T CELL AND MACROPHAGE INTERACTION IN EXPERIMENTAL BLEOMYCIN-INDUCED IDIOPATHIC PULMONARY FIBROSIS

GU YANRONG

UNIVERSITI SAINS MALAYSIA

2025

T CELL AND MACROPHAGE INTERACTION IN EXPERIMENTAL BLEOMYCIN-INDUCED IDIOPATHIC PULMONARY FIBROSIS

by

GU YANRONG

Thesis submitted in fulfilment of the requirements
for the degree of
Doctor of Philosophy

February 2025

ACKNOWLEDGEMENT

As this thesis comes to fruition, my heart brims with a profound sense of gratitude and reflection. It is not merely the culmination of an academic journey that fills me with emotion, but the treasured encounters and experiences with every individual I have met along the way.

First and foremost, I extend my deepest respect and heartfelt thanks to my main supervisor Assoc. Prof. Dr. Badrul Hisham Bin Yahaya. You have been more than an academic guide; you have been a luminary for my life's path. In times of adversity and challenge, it was your sagacious counsel and unwavering support that steadied me. Thank you for your timely and effective guidance in the process of thesis revision. The insights and perspectives I gained during my time there have been instrumental in broadening my horizons and enriching my research. Your fervent passion for academia and your zest for life have profoundly influenced me. I am deeply thanks to my co-supervisor Assoc. Prof. Dr. Rafeezul Bin Mohamed. Thanks for your help in tutoring my thesis writing concern for my life in Malaysia.

I am deeply grateful to my co-supervisors Prof. Yinming Liang, despite being far away in China, have consistently shown care and provided guidance on my research progress. I am immensely grateful to Prof. Toby Lawrence. I am deeply grateful for the guidance you have provided in steering my research topic to fruition. Your expertise and insights have been instrumental in shaping my academic endeavours. Thanks for the professional insights and assistance provided in the field of single-cell transcriptomics. I also extend my thanks to Dr. Rosa Andres Ejarque for the valuable contributions to data analysis.

To my fellow students and friends in Malaysia, thank you for you have been indispensable companions on this academic voyage. The shared intellectual challenges, the journey to discover the enchanting islands of Malaysia and the support offered in times of need are memories I will always cherish.

To my colleagues and friends in China. I sincerely thank all those who offered their unwavering support and assistance during my time in China. Your companionship on these adventures is a treasured part of my journey, and I am grateful for the moments of exploration and camaraderie.

Furthermore, I must also express my profound gratitude to my family, whose unconditional love and support have been my greatest source of strength. As I ventured away from home to immerse myself in scholarly pursuits, the understanding and encouragement from my family ensured I never felt alone. In this thesis section, as I extend my thanks, I also wish to pay a heartfelt tribute to my beloved father. During my journey from China to Malaysia to pursue my studies, my dear father was tragically taken from me by a heart failure triggered by a pulmonary viral infection. It is with deep sorrow that I express my regret for not being able to speak to him one last time. Throughout the writing of this doctoral thesis, I often found myself searching into the mechanisms of pulmonary diseases at night, unable to help but think of my father who is now in heaven. Although I could not be by his side to say goodbye, I sometimes feel his presence, as if he continues to accompany me from above, guiding me through these years.

Lastly, I extend my sincere gratitude to all the experts and scholars who have reviewed this work. Your valuable feedback is greatly appreciated.

TABLE OF CONTENTS

ACKNOWLEDGEMENT ..... **ii**

TABLE OF CONTENTS ..... **iv**

LIST OF TABLES ..... **viii**

LIST OF FIGURES ..... **ix**

LIST OF SYMBOLS ..... **xiii**

LIST OF ABBREVIATIONS ..... **xiv**

LIST OF APPENDICES ..... **xx**

ABSTRAK ..... **xxi**

ABSTRACT ..... **xxiii**

CHAPTER 1 INTRODUCTION **1**

1.1 Research Background 1

1.2 Rationale and importance of the study 5

1.2.1 Hypothesis of the study 7

1.2.2 Objective of the study 8

1.2.2(a) General objective 8

1.2.2(b) Specific objectives 8

CHAPTER 2 LITERATURE REVIEW **9**

2.1 Pulmonary fibrosis diseases 9

2.1.1 The prevalence of pulmonary fibrosis 9

2.1.2 Structure of the respiratory immune system 14

2.1.3 Pathogenesis of pulmonary fibrosis 18

2.1.4 Therapy for pulmonary fibrosis 24

2.2 Animal model of pulmonary fibrosis 28

2.3 Lung immune system 33

2.3.1 First line of the invading insults defence 33

2.3.2 Immune cells in the lung 35
2.4 The role of macrophages and T cells in pulmonary fibrosis disease 39
2.4.1 The role of macrophages in pulmonary fibrosis 40
2.4.2 The role of T cells in pulmonary fibrosis 47
2.4.3 Interaction of T cells and macrophages 51
CHAPTER 3 METHODOLOGY 57
3.1 Experimental design 57
3.2 Mouse model 60
3.3 Diphtheria toxin (DT) treatment 60
3.4 BLM-induced lung fibrosis model 63
3.5 Body weight 65
3.6 Sample collection 65
3.7 Histological analysis 66
3.7.1 Tissue processing, fixation and embedding 66
3.7.2 Hematoxylin and eosin (H&E) staining 67
3.7.3 Masson trichrome staining 68
3.8 Morphometric analysis 69
3.9 Flow Cytometry 70
3.10 RNA extraction 71
3.11 Quantitative Real-Time PCR (qPCR) 72
3.12 Statistical analysis 73
3.13 Single-cell transcriptomic analysis 73
3.13.1 Single-cell transcriptomics sample preparation 74
3.13.2 Single-cell transcriptomics library construction 74
3.13.3 Single-cell transcriptomics data analyses 86
CHAPTER 4 RESULTS 88
4.1 Influence of T cell and macrophage in pulmonary fibrosis 88

4.1.1 Lung morphology in BLM-induced lung fibrosis model 88

4.1.2 Influence of major immune cells in the lung following BLM treatment 89

4.1.3 Influence of T cell subsets and activation in the lung following BLM treatment 93

4.1.4 Influence of T cell subsets in the pulmonary draining lymph node, blood, and spleen following BLM treatment 97

4.1.5 Influence of macrophages in the lung following BLM treatment 101

4.2 Lung macrophages change in T cell deletion mouse model during lung fibrosis 104

4.2.1 Validation of T cell deletion mouse model 104

4.2.2 Body Weight and histological changes in T cell deletion mouse model during lung fibrosis 106

4.2.3 The influence of lung macrophages following BLM treatment in T cell deletion mouse model 110

4.3 Lung macrophages change under T cell deletion after the onset of lung fibrosis 113

4.3.1 Validation of T cell deletion after DT injection 113

4.3.2 Body Weight Changes in lung fibrosis following T cell deletion mouse model 115

4.3.3 Histological changes in lung fibrosis following T cell deletion mouse model 118

4.3.4 Influence of lung macrophage subsets in lung fibrosis following T cell deletion mouse model 120

4.4 Single-cell transcriptomics to analyze the effect of T cells on macrophages in pulmonary fibrosis 122

4.4.1 Single-cell RNA sequencing reveals the diverse immune landscape in the fibrotic lungs of WT and T cell-deficient mice. 122

4.4.2 Validation of T cell deletion at transcriptomic level 126

4.4.3 Cellchat analysis revealed the cell-cell interactions between immune cells in the WT and T cell deficiency mouse during lung fibrosis 128

4.4.4 Cellchat analysis revealed the unique roles of macrophage-associated subclusters in the WT and T cell deficiency mouse during lung fibrosis 134

4.4.5 Gene expression change in IMs population 145

4.4.6 Gene expression validation of M2 phenotype in IMs 149

CHAPTER 5 DISCUSSION 150

5.1 Involvement of immune cells in pulmonary fibrosis mouse models 150

5.2 The role of T cells in pulmonary fibrosis and utilization to T cell-deficient models 154

5.3 Macrophage involved and influenced by T cells in pulmonary fibrosis 163

5.4 Single-cell technology to explore the interactions between T cells and macrophages in pulmonary fibrosis 166

CHAPTER 6 CONCLUSION AND FUTURE RECOMMENDATIONS.... 173

6.1 Conclusion..... 173

6.2 Recommendations for Future Research 176

REFERENCES..... 178

APPENDICES

LIST OF PUBLICATIONS

CONFERENCES PRESENTATION

LIST OF TABLES

Page

Table 2. 1	Properties of AM and IM in steady state	43
Table 3. 1	Sorted sample for experimental groups	71
Table 3. 2	Materials for RT cDNA Mix	76
Table 3.3	Exonuclease I Mix	76
Table 3.4	Random Primer Mix	78
Table 3.5	Extension Enzyme Mix	78
Table 3.6	Random Primer Mix	79
Table 3.7	WTA Index PCR Mix	81
Table 3.8	Sample Tag PCR1 Reaction Mix	82
Table 3.9	Sample Tag PCR2 Reaction Mix	84
Table 3.10	Index PCR Mix	85
Table 4.1	Top 20 highly increased DEGs in IMs of BLM-treated CD3e KO mice compared to WT mice	146

