 7) reported by Young et al.\textsuperscript{696} into the active (day 14) and established fibrotic phases (day 21). Percentage weight loss was used in these studies as a crude indicator for tolerance to BLM challenge, as mice that are more poorly generally lose more body weight. BLM challenge of mice induced a more severe weight loss in HPS1 mice compared to WT controls between days 8 to 11, indicating that HPS1 mice had reduced BLM tolerance (Figure 4.25). A previous study has attributed reduced BLM tolerance of HPS1 mice to elevated BLM-induced pulmonary fibrosis\textsuperscript{696}. However, PSR staining revealed that there was decreased collagen accumulation (and established fibrosis) in HPS1 mice (Figure 4.27). This is in line with gene transcript levels of Col1\(\alpha\)1, and also fibronectin, observed in BLM challenged HPS1 mice, which had a reduced duration of increased levels (day 7 increase only in HPS1 mice, versus day 7 and 14 increase in WT C57BL/6 control), thereby signifying shorter active fibrosis (Figures 4.29 and 4.30). IL-6 transcript levels in BLM challenged HPS1 mice followed a similar trend, suggesting a shorter duration of inflammation as well (Figure 4.28). However, there was a trend suggesting increase in the elevation of IL-6 levels in BLM challenged HPS1 mice compared to WT C57BL/6 controls at day 7 (Figure 4.28). Collectively, it may appear that in contrast to the previous study by Young et al.\textsuperscript{696}, reduced tolerance in HPS1 mice is not due to increased susceptibility to BLM-induced pulmonary fibrosis, but rather as a result of increased inflammation. Further investigation into the change in levels of other pro-inflammatory mediators with proposed roles in pulmonary fibrosis, such as IL-1\(\beta\) and IL-12 p40, have to be carried out to test whether or not increased inflammation does occur in HPS1 mice. The role of inflammation can then be examined by assessing whether or not preventative or therapeutic anti-inflammatory treatment can improve BLM tolerance in HPS1 mice. Alternatively, there may be a difference in kinetics in BLM-induced pulmonary fibrosis between HPS1 mice and WT C57BL/6 controls. HPS1 mice may have an initial burst in collagen production shortly after BLM challenge, which may then be rapidly degraded and resolved. Indeed, protein level analyses revealed that there was a trend suggesting an increase of IL-12 p40 (total) (Figure 4.36), and also elevated fibronectin (Figure 4.37) levels in AM\(\phi\) from BLM-challenged HPS1 mice compared to WT C57BL/6 controls at day 7 that were matched in both strains by day 14. It may be possible that increased macrophage recruitment by IL-12 p40\textsuperscript{123}, and elevated fibroblast migration by fibronectin\textsuperscript{520,521}, may occur earlier in HPS1 mice and contribute to reduced BLM tolerance. Resolution of wound repair requires the coordinated elimination of the persistent irritant, reparation of the alveolar epithelium and endothelium, and clearance of apoptotic and necrotic cells.
and excessive ECM components. There have been few studies that have investigated potential mediators involved in resolution of pulmonary fibrosis. One such mediator that has been identified is Mfge 8, which decreases severity in BLM-induced pulmonary fibrosis by binding and targeting collagen for cellular uptake through its discoidin domains. It would be interesting to assess the levels of Mfge8 to determine whether or not there was indeed an increased resolution of pulmonary fibrosis in BLM-challenged HPS1 mice.

The difference in dosing regimen may also affect development of pulmonary fibrosis in mice. The single high dose regimen used by Young et al. may have incurred a more severe initial tissue injury that resulted in an exaggerated fibrotic response. On the other hand, the repeated low dose regimen used in my studies may have allowed for better tolerance of mice to BLM-induced tissue injury, which resulted in a milder fibrotic response, thereby masking the differences in pulmonary fibrosis between the two strains of mice. Further assessment of the gene transcript and protein levels of other pro-inflammatory mediators with known roles in pulmonary fibrosis, including IL-12 p40 and TNF-α, will have to be carried out to determine whether or not increased inflammation was indeed present in HPS1 mice. Earlier time points will have to be assessed for the expression of various pro-inflammatory and pro-fibrotic mediators in repeated BLM dosing regimen to evaluate the potential difference in kinetics in pulmonary fibrosis between the two strains.

It is also possible that other systemic immune effects may contribute to such reduced BLM tolerance, although this is unlikely as BLM activity has been reported to be restricted to the lung only. Future studies will have to be carried out to evaluate the potential participation of other organs in the increased susceptibility of HPS1 to BLM-challenge, for example the kidneys, that function to remove BLM from the body.

4.6.2.5 There is decreased lymphocytic infiltration in BLM-challenged HPS1 mice as compared to WT controls

There was an increase in the number of AMφ (Figure 4.31) and foamy AMφ (Figure 4.34 and 4.35) in both strains of mice following BLM-challenge compared to saline controls, suggesting that AMφ and foamy AMφ may participate in the pathology of pulmonary fibrosis, and the consequent stress may in turn lead to the weight loss observed in both mouse strains (Figure 4.25). However, there was no difference in the number of AMφ (Figure 4.31) and foamy AMφ (Figure 4.34 and 4.35) observed between BLM-challenged HPS1 mice and WT controls, suggesting that these cell populations are less likely to contribute to the increased weight loss in HPS1 mice. An absence in lymphocytic infiltration into the alveolar space was observed in BLM-challenged HPS1 mice as compared to WT C57BL/6 controls (Figure 4.32), although the role of lymphocytes in pulmonary fibrosis is unclear (Section 1.1.4.5). Further studies will have to be carried out to determine the role of various lymphocytes in
pulmonary fibrosis. For example, DTR can be used to deplete specific subpopulations of T and B lymphocytes in DTR transgenic mice, including all T lymphocytes (CD3), T helper cells (CD4), cytotoxic T lymphocytes (CD8) or B lymphocytes (BLyS), and the corresponding effects on BLM-induced pulmonary fibrosis analysed. Lymphocytes from BLM-challenged WT C57BL/6 controls can also be isolated and introduced into BLM-challenged HPS1 mice and their effects on pulmonary fibrosis assessed.

4.6.2.6 Future directions

4.6.2.6.1 Evaluation of the root cause(s) for reduced BLM tolerance in HPS1 mice

There is evidence suggesting a difference in kinetics for the pathology of BLM-induced pulmonary fibrosis between HPS1 mice and WT C57BL/6 controls prior to and at day 7; further studies at earlier time points may be helpful in distinguishing differences between HPS1 and WT C57BL/6 mice regarding their response to BLM and the development of lung damage. The levels of inflammatory cell infiltration, collagen accumulation, and also protein levels of pro- and anti-fibrotic mediators will be assessed. The role of AMφ with regards to inflammation and fibrosis can also be analysed. Alternatively it may be possible that reduced tolerance to BLM is not caused by more severe pulmonary fibrosis. Further analysis of a potential systemic effect to BLM, or reduced BLM removal by other organs, such as the kidney, in HPS1 mice is required to evaluate whether or not non-pulmonary effects are involved in increased pathology to oropharyngeal BLM-challenge.

4.6.2.6.2 Evaluation of pulmonary fibrosis in HPS1/HPS2 double mutant mice

HPS1 and HPS2 patients develop interstitial pulmonary fibrosis that resembles IPF in terms of disease progression, and its UIP histopathology. This phenomenon is observed in HPS1/HPS2 double homozygous recessive mice, which spontaneously develop interstitial pulmonary fibrosis at nine months to one year of age. It would be of interest to evaluate the events leading up to and during naturally occurring pulmonary fibrosis in mice, which would improve our understanding of the initiation and progression of this devastating disease. There are several parameters to be measured: the levels of pro-inflammatory, pro-fibrotic, tissue remodelling mediators and also alarmins within the lung and BALF that can be assessed at a genetic level by microarrays or at a protein level by multiplex assays; cellular infiltration and collagen accumulation can be assessed by histological analyses; AEC2 hyperplasticity can be evaluated by measurement of development genes that play a role in dysregulated wound healing (e.g. Shh).

Spontaneous pulmonary fibrosis in HPS1/HPS2 mice that results from effective intracellular trafficking is thought to result either directly or indirectly with AMφ activation as mentioned (section 4.1.3).
The susceptibility of AMφ to apoptosis following BLM challenge can also be determined through measuring apoptotic markers, including PTEN and active caspase 3. AMφ may also contribute to the resolution of fibrosis through the efferocytosis of apoptotic cells and phagocytosis of cellular debris and/or irritants, thereby removing the source of inflammation. The phagocytic rate of AMφ can be measured by phagocytosis assays to measure their uptake of apoptotic cells, pathogens and/or latex beads.

4.6.2.7 Summary
Collectively these results suggest that naïve HPS1 mice do not spontaneously develop pulmonary inflammation or fibrosis, but have reduced tolerance to BLM challenge. This reduced BLM tolerance however does not appear to be correlated with more severe pulmonary fibrosis as mentioned in a previous study. This may represent a difference in kinetics of BLM-induced pulmonary fibrosis in HPS1 mice whereby inflammation, fibrosis and resolution is accelerated compared to that in WT C57BL/6 controls. It is interesting to learn what constitutes to hastened resolution of fibrosis in HPS1 mice. Alternatively, this may indicate non-pulmonary effects of BLM that may affect tolerance to this drug. Further studies assessing the cause of reduced BLM tolerance in HPS1 mice will improve our understanding of the other side effects of BLM, which may be manipulated to improve survival in mouse models of BLM-induced pulmonary fibrosis, and also human patients undergoing BLM chemotherapy. Further assessment of spontaneous pulmonary fibrosis in HPS1/HPS2 mice may provide clues to the natural (not artificially generated) course of induction and progression of pulmonary fibrosis in mice.

4.6.3 Conclusion
Together these results show that AMφ display heterogeneity in BLM-induced pulmonary fibrosis. M1-like AMφ accumulate in the late inflammatory phase (day 7), whereas M2-like AMφ accumulate from the early to the late fibrotic phases (days 14 to 21). During BLM-induced pulmonary fibrosis, AMφ secrete mediators that are able to increase Col1α1 mRNA transcripts in pulmonary fibroblasts. Foamy macrophages were also found to accumulate in the alveolar space of BLM-challenged mice. The role of foamy macrophages in pulmonary fibrosis was evaluated in BLM-challenged HPS1 mice due to their alleged elevated number of foamy macrophages, their increased susceptibility to BLM-induced pulmonary fibrosis, and the similarities in pathologies between HPS1 and IPF patients (e.g. UIP histological pattern). However, in the studies here HPS1 mice had reduced tolerance to BLM-challenge that was independent of pulmonary fibrosis, and there was an absence of an increased foamy macrophage accumulation with respect to WT C57BL/6 controls. This suggests that HPS1 mice are not good models for the study of foamy macrophages. Further experiments involving the isolation of foamy macrophages directly by flow cytometry, or perhaps the depletion of foamy
macrophages by inducing cholesterol efflux using high density lipoproteins (e.g. Apo-AI) can reveal the potential role of these cells.
Chapter 5
The role of pathogen recognition receptors on alveolar macrophages in bleomycin-induced pulmonary fibrosis
Chapter 5 | The role of PRRs on AMφ in BLM-induced pulmonary fibrosis

5.1 Introduction

PRRs are expressed on a number of cells of the innate and adaptive immune system, including AMφ, and also resident structural cells, such as AECs. PRRs detect exogenous pathogens or endogenous alarmins and trigger immune responses. PRRs also play a critical role in pulmonary fibrosis through the recognition of alarmins released by necrotic cells following tissue injury, including HA fragments, HMGB1, and uric acid. This may result in further tissue injury induced by chronic inflammation, leading to subsequent dysregulated wound healing. PRRs are also thought to exacerbate pulmonary fibrosis in IPF patients following viral infection. A variety of PRRs are increased on all types of BAL cells in IPF patients, including TLR2, TLR3, TLR7, TLR9 and NLRP3. The importance of these receptors in pulmonary fibrosis has been highlighted in various mouse studies; however, none of these studies have directly addressed the role of PRRs on AMφ.

AMφ express a range of such PRRs capable of recognizing endogenous alarmins or exogenous irritants that can induce chronic inflammation and potential fibrosis within the lung. TLR2 are extracellular PRRs found on the cell surface of macrophages and recognize LMW (<500kDa) HA fragments, which are alarmins that accumulate during tissue injury in IPF patients and within the BLM mouse model of pulmonary fibrosis. TLR2 stimulation by HA fragments promotes the upregulation of pro-inflammatory mediator genes in MH-S cells (a mouse AMφ-derived cell line) including the chemokines CCL3/MIP-1α, CCL4/ MIP-1β, CXCL2/MIP-2 and the cytokine TNF-α. These chemokines actively recruit pro-inflammatory cells and promote inflammation-induced tissue injury. BLM also appears to be a TLR2 ligand, and promotes the production of pro-inflammatory mediators in a human monocytic cell line. HMGB1 is an alarmin that is upregulated in macrophages and injured epithelial cells during acute exacerbation of IPF, and is important in BLM-induced pulmonary fibrosis. HMGB1 can induce a pro-inflammatory response directly through TLR2, or indirectly amplify TLR2 and TLR9 responses in peritoneal macrophages by acting as a molecular chaperone for their respective ligands.

TLR9 are intracellular PRRs found on endosomal surfaces that recognise extracellular histones as alarmins and promote a pro-inflammatory response. In a model of hepatic ischemic/ reperfusion (I/R) injury in rat liver, there was an increase of extracellular histones. Injection of exogenous histones exacerbates I/R injury in wild type (WT) mice, but this exacerbation was absent in TLR9−/− or...
MYD88. TLR7 are also intracellular PRRs that are found on endosomal membranes. TLRs 7 and 9 may be involved in the acute viral exacerbation of pulmonary fibrosis through their anti-viral responses (See Section 1.2.4).

The NLRP3 inflammasome are intracellular PRRs that are found in the cytoplasm of macrophages and promotes a pro-inflammatory response in LPS-primed AMφ challenged by silica, which is known to cause interstitial lung disease, resulting in an increase of IL-1β secretion. The NLRP3 inflammasome can also induce a pro-inflammatory response in LPS-primed peritoneal macrophages from WT mice challenged by silica or asbestosis, but this is abolished in NLRP3−/−, Caspase 1−/− and ASC−/− (components of the NLRP3 inflammasome) mice. The NLRP3 inflammasome can be stimulated by other endogenous alarmins, including extracellular ATP, amyloid-β, ROS and monosodium urate (MSU) crystals.

5.2 Hypothesis
AMφ express a number of PRRs and therefore can be stimulated by a variety of exogenous pathogens or endogenous alarmins to induce pro-inflammatory responses. It was proposed that alarmins are present at elevated levels in pulmonary fibrosis due to passive release by necrotic cells, or active release by immune cells and further promote tissue injury. In this chapter experiments are designed to test the hypothesis that PRR expression on AMφ is increased and when ligated may drive a more pronounced pro-inflammatory response. This outcome may as a consequence generate more extensive tissue injury.

5.3 Aims
The aim of the following studies are to:

1. Assess the mRNA and protein levels of several PRRs that have been reported to be upregulated in BALF of IPF patients, including TLR2, TLR7, TLR9 and NLRP3, in BLM-challenged mice as compared to saline controls. The gene transcript and protein levels of TLR4, a PRR that shares many common ligands with TLR2, including HA fragments and HMGB1, was also assessed.

2. Evaluate the changes of protein level of several pre-selected mediators that are associated with pulmonary fibrosis following challenge of AMφ with respective ligands to their elevated PRRs. This may help identify the possible role of these PRRs in pulmonary fibrosis.

The potential value of the results described in this section is that they may identify key PRRs on AMφ that have increased expression in pulmonary fibrosis and this information may add to our understanding of their role in chronic inflammation that results in tissue remodelling in the lung.
Chapter 5 | The role of PRRs on AMφ in BLM-induced pulmonary fibrosis

5.4 Methods

5.4.1 Multiple oropharyngeal dose protocol for BLM-induced pulmonary fibrosis

BLM-induced pulmonary fibrosis in mice were carried out as described in section 2.2.2. In brief, WT C57BL/6 male mice or male or female HPS1 mice were challenged three times by oropharyngeal instillations of 50μl of 0.13U BLM sulfate (Kyowa Nippon, Kayakin Co Ltd.) or saline controls at 72 hour intervals on Days -7, -4 and -1. Mice were culled on days 7, 14 and 21 post-challenge, which include time points in the ALI phase, early fibrotic phase and the progressive fibrotic stage respectively (Figure 2.1).