LIST OF FIGURES

Page

Figure 1.1 The schematic design of this study 7

Figure 2.1 Categories of ILD 10

Figure 2.2 Global heat maps of adjusted IPF prevalence with specific IPF definitions 13

Figure 2.3 Schematic representation of the airway generations and corresponding anatomical airway structures 15

Figure 2.4 Distribution of lymphatic vessels (green) in the pulmonary lobule ... 17

Figure 2.5 Pathogenesis of idiopathic pulmonary fibrosis (IPF) 19

Figure 2.6 The viral and microbial infections involve in the development of pulmonary fibrosis 22

Figure 2.7 Examples of animal model of pulmonary fibrosis. 32

Figure 2.8 Identification markers of alveolar macrophages and interstitial macrophages 42

Figure 2.9 The classification of T cells 48

Figure 2.10 The interaction between T cells and macrophages 55

Figure 3.1 Flowchart of the study 59

Figure 3.2 Optimise the concentration of diphtheria toxin 62

Figure 3.3 Optimise the concentration of bleomycin. 64

Figure 3.4 Procedure of morphometric analysis 69

Figure 3.5 Schematic graphical illustration of the scRNA experimental design 74

Figure 4.1 Representative histological sections of the lungs from WT mice treated with BLM and PBS 89

Figure 4.2 Flow cytometric gate shows lung immune cells response to BLM-induced lung fibrosis 91

Figure 4.3 Absolute numbers of lung immune cells show their response to BLM-induced lung fibrosis. 93

Figure 4.4 $CD4^+$ T cells activation in the fibrotic lungs 95

Figure 4.5 $CD8^+$ T cells activation in the fibrotic lungs 96

Figure 4.6 T cells in the pulmonary draining lymph nodes response to BLM-induced lung fibrosis 98

Figure 4.7 T cells in the blood response to BLM-induced lung fibrosis 99

Figure 4.8 T cells in the spleen response to BLM-induced lung fibrosis 100

Figure 4.9 Flow cytometric gating analysis of lung macrophages in response to BLM-induced lung fibrosis 102

Figure 4.10 Absolute numbers of lung macrophages show their response to BLM-induced lung fibrosis 103

Figure 4.11 Flow cytometry analysis to confirm the absence of T cells in the lungs of WT and CD3e KO mice 105

Figure 4.12 Body weight of WT and CD3e KO mice following BLM treatment 108

Figure 4.13 Representative histological sections of the lungs from BLM-treated WT and BLM-treated CD3e KO mice 109

Figure 4.14 Flow cytometric gate shows lung macrophages response to BLM-induced lung fibrosis in WT and CD3e KO mice 111

Figure 4.15 Proportion of lung macrophages shows their response to BLM-induced lung fibrosis in WT and CD3e KO mice 112

Figure 4.16 Flow cytometry to test the T cells in the lungs of WT and DT-treated Lat DTR mice 114

Figure 4.17 The schematic of the experimental design in lung fibrosis following T cell deletion mouse model 116

Figure 4.18 Body weight changes in WT and Lat DTR mice with BLM-induced lung fibrosis after DT or PBS treatment 117

Figure 4.19 Representative histological sections of the lungs from WT-BLM and Lat DTR-BLM-DT mice 119

Figure 4.20 Flow cytometric shows lung macrophage response in WT-BLM and Lat DTR-BLM-DT mice 121

Figure 4.21 Default clustering tree of resolution parameters 123

Figure 4.22 Heatmap of SingleR annotation scores derived by differential expression analysis of published datasets from mice lungs 124

Figure 4.23 Nonlinear dimensionality reduction UMAP visualization of identified 17 different clusters after unsupervised clustering 125

Figure 4.24 UMAP visualization of sample groups 126

Figure 4.25 Gene distribution of T cell marker 127

Figure 4.26 CellChat analysis indicates the possible interactions in the fibrotic lungs of WT and CD3e KO mice 129

Figure 4.27 Changes in intercellular communication strength in the fibrotic lungs of WT and CD3e KO mice 130

Figure 4.28 Circular network plot and heat map showing the network centrality analysis of the collagen signalling pathway in the fibrotic lungs of CD3e KO mice 131

Figure 4.29 Heatmap plot showing the communication probabilities of the collagen signalling pathway among the cell populations in the fibrotic lungs of CD3e KO mice 132

Figure 4.30 Circular network plot and heat map showing the communication probabilities of the MIF signalling pathway among the cell populations in the fibrotic lungs of CD3e KO mice 133

Figure 4.31 Heatmap plot showing the communication probabilities of the MIF signalling pathway among the cell populations in the fibrotic lungs of CD3e KO mice 134

Figure 4.32 Scatter graph showing the incoming and outgoing interaction strengths in the fibrotic lungs of WT and CD3e KO mice.....137

Figure 4.33 Scatter graph showing the altered signalling pathways that associate with IMs and iMONs in the fibrotic lungs of WT and CD3e KO mice.....138

Figure 4.34 Heatmap showing the communication probabilities of the THBS signalling pathway among the cell populations in the fibrotic lungs of BLM-treated WT mice and CD3e KO mice 139

Figure 4.35 Circular network plot showing the network centrality analysis of the THBS signalling pathway in the fibrotic lungs of BLM-treated WT mice and CD3e KO mice 140

Figure 4.36 Comparison of Thbs1 expression in immune cells in the fibrotic lungs of WT mice and CD3e KO mice. 141

Figure 4.37 Heatmap showing the communication probabilities of the APP signalling pathway among the cell populations in the fibrotic lungs of BLM-treated WT mice and CD3e KO mice 142

Figure 4.38 Circular network plot showing the network centrality analysis of the APP signalling pathway in the fibrotic lungs of BLM-treated WT mice and CD3e KO mice 143

Figure 4.39 Comparison of App expression in immune cells in the fibrotic lungs of WT mice and CD3e KO mice 144

Figure 4.40 Relative mRNA expression of Arg1 and Fizz1 in the fibrotic lungs of WT and CD3e KO mice.....149

Figure 6.1 T cell depletion exacerbates pulmonary fibrosis by promoting M2 polarization of pulmonary interstitial macrophages.....175

LIST OF SYMBOLS

-	Negative
%	Per cent
+	Positive
°C	Degrees Celsius
µL	Microliter
Gy	Gray
hr	Hour
kg	Kilogram
mg	Milligrams
nM	Nanomolar
min	Min
mL	Millilitres
mm	Millimetres
rpm	Revolutions per min
U	Units
ug	Micrograms
α	Alpha
β	Beta
γ	Gamma
δ	Delta
ε	Epsilon
ζ	Zeta
nM	Nanomolar

LIST OF ABBREVIATIONS

2.4G2	Monoclonal antibody against Fc receptor γIIb
Adgre1	Adhesion G protein-coupled receptor E1
ADP-ribose	Adenosine diphosphate ribose
AECs	Alveolar epithelial cells
AHR	Aryl hydrocarbon receptor
AIF1	Allograft inflammatory factor 1
ALRs	AIM2-like receptors
AmDCs	Airway mucosal dendritic cells
AMPure XP	AMPure XP beads for magnetic bead purification
AMs	Alveolar macrophages
APCs	Antigen-presenting cells
APP	Amyloid precursor protein
Arg1	Arginase 1
Axl	Tyrosine-protein kinase receptor UFO
BALF	Bronchoalveolar lavage fluid
BALT	Bronchus-associated lymphoid tissue
BLM	Baseline mean
BMDM	Bone marrow-derived macrophages
C1QB+	Complement component 1
Car4	Carbonic anhydrase 4
CC	Chemokine (C-C motif) ligand
Ccr2	Chemokine (C-C motif) receptor 2
CD	Cluster of differentiation
CD3e	CD3 epsilon chain
cDC	Conventional dendritic cell
cDC1	Conventional dendritic cell type 1
cDC2	Conventional dendritic cell type 2
cDNA	Complementary DNA
CHI3L1	Chitinase 3-like 1
CLP	Common lymphoid progenitor
CLRs	C-type lectin receptors

CMV	Cytomegalovirus
COPD	Chronic obstructive pulmonary disease
COVID-19	Coronavirus disease 2019
CPI	Composite Physiologic Index
CSF1	Colony-stimulating factor 1
Csf1r	Colony-stimulating factor 1 receptor
CTGF	Connective tissue growth factor
CTLA4	Cytotoxic T-lymphocyte-associated protein 4
CTLs	Cytotoxic T lymphocytes
CTSK	Cathepsin K
Cx3cr1	Chemokine (C-X3-C motif) receptor 1
CXCL	Chemokine (C-X-C motif) ligand
CXCR2	C-X-C motif chemokine receptor 2
DEGs	Differentially expressed genes
DLCO	Diffusing capacity of the lung for carbon monoxide
DN	Double negative
dNTP	Deoxyribonucleotide triphosphate
DP	Double positive
DSP	Desmoplakin gene
DT	Diphtheria toxin
DTR	Diphtheria toxin receptor
DTT	Dithiothreitol
EAE	Experimental autoimmune encephalomyelitis
EBV	Epstein-Barr virus
ECM	Extracellular matrix
EDTA	Ethylenediaminetetraacetic acid
EF-2	Elongation factor-2
ETP	Early thymic progenitor
FABP4	Fatty acid-binding protein 4
FACS	Fluorescence-activated cell sorting
FBS	Fetal bovine serum
Fc receptors	Fc receptors
FDR	False discovery rate
FEV1	Forced Expiratory Volume in 1 second

FGF	Fibroblast growth factor
FGFR	Fibroblast growth factor receptor
FITC	Fluorescein isothiocyanate
FIZZ1	Found in inflammatory zone 1
FOXP3	Forkhead box P3
Fra-2	Fos-related antigen 2
Fra-2 Tg	Transgenic for Fos-related antigen 2
FVC	Forced vital capacity
GAP	Gender-Age-Physiology index
GPC4	Glypican 4
GSK3β	Glycogen synthase kinase 3 beta
GTPase rac2	GTPase
H&E	Haematoxylin and eosin
HIV	Human immunodeficiency virus
HRCT	High-resolution computed tomography
HSC	Hematopoietic stem cell
HTLV	Human T-lymphotropic virus
IACUC	Institutional Animal Care and Use Committee
iDC	Immature dendritic cell
IFNs	Type I interferons
Ig	Immunoglobulin
IGF1	Insulin-like growth factor 1
IL	Interleukin
ILCs	Innate lymphoid cells
ILD	Interstitial lung disease
IM	Interstitial macrophage
iMON	Inflammatory monocyte
INF-α	Interferon-alpha
INHBA	Inhibin beta A
IPF	Idiopathic pulmonary fibrosis
ITGAM	Integrin alpha M
KO	Knockout
LAT	Linker for activation of T cells
LCK	Lymphocyte-specific protein tyrosine kinase

LGMN	Legumain
LIPA	Lipase A
LPS	Lipopolysaccharide
Lyve1	Lymphatic vessel endothelial hyaluronan receptor 1
Lyz2	Lysozyme 2
M1	Classically activated macrophages
M2	Alternatively activated macrophages
Masson	Masson's trichrome stain
MDMs	Monocyte-derived macrophages
MERS	Middle East respiratory syndrome
MERTK	Mer tyrosine kinase
MHC	Major histocompatibility complex
MHV-68	Murine gammaherpesvirus 68
MIF	Macrophage migration inhibitory factor
miRNA	MicroRNA
Mmp12	Matrix metalloproteinase 12
MPP	Multipotent progenitor
Mrc1	Mannose receptor C type 1
MUC5B	Mucin-5B
MyD88	Myeloid differentiation primary response 88
NAD	Nicotinamide adenine dinucleotide
NAMs	Airway-associated macrophages
NETs	Neutrophil extracellular traps
NK	Natural killer
NLRs	NOD-like receptors
NOD/SCID	Non-obese diabetic/severe combined immunodeficiency
NSIP	Nonspecific interstitial pneumonitis
PAMPs	Pathogen-associated molecular patterns
PBS	Phosphate-buffered saline
PCA	Principal component analysis
PCR	Polymerase chain reaction
PD	Parkinson's disease
pDC	Plasmacytoid dendritic cell
PDGF	Platelet-derived growth factor

PDGFR	Platelet-derived growth factor receptor
PF	Pulmonary fibrosis
PG	Prostaglandin
PLA2G7	Phospholipase A2 group VII
Plp	Proteolipid protein
poly(I:C)	Polyinosinic-polycytidylic acid
PPARγ	Peroxisome proliferator-activated receptor gamma
PR8	PR8 influenza virus strain
PRRs	Pattern recognition receptors
PTEN	Phosphatase and tensin homolog
qPCR	Quantitative polymerase chain reaction
RA	Rheumatoid arthritis
RLRs	RIG-I-like receptors
RNA	Ribonucleic acid
ROS	Reactive oxygen species
RPE	Retinal pigment epithelium
RT	Reverse transcription
SARS-CoV-2	Severe acute respiratory syndrome coronavirus 2
scRNA	Single-cell RNA
SFK	Src family kinase
SFTPA2	Surfactant protein A2
SFTPC	Surfactant protein C
SMA	Smooth muscle actin
SOCS3	Suppressor of cytokine signalling 3
SP	Single positive
SPARC	Secreted protein acidic and rich in cysteine
SPP1	Secreted phosphoprotein 1
SSC	Side scatter
T-bet	T-box transcription factor T-bet
TCR	T cell receptor
Tcrb	T cell receptor beta chain
TERC	Telomerase RNA component
TERT	Telomerase reverse transcriptase
TGF	Transforming growth factor

Th	Helper T cell
THBS	Thrombospondin
TIMP	Tissue inhibitor of metalloproteinases
TLRs	Toll-like receptors
TNF	Tumour necrosis factor
Treg	Regulatory T cell
UIP	Usual interstitial pneumonia
UMAP	Uniform manifold approximation and projection
UMI	Unique molecular identifier
VEGF	Vascular endothelial growth factor
VEGFR	Vascular endothelial growth factor receptor
VISTA	V-domain Ig suppressor of T cell activation
Wnt/β-catenin	Wnt signalling pathway and β-catenin
WT	Wild type
WTA	Whole transcriptome amplification
ZAP-70	Zeta-chain (TCR) associated protein 70

LIST OF APPENDICES

Appendix A SUPPLEMENTARY TABLES

Appendix B ETHICS APPROVAL LETTERS

INTERAKSI SEL T DAN MAKROFAJ DALAM FIBROSIS PULMONARI IDIOPATIK ARUHAN BLEOMYCIN SECARA EKSPERIMEN

ABSTRAK

Fibrosis pulmonari adalah penyakit progresif dan tidak dapat dipulihkan dengan etiologi yang kurang difahami dan kadar kematian yang tinggi. Interaksi sel dalam peraturan imun memainkan peranan penting dalam perkembangan penyakit. Walaupun makrofaj telah dikaji secara meluas dalam model fibrosis paru-paru, interaksi mereka dengan sel-sel lain, terutamanya dengan sel-sel T masih tidak diketahui. Terutamanya, perbincangan silang antara imuniti semula jadi dan imuniti penyesuaian sangat menarik. Kajian sekarang dijalankan untuk menentukan kesan interaksi antara makrofaj dan sel T ke atas kamajuan fibrosis pulmonari. Pertama, peningkatan dan pengakatifan sel T disahkan dalam model tikus fibrosis pulmonari yang disebabkan oleh bleomycin (BLM). Kemudian, impak sel T ke atas kemajuan penyakit disiasat menggunakan tikus yang kekurangan sel T, CD3e *knockout* (CD3e KO). Yang menghairankan, ketiadaan sel T mengakibatkan fibrosis paru-paru yang lebih teruk, disertai dengan peningkatan ketara dalam makrofaj interstisial (IMs). Penemuan ini disahkan lagi apabila ketiadaan sel-sel T 2 minggu selepas pemodelan fibrosis paru-paru menggunakan toksin difteria (DT) yang disebabkan oleh pengurangan sel T lengkap. Dalam kedua-dua senario, kajian ini mendapati peningkatan dalam makrofaj interstisial (IM) pulmonari dan keterukan fibrosis paru-paru. Tambahan pula, analisis kajian ini terhadap penjujukan RNA sel tunggal (scRNA-seq) mendedahkan pengayaan spesifik Kolagen dan Faktor Perencatan

Migrasi Makrofaj (MIF) laluan isyarat dalam kumpulan fibrosis paru-paru pengurangan sel T. Terutama, IM dan monosit radang (iMONs) muncul sebagai dua jenis sel yang diubah isyarat paling dominan jika tiada sel T. Secara khusus, isyarat Thrombospondin (THBS) telah diubah dalam IM, manakala isyarat Amyloid Precursor Protein (APP) diubah dalam iMONs. Tambahan pula, kekurangan sel T dalam fibrosis pulmonari menyebabkan polarisasi IM ke arah fenotip M2. Kesimpulannya kajian ini mengenal pasti peranan perlindungan sel-sel T dalam fibrosis, sebelum dan selepas permulaannya. Terutama, ketiadaan sel T menyebabkan percambahan dan polarisasi fibrosis pulmonari IMs. Tambahan pula, kekurangan sel T dalam fibrosis paru-paru membawa kepada polarisasi IM ke arah fenotip M2 dengan mengesan ekspresi gen penanda M2, Arg1, Chil3, Fizz1. Oleh itu, ketiadaan sel T mengakibatkan percambahan dan polarisasi IM dalam fibrosis paru-paru. Kesimpulannya, kajian ini mengenal pasti peranan pelindung sel T dalam fibrosis, sebelum dan selepas permulaannya.