5.4.2 Flow cytometry

3 x 10^5 cells/ml of BAL cells were separated into 3 tubes (i.e. 1 x 10^5 cells/tube), one for positive staining for proteins of interest (surface: CD11c, TLR2, and TLR4; intracellular: TLR7, TLR9, NLRP3), one for unstained negative control, and one for isotype control of nonspecific antibody binding. For each tube, cells were resuspended at 1x10^5 cells per 100 μl Fluorescence Activated Cell Sorting (FACS) analysis buffer (DPBS + 0.1% FCS), 1% of Fc Receptor (FcR) block (Miltenyi Biotech) and 5% mouse serum per 100μl reaction. FcR block was added to prevent non-specific binding of the Fc (constant) region of detection antibodies/isotype controls to cell surface FcR that may generate a false positive signal. Mouse serum was added to prevent non-specific binding of the variable region of detection antibodies/isotype controls.

In the preliminary study, cells for staining of proteins of interest were incubated with FACS analysis buffer, 2 μg/ml CD11c-APC, 2 μg/ml TLR2-eFluor 660®, 2 μg/ml TLR4-APC for 30 minutes in the dark at 4°C. For the staining for isotype control, cells were incubated with 2 μg/ml Armenian hamster IgG-APC, 2 μg/ml Rat IgG2b-eFluor®, or 2 μg/ml Rat IgG2a-APC for 30 minutes in the dark at 4°C. For unstained controls, cells were incubated in FACS analysis buffer only for 30 minutes in the dark at 4°C.

BAL cells were then washed in 2 ml DPBS and centrifuged at 500 g for 10 minutes at 4°C. The supernatant was then discarded and BAL cells were fixed and permeabilised with 300 μl BD Cytofix/Cytoperm™ (BD Biosciences) for 20 minutes in the dark at 4°C. BAL cells were then washed with 2 ml BD Perm/Wash™ (BD Biosciences). Cells were then resuspended in 500 μl 1 x Cell Fix (BD Biosciences) (diluted 1:10 from 10x stock solution), and kept in the dark at 4°C until further analysis.

For intracellular staining, permeabilised BAL cells were stained with primary (2 μg/ml TLR7-rabbit or 2 μg/ml TLR9-Biotin) or directly conjugated antibodies (10 μl/10^6 cells NLRP3-APC) for 30 minutes in the dark at 4°C, and then washed with 2 ml BD Perm/Wash™ (BD Biosciences). For isotype controls, cells were incubated with primary (2 μg/ml rabbit control IgG or 2 μg/ml RatIgG2A-Biotin) or directly
conjugated antibodies (10 μl/10^6 cells Rat IgG2A-APC) for 30 minutes in the dark at 4°C. This step was repeated for staining with secondary antibodies (2 μg/ml mouse anti-rabbit or Streptavidin-APC respectively for TLR7 or TLR9, or their respective isotype control staining). Stained cells were then resuspended in 500 μl 1 x Cell Fix (BD Biosciences), and kept at 4°C until further analysis. The staining procedures for AMφ are summarised in table 5.1 below.

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<td>TLR4</td>
</tr>
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</tr>
<tr>
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</tr>
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</tr>
<tr>
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<td>Anti-Mouse CD282 (TLR2) eFluor® 660</td>
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<td>N/A</td>
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<td></td>
<td>N/A</td>
<td>Rat Anti-Mouse CD289 (TLR9) Biotin</td>
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<tr>
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<td>N/A</td>
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</tr>
<tr>
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</tr>
<tr>
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<td>2°</td>
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Table 5.1 Summary of antibodies used for staining BAL cells in preliminary flow cytometry study.

In the follow-up study, AMφ were stained for the cell surface proteins CD11c, TLR2, MHC-II and MRC1 with directly conjugated antibodies, including 2 μg/ml anti-mouse CD11c-APC/Cy7, 2 μg/ml anti-mouse TLR2 eFlour660®, 2 μg/ml MHC-II-FITC and 2 μg/ml MRC1-PerCP/Cy5.5. For the isotype control, cells were incubated with 2 μg/ml Armenian hamster IgG-APC, 2 μg/ml Rat IgG2b Isotype Control eFluor® 660, 2 μg/ml Rat IgG2b-FITC and 2 μg/ml Rat IgG2a-PerCP/Cy5.5 in the same cell surface staining protocol mentioned above. Unstained controls were also prepared by the incubation of AMφ in FACS analysis buffer in the absence of antibodies as with the aforementioned protocols.
Anti-Rat/anti-Hamster IgK compensation kit (BD Biosciences) was used on the day of flow cytometric analysis to compensate for fluorophore spectral overlap in multi-colour staining. The set consists of two populations of microparticles, including the BD CompBeads Anti-Rat/Hamster Ig, κ particles (positive control), which bind any rat or hamster κ light chain-bearing immunoglobulin, and the BD CompBeads Negative Control (FBS) Particles (negative control), which has no binding capacity. When mixed together with a fluorochrome-conjugated rat or hamster antibody, the BD-CompBeads provide distinct positive and negative (background fluorescence)-stained populations that can be used to set compensation levels manually. Anti-Rat/anti-Hamster IgK compensation kit (BD Biosciences) was prepared according to manufacturer’s instructions. In brief, 60 μl of BD CompBeads Anti-Rat/Hamster Ig, κ particles and 60 μl of BD CompBeads Negative Control (FBS) particles were added to 100 μl FACS buffer (+0.1 % FBS), and 20 μl of pre-diluted antibody stock (where final concentration in 220 μl is equivalent to that used in experiments) was subsequently added. This process was carried out separately for every different type of fluorophore that was used in multicolour experiments. The BD CompBeads Anti-Rat/Hamster Ig, κ particles/BD CompBeads Negative Control (FBS) particles/antibody mixture was then incubated in the dark at 4°C for 30 minutes. The mixture was then washed with 2 ml FACS buffer and centrifuged at 500 g for 10 minutes. The supernatant containing unbound antibodies was discarded, and the pellet containing Compbeads was resuspended in 500 μl FACS buffer.

FACS data was read by FACS CANTO II (BD Biosciences). Prior to recording data for samples, compensation for spectral overlap was determined from the pre-stained BD CompBeads Anti-Rat/Hamster Ig, κ particles/BD CompBeads Negative Control (FBS) particles using the compensation setup on the FACS CANTO II software (BD Biosciences). FACS data was analyzed by Flowjo (Tree Star Inc). AMφ were defined as SSC<sup>hi</sup> FSC<sup>hi</sup> CD11c<sup>+</sup>, and the expression of the protein of interest was gated with respect to their respective isotype controls. Data was presented as median fluorescence intensity (MFI) and % positive with respect to isotype controls.

5.4.3 AMφ enrichment and culture
AMφ were isolated from BAL of BLM-challenged mice or saline controls at days 7, 14 and 21 following two PBS washes in mice, and enriched by plastic adherence to tissue culture plates, the details of which are described in detail in sections 2.2.4, 2.2.5 and 2.2.6. 1 x 10<sup>5</sup> AMφ per well in a 48-well plate that were enriched by plastic adherence for 90 minutes were further cultured in fresh macrophage complete media for 4 hours for mRNA level analysis of various PRRs. Total RNA extraction, reverse transcription, and qRT-PCR for mRNA level analysis were carried out as described at sections 2.2.11, 2.2.12 and 2.2.13 respectively.
5.4.4 TLR2 and 9 stimulation of AMϕ isolated from saline or BLM-challenged mice

$1 \times 10^5$ AMϕ per well in a 6-well plate were enriched by plastic adherence for 90 minutes as mentioned above (section 5.4.3). AMϕ were then stimulated with 10 ng/ml of TLR2 ligand, Pam2CSK4 (P2C) (Invivogen), 10ng/ml of TLR9 ligand, CpG-ODN (A) (Invivogen), or 10 ng/ml CpG-ODN (A) control (Invivogen) in fresh culture media for 24 hours. Measurements of proteins of interest in culture supernatant was measured as mentioned in sections 2.2.14 (fibronectin enzyme linked immunosorbent assay (ELISA) and 2.2.15 [Meso-Scale Discovery (MSD)].

5.4.5 Statistical analyses

All statistical analysis was carried out using GraphPad Prism 6.0 software (GraphPad software, CA, USA). Normality distribution was tested by visual inspection of data as there are insufficient samples for normality testing. Two-way ANOVA was used to compare time-course curves followed by Sidak’s multiple comparison test to determine the significance. P values of less than 0.05 were deemed statistically significant with *** $p < 0.001$, ** $p < 0.01$ and * $p < 0.05$. Error bars represent standard deviation (SD) as indicated in the figure legends.
5.5 Results

5.5.1 The mRNA and protein level analyses of PRRs on AM\(\phi\) isolated from saline or BLM-challenged mice

In the following investigation, the mRNA and protein levels of the selective PRRs on AM\(\phi\) from BLM-challenged mice or saline controls was investigated. This analysis was designed to test the hypothesis that exposure to BLM might activate AM\(\phi\) via PRRs and that this may contribute to the pathology that occurs in pulmonary fibrosis. Stimulation of cells via their PRRs lead to the release of pro-inflammatory mediators (e.g. TNF-\(\alpha\)), which may in turn upregulate PRR mRNA levels in a positive feedback loop\(^1\,^2\). For example, macrophages stimulated by peptidoglycan or LPS, which are TLR2 and TLR4 ligands respectively, have increased mRNA levels of TLR2 in a NF\(\kappa\)B -dependent manner\(^1\). Stimulation of P815 cells (mastocytoma cell line) with IL-12 increases TLR2 mRNA levels in a PI3K-dependent manner\(^2\). It is possible that TLRs that are increased in levels as a result of such inflammation have a greater response to their respective ligands \textit{in vivo} and therefore have a larger contribution to pathology in pulmonary fibrosis.

The mRNA level analysis revealed that TLR9 was significantly increased by 3-fold (p<0.01) at day 7 after the last dose of BLM. Although there was a trend indicating a 2-fold increase in the mRNA levels of TLR9 in AM\(\phi\) from BLM-challenged at days 14 and 21 compared to the saline controls it was not significant (Figure 5.2). This suggests that TLR9 mediated activation of AM\(\phi\) may play a more important role in inflammation than fibrosis. In contrast to TLR9 mRNA levels no increases in TLRs 2, 4 and 7, and NLRP3 specific transcripts was detected (Figure 5.1).
Chapter 5 | The role of PRRs on AMφ in BLM-induced pulmonary fibrosis

Figure 5.1 Selected Pathogen Recognition Receptor (PRR) mRNA levels in alveolar macrophages (AMφ) from bleomycin (BLM)-challenged mice compared to saline controls. 8-10 week old male mice were challenged via an oropharyngeal route three times at 72 hour intervals with either saline, or 0.13 International Units (IU) BLM, and were culled at days 7, 14 and 21 respectively. 1x10⁵ AMφ were seeded per well of a 48-well tissue culture plate and enriched by plastic adherence for 90 minutes. Isolated AMφ were further cultured in fresh macrophage complete media for 4 hours. PRR mRNA levels in AMφ was determined by qRT-PCR. Each sample was run in triplicates and the average was used to determine mean of sample repeats. Data is represented as mean ± SD; n=3 from two separate studies. Two way ANOVA with Sidak post-test was carried out. BD, Below Detection; BLM, Bleomycin; NS, Not significant.

Figure 5.2 TLR9 mRNA levels in AMφ from BLM-challenged mice compared to saline controls. 8-10 week old male mice were challenged via an oropharyngeal route three times at 72 hour intervals with either saline, or 0.13 IU BLM, and were culled at days 7, 14 and 21 respectively. 1x10⁵ AMφ were seeded per well of a 48-well tissue culture plate and enriched by plastic adherence for 90 minutes. Isolated AMφ were further cultured in fresh macrophage complete media for 4 hours. TLR9 mRNA levels in AMφ was determined by qRT-PCR. Each sample was run in triplicates and the average was used to determine mean of sample repeats. Data is represented as mean ± SD; saline (day 7), n=5; saline (day 14), n=3; saline (day 21), n=3; BLM (day 7), n=9; BLM (day 14), n=7; BLM (day 21), n=5 from two separate studies. Two way ANOVA with Sidak post-test was carried out; **: p< 0.0. BD, Below Detection; BLM, Bleomycin; NS, Not significant.
TLR2 and TLR4 are expressed on the cell surface and binds to bacterial peptidoglycans and LPS respectively. TLR7 and TLR9 are intracellular and found on endosomal surfaces, and binds to viral RNA and CpG-DNA respectively. The NLRP3 inflammasome is also intracellular and found in the cytoplasm, and is activated by binding to endogenous alarmins (e.g. ROS, uric acid). Preliminary flow cytometric analysis was performed in order to evaluate the surface protein levels of extracellular TLRs 2 and 4, and intracellular protein levels of intracellular TLRs 7 and 9, and the NLRP3 inflammasome, to investigate if their level was modulated following exposure to BLM.

The results showed that TLR2 was constitutively expressed on the surface of all AMφ; however, there was an increase in this baseline level as determined by MFI on the AMφ at days 7, 14 and 21 post BLM challenge. Thus it appears that cell surface protein levels of TLR2, which is present on AMφ in control mice is enhanced following challenge with BLM (Figure 5.3).

In order to validate this result for TLR2 surface protein levels a repeat experiment was performed in which a 2.3-fold increase in MFI (from 1500 to 3500; \( p<0.001 \)) on AMφ from the BLM-challenged mice was also observed compared to saline controls (\( p<0.001 \)) (Figures 5.4 and 5.5). The increase in surface protein but not transcript levels observed for TLR2 may be due to post-transcriptional or post-translational regulation; a possibility is the mobilisation of internal stores of preformed TLR2 onto the cell surface upon appropriate stimuli. MicroRNAs (miRNA) may also inhibit the translation of TLR2 gene transcript under normal conditions by targeting it for degradation, but this process may be inhibited under fibrotic conditions. It is unclear whether or not the increasing surface protein levels of TLR2 in AMφ where they are already highly expressed would matter physiologically. The potential increased response to TLR2 stimulation in AMφ owing to increased TLR2 surface protein levels was evaluated in section 5.5.2.2. The physiological role of the increased surface protein levels of TLR2 in AMφ of mice in promoting BLM-induced pulmonary fibrosis can also be tested by inhibiting TLR2 using concentration gradient of pharmacological inhibitors or neutralising antibodies.

Flow cytometric analysis also revealed an increase in the number of AMφ expressing TLR9 from 4% to 25% upon BLM challenge at day 7, but this elevation returned to saline control levels by day 14. These results are concurrent with the mRNA level data, and suggest that TLR9 mediated signalling may play a more important role in the inflammatory response initiated by BLM. It was observed that AMφ showed a biphasic increase in the intracellular protein levels of NLRP3: there was an increase from 22% to 67% of AMφ expressing NLRP3 at day 7 upon BLM challenge, and from 36% to 59% at day 21. This suggests that NLRP3 may play a role in the initiation of pulmonary fibrosis through inflammation induced tissue injury, and maintenance of established fibrosis, but has no role in active fibrosis. No increased surface protein level for TLR4 or intracellular protein level for TLR7 was
observed in BLM-challenged mice compared to saline controls (Figure 5.3). It was observed that the median fluorescence intensity (MFI) for the AMφ stained with TLR7-specific antibodies is lower than that of the corresponding isotype control at days 7 and 21. This indicates that there was more binding for isotype controls than TLR7-specific antibodies. The large amount of non-specific binding by the isotype control may be due to rabbit polyclonal antibodies versus a monoclonal antibody being used. Perhaps a monoclonal antibody would provide greater specificity in binding in the future.

The median fluorescence intensity (MFI) for isotype controls for the intracellular proteins, including TLR7, TLR9 and NLRP3 were high despite the usage of FcR to eliminate non-specific binding on the cell surface. This is presumably because isotype controls are optimised towards cell surface proteins. Isotype controls may therefore not behave as expected intracellularly than that on the cell surface because of inherent difference in amino acid composition to different amount of fluorophore conjugated to the isotype control versus the experimental antibody.