T CELL AND MACROPHAGE INTERACTION IN EXPERIMENTAL BLEOMYCIN-INDUCED IDIOPATHIC PULMONARY FIBROSIS

ABSTRACT

Pulmonary fibrosis is a progressive and irreversible disease with poorly understood aetiology and high mortality rates. The cell interactions in immune regulation play a pivotal role in disease progression. While macrophages have been extensively studied in lung fibrosis models, their interactions with other cells, especially with T cells remain largely unknown. Particularly, the crosstalk between innate immunity and adaptive immunity is of great interest. The current study was performed to determine the effect of macrophages and T cells interaction on pulmonary fibrosis progression. Firstly, the increase and activation of T cells were validated in a mouse model of bleomycin (BLM)-induced pulmonary fibrosis. Then, the impact of T cells on disease progression was investigated using CD3e knockout (CD3e KO) T cell-deficient mice. Surprisingly, the absence of T cells resulted in markedly more severe lung fibrosis, accompanied by a significant increase in interstitial macrophages (IMs). These findings were further confirmed when T cells were depleted 2 weeks after lung fibrosis modelling using diphtheria toxin (DT)-induced complete T cell depletion (Lat DTR mice). In both scenarios, it was observed an increase in pulmonary IMs and exacerbation of lung fibrosis. Additionally, the analysis of single-cell RNA sequencing (scRNA-seq) revealed the specific enrichment of collagen and Macrophage Migration Inhibitory Factor (MIF) signalling pathways in the T cell depletion lung fibrosis group. Notably, IMs and inflammatory monocytes (iMONs) emerged as the two most dominant signalling-altered cell types in the absence of T cells. Specifically, Thrombospondin (THBS)

signalling was altered in IMs, while Amyloid Precursor Protein (APP) signalling was altered in iMONs. Furthermore, the lack of T cells in pulmonary fibrosis led to the polarization of IMs towards an M2 phenotype by detected the M2 markers gene expression, Arg1, Chil3, Fizz1. Thus, the absence of T cells resulted in the proliferation and polarization of IMs in pulmonary fibrosis. In conclusion, this study identified the protective role of T cells in fibrosis, both prior to and following its onset.

CHAPTER 1

INTRODUCTION

1.1 Research Background

Idiopathic pulmonary fibrosis (IPF) is an irreversible chronic lung disease mainly characterized by increased collagen deposition and collapse of lung structure (Snijder *et al.*, 2019). Characterized by tissue histopathology of lung tissue scarring and usual interstitial pneumonia (UIP) (Glass *et al.*, 2022). IPF is a form of fibrotic interstitial lung disease (ILD) without an identifiable triggering factor, characterized by spontaneous, progressive fibrosis. Pulmonary fibrosis is the fibrotic outcome of ILDs. Studies on the genetic basis of fibrotic ILDs have shown that genes associated with an increased risk of IPF are also related to the risk of other fibrotic ILDs (Podolanczuk *et al.*, 2023). The prevalence of IPF is highest in North America, followed by the Asia-Pacific region, with comparatively lower incidence rates observed in Europe (Maher *et al.*, 2021). Pulmonary fibrosis leads to a deterioration in lung function and impaired gas exchange, manifesting clinically as dry cough and dyspnea, which significantly reduces the quality of life for the patients (Henderson *et al.*, 2020; Shenderov *et al.*, 2021). For clinical management, assessment methods like the GAP (Gender-Age-Physiology index) and CPI (Composite Physiologic Index) are vital for treatment. The course of IPF varies greatly among individuals, with some patients experiencing acute respiratory failure, a condition referred to as acute exacerbation of idiopathic pulmonary fibrosis. The occurrence of acute exacerbation is associated with an extremely high mortality rate (Luo and Xiang, 2024).The clinical manifestations of pulmonary fibrosis are characterized by an irreversible progression, poor prognosis, resistance to drug treatment, and a high mortality rate (Wong *et al.*, 2020).

The clinical presentation of IPF patients typically consists of nonspecific symptoms such as exertional dyspnea, dry cough, and fatigue, leading to a high risk of misdiagnosis or delayed diagnosis (Richeldi *et al.*, 2017). Currently, treatment options for IPF are very limited. They primarily include oxygen therapy and anti-fibrotic treatments during the early stages of the disease, and lung transplantation in the end stages (Raghu *et al.*, 2022).

Pirfenidone and nintedanib are currently the most effective anti-fibrotic drugs with proven clinical efficacy, as they can slow down the decline in lung function. However, they are unable to halt or reverse the decline in lung function (Flaherty *et al.*, 2019; Gerckens *et al.*, 2021). Both pirfenidone and nintedanib have some side effects, with pirfenidone causing gastrointestinal disorders, anorexia, and photosensitivity, while nintedanib can cause diarrhea and nausea (Vietri *et al.*, 2020; Imai *et al.*, 2024). The price of these two medications is exorbitantly high, presenting a significant financial barrier. Furthermore, the optimal timing for initiating treatment remains uncertain (Lederer and Martinez, 2018). Lung transplantation remains the only effective treatment for IPF, but approximately half of IPF patients live more than 5 years after transplantation (Balestro *et al.*, 2019). This shortened lifespan may be attributable to the age of the patient, the condition of the donor's lung, the timing of the referral for transplantation, and the surgical approach (Gu *et al.*, 2022). However, the limited availability of donors, high surgical costs, and the constraints associated with post-transplantation treatment and care hinder the widespread adoption of lung transplantation (Kapnadak and Raghu, 2021). Despite being considered a rare disease, the high mortality rate of IPF imposes a significant burden on society and the economy. The annual per capita cost for IPF patients in North America is approximately three times higher than the national medical

expenditure, with a marked increase in the use of societal healthcare resources. As a result, IPF represents an increasingly significant threat to the global public health sector (Maher *et al.*, 2021).

Pulmonary fibrosis results from a series of tissue repair in response to various stimuli, such as bacteria (Ichikawa *et al.*, 2019), viruses (Li *et al.*, 2022a), and dust (Zhang *et al.*, 2019b). Recurrent damage imposed upon susceptible alveolar epithelial cells (AECs) is associated with an escalation in cellular demise, a disruption in the re-epithelialization process, and pathological engagements with fibroblasts. These pathological interactions foster a state of persistent activation, characterized by excessive collagen and matrix synthesis leading to widespread scar in the lungs. In addition, high deposition of extracellular matrix (ECM) and aberrant fibroblast activity have been previously detected in the lungs of IPF patients (Kropski and Blackwell, 2019; Henderson *et al.*, 2020). Although the mechanisms of pulmonary fibrosis induction are not yet well understood, numerous studies have shown that many factors are linked to fibrosis, such as inflammation (Savin *et al.*, 2022), infection (Huang and Tang, 2021), autoimmune disorder (Diesler and Cottin, 2022), and tumour (Tzouvelekis *et al.*, 2019). The disorder response triggered by these diseases break the tissue repair balance and leads to fibrosis occurrence. The need to investigate the mechanisms of pulmonary fibrosis and to find effective targets for its control has become more urgent. The average survival time after IPF diagnosis is generally three to five years without effective treatment (Glassberg, 2019).

Compelling evidence indicates that immune dysregulation plays a crucial role in promoting the development of disease in IPF patients and pulmonary fibrosis

mouse models (Heukels *et al.*, 2019a; Shenderov *et al.*, 2021). Both innate and adaptive immune system participate in the process of pulmonary fibrosis. There exists communication between these immune cells. Macrophages, as a central player in innate immunity, are characterized by heterogeneous and high plasticity (Huang *et al.*, 2023). Macrophages in the lung are generally divided into two classical categories, alveolar macrophages (AMs) and interstitial macrophages (IMs) (Gu *et al.*, 2022). Substantial evidence suggests that macrophages play an important role in the development of lung fibrosis (Ucero *et al.*, 2019; Wang *et al.*, 2021). The lungs are chronically exposed to external particulates and pathogens, yet they possess an intricate array of protective mechanisms, including the mucous immune system, to counteract these threats (Kageyama *et al.*, 2024). The complexities of the lung tissue and the heterogeneity of macrophages present challenges in studying their roles in pulmonary fibrosis (Amit *et al.*, 2016; Wynn and Vannella, 2016). Recent technological advancements have contributed to the exploration of lung macrophages, with IMs gaining recognition for their various functions in pulmonary fibrosis (Gu *et al.*, 2022). However, the specific impact of IMs on the disease and the underlying regulatory factors remains unknown.

T cells are one of the most important compositions of adaptive immunity (He *et al.*, 2022). Substantial evidence revealed that T cells are involved in pulmonary fibrosis (Zhang and Zhang, 2020; Yuan *et al.*, 2021). CCR2 (C – C motif chemokine receptor 2)$^{+}$CD4^{+} T cell plays a protective role in lung fibrosis (Milger *et al.*, 2017), regulatory T cells (Tregs) effect Th17 cells, CD8^{+} T cells, CD4^{+}CD28^{-} and CD4^{+}CD28^{+} T cell in pulmonary fibrosis (Chakraborty *et al.*, 2018), PD-1 (Programmed death-1)$^{+}$CD4^{+} T cells promote pulmonary fibrosis (Celada *et al.*, 2018). The involvement of T cells in IPF is multifaceted, with different subsets of T

cells that may potentially have diverse functions, such as profibrotic or antifibrotic. The effect of T cells on pulmonary fibrosis has not yet been clearly concluded (Shenderov *et al.*, 2021).

Furthermore, intercellular communication is essential in the progression of lung fibrosis disease. Macrophages and T cells are representative of innate and acquired immunity respectively and are involved in pulmonary fibrosis (Shenderov *et al.*, 2021). In terms of immune cell interactions, more attention has been paid to the role of macrophages in presenting antigens to T cells (Guerriero, 2019; Wardell *et al.*, 2021). However, how T cells impact on macrophages, as well as how macrophages respond to T cells in pulmonary fibrosis, remain largely unknown. In the lungs of virus-infected patients, single-cell omics analysis has indicated a potential interaction between T cells and macrophages with the aggravation of the disease (Zhao *et al.*, 2021). However, whether there are interactions between T cells and macrophages in pulmonary fibrosis, and whether T cells act on macrophages, remains unclear. In light of the intricate relationship between these two immune cell types is pivotal to the pathogenesis of the disease, and understanding their interactions is essential for developing targeted therapeutic strategies. So far, the current gap in the understanding of the T cell-macrophage interactions in pulmonary fibrosis is a critical area that warrants immediate attention. By investigating these interactions, it may uncover new insights into pulmonary fibrosis's pathophysiology and identify innovative approaches to treat this devastating disease.

1.2 Rationale and importance of the study

IPF presents a significant clinical challenge due to its obscure aetiology, the irreversible progression of the disease, and the high mortality rates. Therefore,

understanding the pathogenesis of the disease and exploring clinical targets for treatment are urgent tasks. Animal models are crucial for advancing the understanding of pulmonary fibrosis. They provide a platform for basic research, enabling the researchers to explore the disease's development and pinpoint therapeutic targets. A variety of animal models have been developed to shed light on the causes of lung fibrosis and to uncover new drug targets. The most extensively utilized method for modelling is the Bleomycin (BLM)-induced model, which is regarded as the most representative of the pathological alterations observed in IPF (Ishida *et al.*, 2023). Therefore, this study employed a BLM-induced pulmonary fibrosis disease model to investigate the role of immune cells in pulmonary fibrosis.

The utilization of genetically edited mice within the realm of immunology is of profound significance, offering a potent instrument for elucidating the intricacies of immune response mechanisms. These mice models are instrumental in examining the roles of specific genes or signalling pathways within the immune response. Furthermore, by editing particular immune cells, it can gain a deeper understanding of their specific functions within the immune reaction. In this study, an animal model with specifically ablated T cells is used to investigate pulmonary fibrosis, addressing a significant gap in the understanding of immunoregulation in the disease. The research focuses on examining the impact of T cell deficiency on disease progression and the resulting macrophage immune response. To delve deeper into the interplay between immune cells under pathological conditions, this research employed single-cell RNA sequencing technology to offer an unprecedented resolution. This advanced technique allows researchers to examine the states and types of individual cells, thereby providing a detailed and nuanced view of the cellular landscape. By dissecting the heterogeneity at the single-cell level, this technology has unveiled

unexpected insights into the complex interactions between various cell types during the pathogenesis of diseases (Henderson *et al.*, 2020). In the context of pulmonary fibrosis, single-cell RNA sequencing has elucidated the intricate dynamics between T cells and macrophages during the lung fibrotic process. This exploration aims to provide a foundational understanding that could pave the way for the development of targeted immunotherapies for pulmonary fibrosis in the future. The schematic design of this study is shown (Figure 1.1).

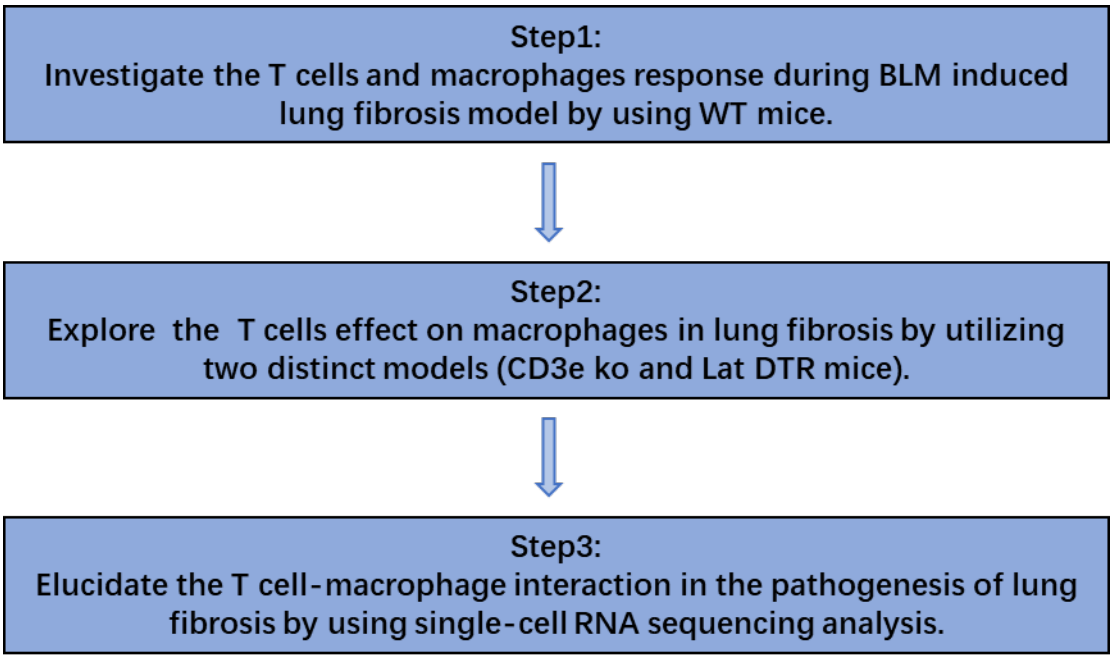

Figure 1.1 The schematic design of this study.

1.2.1 Hypothesis of the study

(1) T cells activation and expansion have an important influence on disease progression in pulmonary fibrosis.

(2) Macrophages are key cellular components for immune response and play a vital role in airway remodelling in pulmonary fibrosis.

(3) During lung fibrosis, the crosstalk between T cell and macrophage will affect the fibrosis and repair process.

1.2.2 Objective of the study

1.2.2(a) General objective

To study the interaction between T cells and macrophages in mice model of idiopathic pulmonary fibrosis.

1.2.2(b) Specific objectives

(1) To characterise the T cell and macrophage populations in a bleomucin-induced mouse model of pulmonary fibrosis.

(2) To evaluate the impact of T cell depletion on the progression of pulmonary fibrosis using CD3e KO and Lat-DTR T cell-deficient mouse models.