Collectively these data suggest that TLRs2 and 9 are increased in AMφ during BLM-induced pulmonary fibrosis, and therefore may play a role in pathological response resulting in fibrosis within the lung.
Figure 5.3 PRR protein levels in AMφ from BLM-challenged mice compared to saline controls. 8-10 week old C57BL/6 male mice were challenged via an oropharyngeal route three times at 72 hour intervals with either saline, or 0.13 IU BLM, and were culled at days 7, 14 and 21 after the last dose. BAL cells were then harvested and stained with cluster of differentiation (CD)11c-PE (2 μg/ml), and toll like receptor (TLR)2-eFluor660® (2 μg/ml), TLR4-APC (2 μg/ml) at the cell surface, or intracellular staining for TLR7-Rabbit (2 μg/ml) + mouse anti-rabbit-APC (2 μg/ml), TLR9-Biotin (2 μg/ml) + Streptavidin-APC (2 μg/ml), or NLRP3-APC (10 μl/10⁶ cells) antibodies. AMφ were identified as Side Scatter (SSC)⁹⁺ Forward Scatter (FSC)⁹⁺ CD11c⁺ and autofluorescent in the FL1 channel, and analysed for changes in protein levels of TLR2, TLR4, TLR7, TLR9 and NLRP3. Data is from a single pilot study. BLM: Bleomycin.
Figure 5.4 TLR2 cell surface protein levels AMϕ from BLM-challenged mice compared to saline controls. 8-10 week old C57BL/6 male mice were challenged via an oropharyngeal route three times at 72 hour intervals with either saline, or 0.13 IU BLM, and were culled at days 7, 14 and 21 after the last dose. BAL cells were then harvested and stained with TLR2-eFluor660® (2 μg/ml), CD11c-APC/Cy7 (2 μg/ml), MHC-II-FITC (2 μg/ml) and MRC1-PerCP/Cy5.5 (2 μg/ml) antibodies. AMϕ were identified as SSChi FSChi CD11c+ and autofluorescent in the FL1 channel, and analysed for changes in protein levels of TLR2, MHC-II (IA/IE), MRC1 cell surface protein levels by flow cytometry. Representative images are shown; n=6 (saline day 7), n=7 (BLM day 7), n=8 (saline and BLM days 14 and 21) from two separate studies. BLM: Bleomycin; SAL: Saline. Histograms of individual repeats are shown in appendix 11.
Figure 5.5 Median fluorescence intensity (MFI) and % population positive of TLR2 cell surface protein levels on AMφ from BLM-challenged mice compared to saline controls. 8-10 week old C57BL/6 male mice were challenged via an oropharyngeal route three times at 72 hour intervals with either saline, or 0.13 IU BLM, and were culled at days 7, 14 and 21 after the last dose. BAL cells were then harvested and stained with TLR2-eFluor660® (2 μg/ml), CD11c-AP/Cy7 (2 μg/ml), MHC-II-FITC (2 μg/ml) and MRC1-PerCP/Cy5.5 (2 μg/ml) antibodies. AMφ were identified as SSC<sup>hi</sup> FSC<sup>hi</sup> CD11c<sup>+</sup> and autofluorescent in the FL1 channel, and analysed for changes in protein level of TLR2, MHC-II (IA/IE), MRC1 cell surface protein levels by flow cytometry. Representative images are shown; n=6 (saline day 7), n=7 (BLM day 7), n=8 (saline and BLM days 14 and 21) from two separate studies; ****: p<0.001. BLM: Bleomycin; SAL: Saline.
5.5.2 The effect of TLR stimulation on AMφ phenotypes

5.5.2.1 TLR stimulation of AMφ from naïve mice

Having identified that TLR2 surface protein level and TLR9 mRNA level were elevated in AMφ following BLM challenge the potential role of these TLRs in pulmonary fibrosis was then investigated. To this end, AMφ from naïve mice were challenged with media in the control group, P2C (a synthetic diacetylated lipoprotein that acts as a TLR2 ligand), CpG-ODN (A) (a TLR9 ligand), or non-CpG ODN (GpC-ODN) [a non-TLR9 specific control for CpG-ODN (A)]. The production of several mediators with reported roles in pulmonary fibrosis, including IL-1β, TNF-α, CXCL1, IL-10, and IL-12 p40 (total) were then analysed.

From the results it was observed that TLR2 stimulation of AMφ significantly increased the production of the pro-inflammatory mediators. IL-1β levels were elevated by about 4-fold from 7 pg/ml to 27 pg/ml (p<0.01), similarly protein levels of TNF-α was increased by about 7-fold from 2,400 pg/ml to 18,000 pg/ml (p<0.005). CXCL1 was amplified by 12-fold from 840 pg/ml to 10,000 pg/ml (p<0.001). Production of IL-10 was also escalated by around 8-fold from 33 pg/ml to 260 pg/ml (p<0.05), possibly as part of a negative feedback loop activated to limit inflammation (Figure 5.6). Activation via TLR9 significantly increased the production of IL-12 p40 (total) by 3.7-fold from 469 pg/ml to 1,740 pg/ml (p<0.01). However, it had little effect on the protein levels of other pro-inflammatory mediators investigated (Figure 5.6). Therefore, TLR9 stimulation of AMφ may trigger a predominantly pro-inflammatory response indirectly through the induction of a T4 response.

Collectively these data demonstrate that activation of AMφ via different TLRs trigger different mediator profiles which could potentially drive pulmonary fibrosis through either interacting or independent mechanisms. For example, IL-12 p40 can promote IL-1β induced fibrosis in mice via the IL-17A pathway or recruit macrophages in silica-induced pulmonary fibrosis. On the other hand, long term lung-specific overexpression of IL-10 in mice induced pulmonary fibrosis through fibrocyte recruitment and M2-like macrophage activation through a CCR2/CCL2 axis.
Figure 5.6 Protein level analysis of pro-inflammatory mediator biosynthesis in AMΦ from naïve mice following TLR2 or TLR9 stimulation. 1x10^5 AMΦ were seeded per well of a 48-well tissue culture plates for 90 minutes, and then stimulated with macrophage complete media, 10 ng/ml Pam2CSK4 (P2C), 3μM CpG-ODN (A) (CpG) or 3μM CpG-ODN (A) Control (Ctrl) in fresh macrophage complete media for 24 hours. IL-1β, TNF-α, CXCL1, IL-10 and IL-12 p40 (total) was determined by Meso Scale Discovery (MSD) of culture supernatants. Each sample was run in duplicate in MSD determination of protein levels, and the average was used to calculate sample repeat mean. Data are represented as mean ± SD; n=3 from three separate studies. One way ANOVA with Tukey post-test was carried out; *: p<0.05, **: p< 0.01, ***: p<0.005, ****: p<0.001. BD, Below Detection; ND, Not Detected. IL-12 p40 (total) includes free IL-12 p40, IL-12 p40 homodimers and IL-12 p40 heterodimers (IL-12 p70 and IL-23). Standard curves for the MSD of the above proteins are found in appendix 9.
5.5.2.2 TLR stimulation of AMφ from saline or BLM-challenged mice

Having identified that different TLR stimulation induces a different mediator response in naïve mice, it was next investigated whether or not the increased surface protein levels of TLRs 2 and intracellular protein levels of TLR9 observed in AMφ isolated from BLM-challenged mice result in an enhanced response to the corresponding ligands and produce increased amounts of associated mediators.

In order to investigate these issues AMφ isolated at different stages of BLM-induced pulmonary fibrosis in mice or saline controls were stimulated with TLRs 2 or 9 ligands and assessed for their responsiveness by their production of the pro-inflammatory mediators investigated in the previous section (section 5.5.2.1), including IL-1β, TNF-α, CXCL1, IL-10 and IL-12 p40 (total). The key comparison here is the fold change in pro-inflammatory mediator levels in AMφ culture supernatant, between BLM-challenged mice and the corresponding saline control, following TLR2 or TLR9 stimulation. It was presumed that an increased response to TLR ligands would induce a greater fold change in protein levels of pro-inflammatory mediators in AMφ. The panel of mediators was extended to assess the effect of TLRs 2 or 9 stimulation on pro-fibrotic mediators, such as fibronectin, and other mediators that have been reported to be involved in pulmonary fibrosis, including IL-6, CCL2/MCP-1, CCL3/MIP-1α, CCL4/MIP-1β, CCL5/RANTES, and also CCL20/MIP-3α.

AMφ from BLM-challenged mice have an increased response to TLR2 mediated signalling, and the levels of a number of pro-inflammatory mediators induced following P2C stimulation were increased as compared to their saline controls (Table 5.1). IL-1β levels in the culture supernatant of P2C-stimulated AMφ from BLM-challenged mice as compared to their corresponding saline controls were significantly increased by 8.6-fold at day 7 (p<0.001), 5.87-fold at day 14 (p<0.001), and 322.5-fold at day 21 (p<0.05) (Figure 5.7).

A significant enhancement in TNF-α protein levels in P2Gstimulated AMφ from BLM- versus saline challenge mice by 2.90-fold (p<0.01) was detected at day 21, but no such amplification was observed at days 7 and 14 (Figure 5.8).

P2C stimulation induced increase in the protein levels of CXCL1 were also significantly elevated in AMφ from BLM-challenged mice as compared to saline controls by 2.53-fold at day 7 (p<0.001), 1.51-fold at day 14 (p<0.005) and 3.09-fold (p<0.001) at day 21 (Figure 5.9).
Similarly levels of IL-10 following P2C stimulation was significantly increased in AMϕ from BLM-challenged mice as compared to saline controls by 24.8-fold at day 7 (p<0.001), 10.3-fold at day 14 (p<0.01) and 23.3-fold (p<0.001) at day 21 (Figure 5.10).

There was also a significant difference in IL-12 p40 (total) production between P2C-stimulated AMϕ from saline or BLM-challenged mice by 5.00-fold at day 7 (p<0.001); 4.00-fold at day 14 (p<0.005) and 7-fold at day 21 (p<0.01) (Figure 5.11). However, this increase in IL-12 p40 (total) production may be due to direct effects of BLM as opposed to TLR2 stimulation, as P2C has little effect on IL-12 p40 (total) production (Figure 5.6). Alternatively in contrast to the saline controls BLM exposure might increase the sensitivity of AMϕ to TLR2 ligation ex vivo. However as noted above there was an increased response to TLR2 stimulation in saline controls at day 14 by around 2- to 3-fold as compared to its counterparts at other time points, although the reason for this is unclear. TLR2 stimulation had no effect on the production of the M2-associated protein fibronectin in both saline controls and BLM-challenged mice (Figure 5.12).
Chapter 5 | The role of PRRs on AMφ in BLM-induced pulmonary fibrosis

Table 5.2 Pro-inflammatory mediator secretion levels and fold change between AMφ from BLM-challenged mice or saline controls at various time points following TLR2 stimulation. 8-10 week old male C57BL/6 mice were challenged via an oropharyngeal route three times at 72 hour intervals with either saline, or 0.13 IU BLM, and were culled at days 7, 14 and 21 after the last dose. 1x10⁵ AMφ were seeded per well of a 48-well tissue culture plate for 90 minutes, and enriched AMφ were then further stimulated with media or 10 ng/ml P2C for 24 hours for protein level analysis. Pro-inflammatory mediator secretion was determined by MSD of culture supernatants. Each sample was run as duplicates for the determination of protein levels by MSD, and the average was taken to calculate the mean of sample repeats. N=3 from three separate studies, and data are represented as mean. Two way ANOVA with Sidak post-test was carried out.

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<th>Day 14</th>
<th>Fold Change</th>
<th>P value</th>
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<td>Saline (pg/ml)</td>
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Figure 5.7 Analysis of Interleukin (IL)-1β protein levels following TLR2 stimulation in AMφ from BLM-challenged mice compared to saline controls. 8-10 week old male C57BL/6 mice were challenged via an oropharyngeal route three times at 72 hour intervals with either saline, or 0.13 IU BLM, and were culled at days 7, 14 and 21 after the last dose. 1x10^5 AMφ were seeded per well of a 48-well tissue culture plate for 90 minutes, and enriched AMφ were then further stimulated with media or 10 ng/ml P2C for 24 hours for protein level analysis. IL-1β protein levels was determined by MSD of culture supernatant. Each sample was run as duplicates for the determination of protein levels by MSD, and the average was taken to calculate the mean of sample repeats. Data is represented as mean ± SD; n=6 (saline day 7); n=7 (BLM day 7, saline days 14 and 21), n=8 (BLM days 14 and 21) from two separate studies. Two way ANOVA with Sidak post-test was carried out; *: p<0.05, **: p<0.01, ***: p<0.005, ****: p<0.001. BD, Below Detection; BLM, Bleomycin; NS, Not significant. Standard curves for the MSD of IL-1β is found in appendix 9.
Figure 5.8 Analysis of Tumour Necrosis Factor (TNF-α) protein levels following TLR2 stimulation in AMφ from BLM-challenged mice compared to saline controls. 8-10 week old male C57BL/6 mice were challenged via an oropharyngeal route three times at 72 hour intervals with either saline, or 0.13 IU BLM, and were culled at days 7, 14 and 21 after the last dose. 1x10⁵ AMφ were seeded per well of a 48-well tissue culture plate for 90 minutes, and enriched AMφ were then further stimulated with media or 10 ng/ml P2C for 24 hours for protein level analysis. TNF-α protein levels was determined by MSD of culture supernatant. Each sample was run as duplicates for the determination of protein levels by MSD, and the average was taken to calculate the mean of sample repeats. Data is represented as mean ± SD; n=6 (saline day 7); n=7 (BLM day 7, saline days 14 and 21), n=8 (BLM days 14 and 21) from two separate studies. Two way ANOVA with Sidak post-test was carried out; **: p < 0.01, ****: p < 0.001. BD, Below Detection; BLM, Bleomycin; NS, Not significant. Standard curves for the MSD of TNF-ds found in appendix 9.
Figure 5.9 Analysis of CXC Ligand (CXCL)1 protein levels following TLR2 stimulation in AMφ from BLM-challenged mice compared to saline controls. 8-10 week old male C57BL/6 mice were challenged via an oropharyngeal route three times at 72 hour intervals with either saline, or 0.13 IU BLM, and were culled at days 7, 14 and 21 after the last dose. 1x10^5 AMφ were seeded per well of a 48-well tissue culture plate for 90 minutes, and enriched AMφ were then further stimulated with media or 10 ng/ml P2C for 24 hours for protein level analysis. CXCL1 protein levels was determined by MSD of culture supernatant. Each sample was run as duplicates for the determination of protein levels by MSD, and the average was taken to calculate the mean of sample repeats. Data is represented as mean ± SD; n=6 (saline day 7); n=7 (BLM day 7, saline days 14 and 21), n=8 (BLM days 14 and 21) from two separate studies. Two way ANOVA with Sidak post-test was carried out; *: p<0.05, **: p<0.01, ***: p<0.005, ****: p<0.001. BD, Below Detection; BLM, Bleomycin; NS, Not significant. Standard curves for the MSD of CXCL1 is found in appendix 9.
Figure 5.10 Analysis of IL-10 protein levels following TLR2 stimulation in AM\(\phi\) from BLM-challenged mice compared to saline controls. 8-10 week old male C57BL/6 mice were challenged via an oropharyngeal route three times at 72 hour intervals with either saline, or 0.13 IU BLM, and were culled at days 7, 14 and 21 after the last dose. 1x10^5 AM\(\phi\) were seeded per well of a 48-well tissue culture plate for 90 minutes, and enriched AM\(\phi\) were then further stimulated with media or 10 ng/ml P2C for 24 hours for protein level analysis. IL-10 protein levels was determined by MSD of culture supernatant. Each sample was run as duplicates for the determination of protein levels by MSD, and the average was taken to calculate the mean of sample repeats. Data is represented as mean ± SD; n=6 (saline day 7); n=7 (BLM day 7, saline days 14 and 21), n=8 (BLM days 14 and 21) from two separate studies. Two way ANOVA with Sidak post-test was carried out; **: p<0.01, ****: p<0.001. BD, Below Detection; BLM, Bleomycin; NS, Not significant. Standard curves for the MSD of IL-10 is found in appendix 9.
Figure 5.1 Analysis of IL-12 p40 (total) protein levels following TLR2 stimulation in AMφ from BLM-challenged mice compared to saline controls. 8-10 week old male C57BL/6 mice were challenged via an oropharyngeal route three times at 72 hour intervals with either saline, or 0.13 IU BLM, and were culled at days 7, 14 and 21 after the last dose. 1x10⁵ AMφ were seeded per well of a 48-well tissue culture plate for 90 minutes, and enriched AMφ were then further stimulated with media or 10 ng/ml P2C for 24 hours for protein level analysis. IL-12 p40 (total) protein levels was determined by MSD of culture supernatant. Each sample was run as duplicates for the determination of protein levels by MSD, and the average was taken to calculate the mean of sample repeats. Data is represented as mean ± SD; n=6 (saline day 7); n=7 (BLM day 7, saline days 14 and 21), n=8 (BLM days 14 and 21) from two separate studies. Two way ANOVA with Sidak post-test was carried out; *: p<0.05, **: p<0.01, ***: p<0.005, ****: p<0.001. BD, Below Detection; BLM, Bleomycin; NS, Not significant. IL-12 p40 (total) includes free IL-12 p40, IL-12 p40 homodimers and IL-12 p40 heterodimers (IL-12 p70 and IL-23). Standard curves for the MSD of IL-12 p40 (total) is found in appendix 9.
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Figure 5.1 Analysis of fibronectin protein levels following TLR2 stimulation in AMφ from BLM-challenged mice compared to saline controls. 8-10 week old male C57BL/6 mice were challenged via an oropharyngeal route three times at 72 hour intervals with either saline, or 0.13 IU BLM, and were culled at days 7, 14 and 21 after the last dose. 1x10^5 AMφ were seeded per well of a 48-well tissue culture plate for 90 minutes, and enriched AMφ were then further stimulated with media or 10 ng/ml P2C for 24 hours for protein level analysis. Fibronectin protein levels was determined by ELISA of culture supernatant. Each sample was run as duplicates for the determination of protein levels by MSD, and the average was taken to calculate the mean of sample repeats. Data is represented as mean ± SD; n=6 (saline day 7); n=7 (BLM day 7, saline days 14 and 21), n=8 (BLM days 14 and 21) from two separate studies. Two way ANOVA with Sidak post-test was carried out. BD, Below Detection; BLM, Bleomycin; NS, Not significant. Standard curves for the ELISA of fibronectin is found in appendix 9.
There is a significant enhancement of IL-6 protein levels by 11.6-fold at day 7 (p<0.01), 9.61-fold at day 14 (p<0.01) and 27.5-fold (p<0.01) at day 21 in P2C stimulated BLM-challenged mice as compared to saline controls (Figure 5.13).

As regards to CCL2 protein levels, there was a non-significant trend indicating an elevated response by 3.95-fold (p>0.05) at day 7 (Figure 5.14) between P2C stimulated AMφ from BLM-challenged mice as compared to the corresponding saline control. However, there was a significant increase in CCL2 production by 2.06-fold at day 14 (p<0.05) and 5.23-fold at day 21 (p<0.001) in P2C stimulated AMφ from BLM- versus saline-challenged mice (Figure 5.14).

A significant rise in CCL4 protein levels, in P2C stimulated AMφ from BLM-challenged mice as compared to saline controls, by 2.30-fold was observed at day 7 (p<0.05), and 3.39-fold (p<0.001) at day 21 (Figure 5.16). There is also a non-significant trend suggesting an enhancement of CCL4 levels, by 1.73-fold (p>0.05), at day 14 (Figure 5.16).

The levels of CCL5 in the culture supernatant of P2C-stimulated AMφ isolated from BLM-challenged mice as compared to the saline controls increased significantly from by 6.09-fold; p<0.01 at day 7, 4.28-fold at day 14 (p<0.001), and 4.43-fold at day 21 (p<0.001) (Figure 5.17).

It was also noted that CCL20 protein was significantly increased by 4.24-fold at day 7 (p<0.05), but the levels were below the limit of detection at days 14 and 21(Figure 5.18). These increases in the response to TLR2 stimulation with P2C corresponds to increased TLR2 surface protein levels in these cells observed from flow cytometric analysis (Figure 5.4 and 5.5).
Figure 5.1 Analysis of IL-6 protein levels following TLR2 stimulation in AMφ from BLM-challenged mice compared to saline controls. 8-10 week old male C57BL/6 mice were challenged via an oropharyngeal route three times at 72 hour intervals with either saline, or 0.13 IU BLM, and were culled at days 7, 14 and 21 after the last dose. 1x10⁵ AMφ were seeded per well of a 48-well tissue culture plate for 90 minutes, and enriched AMφ were then further stimulated with media or 10 ng/ml P2C for 24 hours for protein level analysis. IL-6 protein levels was determined by MSD of culture supernatant. Each sample was run as duplicates for the determination of protein levels by MSD, and the average was taken to calculate the mean of sample repeats. Data is represented as mean ± SD; n=6 (saline day 7); n=7 (BLM day 7, saline days 14 and 21), n=8 (BLM days 14 and 21) from two separate studies. Two way ANOVA with Sidak post-test was carried out, **: p< 0.01, ***: p<0.005. BD, Below Detection; BLM, Bleomycin; NS, Not significant. Standard curves for the MSD of IL-10 is found in appendix 10.
Figure 5.14 Analysis of CCL2 protein levels following TLR2 stimulation in AM\(\phi\) from BLM-challenged mice compared to saline controls. 8-10 week old male C57BL/6 mice were challenged via an oropharyngeal route three times at 72 hour intervals with either saline, or 0.13 IU BLM, and were culled at days 7, 14 and 21 after the last dose. 1x10^5 AM\(\phi\) were seeded per well of a 48-well tissue culture plate for 90 minutes, and enriched AM\(\phi\) were then further stimulated with media or 10 ng/ml P2C for 24 hours for protein level analysis. CCL2 protein levels was determined by MSD of culture supernatant. Each sample was run as duplicates for the determination of protein levels by MSD, and the average was taken to calculate the mean of sample repeats. Data is represented as mean ± SD; n=6 (saline day 7); n=7 (BLM day 7, saline days 14 and 21), n=8 (BLM days 14 and 21) from two separate studies. Two way ANOVA with Sidak post-test was carried out; *: p<0.05, **: p<0.01, ****: p<0.001. BD, Below Detection; BLM, Bleomycin; NS, Not significant. Standard curves for the MSD of CCL2 is found in appendix 10.
Figure 5.15 Analysis of CCL3 protein levels following TLR2 stimulation in AMφ from BLM-challenged mice compared to saline controls. 8-10 week old male C57BL/6 mice were challenged via an oropharyngeal route three times at 72 hour intervals with either saline, or 0.13 IU BLM, and were culled at days 7, 14 and 21 after the last dose. 1x10⁵ AMφ were seeded per well of a 48-well tissue culture plate for 90 minutes, and enriched AMφ were then further stimulated with media or 10 ng/ml P2C for 24 hours for protein level analysis. CCL3 protein levels was determined by MSD of culture supernatant. Each sample was run as duplicates for the determination of protein levels by MSD, and the average was taken to calculate the mean of sample repeats. Data is represented as mean ± SD; n=6 (saline day 7); n=7 (BLM day 7, saline days 14 and 21), n=8 (BLM days 14 and 21) from two separate studies. Two way ANOVA with Sidak post-test was carried out; **: p<0.01, ***: p<0.005. BD, Below Detection; BLM, Bleomycin; NS, Not significant. Standard curves for the MSD of CCL3 is found in appendix 10.
Figure 5.16 Analysis of CCL4 protein levels following TLR2 stimulation in AMφ from BLM-challenged mice compared to saline controls. 8-10 week old male C57BL/6 mice were challenged via an oropharyngeal route three times at 72 hour intervals with either saline, or 0.13 IU BLM, and were culled at days 7, 14 and 21 after the last dose. 1x10^5 AMφ were seeded per well of a 48-well tissue culture plate for 90 minutes, and enriched AMφ were then further stimulated with media or 10 ng/ml P2C for 24 hours for protein level analysis. CCL4 protein levels was determined by MSD of culture supernatant. Each sample was run as duplicates for the determination of protein levels by MSD, and the average was taken to calculate the mean of sample repeats. Data is represented as mean ± SD; n=6 (saline day 7); n=7 (BLM day 7, saline days 14 and 21), n=8 (BLM days 14 and 21) from two separate studies. Two way ANOVA with Sidak post-test was carried out; *: p<0.05, ***: p<0.005, ****: p<0.001. BD, Below Detection; BLM, Bleomycin; NS, Not significant. Standard curves for the MSD of CCL4 is found in appendix 10.
Figure 5.1 Analysis of CCL5 protein levels following TLR2 stimulation in AMφ from BLM-challenged mice compared to saline controls. 8-10 week old male C57BL/6 mice were challenged via an oropharyngeal route three times at 72 hour intervals with either saline, or 0.13 IU BLM, and were culled at days 7, 14 and 21 after the last dose. 1x10⁵ AMφ were seeded per well of a 48-well tissue culture plate for 90 minutes, and enriched AMφ were then further stimulated with media or 10 ng/ml P2C for 24 hours for protein level analysis. CCL5 protein levels was determined by MSD of culture supernatant. Each sample was run as duplicates for the determination of protein levels by MSD, and the average was taken to calculate the mean of sample repeats. Data is represented as mean ± SD; n=6 (saline day 7); n=7 (BLM day 7, saline days 14 and 21), n=8 (BLM days 14 and 21) from two separate studies. Two way ANOVA with Sidak post-test was carried out; **: p< 0.01, ****: p<0.001. BD, Below Detection; BLM, Bleomycin; NS, Not significant. Standard curves for the MSD of CCL5 is found in appendix 10.
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Figure 5.18 Analysis of CCL20 protein levels following TLR2 stimulation in AMφ from BLM-challenged mice compared to saline controls. 8-10 week old male C57BL/6 mice were challenged via an oropharyngeal route three times at 72 hour intervals with either saline, or 0.13 IU BLM, and were culled at days 7, 14 and 21 after the last dose. 1x10⁵ AMφ were seeded per well of a 48-well tissue culture plate for 90 minutes, and enriched AMφ were then further stimulated with media or 10 ng/ml P2C for 24 hours for protein level analysis. CCL20 protein levels was determined by MSD of culture supernatant. Each sample was run as duplicates for the determination of protein levels by MSD, and the average was taken to calculate the mean of sample repeats. Data is represented as mean ± SD; n=6 (saline day 7); n=7 (BLM day 7, saline days 14 and 21), n=8 (BLM days 14 and 21) from two separate studies. Two way ANOVA with Sidak post-test was carried out; *: p<0.05. BD, Below Detection; BLM, Bleomycin; NS, Not significant. Standard curves for the MSD of CCL20 is found in appendix 10.
TLR9 stimulation with CpG-ODN (A) induced only a minimal change in pro-inflammatory mediators and also fibronectin levels in the saline controls, but this was increased in BLM-challenged animals (Table 5.2). It was observed that IL-1β levels were significantly increased from 2.14 pg/ml to 17.7 pg/ml at day 7 (8.28-fold; p<0.005), and from 1.76 pg/ml to 14.8 pg/ml at day 14 (8.42-fold; p<0.001) (Figure 5.19) in CpG-ODN (A) stimulated AMφ from saline controls as compared to BLM-challenged mice. There was also a trend suggesting an escalation in IL-1β from 1.24 pg/ml to 104 pg/ml at day 21 (83.8-fold; p>0.05). TNF-α levels were also significantly elevated from 111 pg/ml to 421 pg/ml at day 7 (3.80-fold; p<0.05) (Figure 5.20). In the fibrotic phases, a trend suggesting a small rise in TNF-α protein was revealed, namely an increase from 187 pg/ml to 594 pg/ml at day 14 (3.18-fold; p>0.05) and 167 pg/ml to 1,170 pg/ml at day 21 (6.99-fold; p>0.05) (Figure 5.20). Similarly, there was a significant amplification of CXCL1 protein levels from 322 pg/ml to 2,670 pg/ml at day 7 (8.29-fold; p<0.001), 545 pg/ml to 3,180 pg/ml at day 14 (5.84-fold; p<0.001) and 288 pg/ml to 3,960 pg/ml at day 21 (13.8-fold; p<0.001) (Figure 5.21). Moreover, IL-10 was detected rising significantly from 6.68 pg/ml to 112 pg/ml at day 7 (16.8-fold; p<0.001), 7.66 pg/ml to 179 pg/ml at day 14 (23.3-fold; p<0.05) and 6.61 pg/ml to 90 pg/ml (13.7-fold; p<0.05) (Figure 5.22). The increased response to CpG stimulation in AMφ was concurrent with a significant elevation in TLR9 mRNA levels observed at day 7, and also is consistent with the trend suggesting increase in TLR9 mRNA levels in the fibrotic phases at days 14 and 21 (Figure 5.2). There was no such increase in protein levels of TLR9 in the fibrotic phases (days 14 and 21) as observed by flow cytometry (Figure 5.3). This may result from the fact that TLR9 is weakly expressed in AMφ, and the increased levels of TLR9 in the fibrotic phases may be too small to be accurately detected by flow cytometry.

There was also a significant increase in IL-12 p40 (total) production between P2C-stimulated AMφ from saline or BLM-challenged mice, rising from 477 pg/ml to 4870 pg/ml at day 7 (10-fold; p<0.001); 200 pg/ml to 5,780 pg/ml at day 14 (29-fold; p<0.05) and 764 pg/ml to 8,080 pg/ml at day 21 (11-fold; p<0.05); however, this increase may be in part be due to BLM-challenge of mice rather than TLR9 stimulation per se (Figure 5.23).

CpG-ODN (A) stimulation led to a decrease of fibronectin protein levels in AMφ isolated from BLM-challenged mice. However, this reduction was also observed for AMφ challenged with the non-CpG control (Figure 5.24). This suggests that oligonucleotides in general, rather than CpG-ODN (A), suppressed fibronectin protein level in a non-TLR9 specific manner. Although guanine-phosphorothiate-cytosine (GpC)-ODN, the negative control used for these studies, has been demonstrated to be non-stimulatory ligands of TLR923, little is known about the other properties of this ligand. However, it may be possible that GpC-ODN may bind to other PRRs that recognize nucleic acids to trigger an immune response. For example, GpC-ODN was reported to have...
immunosuppressive activities on plasmacytoid DCs (pDC) in a TLR7 manner. GpC-ODN may act on AMφ via such nucleic acid binding PRRs to suppress fibronectin protein level.