(3) To elucidate the effects of T cell absence on macrophage subpopulations response during the development of pulmonary fibrosis.

(4) To analyse the transcriptome profiles of immune cell populations in T cell-sufficient and T cell-deficient fibrotic lungs using single cell RNA sequencing, focusing on macrophage associated signaling pathways.

(5) To identify potential mechanisms underlying the interaction between T cells and macrophages in pulmonary fibrosis, focusing on interstitial macrophage functional shift.

CHAPTER 2

LITERATURE REVIEW

2.1 Pulmonary fibrosis diseases

2.1.1 The prevalence of pulmonary fibrosis

Pulmonary fibrosis is an irreversible chronic lung disease mainly characterized by increased collagen deposition and collapse of lung structure (Snijder *et al.*, 2019). The main manifestations are a progressive decline in lung function, increasing pulmonary fibrosis tested by high-resolution computed tomography (HRCT) (Cottin *et al.*, 2019; Wong *et al.*, 2020; Selman and Pardo, 2021).

Interstitial lung disease (ILD) is a heterogeneous disease that mainly involve inflammation and fibrosis in the interstitium, alveoli, and fine bronchi of the lungs (Wong *et al.*, 2020). In addition, most ILDs have a tendency towards progressive fibrosis, referred to as progressive fibrosing ILD (PF-ILD) (Flaherty *et al.*, 2017). Of note, the term "PF-ILD" refers to a group of ILDs with analogous clinical manifestations rather than a definite clinical case (Renzoni *et al.*, 2021; Selman and Pardo, 2021). These progressive fibrosing lung diseases include connective tissue disease-associated ILD, fibrotic hypersensitivity pneumonitis, unclassifiable ILD, idiopathic non-specific interstitial pneumonia, and rarely sarcoidosis, organizing pneumonia, and ILD associated with occupational exposures (Cottin *et al.*, 2019; Wong *et al.*, 2020). This means that some common predisposing factors such as viruses, antigenic substances, and environmental exposures will trigger ILD patients to develop clinical manifestations of pulmonary fibrosis. The subtypes of ILD can be classified into four categories (Figure 2.1) (Kreuter *et al.*, 2021).

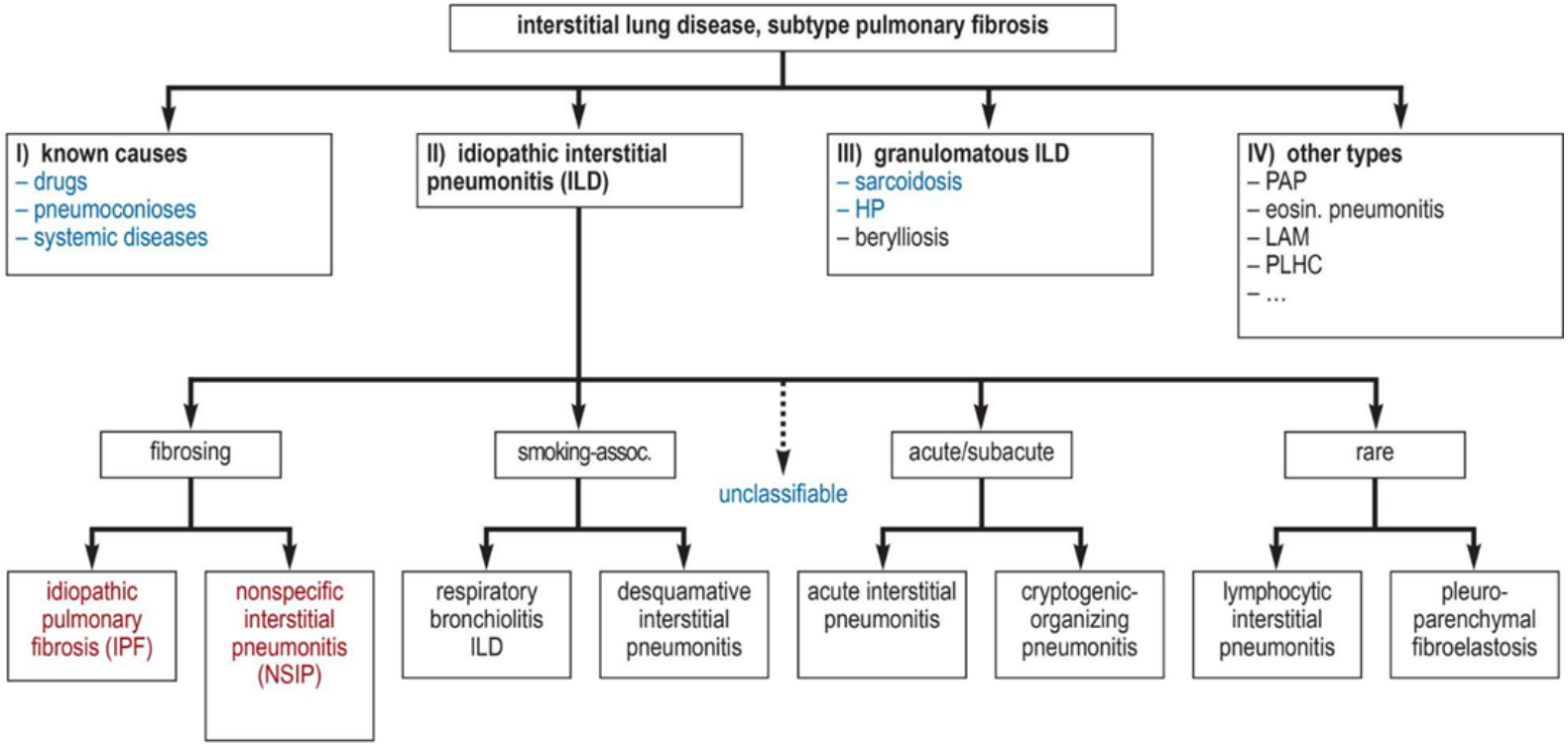

Figure 2.1 Categories of ILD. Within these categories, there are types that always take a fibrosing course (red): IPF and idiopathic NSIP.There are other entities that can take a fibrosing course (blue): drug-associated ILD, collagenosis-associated ILD, pneumoconioses, unclassifiable ILD, fibrosing exogenous allergic alveolitis, fibrosing sarcoidosis.HP, hypersensitivity pneumonitis; ILD, interstitial lung disease; LAM, lymphangioleiomyomatosis; PAP, pulmonary alveolar proteinosis;PLHC, pulmonary Langerhans-cell histiocytosis. Image adapted from (Kreuter et al., 2021).

Idiopathic pulmonary fibrosis (IPF) has been widely utilized since the 1970s and is associated with the clinical condition known as typical usual interstitial pneumonia (UIP), is one of the most typical and severe types of ILD (Kropski and Blackwell, 2019). IPF is characterized by UIP on both histopathology and HRCT of the chest (Thiessen *et al.*, 2019). UIP pattern is heterogeneous interstitial fibrosis, fibroblast foci, myogenic metaplasia, and marked destruction of architecture. Nonspecific interstitial pneumonitis is characterized by NSIP pattern, with uniform, chronic interstitial pneumonitis, and little interstitial fibrosis (Kreuter *et al.*, 2021). According to the guidelines, the diagnosis of IPF must exclude systemic diseases or exposure factors known to cause interstitial lung disease (ILD), such as connective tissue diseases, medications, and factors in the work and home environment (Bartold *et al.*, 2024). During the initial assessment of patients suspected of having IPF, it is necessary to identify these known causes of ILD, which may lead to a diagnosis of ILD associated with connective tissue disease, fibrotic hypersensitivity pneumonitis, pneumoconiosis, drug-induced ILD, or other ILDs (Glassberg, 2019). Initial pulmonary function tests might not reveal significant abnormalities. However, the defining features of IPF are restrictive ventilatory impairment and a reduction in diffusion capacity, which ultimately leads to permanent loss of lung function (Heukels *et al.*, 2019b). IPF is typically characterized by a gradual and insidious onset of symptoms, including a dry cough, progressive dyspnea, exertional breathlessness, and a decline in exercise tolerance (Rozenberg *et al.*, 2020). The disease is irreversible and has a chronic course that can span from months to years' post-diagnosis. On physical examination, fine crackles reminiscent of Velcro may be auscultated (Moran-Mendoza *et al.*, 2021). Approximately 10%-20% of IPF patients face acute exacerbations annually, which are characterized by acute or subacute worsening of respiratory

symptoms, accompanied by new ground-glass opacities and consolidations on HRCT superimposed on the existing disease pattern. These exacerbations accelerate the disease's progression and are associated with a heightened risk of mortality (Biondini *et al.*, 2020; Suzuki *et al.*, 2020).