Collectively these results demonstrate that the increased surface protein level of TLR 2 and elevated mRNA level of 9 in AMφ from BLM-challenged mice compared to saline controls, is correlated with an increased pro-inflammatory response to their respective ligands. This suggests that the elevated levels of TLRs in AMφ of BLM-challenged mice are functional and may result in an increased AMφ response to endogenous alarmins, such as HMGB1, that are also ligands to TLR2 or TLRs 2 and 9 respectively. Alternatively stimulation of TLRs does not appear to modulate the levels of the ECM component fibronectin.
Table 5.3 Pro-inflammatory mediator secretion levels and fold change between AMφ from BLM-challenged mice or saline controls at various time points following TLR9 stimulation. 8-10 week old C57BL/6 male mice were challenged via an oropharyngeal route three times at 72 hour intervals with either saline, or 0.13 IU BLM, and were culled at days 7, 14 and 21 after the last dose. 1x10^5 AMφ were seeded per well of a 48-well tissue culture plate for 90 minutes, and enriched AMφ were then further stimulated with media or 10 ng/ml CpG-ODN (A) or 10 ng/ml CpG-ODN (A) control (Ctrl) for 24 hours for protein level analysis. Each sample was run as duplicates for the determination of protein levels by MSD, and the average was taken to calculate the mean of sample repeats. Two way ANOVA with Sidak post-test was carried out. Data are represented as mean; n=3 from three separate studies.
Figure 5.19 Analysis of IL-1β protein levels following TLR2 stimulation in AMφ from BLM-challenged mice compared to saline controls. 8-10 week old male C57BL/6 mice were challenged via an oropharyngeal route three times at 72 hour intervals with either saline, or 0.13 IU BLM, and were culled at days 7, 14 and 21 after the last dose. 1x10^5 AMφ were seeded per well of a 48-well tissue culture plate for 90 minutes, and enriched AMφ were then further stimulated with media or 10 ng/ml P2C for 24 hours for protein level analysis. IL1β protein levels was determined by MSD of culture supernatant. Each sample was run as duplicates for the determination of protein levels by MSD, and the average was taken to calculate the mean of sample repeats. Data is represented as mean ± SD; n=6 (saline day 7); n=7 (BLM day 7, saline days 14 and 21), n=8 (BLM days 14 and 21) from two separate studies. Two way ANOVA with Sidak post-test was carried out; **: p<0.01, ****: p<0.001. BD, Below Detection; BLM, Bleomycin; NS, Not significant. Standard curves for the MSD of IL-1β is found in appendix 9.
Figure 5.20 Analysis of TNF-α protein levels following TLR2 stimulation in AMφ from BLM-challenged mice compared to saline controls. 8-10 week old male C57BL/6 mice were challenged via an oropharyngeal route three times at 72 hour intervals with either saline, or 0.13 IU BLM, and were culled at days 7, 14 and 21 after the last dose. 1x10^5 AMφ were seeded per well of a 48-well tissue culture plate for 90 minutes, and enriched AMφ were then further stimulated with media or 10 ng/ml P2C for 24 hours for protein level analysis. TNF-α protein levels was determined by MSD of culture supernatant. Each sample was run as duplicates for the determination of protein levels by MSD, and the average was taken to calculate the mean of sample repeats. Data is represented as mean ± SD; n=6 (saline day 7); n=7 (BLM day 7, saline days 14 and 21), n=8 (BLM days 14 and 21) from two separate studies. Two way ANOVA with Sidak post-test was carried out; *: p<0.05, ***: p<0.005. BD, Below Detection; BLM, Bleomycin; NS, Not significant. Standard curves for the MSD of TNF-αifound in appendix 10.
Figure 5.2 Analysis of CXCL1 protein levels following TLR2 stimulation in AMφ from BLM-challenged mice compared to saline controls. 8-10 week old male C57BL/6 mice were challenged via an oropharyngeal route three times at 72 hour intervals with either saline, or 0.13 IU BLM, and were culled at days 7, 14 and 21 after the last dose. 1x10^5 AMφ were seeded per well of a 48-well tissue culture plate for 90 minutes, and enriched AMφ were then further stimulated with media or 10 ng/ml P2C for 24 hours for protein level analysis. CXCL1 protein levels was determined by MSD of culture supernatant. Each sample was run as duplicates for the determination of protein levels by MSD, and the average was taken to calculate the mean of sample repeats. Data is represented as mean ± SD; n=6 (saline day 7); n=7 (BLM day 7, saline days 14 and 21), n=8 (BLM days 14 and 21) from two separate studies. Two way ANOVA with Sidak post-test was carried out; ****: p<0.001. BD, Below Detection; BLM, Bleomycin; NS, Not significant. Standard curves for the MSD of CXCL1 is found in appendix 9.
Figure 5.2 Analysis of IL-10 protein levels following TLR2 stimulation in AMφ from BLM-challenged mice compared to saline controls. 8-10 week old male C57BL/6 mice were challenged via an oropharyngeal route three times at 72 hour intervals with either saline, or 0.13 IU BLM, and were culled at days 7, 14 and 21 after the last dose. 1x10⁵ AMφ were seeded per well of a 48-well tissue culture plate for 90 minutes, and enriched AMφ were then further stimulated with media or 10 ng/ml P2C for 24 hours for protein level analysis. IL-10 protein levels was determined by MSD of culture supernatant. Each sample was run as duplicates for the determination of protein levels by MSD, and the average was taken to calculate the mean of sample repeats. Data is represented as mean ± SD; n=6 (saline day 7); n=7 (BLM day 7, saline days 14 and 21), n=8 (BLM days 14 and 21) from two separate studies. Two way ANOVA with Sidak post-test was carried out; *: p<0.05, **: p<0.01, ****: p<0.001. BD, Below Detection; BLM, Bleomycin; NS, Not significant. Standard curves for the MSD of IL-10 is found in appendix 9.
Figure 5.23 Analysis of IL-12 p40 (total) protein levels following TLR2 stimulation in AMφ from BLM-challenged mice compared to saline controls. 8-10 week old male C57BL/6 mice were challenged via an oropharyngeal route three times at 72 hour intervals with either saline, or 0.13 IU BLM, and were culled at days 7, 14 and 21 after the last dose. 1x10⁶ AMφ were seeded per well of a 48-well tissue culture plate for 90 minutes, and enriched AMφ were then further stimulated with media or 10 ng/ml P2C for 24 hours for protein level analysis. IL-12 p40 (total) protein levels was determined by MSD of culture supernatant. Each sample was run as duplicates for the determination of protein levels by MSD, and the average was taken to calculate the mean of sample repeats. Data is represented as mean ± SD; n=6 (saline day 7); n=7 (BLM day 7, saline days 14 and 21), n=8 (BLM days 14 and 21) from two separate studies. Two way ANOVA with Sidak post-test was carried out; ***: p<0.005, ****: p<0.001. BD, Below Detection; BLM, Bleomycin; NS, Not significant. IL-12 p40 (total) incudes free IL-12 p40, IL-12 p40 homodimers and IL-12 p40 heterodimers (IL-12 p70 and IL-23). Standard curves for the MSD of IL-12 p40 (total) is found in appendix 9.
Figure 5.24 Analysis of fibronectin protein levels following TLR2 stimulation in AMφ from BLM-challenged mice compared to saline controls. 8-10 week old male C57BL/6 mice were challenged via an oropharyngeal route three times at 72 hour intervals with either saline, or 0.13 IU BLM, and were culled at days 7, 14 and 21 after the last dose. 1x10^5 AMφ were seeded per well of a 48-well tissue culture plate for 90 minutes, and enriched AMφ were then further stimulated with media or 10 ng/ml P2C for 24 hours for protein level analysis. Fibronectin protein levels was determined by ELISA of culture supernatant. Each sample was run as duplicates for the determination of protein levels by ELISA, and the average was taken to calculate the mean of sample repeats. Data is represented as mean ± SD; n=6 (saline day 7); n=7 (BLM day 7, saline days 14 and 21), n=8 (BLM days 14 and 21) from two separate studies. Two way ANOVA with Sidak post-test was carried out; **: p<0.01, ***: p<0.005, ****: p<0.001. BD, Below Detection; BLM, Bleomycin; NS, Not significant. Standard curves for the MSD of fibronectin is found in appendix 9.
5.5.3 HMGB1 levels in BALF from saline or BLM challenged mice

From the above studies, it was demonstrated that AMφ isolated from BLM challenged mice have increased surface protein and mRNA levels of TLRs 2 and 9 respectively, which corresponded to the enhanced pro-inflammatory responses they displayed to their respective ligands when stimulated \textit{ex vivo}. It was next investigated whether or not any naturally occurring ligands to these TLRs accumulated \textit{in vivo} in the BLM disease model. One such ligand is the alarmin HMGB1, a nucleoprotein that is passively released by necrotic cells\textsuperscript{647}, or actively released by activated macrophages\textsuperscript{648–652}. HMGB1 can stimulate TLRs 2, and 4\textsuperscript{607–610}, and also act as a molecular chaperone for TLR 2, 4, and 9 ligands, escorting them to their respective receptors to amplify the pro-inflammatory response\textsuperscript{611,653,820}. HMGB1 also appears to be important in pulmonary fibrosis: HMGB1 protein levels are elevated in macrophages and injured epithelial cells in IPF patients with acute exacerbations\textsuperscript{651}. An increase in HMGB1 levels was also detected in the BALF of mice challenged with a single BLM dose, and clearance of HMGB1 with therapeutic antibodies attenuated the pathophysiological responses\textsuperscript{654}.

In the following study, whether or not there was an accumulation of HMGB1 in BALF was assessed in mice challenged with the novel repeat BLM dosing regimen or saline controls. The results reveal that HMGB1 levels were elevated in BALF from BLM challenged mice as compared to saline controls, escalating from 116 ng/ml to 257 ng/ml at day 7 (2.22-fold; \(p<0.01\)), and from 112 ng/ml to 237 ng/ml at day 14 (2.11-fold; \(p<0.01\)). HMGB1 levels also appeared to be marginally increased, albeit at non-significant levels, from 122 ng/ml to 152 ng/ml (1.23; \(p>0.05\)) at day 21 (Figure 5.25). These results suggest that there are changes in the levels of a select alarmin in mice following the onset of fibrosis and reinforce the importance of PRR signalling in the pathophysiology of pulmonary fibrosis.

![Figure 5.25 Analysis of HMGB1 protein levels in BALF isolated from BLM-challenged mice compared to saline controls. 8-10 week old male C57BL/6 mice were challenged via an oropharyngeal route three times at 72 hour intervals with either saline, or 0.13 IU BLM, and were culled at days 7, 14 and 21 after the last dose. BALF was collected by 2 x 0.5 ml DPBS washes of the mouse lungs. Data is represented as mean ± SD; n=5 (saline days 7, 14 and 21); n=6 (BLM days 7, 14 and 21) from two separate studies. Each sample was run as duplicates for the determination of protein levels by ELISA, and the average was taken to calculate the mean of the sample repeats. Two way ANOVA with Sidak post-test was carried out; **: \(p<0.01\). BD, Below Detection; BLM, Bleomycin; NS, Not significant.](image)}
5.6 Discussion

The mRNA levels and flow cytometric analyses of various PRRs from AM\(\Phi\) at different stages of BLM-induced pulmonary fibrosis and their functional outcomes were determined in this chapter. The mRNA level analysis revealed that TLR9 gene transcripts were increased, whereas from flow cytometry it was noted that TLR2 surface protein expression was elevated in AM\(\Phi\) isolated from BLM challenged mice. An amplified response was observed when AM\(\Phi\) from BLM-challenged mice were stimulated with TLRs 2 or 9 ligands. There were also increased levels of the alarmin HMGB1, which is a ligand for TLRs 2, 4 and 9, in the BAL of BLM-challenged mice.

In the following section, experimental limitations and their implications to analyses will first be discussed in section 5.6.1. Experimental results will be discussed in sections 5.6.2-5.6.4, and this will be followed by future directions of the studies in section 5.7 and conclusions to the chapter at section 5.8.

5.6.1 Experimental limitations

There are several experimental limitations to these studies. Preliminary studies were carried out for the flow cytometry analyses of increased surface expression of TLRs 2 and 4, or enhanced intracellular section for TLRs 7, 9 or NLRP3. Further repeats will have to be carried out validate the observations made.

There were high MFI for isotype controls for intracellular staining in flow cytometry studies. It was later discovered that isotype controls were optimised for extracellular use, and may have different binding activities when exposed to an intracellular environment. It may therefore be more suitable to employ a negative control in the form of FMO, where all antibodies but the one to be controlled is present.

The majority of these studies BLM-challenged mice were compared to their respective saline controls, whereas data of constitutive expression from naïve mice was absent. Whereas it was assumed that saline challenge does not elicit any responses within the lung, mRNA and protein levels of AM\(\Phi\)-derived mediators of interest from naïve controls should still be evaluated to determine their basal expression.

As discussed before IL-12 p40 (total) measures different forms of IL-12 p40 and was found to be increased in TLR2 and TLR9 stimulated AM\(\Phi\) isolated from BLM-challenged mice, although the exact oligomer form it is increased in is unknown. As different IL-12p40 isoforms have different functions, it is important to determine the specific isoform that is increased to determine its function, and
further protein analyses of specific IL-12 isoforms will have to be carried out. In light of these experimental limitations, results are discussed in the sections below (section 5.6.2 - 5.6.4).

5.6.2 TLR2 surface protein and TLR9 gene transcript levels are increased in BLM-challenged mice

The mRNA and protein levels of various PRRs on AM\(\phi\), including TLRs 2, 4, 7 and 9, and NLRP3, were determined by qRT-PCR and flow cytometry respectively. This is the first time that the mRNA and protein levels of various PRRs is systematically assessed on AM\(\phi\) specifically and over a time course, rather than collectively over the whole lung at a single time point in BLM-challenged mice. This may thus allow the determination of the potential participation of various PRRs on AM\(\phi\) phenotype at different stages of pulmonary fibrosis. Results have indicated a role for TLRs 2 and 9 in the pathology of pulmonary fibrosis. There was increased TLR2 surface protein (Figures 5.3 and 5.4) but not in mRNA levels (Figure 5.1). As regards TLR9 mRNA levels (Figure 5.2) they were significantly elevated during the inflammatory phase (day 7), and had a non-significant trend suggesting elevation at the active (day 14) and established fibrotic phases (day 21) in BLM-challenged mice compared to saline controls, but flow cytometry indicated no change in intracellular expression was observed between the two groups. These results may represent errors in gating owing from the use of isotype control that are optimised for extracellular use (see section 5.6.1), although it is also likely that protein expression is not increased.

The increase in TLR2 surface protein levels but not mRNA levels suggest that it is regulated at the post-transcriptional and/or post-translational level, which may reflect its function in the lung microenvironment. TLR2 is constitutively highly expressed and participates in tissue homeostasis in addition to innate immunity to extracellular bacteria. For example, TLRs 2 and 4 have an anti-apoptotic role on AECs through the recognition of HMW HA\(^8\)\(^{16}\). This PRR may also recognize airway microbiota to influence the nature of the host immune response to ‘invader pathogens’ and thereby maintain pulmonary homeostasis\(^8\)\(^{31,832}\). For example, *Staphylococcus aureus* (*S. aureus*), the most common commensal bacteria found in the human upper respiratory tract\(^8\)\(^{33}\) negatively regulates influenza-mediated ALI in a TLR2-dependent manner\(^8\)\(^{34}\). Under normal conditions, these airway microbiota may also continuously enter the alveoli upon breathing\(^8\)\(^{34}\).

A potential post-translational regulatory mechanism is the transmigration of TLR2 from the endoplasmic reticulum (ER) where it is synthesized onto the cell surface. ER-intrabodies are artificially generated intracellular antibodies (intrabodies) consisting of an N-terminal ligand-binding domain that is fused to a C-terminal ER-retention domain. ER-intrabodies are therefore expressed

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and retained within the ER, and can act to inhibit protein surface expression and/or secretion. Adenoviral gene transfer allowed overexpression of anti-TLR2 ER-intrabodies (αT2ib) in human embryonic kidney (HEK) 293 cells, RAW264.7 (a murine monocyte cell line), and BMMφ, which abolished TLR2 surface expression and attenuated TLR2-dependent cell activation to Pam3CSK4 (a synthetic triacylated lipoprotein), thereby indicating the importance of TLR2 surface expression to the responsiveness to extracellular PAMPs. To my knowledge there have been no reports on naturally occurring regulation of TLR2 activity through alteration of its surface expression. However, the decreased surface expression of another TLR, TLR4 plays a significant role in dampening of the inflammatory response following LPS tolerance. Further studies will have to be carried out to determine the role of TLR2 surface expression in BLM-induced pulmonary fibrosis by blocking their transmigration to the cell surface, which can be achieved by the overexpression of αT2ib through adenoviral gene transfer in vivo systematically. The specific role of TLR2 surface expression on hematopoietic cells in pathology can also be determined through the assessment of changes in BLM-induced pulmonary fibrosis in adoptive transfer experiments of bone marrow overexpressing αT2ib into non-transfected mice, or vice versa, as compared with non-transfected and transfected controls.

The increase in TLR9 mRNA levels suggest that it is regulated at a transcriptional level, which may also reflect on its functionality. TLR9 is responsible for the recognition of CpG-ODN in intracellular viruses, but does not appear to display this homeostatic role. They may therefore be found to be weakly expressed on AMφ at basal levels in mice and humans and only induced during viral infection. Preliminary intracellular flow cytometry study of TLR9 revealed a small increase at day 7 in BLM-challenged mice that returned to basal levels at days 14 and 21. The lack of intracellular TLR9 protein expression may be due to the use of isotype controls optimised for extracellular studies as negative controls, although it may be possible that TLR9 protein expression is unchanged at days 14 and 21 owing to post-transcriptional inhibition. Further studies of TLR9 using FMO as negative control will have to be carried out to evaluate its protein expression in BLM-challenged mice versus saline controls.

5.6.3 AMφ from BLM-challenged mice have increased response to TLR2 and TLR9 ligands
Stimulation of AMφ from naïve mice with P2C and CpG-ODN (A), the ligands of TLR2 and TLR9 respectively, have revealed different protein expression profiles. TLR9 stimulation induced a strong increase in the levels of the pro-inflammatory cytokine IL-12 p40 (total), whereas TLR2 stimulation resulted in a moderate increase in both pro-inflammatory (IL-1β, TNF-α, and CXCL1) and anti-inflammatory (IL-10) mediator levels (Figure 5.6). These results indicate that TLRs not only recognize
specific ligands, but are also capable of generating distinct expression profiles that may bring about a certain degree of specificity to the pulmonary innate immune system. This is similar to previous observations made in pDCs\textsuperscript{839–841}. Stimulation of AMφ from BLM-challenged mice with P2C or CpG-ODN (A) revealed an amplified response to TLR2 and TLR9 respectively, with an increased production of pro- and anti-inflammatory mediators in both cases, but had no effect on fibronectin levels (Tables 5.1 and 5.2; Figures 5.7 to 5.24). However, TLR2-stimulated AMφ displayed a more robust increase in TNF-α (Figure 5.8) and CXCL1 (Figure 5.9) protein levels, whereas TLR9-stimulated AMφ had a stronger increase in IL-12 p40 (total) levels (Figure 5.23). This suggests that stimulation of AMφ via different TLRs may contribute to pulmonary fibrosis slightly differently. TNF-α facilitates IL-1β mediated increase in pro-inflammatory (e.g. CXCL1, CXCL2) and pro-fibrotic mediators (e.g. TGF-β, PDGF) that may contribute to tissue injury and fibrosis respectively\textsuperscript{376,377}. The chemokine CXCL1 is the mouse analogue of human CXCL8, and may play a role in angiogenesis\textsuperscript{842}. As highlighted before IL-12 p40 (total) measures monomeric and homodimeric IL-12 p40, and also heterodimeric IL-12 p70 (p40 + p35) and IL-23 (p40 + p19) and each of these may play a role in pulmonary fibrosis. IL-12 p40 is chemotactic for macrophages \textit{in vitro} and \textit{in vivo}\textsuperscript{383,384}, and was proposed to promote silica-induced pulmonary fibrosis through the macrophage recruitment\textsuperscript{124}. IL-12 p40 facilitates IL-17A mediated BLM- and IL-1β-induced pulmonary fibrosis\textsuperscript{125}. IL-23 may promote the differentiation of IL-17 producing T\textsubscript{H}17 cells\textsuperscript{386–388}, and IL-17A can mediate BLM\textsuperscript{125,385} or IL-1β induced pulmonary fibrosis\textsuperscript{125}. On the other hand, IL-12 p70 protects from BLM-induced pulmonary fibrosis\textsuperscript{382}, potentially through the induction of IFN-γ\textsuperscript{382}, thereby shifting the cytokine balance away from a pro-fibrotic T\textsubscript{H}2 response. It is likely that TLR stimulation promotes the increased expression of IL-12 p40 in the form of the bioactive IL-12 p70\textsuperscript{839}. It has been reported that TLR2 promotes, whereas TLR9 protects from BLM-induced pulmonary fibrosis in mice, which may be attributed to the different levels of IL-12 p70 generated in differentially stimulated AMφ. Further protein expression analyses of these different forms of IL-12 oligomers will have to be carried out to confirm this hypothesis.

The stimulation of AMφ from BLM-challenged mice with P2C also increased the expression of other mediators with reported roles in IPF, namely IL-6, CCL2, CCL4, and CCL5 (Figure 5.13 to 5.17), which reinforces the potential role for TLR2 in pulmonary fibrosis. Further experiments will have to be carried out to assess the expression of these proteins following TLR9 stimulation. Together these studies suggest multiple potential roles for AMφ activation by TLR stimulation in pulmonary fibrosis. These suggestions have to be verified by various studies, including chemoattraction, angiogenesis and/or fibroblast activation studies, of hematopoietic cells, endothelial cells and/or fibroblasts respectively, which are co-cultured with supernatant from AMφ isolated from BLM-challenged mice and stimulated by either TLR2 or TLR9 ligands \textit{ex vivo}.
5.6.4 The alarmin HMGB1 is increased in BALF of BLM-challenged mice

There were increased levels of HMGB1 detected in BALF of BLM-challenged mice compared to saline controls (Figure 5.25). HMGB1 is an alarmin\(^647\) that plays an important role in pulmonary fibrosis in IPF patients\(^651\) and BLM-challenged mice\(^654\). HMGB1 may promote an inflammatory response directly through binding to TLRs 2 or 4\(^607–610\), or indirectly by chaperoning ligands of TLRs 2, 4 and 9 to their respective receptors\(^611,653,820\). Further immunofluorescence studies of lung histological sections and also coimmunoprecipitation studies from BLM-challenged mice can be carried out to evaluate binding of HMGB1 to various TLRs in pulmonary fibrosis. Other alarmins, such as LMW HA fragments, extracellular histones and HSP70 may also be present in BLM-induced lung injury and fibrosis. The contributions of these alarmins to pulmonary fibrosis can be evaluated by measuring their levels in BALF and lung from BLM-challenged mice. Alarmin-specific antibodies can then be administered into the lung at various stages of pathology to remove these alarmins, and the changes to collagen accumulation and fibrosis assessed.

Given that endogenous alarmins are present in BALF at elevated levels, and there was also an increase in TLRs 2 and 9 expressions that correlated with amplified responses to their corresponding ligands in AM\(\Phi\) \textit{ex vivo}, it was perhaps therefore surprising that there was little intrinsic increase of pro-inflammatory mediators observed in AM\(\Phi\) from BLM-challenged mice. There may be several non-exclusive reasons for this outcome. First, immunosuppressive mediators, including IL-10\(^126,587,843\) and TGF-\(\beta\) are prevalent within the fibrotic lung and may dampen AM\(\Phi\) response to TLR stimulation. Second, there may be tolerance of TLRs\(^347\) to persistent alarmins \textit{in vivo}. However, these reasons are unlikely as the results above have revealed that TLRs, including TLRs 2 and 9 at least, commence a functional response to commercially available ligands. Another reason may be that alarmins found \textit{in vivo} may have a weaker pro-inflammatory capacity compared to commercially available TLR ligands \textit{in vitro}. To better evaluate tissue injury induced AM\(\Phi\) activation, AM\(\Phi\) from naive mice can be stimulated by various alarmins \textit{ex vivo} and their expression profile of various pro-inflammatory and pro-fibrotic mediators can be characterised. The responsiveness of AM\(\Phi\) from BLM-challenged mice to these alarmins can then be assessed \textit{ex vivo}. It may also be possible that alarmins act on AM\(\Phi\) in a paracrine manner, such that only AM\(\Phi\) in the vicinity of tissue injury is affected. In these studies, AM\(\Phi\) were isolated from across the whole lung from BLM-challenged mice, which was spatially heterogeneous and included both fibrotic and normal regions. It is likely that the protein expression of activated disease-state AM\(\Phi\) in fibrotic areas is masked by those from non-fibrotic areas. The distribution of alarmins in binding to various cells can be evaluated by immunohistochemistry. Protein expression of disease-state AM\(\Phi\) can be more specifically characterised by isolating them specifically by LCM from fibrotic areas.
5.7 Future directions

Although it was observed that TLR2 surface expression and TLR9 gene transcript expression were increased in AMφ of BLM-challenged mice, which corresponded to an increased response to their respective ligands, whether or not these responses translated into a pro- or anti-fibrotic response is unclear. To further evaluate the specific role of TLRs of AMφ in pulmonary fibrosis, the effects on BLM-induced pulmonary fibrosis, following adoptive transfer of AMφ from TLR2−/− or TLR9−/− mice into WT C57BL/6 mice that are depleted of AMφ (by liposomal chondrate), can be evaluated with respect to cellular infiltration, collagen accumulation and pro-fibrotic mediator levels. It is also possible to inhibit TLR2 and/or TLR9 of AMφ in BLM-challenged mice or saline controls in vivo through the application of pharmacological inhibitors and/or neutralising antibodies. The role of TLR2 and/or TLR9 can be determined through the comparison of the phenotype of AMφ isolated from TLR inhibited mice versus uninhibited controls. The effect of various alarmins that are found in the fibrotic lung, such as LMW HA fragments and HMGB1, on AMφ phenotype with respect to their protein expression of pro-inflammatory and pro-fibrotic mediators, and also their subsequent effects on fibroblast collagen expression, monocyte chemotaxis and or EMT can be assessed in co-culture studies.

5.8 Conclusion

Collectively these studies have for the first time identified TLRs 2 and 9 as the potential contributing PRRs on AMφ to BLM-induced pulmonary fibrosis, and demonstrated a corresponding increase in functional activity of AMφ in response to the ligands. These TLRs may contribute to pathology through the binding of endogenous alarmins, such as HMGB1, and release of a number of pro-inflammatory mediators. The protective role of TLR9 stimulation in BLM-induced pulmonary fibrosis may be in part brought about by the T\textsubscript{h}1 -inducing cytokine IL-12 p70 that suppresses the pro-fibrotic T\textsubscript{h}2 response.
Chapter 6

General Discussion
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In the following chapter, I will first review the key findings regarding the heterogeneity of AMφ in vitro and in vivo discovered in this thesis in section 6.1, the results of which will be summarised into a model of AMφ activation in section 6.2. I will then discuss the various experimental limitations to these studies and follow-up studies in section 6.3. Future work to further advance the current findings will be discussed in section 6.4, and the thesis will be concluded in section 6.5.

6.1 Significance of finding and key results

AMφ have a known role in IPF through the release of various pro-inflammatory and pro-fibrotic mediators. Differential expression of these mediators may be accomplished by different macrophage subsets, which have been predetermined by selecting the in vitro culture conditions for BMMφ and MDM. Recent studies in IPF patients and rodent models of pulmonary fibrosis have reported the accumulation of M2-associated markers in BAL and lung, and M2 macrophages were therefore suggested to play a role in pathology. However, several caveats have put these findings into question.

The translatability of macrophage subset-associated markers from artificially generated BMMφ in vitro into terminally differentiated AMφ in vivo is unclear. Whilst BMMφ may provide a useful model for the study of macrophage behaviour, they are artificially generated through the culture of bone marrow cells with M-CSF in mice, or GM-CSF in humans. These may not truly represent terminally differentiated AMφ that reside within the alveolar space, which are constantly exposed to a variety of homeostatic signals, exogenous pathogens, and also endogenous surfactants and growth factors within the lung. AMφ may therefore have different phenotypes to BMMφ such that markers identified within the two populations are not directly interchangeable. For example, CD11b is a well characterised macrophage marker that is identified in BMMφ and found in most tissue macrophages, but this is absent in AMφ and CD11c, a common dendritic cell marker, is found instead. The use of BMMφ subset markers for AMφ has not been limited to studies of pulmonary fibrosis, but also asthma, COPD, and lung cancer. There is thus a need to validate macrophage subset markers identified from BMMφ in AMφ.

Previous studies of AMφ heterogeneity in pulmonary fibrosis have assessed either M1- or M2-associated markers. However, in an in vivo environment a multitude of inducing signals, including cytokines, growth factors, microbes, hypoxia and alarmins are present. Different signals may have accumulative effects on influencing AMφ phenotypes. For example, in vitro studies have revealed that IFN-Υ may amplify the pro-inflammatory phenotype induced by LPS stimulation. IL-4 priming overnight prior to the addition of LPS amplifies the inflammatory response, whereas simultaneous
addition of IL-4 with LPS suppresses it\textsuperscript{844}. IL-10 is also reported to amplify IL-4 induced M2 phenotype\textsuperscript{845}. Therefore in an \textit{in vivo} environment, AM\(\phi\) are unlikely to fall neatly into the category of a particular M1 or M2 subset, but rather display hybrid phenotypes. A simultaneous assessment of M1 and M2-associated markers on AM\(\phi\) is therefore required to better characterise its phenotype. Another caveat of previous studies is that the phenotypes of AM\(\phi\) were in general only characterised at a single time point in pulmonary fibrosis. As pulmonary fibrosis is a progressive disease that displays temporal heterogeneity, the changes of AM\(\phi\) phenotype at different stages of pathology may be neglected. In pulmonary fibrosis, M1 macrophages were hypothesised to participate in initial inflammation induced tissue injury, whereas M2 and M2-like macrophages were involved in dysregulated wound healing and fibrosis. An analysis of AM\(\phi\) phenotype over a time course is therefore required to assess the kinetics of AM\(\phi\) contribution to the different stages of pathology.

In addition to these potential limitations in the analysis of AM\(\phi\) phenotypes in pulmonary fibrosis, the contribution of PRRs in selecting/modifying AM\(\phi\) phenotypes in the pathology has not been addressed. Several TLRs, including TLRs 2, 3, 7, 8 and 9 are increased in the BAL cells of IPF patients. Rodent models of pulmonary fibrosis have also identified pathogenic roles for TLR2 and the NLRP3 inflammasome, whereas TLR9 in contrary to its human orthologue was reported to be protective in mice. These PRRs may promote a pro-inflammatory response and alter AM\(\phi\) phenotypes that are induced by cytokine priming. As extensive tissue injury occurs in pulmonary fibrosis, a large amount of alarmins are likely to be present and capable of modifying AM\(\phi\) phenotypes through ligation to their respective PRRs. The expression and functional activity of various PRRs on AM\(\phi\) specifically is therefore of interest to fully appreciate the activation of these cells.

HPS is a collection of autosomal recessive genetic disorders that is characterized by abnormal biogenesis and/or function of LROs found in specialised secretory cells, including melanosomes, platelets, epithelial cells and macrophages\textsuperscript{690–692}. HPS1 is a genetic disease prevalent in Northwest Puerto Ricans, and is commonly associated with interstitial pulmonary fibrosis, termed HPSPF, that resembles IPF in histopathology and progression. Foamy macrophages are often found in HPS1 patients with or without HPSPF, although their role in pathology is unclear. HPS1 mice provide in part a disease model of HPS1 patients, although unlike their human counterparts these do not spontaneously develop pulmonary fibrosis. Nevertheless HPS1 mice were reported to have increased susceptibility to BLM-induced pulmonary fibrosis at the late inflammatory phase (day 7)\textsuperscript{696}. The effect of BLM-induced pulmonary fibrosis in HPS1 mice has however not been assessed at later time points in this study where fibrosis is more prominent\textsuperscript{696}. It was therefore interesting to evaluate
the severity of BLM-induced pulmonary fibrosis at the active (day 14) and established (day 21) fibrotic phases.

The first significant finding was that terminally-differentiated AMφ displayed plasticity in vitro, and are able to respond to priming signals, including IFN-γ and IL-13. IFN-γ induced a ‘M1-like’ phenotype that is pro-inflammatory, whereas IL-13 promoted a ‘M2-like’ phenotype that is pro-wound healing. Preliminary experiments also revealed that AMφ promoted a wound-healing phenotype in response to TGF-β and hypoxia. LPS stimulation either augmented or suppressed priming induced AMφ phenotypes. These results revealed for the first time the plasticity of terminally differentiated AMφ in vitro, and validated the use of several subset-associated markers for future studies of AMφ heterogeneity in vivo.

The second significant finding was that different AMφ subsets accumulated at various stages of BLM-induced pulmonary fibrosis. M1-like AMφ were found at the late inflammatory phase (day 7), whereas a M1/ M2-like hybrids were present from day 7 to the established fibrotic phase (day 21). These results demonstrate here for the first time that AMφ are able to alter their phenotype during the progression in pulmonary fibrosis and potentially contribute to different aspects of disease. It is shown here as well that AMφ do not necessarily fall into a M1-like or M2-like phenotype ex vivo, but rather have a mixed phenotype that is in between these two polarization extremities.

The third significant finding is that TLRs 2 and 9 were elevated, at the surface protein and gene transcript level respectively, in AMφ in BLM-induced pulmonary fibrosis, and this corresponded with an increased response to their ligands, P2C and CpG-ODN (A) respectively. The alarmin HMGB 1, which exerts its effects through the ligation of TLRs 2 and 4, or acting as a molecular chaperone to ligands of TLRs 2, 4 and 9 to their respective receptors, was also elevated in the BAL of BLM-challenged mice. These results implicate a role for TLRs 2 and 9 in influencing AMφ phenotype in BLM-induced pulmonary fibrosis.

The fourth major finding is that HPS1 mice appear to have reduced tolerance to BLM challenge between days 8 to 11, but this does not appear to be correlated with more severe pulmonary fibrosis. These results suggest that the susceptibility of HPS1 mice to BLM-induced pulmonary fibrosis requires reviewing, and broaden future studies to explore other mechanisms that may contribute to reduced tolerance to this chemotherapeutic agent.