The prevalence rate of IPF is highest in the Asia-Pacific region (0.57 to 4.51), followed by Europe (0.33 to 2.51), with comparatively lower incidence rates observed in North America (2.40 to 2.98) (Figure 2.2) (Maher *et al.*, 2021). In a Malaysian case study examining idiopathic pulmonary fibrosis (IPF), a striking 85% prevalence was observed among male patients. The mean age at diagnosis was 67 years, with a significant 46% of the cohort reporting a history of smoking. An analysis of the ethnic distribution of IPF patients revealed a higher incidence among individuals of Indian ethnicity, followed by those of Malay ethnicity, with the lowest representation observed in the Chinese population (Aflah *et al.*, 2019).

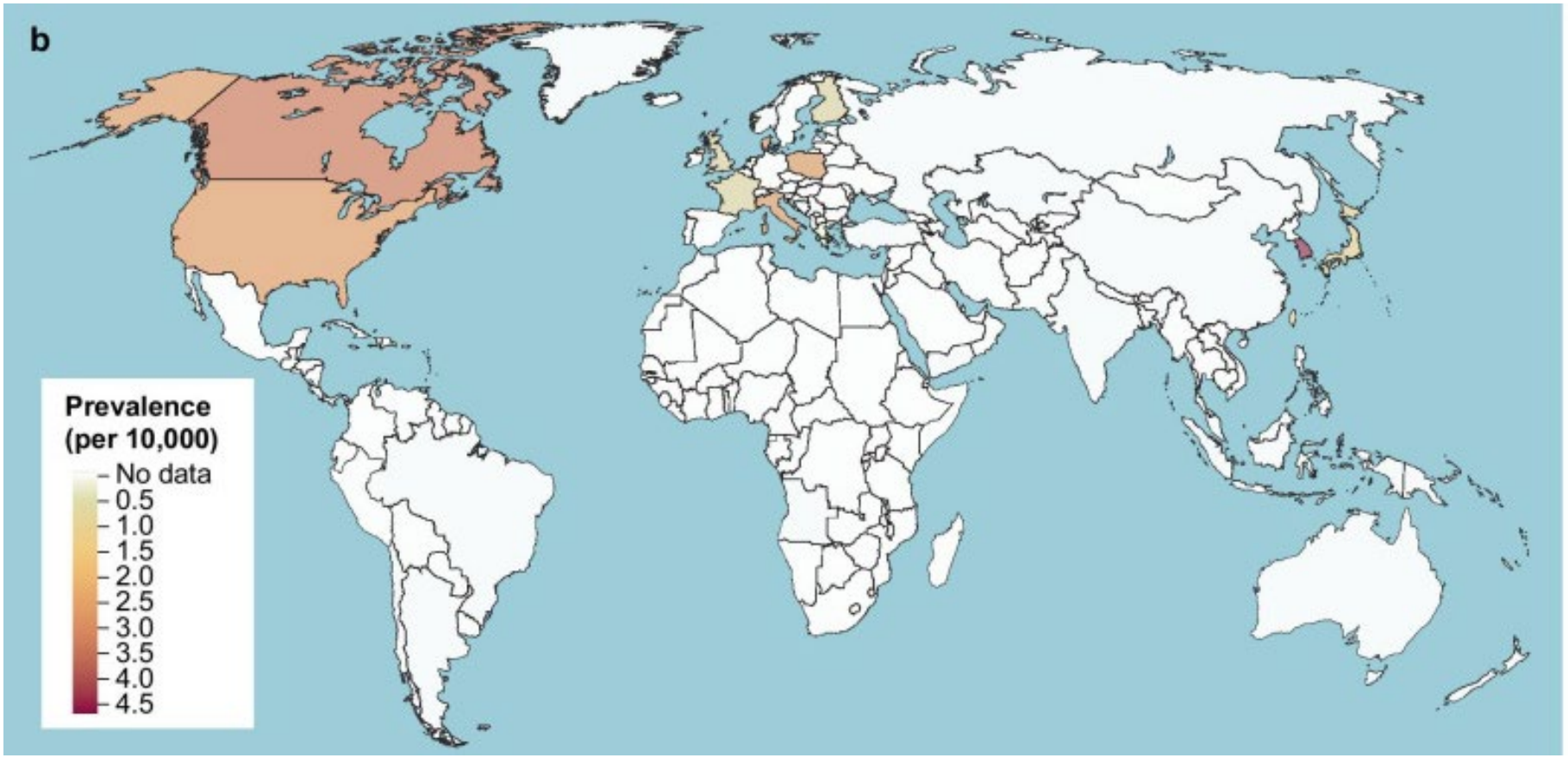

Figure 2.2 Global heat maps of adjusted IPF prevalence with specific IPF definitions. Image adapted from (Maher et al., 2021).

2.1.2 Structure of the respiratory immune system

The respiratory immune system can be anatomically delineated into two primary segments: the upper and lower respiratory tracts. The upper respiratory tract includes the nasopharynx and larynx, which play crucial roles in respiratory immunity by trapping large particles and initiating innate immune responses (Planer and Morrisey, 2023). The lower respiratory tract, encompassing the trachea, large airways, and small airways, culminates in the alveoli where gas exchange occurs. The airways are lined with epithelial cells and undergo multiple bifurcations, the large airways have a diameter greater than 2-3 millimetres, while the small airways are defined by a diameter of fewer than 2 millimetres. The small airways contribute minimally to the total airway resistance and exhibit structural differences from the large airways, such as the distribution of smooth muscle, the absence of cartilage, and the presence of surfactant lining (Bustamante-Marin and Ostrowski, 2017). The small airways consist of the bronchioles, terminal bronchioles, and respiratory bronchioles, which are connected to the alveolar ducts and alveolar sacs (Figure 2.3) (Usmani *et al.*, 2021). The alveolar sacs are clusters of alveoli located at the distal end of the alveolar ducts, with the alveolar ducts linking the alveolar sacs to the respiratory bronchioles, forming the respiratory zone responsible for gas exchange (Ahookhosh *et al.*, 2020).