6.2 Model of AMφ activation in pulmonary fibrosis

From the above studies, it was revealed that AMφ are able to respond to priming and/or stimuli to alter their mRNA and protein levels. Under normal conditions AMφ are constantly stimulated by
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exogenous pathogens, but homeostasis is maintained by endogenous anti-inflammatory mediators (e.g. TGF-β) secreted by AECs.

During initial tissue injury, inflammatory cells are recruited via pro-inflammatory chemokines released from tissue resident AEC and AMφ that are activated by alarmins via their TLRs in an attempt to clear cellular debris and the source of irritation. Fibroblasts and fibrocytes should also be recruited during acute inflammation to aid in ECM deposition and wound closure.

In the case of IPF, tissue injury is thought to be prolonged, similar to that observed in silicosis/asbestosis. Under these circumstances acute inflammation may become chronic in an IL-6 dependent manner, which promotes the accumulation of long-lived macrophages and lymphocytes in place of short-lived granulocytes. This is particularly important to protect from further tissue damage as neutrophils produce large quantities of ROS and RNS that may lead to further tissue damage. The lung environment may switch from predominantly pro-inflammatory into an exaggerated wound-healing response, in a frustrated attempt to repair the injured tissue. There are several potential causes for such a switch in environment, perhaps due to anti-inflammatory mediators (e.g. TGF-B, IL-10) that are released as a negative feedback to the pro-inflammatory response, dampening of the pro-inflammatory response through prolonged TLR activation and also anti-inflammatory mediator engagement, and in the case of APCs, efferocytosis of apoptotic cells.

The gene and protein expression of pulmonary macrophages may adapt to the aforementioned environmental cues to generate different subsets that are specialised in different functions. In acute inflammation, AMφ are more likely than not to be activated via PRR stimulation by alarmins released during tissue injury, and inflammation is further amplified through AMφ activation by pro-inflammatory mediators. Indeed in Chapter 4, it was observed that AMφ isolated from day 7 of BLM-induced pulmonary fibrosis had a M1-like phenotype. In normal wound healing however, AMφ may adopt a wound-healing M2-like phenotype following priming by anti-inflammatory mediators (e.g. TGF-β). On the other hand in chronic inflammation/ fibrosis, a M1/M2-like hybrid may result from simultaneous PRR stimulation by persistent tissue injury and the presence of excessive pro-wound healing/ pro-fibrotic mediators. These M1/M2 hybrid macrophages may further contribute to the non-resolving cycle of chronic inflammation and fibrosis through the simultaneous release of pro-inflammatory and pro-fibrotic mediators.

6.3 Experimental limitations

As aforementioned in the previous chapters, there are several limitations with respect to experimental repeats, sample numbers, statistical analyses, and controls. Due to the wide preliminary scope of PhD project, there are several studies in this thesis, including *in vitro* mRNA and/or protein level analysis studies of IFN-Υ or IL-13-primed BMMφ and TGF-β and hypoxia-primed
AMφ in Chapter 3, and also evaluation of levels of TLR4, TLR7, TLR9 and NLRP3 on AMφ in Chapter 5. There are also insufficient sample numbers for the evaluation of naïve, saline or BLM-challenged HPS1 mice in Chapter 4, the experiments of which have to be repeated to confirm the results observed.

Due to the lack of sample numbers, normality testing was based on visual inspection of data rather than statistical testing [e.g. Kolmogorov-Smirnov test (n=5), Shapiro-Wilk test (n=7), and D’Agostino test (n=8)]. This is a crude way of the assessment of normality and further repeats will have to be carried out to confirm parametric/ non-parametric distribution to determine the most appropriate statistical testing to be used.

For the limitations to experimental controls, there is a lack of baseline controls from naïve mice in the studies of AMφ heterogeneity in vivo in BLM-induced WT C57BL/6 and HPS1 mice (Chapter 4), and also the studies of the role of PRR expressed on AMφ in BLM-induced pulmonary fibrosis (Chapter 5). It was assumed in the previous experiments that saline was inert and therefore saline controls would behave in a similar fashion as naïve mice. However, this assumption needs to be reaffirmed to determine constitutive expression of various proteins of interest by AMφ in unchallenged mice.

For flow cytometry studies, there was also a lack of fluorescence minus one (FMO) controls, which are cells stained with all antibodies against the proteins of interest but the one that is being controlled. Instead isotype controls was used for gating for positively stained cells. As aforementioned, isotype controls may serve as good negative controls against non-specific antibody binding, but ultimately they have different specificity to the actual protein of interest-specific antibodies under use, and therefore are not the best candidates for negative control against cells unstained for that particular protein of interest. While spectral shift was very pronounced for MHC-II in IFN-Υ-primed AMφ in chapter 3, and also TLR2 for AMφ from BLM-challenged mice in chapter 5, flow cytometry results in chapters 3, 4 and 5 will therefore have to be repeated using FMO for a more accurate measurement of the changes in the respective protein levels.

For histology staining, results were analysed qualitatively but no quantification assessment was carried out. While drastic changes with respect to cellular infiltration and collagen accumulation was observed for H&E and PSR staining respectively between saline and BLM-challenged WT C57BL/6 mice, further assessment may be required to detect subtle differences in these parameters between BLM-challenged WT C57BL/6 and HPS1 mice (Chapter 4).
For the assessment of the protein levels of IL-12 family members, IL-12 p40 (total) MSD that measures various IL-12 p40 containing isoforms was used. These include the pro-inflammatory IL-12 p70, IL-23, and also the anti-inflammatory IL-12 p40 monomer/dimers. Further measurements of the protein levels of these isoforms have to be carried out to determine the role of IL-12 p40 in pulmonary fibrosis.

For the study between HPS1 and WT C57BL/6 mice, HPS1 mice of mixed gender was used due to lack of mouse numbers. Further experiments using WT C57BL/6 and HPS1 mice of matching gender will have to be carried out to eliminate any potential inherent gender related differences.

Finally, the current experiments are limited to the late inflammatory phase (day 7), early fibrotic phase (day 14) and late fibrotic phase (day 21) in WT C57BL/6 mice. It may be of interest to evaluate later time points to investigate potential spontaneous resolution of fibrosis in BLM-challenged WT C57BL/6 mice, or perhaps assess earlier time points in BLM-challenged HPS1 mice to evaluate the reasons behind their reduced BLM tolerance during BLM-induced inflammation (Chapter 4).

6.4 Future work

6.4.1 Analysis of AMΦ transcriptome and proteome ex vivo

Current studies analysing the role of AMΦ in pulmonary fibrosis have focused on evaluating the expression of genes with known roles in pathology, using qRT-PCR in AMΦ. Whilst these studies have pushed forward our understanding in their contributions to pulmonary fibrosis, the process involved are low throughput. I therefore propose the use of high throughput sequencing in the analysis of AMΦ genotype using microarray and/or next generation sequencing (NGS) studies.

In a recent study, the transcriptome was evaluated in the lungs of BLM-challenged mice and compared to that in patients using two separate IPF gene expression datasets available in Gene Expression Omnibus (GEO): GSE2052\textsuperscript{846} and GSE10667\textsuperscript{847}. GSE2052 assayed lung tissue from IPF patients, and also non-IPF controls. GSE10667 sampled lung tissue from IPF patients with slow progression, acute exacerbations and also non-IPF patients. It was observed from these studies that gene sets from BLM-challenged mice that were upregulated in the active (day 14) and the established fibrotic phases (days 21-35), were also upregulated in IPF patients compared to non-IPF controls. Analysis of leading edge genes (genes that contribute most to the enrichment of BLM signatures in the clinical fibrosis expression profiles) show an upregulation of many cell cycle-related genes [cell division cycle proteins (CDC), cyclin-dependent kinases (CDKs)], fibrosis related pathways (PDGF-, integrin-, and syndecan-mediated signalling) gene sets, and other pro-fibrotic mediators (MMPs, collagen, TGF-β). It would be interesting to evaluate the specific contributions of AMΦ to
these processes in a large scale through microarray analysis and subsequent comparison to the 

6.4.2 Phenotype comparison of macrophages in pulmonary fibrosis with Tumour Associated 

Macrophages (TAM)

In the current literature macrophage heterogeneity in pulmonary fibrosis have been widely 

described as M1-like and M2-like. However, due to the wide range of signals within the lung, 
pulmonary macrophages are more likely than not to fall within the spectrum of these two opposing 
phenotypes, and the actual repertoire of macrophage phenotypes in vivo may be much broader than 
M1-like versus M2-like. It is therefore more appropriate to assign macrophage subsets based on 
their functional phenotype in vivo, rather than an artificially generated one predetermined in vitro. 
To this end, functional phenotypes identified in TAMs may serve as a useful guidance for such 
characterisation in AMφ in pulmonary fibrosis.

TAM stimulate tumour cell migration, invasion, intravastation, and also the angiogenic response 
required for tumour growth and metastasis. Various subpopulations of TAM with different 
functions have been identified and characterised into different subpopulations based on in vitro 
characterised or ex vivo determined functional subsets, although it is unclear whether or not these 
are distinct or overlapping entities (Table 6.1). Events such as tissue invasion and neoangiogenesis 
occur in pulmonary fibrosis as well as tumourigenesis. The information obtained from TAMs may 
therefore be useful to further define functional subsets of macrophages in these similar events at 
different stages of pulmonary fibrosis.

<table>
<thead>
<tr>
<th>Macrophage subpopulation</th>
<th>Markers</th>
</tr>
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<tbody>
<tr>
<td>M1-like macrophages (Tumoricidal)</td>
<td>IL-12⁺, MHCI⁺, TNF-α⁺, CD80/86⁺, ROS, RNS⁸⁵¹,⁸⁵²</td>
</tr>
<tr>
<td>M2-like macrophages (Tumorigenic)</td>
<td>Arginase⁺, EGF⁺, VEGF⁺, FGF⁺⁸⁵¹,⁸⁵²</td>
</tr>
<tr>
<td>Invasive macrophages</td>
<td>WNT⁺, EGF⁺, VEGF⁺, CSFR1⁺⁸⁵³–⁸⁵⁶</td>
</tr>
<tr>
<td>Angiogenic macrophages</td>
<td>VEGF⁺, Tie2⁺, TNF-α⁺, IL-1β⁺, CXCL8⁺, PDGF⁺, FGF⁺⁸⁴⁸,⁸⁵⁷,⁸⁵⁸</td>
</tr>
<tr>
<td>Metastasis-associated macrophages</td>
<td>VEGFR1⁺, VEGF⁺, CXCR4⁺, CCR2⁺, Tie2⁺, intergrin α4⁸⁵⁹,⁸⁶⁰</td>
</tr>
</tbody>
</table>

Table 6.1 Subpopulations of macrophages in cancer biology. Arg-2, Arginase-2; CCL, CC-chemokine Ligand; 
CCR, CC-chemokine receptor; CD, cluster of differentiation; CXCL, CXC-chemokine ligand; CXCR, CXC- 
chemokine receptor; EGF, Epidermal Growth Factor; IL, Interleukin; PDGF, platelet derived growth factor; 
PGE₂, prostaglandin E₂; Tie2, receptor Tyrosine kinase with Ig-like and EGF-like domains; TNF-α, Tumour 
Necrosis Factor-α; VEGF, vascular endothelial growth factor; VEGFR, VEGF receptor.
6.4.3 Interstitial macrophages (IMφ) in pulmonary fibrosis

The lung is occupied by two populations of pulmonary macrophages, including AMφ residing in the alveolar space, and interstitial macrophages (IMφ) occupying the lung interstitium. Given that these two populations of macrophages behave differently (described further below), I propose the analysis of IMφ heterogeneity in rodent models of pulmonary fibrosis.

Current studies of the role of macrophages, including those in this thesis, have largely relied on AMφ, presumably due to the ease of isolation of these cells. Whereas AMφ can be easily isolated through washings of the lung with DPBS, the isolation of IMφ involves homogenisation of the lung, followed by separation from other lung populations through fluorescence or magnetic activated cell sorting (FACS or MACS respectively). While AMφ are undoubtedly key players in the pathogenesis of pulmonary fibrosis, IMφ may possess a more central role as they are located within the interstitium where fibrosis occurs.

IMφ and AMφ possess different phenotypes and play different roles within the lung. AMφ are long-lived tissue resident cells that act as the first line of defence against invading pathogens, with a turnover rate of around 40% per year\(^273,274\). AMφ are constantly exposed to a hyperoxic environment and are in intimate contact with both air- and blood-borne materials, and function as a primary defence of the lung against inhaled particulate matter, microorganisms and environmental toxins\(^861–863\). These cells express great capacity in providing non-specific innate immunity, and express greater functional activity to inflammation and anti-microbial defence, including increased chemotaxis, phagocytosis, cytotoxicity and release of reactive oxygen and nitrogen intermediates, Prostaglandin E (PGE), TNF-α and Interferon (IFN)\(^864\). Mouse studies has shown that although AMφ are capable of transporting antigens to the lymph nodes\(^865\), they are poor APCs\(^866–868\).

IMφ act as the intermediate between the innate and adaptive response, and are shorter lived than AMφ, with a turnover rate of 21 days\(^176,274\). IMφ have pronounced immunoregulatory activity, and express greater quantities of the C3 Receptor and intracellular adhesion molecule 1, and more active in secreting IL-1 and IL-6, and exhibit greater MHCII along with stronger antigen-presenting capacity\(^275,869–881\). Moreover, IMφ, but not AMφ, produce high levels of Interleukin (IL)-10 and therefore inhibit dendritic cell migration\(^882\).

As mentioned in the general introduction, IL-1β, IL-6 (section 1.2.3.1) and IL-10 (section 1.2.3.2.3) are important mediators in the fibrotic response. IL-1β promotes tissue injury\(^275\) and the expression of angiogenic mediators (CXCL1, CXCL2, and CXCL8)\(^376\) and pro-fibrotic mediators (TGF-β and PDGF)\(^375\). IL-6 may promote collagen expression\(^398–400\) and also fibroblast differentiation into myofibroblasts\(^402\). IL-10 may protect from further tissue injury through its anti-inflammatory
properties in suppressing TNF-α and ROS production in macrophages, but promote fibrosis through fibrocyte recruitment and M2-like macrophage activation via a (CCR2)/CCL2 axis.

It is therefore of interest to evaluate the specific contributions of IMϕ in interstitial pulmonary fibrosis. IMϕ can be isolated by FACS or MACS of lung homogenates by positively selecting their specific cell surface markers, such as CD11c and MHC-II (appendices 12 and 13) following the removal of AMϕ by BAL. A broad comparison of AMϕ versus IMϕ in BLM-induced pulmonary fibrosis can be compared. On the other hand, it is also possible to isolate and assess the specific genotype and phenotype of different IMϕ subpopulations using flow cytometry. Assessment of the role of IMϕ in BLM-induced pulmonary fibrosis will help us further understand the different roles of these cells in pathology.

6.4.4 Isolation of diseased from non-diseased tissues in pulmonary fibrosis

A particular difficulty for studying AMϕ transcriptome ex vivo is to separate the heterogeneous subpopulations, such that the same functional subset, rather than a pooled sample of different subsets are analysed. As demonstrated in Chapter 2, macrophage subsets pre-defined in vitro, namely M1-like and M2-like macrophages, may be insufficient to fully represent the complexity of macrophage genotype in vivo. However, these may serve as a good starting point of which to pre-sort AMϕ for further analyses. A particular caveat of isolation of macrophage subsets is that there are insufficient numbers for kinetic microarray analysis. On the other hand, emerging technologies for single cell/low quantity RNA analysis may be able to bypass the use of markers altogether. Cells of interest may be isolated from different histopathological regions using LCM (appendix 14), and their specific role can be assessed. There are at present several types of single cell/low quantity RNA analysis technologies, including single cell RNA-seq, NanoString nCounter system, and microfluidic platform by Fluidigm for single-cell gene expression analyses. Target genes identified in animal models can then be translated and validated in human tissue using qRT-PCR. Protein analysis by mass cytometry also allows the simultaneous assessment of a large panel of proteins expressed within a single cell, which would improve our distinction of different subpopulations of cells within a sample.

6.4.5 Investigation of resolution in BLM-induced pulmonary fibrosis

Current studies of BLM-induced pulmonary fibrosis have focused on the assessment of the initiation and progression of pathology. BLM-induced pulmonary fibrosis by a single intratracheal dose has been reported to resolve over time. There was a decrease in the gene expression of pro-inflammatory and pro-fibrotic mediators, a reduced cellular infiltration, hydroxyproline (collagen precursor) content, and collagen accumulation, with minimal disruption to alveolar architecture, suggesting that chronic inflammation, fibrosis and tissue remodelling are
spontaneously reversible. However, the resolution pulmonary fibrosis in mice have not been
extensively investigated, primarily because this phenomenon is not observed in IPF patients\textsuperscript{1,202,242}.
Nevertheless, study of the resolution phase may be useful to better understand the criterion for
reversing pathology, and perhaps identify target genes or proteins that can be manipulated to
promote this anti-fibrotic activity.

Chronic inflammation arises from non-resolving acute inflammation that is caused by the presence
of a persistent irritant, and is related to dysregulated wound healing that progresses into tissue
fibrosis. It is unclear how this process may be spontaneously inhibited in models of resolving fibrosis,
although it is possible that the source of irritation is simply cleared by mononuclear phagocytes. For
example, removal of the injury stimulus has been shown to improve liver fibrosis in alcoholic liver
disease\textsuperscript{884}, viral hepatitis\textsuperscript{885-887}, biliary obstruction\textsuperscript{888} and autoimmune hepatitis\textsuperscript{889}. In a CCl\textsubscript{4}-induced
model of liver fibrosis, rats that are challenged for 4 to 8 weeks develop fibrosis but undergo
spontaneous resolution\textsuperscript{347,890,891}, whereas when the injury is continued for a longer time (>12 weeks),
cirrhosis (advance fibrosis) develops and in the absence of ongoing injury there is only partial
reversal with remodelling\textsuperscript{890}. Similarly as aforementioned in local administration models of BLM-
induced pulmonary fibrosis, resolution was observed in single intratracheal but not multiple
repetitive endotracheal dose models. The number of apoptotic cells, levels of alarmins and also
efferocytotic activity of macrophages can be assessed to determine clearance of inducers of chronic
inflammation, and their correlation with reversal of chronic inflammation can also be evaluated.

The progressive nature of fibrosis in IPF patients is largely accredited to the long-lived
myofibroblasts that are resistant to apoptosis and the failure of macrophages to resolve the
aberrant wound healing/ fibrotic response. Fibrosis may be attenuated through the inhibition of
fibroblast proliferation and activation and/or active removal of collagen secreting cells\textsuperscript{85,892,893}.
Indeed in BLM-induced pulmonary fibrosis, there is decreased levels of G0/G1 switch gene 2 (G0s2)
transcripts during the resolution phase. G0s2 belongs to the family of peroxisome proliferator-
activated receptors (PPARs) that promotes the reduction of fibroblast proliferation and its
differentiation into myofibroblasts\textsuperscript{894}. In CCl\textsubscript{4}-induced liver fibrosis, apoptosis of the hepatic stellate
cell, the primary collagen-secreting cell in the liver, was observed during the resolution phase\textsuperscript{347}. The
proliferation and apoptotic rate of myofibroblasts in BLM-induced pulmonary fibrosis should be
evaluated to confirm these hypotheses. The signalling pathways associated with myofibroblast
survival and resistance to apoptosis can also be targeted to evaluate their importance in the
progression of fibrosis.
There are a number of mediators that may be involved in the reversal of tissue fibrosis, in particular those that play a role in ECM turnover such as MMPs. It has been described earlier that MMPs (e.g. MMP-9, MMP-12) play a pro-fibrotic role, in aberrant tissue remodelling and also activation of pro-fibrotic precursors (e.g. latent TGF-β) in pulmonary fibrosis. However, other MMPs may also play a role in resolution through the degradation of excess ECM deposits. The levels of MMPs have not been extensively evaluated at the resolution phase, and it would be useful to identify candidates that lead to successful reversal of pulmonary fibrosis. Other mediators may include anti-fibrotic proteins, collagen uptake proteins (e.g. mfge8), and also the phagocytotic activity of macrophages of degraded ECM components.

There is much debate on the degree of reversibility of tissue fibrosis, which may depend on the maturity of the scar tissue: it has been observed that sufficiently advanced fibrosis becomes irreversible. There are several unique features of advance fibrosis that may contribute to their resistance to resolution: the paucity of cellular infiltration within the scar tissue, and greater cross-linking of ECM components. Inflammatory cellular influx is required to resolve fibrosis353, possibly by their release of ECM degrading enzymes (e.g. MMPs)891 and phagocytosis of ECM components. For example, macrophages are reported to play a role in the resolution of pulmonary fibrosis. Depletion of macrophages in CD11b-DTR mice at the onset of fibrosis resolution could retard ECM degradation and the loss of HSCs in a CCl4-induced model of liver fibrosis348. Depletion of AMφ using liposomal clodronate from days 42-46 slows ECM degradation and reversal of fibrosis when assessed at day 56 in BLM-induced pulmonary fibrosis175. The genotype and phenotype of these resolution phase macrophages should be characterised ex vivo to help better understand their role in the reversal of fibrosis. ECM cross-linking may stiffen the matrix and result in the concealment of epitopes important for integrin-mediated regeneration and stabilisation of the physical properties of the matrix895. For example, the copper-dependent enzyme lysyl oxidase(LOX)-like 2 (LOXL2) facilitates the cross-linking of collagen and elastin896. LOXL2 is increased in levels in IPF patients and also human hepatitis C-associated liver fibrosis896. Treatment of single intratracheal dose BLM-challenged mice with copper chelator tetrathiomolybdate reduced LOX (LOX, LOXL1 and LOXL2), collagen-1, MMP (MMPs2 and 8) and TIMP1 protein expression and regressed fibrosis897. Inhibition of LOXL2 with an anti-LOXL2 monoclonal antibody prevented fibrosis and α-SMA+ myofibroblast accumulation in CCl4-induced liver fibrosis896. Prevention and treatment with an anti-LOX2 monoclonal antibody also reduced collagen cross-linking, accumulation, and myofibroblast accumulation in a single intratracheal BLM dose model of pulmonary fibrosis in mice896. The importance of ECM cross-linking in driving pulmonary fibrosis can be determined by comparing that in fibrotic lesions of resolvable (i.e. single dose) versus unresolvable (i.e. multiple dose) models of BLM-induced pulmonary fibrosis.
6.5 Future directions

In conclusion, to tackle the role of AMφ in IPF it is essential to learn more about AMφ heterogeneity in those isolated directly from human patients. To increase our understanding of the role of AMφ in interstitial lung diseases it will be necessary to conduct more ex vivo studies to assess macrophage functional response, such as efferocytosis of labelled apoptotic cells, response to other alarmins, and how this correlates with the gene expression and phenotype in vivo in a range of animal models that addresses different responses. For example, BLM-induced pulmonary fibrosis directly induces AEC injury and apoptosis, and the subsequent activation of macrophages by alarmins and apoptotic AECs is critical to the fibrotic response. Asbestos and silica fibres directly activate macrophages – their large size and resistance to removal results in ‘frustrated phagocytosis’ by macrophages triggers a pro-inflammatory response by NLRP3 activation666, leading to peripheral damage to AECs that in turn undergo apoptosis to further activate macrophages through their release of alarmins. It would therefore be interesting to dissect some of the downstream signalling pathways involved, as means to target these responses.

In addition to the role of AMφ, there are several other problems surrounding IPF that needs to be addressed. The mechanism for the initiation of IPF is still not fully understood. As aforementioned in the general introduction there are two prevailing hypothesis, including the inflammation-driven model (section 1.1.4.2) and the AEC injury driven model (section 1.1.4.3). However, whether or not these two pathways are interdependent or independent is unknown. In other words, pulmonary fibrosis may be initiated by AEC injury and/or chronic inflammation in separate processes, or AEC injury may lead to chronic inflammation that initiates pulmonary fibrosis (or vice versa). It is also unclear what constitutes the transition from initial tissue injury and dysregulated wound healing to active fibrosis. It was aforementioned that IL-6 induced shift in immune cell recruitment, from granulocytes to monocytes and lymphocytes90, which may play a role in the switch from acute to chronic inflammation and promote the expression of wound-healing/ pro-fibrotic mediators within the lung. It would be interesting to assess whether or not other mechanisms are at play to facilitate this transition. To this end, the HPS1/HPS2 mice may be of use to investigate these processes, as naturally occurring mutations in these mice lead to spontaneous development of pulmonary fibrosis 9 months710. It would be interesting to study the kinetics with respect to various pro-inflammatory, pro-fibrotic and tissue remodelling mediators, and also alarmin levels in the BAL and lung tissue.

The reversibility of pulmonary fibrosis is also unclear, and may be dependent on the ability of collagen-secreting myofibroblasts to apotose, the amount of cross-linking in ECM components and clearance of excessive ECM components, the persistent irritant, alarmins and apoptotic cells (section
6.4.5). The criterion for resolution may be investigated through comparing resolvable models of pulmonary fibrosis with unresolvable ones (e.g. single vs. multiple oropharyngeal dose of BLM).

More recently, it was discovered that epigenetics may also play a role in IPF pathology. For example, effective histone acetylation is responsible for the repression of two anti-fibrotic genes, cyclooxygenase-2 and CXCL10/IFN-γ induced protein 1(IP-10). Different levels of DNA methylation of 3 CpG islands in the promoter of α-SMA in fibroblasts, myofibroblasts, and AEC2 were shown to correlate with α-SMA gene expression in these cells. Silencing with pharmacologic inhibitors or siRNA, or overexpression of DNA methyltransferase increased or reduced α-SMA expression, but both affected TGF-β1-induced myofibroblast differentiation. A number of gene expression profiles have also identified the change in levels of miRNA with roles in fibroproliferation, EMT and TGF-β signalling, including the downregulation of let-7d and miR-29, and upregulation of miR-155 and miR-21, in IPF patients compared to controls. Evaluation of the epigenomic profile may provide novel therapeutics for the treatment for this devastating disease.

This thesis therefore ends with the proposal that a combination of murine models and studies in human patients will provide a thorough evaluation of the role of AMφ and also the course of pathology in this devastating disease, which may provide novel therapeutic implications in the future.
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Appendix 1 – Standard curve for MSD analyses of fibrosis-associated mediators in differentially primed AMφ from naïve mice (Figure 3.10)

IL-6

CCL2 (MCP-1)

CCL3 (MIP-1α)

CCL4 (MIP-1β)

CXCL2 (MIP-2)

CCL5 (RANTES)
Appendix 2 – Standard curve for ELISA/MSD analyses of macrophage subset-associated mediators in differentially primed AMϕ from naïve mice (Figure 3.12, 3.14 and 3.16)
Appendix 3- Flow cytometry for differentially primed AMΦ (Figures 3.13-3.14)

24 hours

![Graphs for MRC1 and MHCII at 24 hours](image)

72 hours

![Graphs for MRC1 and MHCII at 72 hours](image)
Appendix 4 – Standard curve for ELISA/MSD analyses of macrophage subset-associated mediators in AM\(\phi\) from saline or BLM-challenged mice (Figure 4.11)
Appendix 5 – Flow cytometry analysis of MRC1 and MHC-II in CD11c^+ FSC^hi SSC^hi AMφ (Figure 4.11)

**Saline (Day 7)**

![Flow cytometry plots for MRC1 and MHC-II](image)

- MRC1 vs. MHCII plots for different quadrants.
- Data points and gating strategies are shown.
- Statistical information is indicated for each quadrant.
BLM (Day 7)
Saline (Day 14)
Saline (Day 14)
BLM (Day 14)
Saline (Day 21)

[Flow cytometry plots showing MRC1 and MHCII levels on Day 21]
Saline (Day 21)
BLM (Day 21)
BLM (Day 21)
Appendix 6 – Standard curve for MSD analyses of fibrosis-associated mediators in differentially primed AMφ from saline or BLM-challenged mice (Figure 4.14)
Appendix 7 – Standard curve for MSD analyses of TNF-α and CXCL1, and ELISA analysis of fibronectin from WT C57BL/6 or HPS1 mice (Figure 4.23 and 4.24)
Appendix 8 - Standard curve for MSD analyses of IL-12 p40 and fibronectin from WT C57BL/6 or HPS1 mice (Figure 4.36 and 4.37)
Appendix 9 – Standard curve for MSD analyses of IL-1β, TNF-α, CXCL1, IL-10, IL-12 p40 (total) and ELISA analysis of fibronectin from saline or BLM-challenged (Figure 5.6 – 5.14; Figures 5.21 – 5.26)
Appendix 10 – Standard curve for MSD analyses of IL-6, CCL2, CCL3, CCL4, CCL5 and CCL20 from saline or BLM-challenged WT C57BL/6 mice (Figure 5.15 – 5.20)

IL-6

CCL2 (MCP-1)

CCL3 (MIP-1α)

CCL4 (MIP-2)

CCL5 (RANTES)

CCL20 (MIP-3α)
Appendix 11 – Flow cytometry data for TLR2 stained AMΦ isolated from saline or BLM-challenged mice (Figure 5.4)

Saline (Day 7)
Bleomycin (Day 7)

<table>
<thead>
<tr>
<th>BLM</th>
<th>Isotype Control</th>
<th>Unstained Control</th>
</tr>
</thead>
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Saline (Day 14)
Bleomycin (Day 14)
Saline (Day 21)
Bleomycin (Day 21)

<table>
<thead>
<tr>
<th>BLM</th>
<th>Isotype Control</th>
<th>Unstained Control</th>
</tr>
</thead>
</table>

![Graphs showing cell count distributions for BLM, Isotype Control, and Unstained Control](image)

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Appendix 12 – Characterisation of surface protein markers in mouse pulmonary IMϕ

Interstitial Macrophages

Appendix 12. Characterisation of surface protein markers of pulmonary interstitial macrophages (IMϕ). A 12 week old male C57BL/6 mice were culled and lungs were washed 5 times with DPBS to remove AMϕ, and then homogenised in digestion media (RPMI 1640 + HEPEs + 1% Penicillin / Streptomycin + 5% fetal bovine serum + 1.0 mg/ml collagenase A +25 U/ml DNase1) for 60 minutes at 37 °C. Cells within the lung homogenate were then resuspended in FACS buffer (DPBS + 0.5% mouse serum) and stained for the extracellular (surface) proteins, including CD11b, CD11c, and MHC-II (IA/I E) with anti-CD11b-PE/Cy7 (2 μg/ml), anti-CD11c-PE (2 μg/ml) and anti-MHC-II (IA/I E)-FITC (5 μg/ml), and intracellular macrophage-specific markers F4/80 and CD 68 with anti-F4/80-Pacific Blue (1 μg/ml) and anti-CD68-PerCP-5.5 (1 μg/ml). IMϕ were identified as SSC<sup>+</sup>FSC<sup>+</sup>F4/80<sup>+</sup>, and their surface expression of CD11b, CD11c and MHC-II was determined as shown above.
Appendix 13 – Verification of surface protein markers in mouse pulmonary IMφ

A 10 week old male C57BL/6 mouse was culled and lungs were washed 5 times with DPBS to remove AMφ, and then homogenised in digestion media (RPMI 1640 + HEPEs + 1% Penicillin/Streptomycin + 5% fetal bovine serum + 1.0 mg/ml collagenase A + 25 U/ml DNase1) for 60 minutes at 37°C. Cells within the lung homogenate were then resuspended in FACS buffer (DPBS + 0.5% mouse serum) and stained for the extracellular (surface) proteins, including CD11b, CD11c, and MHC-II (IA/IE) with anti-CD11b-PE/Cy7 (2 μg/ml), anti-CD11c-PE (2 μg/ml) and anti-MHC-II (IA/IE)-FITC (5 μg/ml), and intracellular macrophage-specific markers F4/80 and CD68 with anti-F4/80-Pacific Blue (1 μg/ml) and anti-CD68-PerCP-5.5 (1 μg/ml). Selection of SSC<sup>−</sup>FSC<sup>−</sup> CD11c<sup>+</sup> MHC-II<sup>+</sup> pulmonary cells revealed that these have a high expression of the macrophage-specific markers F4/80<sup>+</sup> and CD68<sup>+</sup>.
Appendix 14 – Laser Capture Microdissection (LCM) of mouse lung tissue

A 12 week old mouse was culled and its lungs was then inflated with Optimal Cutting Temperature (OCT) and snap frozen in liquid nitrogen. 10μm frozen lung sections were sliced and stained with haematoxylin and eosin (H&E), and then used for LCM as shown above.