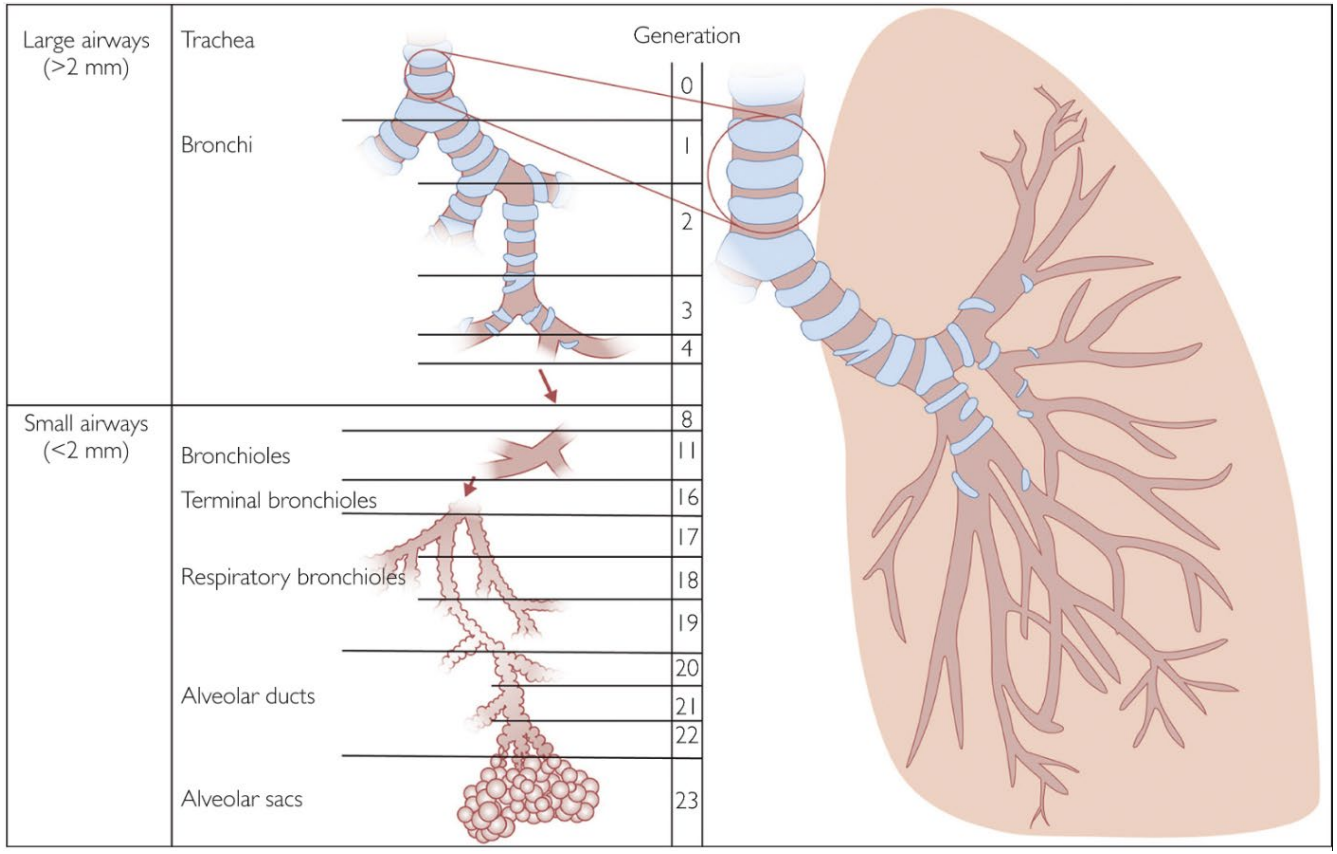

Figure 2.3 Schematic representation of the airway generations and corresponding anatomical airway structures. The trachea is the main airway that bifurcates into the right and left primary bronchi, which enter the lungs at the hilum and further divide into bronchioles. The human airways, lined with epithelial cells, undergo approximately 23 successive bifurcations starting from the trachea, progressively decreasing in diameter and length with each generation until they lead to the alveoli at the end of the respiratory bronchioles. Large airways, with a diameter greater than 2-3 mm, include the trachea and bronchi, while small or peripheral airways, less than 2 mm in diameter, begin to emerge around the eighth generation of this branching process. Image adapted from (Usmani et al., 2021).

In the pulmonary system, lymphatic vessels are categorized based on their anatomical location into three distinct types: pleural lymphatics, which are situated adjacent to the pleural surface; interlobular lymphatics, found within the interlobular septa; and intralobular lymphatics, which penetrate deeper into the lung tissue, residing within the lobules themselves. The deep intralobular lymphatic vessels can be further subdivided into several distinct components: the peribronchial lymphatics, which are associated with the bronchial vascular bundles; the perivascular lymphatics, encircling the pulmonary blood vessels; the bronchiolar lymphatics, typically surrounding the terminal and respiratory bronchioles; and the alveolar septal lymphatics, located within the interstitial spaces of the alveoli (Figure 2.4) (Weber *et al.*, 2018). The intricate network of lymphatic vessels reflects that facilitates the drainage of lymphatic fluid from various regions of the lung, playing a crucial role in immune function and fluid balance.

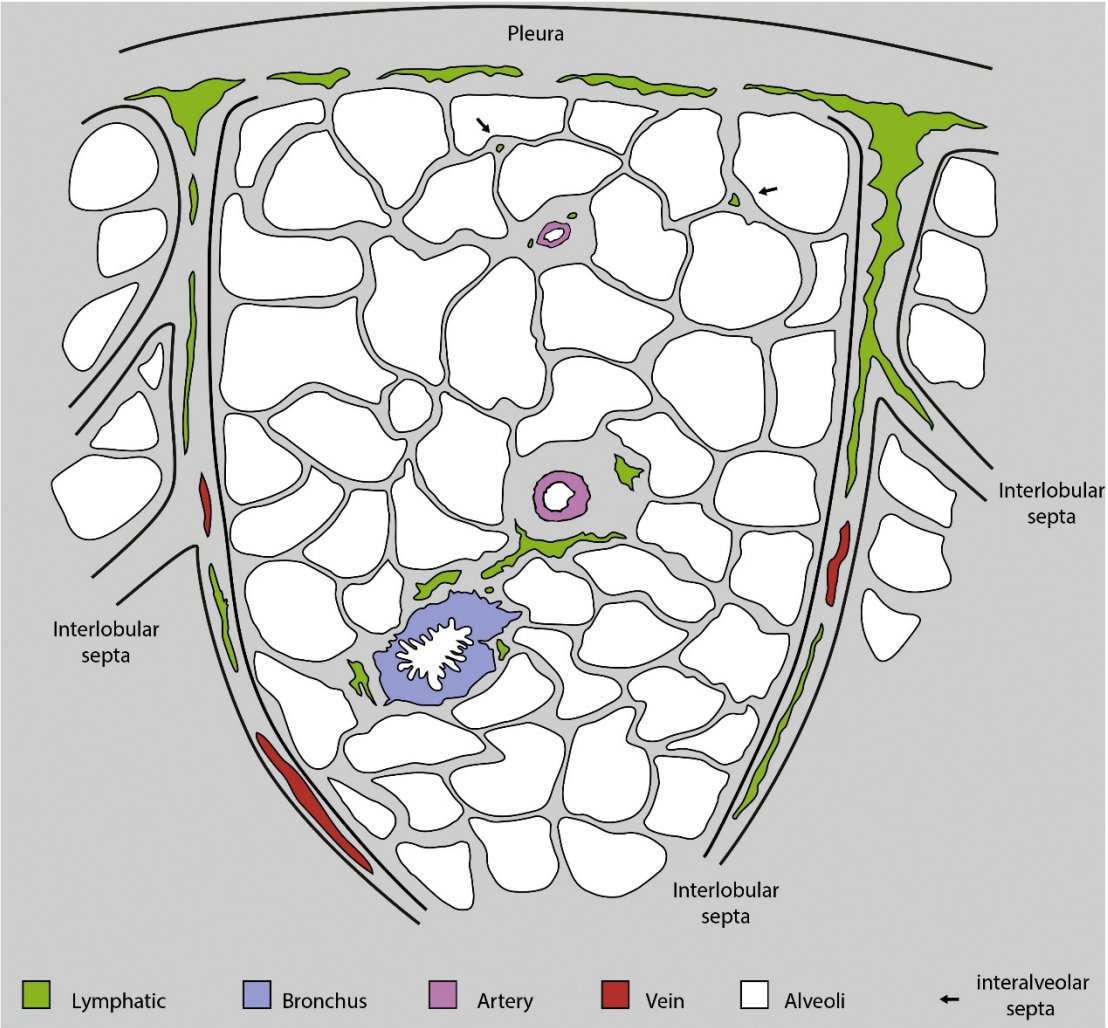

Figure 2.4 Distribution of lymphatic vessels (green) in the pulmonary lobule. Most vessels are located in the pleura, in the interlobular septa and in association with bronchovascular bundles. Lymphatic vessels are also present in interalveolar septa, in association with arterioles and only occasionally independent of blood vessels. Arrows indicate lymphatic vessels independent of blood vessels in interalveolar septa. Image adapted from (Weber et al., 2018).

The airway mucosa also includes potential inductive sites, bronchus-associated lymphoid tissue (BALT). Classic BALT consists of discrete aggregates of lymphoid cells beneath the epithelium, similar to tonsillar tissue and Peyer's patches in the intestine. The distribution of BALT varies among different species, with a higher prevalence of BALT observed in infants in humans, and most BALT containing independent lymphoid follicles. Importantly, in murine influenza virus infection models, although induced BALT lacks the structural organization of lymphoid tissue, it can generate protective immune responses. This suggests that BALT plays a crucial

role in local immune homeostasis during early life stages when central lymphoid structures are not yet functionally matured (Pabst, 2022).

2.1.3 Pathogenesis of pulmonary fibrosis

Fibrosis is a routine and essential procedure in the host's process of repairing injury and healing wounds. The alveolar epithelium is impacted by an initial trigger. Activated lung fibroblasts and myofibroblasts are the main effector cells involved in fibrosis formation. Lung fibroblasts recruit inflammatory cells to the site of injury by continuously releasing chemotactic factors, such as CCL19 and CXCL13 (Kalafatis *et al.*, 2021). Myofibroblasts synthesize ECM at the site of damage and rapidly undergo apoptosis at the end of the repair phase (Moss *et al.*, 2022). However, myofibroblasts in IPF exhibit anti-apoptotic abilities, leading to excessive ECM deposition. The various growth factors and cytokines (such as TGF-β, CTGF, PDGF, IGF1) contained within the ECM affect biological processes such as cell differentiation, proliferation, adhesion, and migration, exacerbating the degree of lung fibrosis and cause destruction of the alveolar structure and remodelling of lung parenchyma (Mei *et al.*, 2021). Additionally, fibroblasts, as precursors of lung myofibroblasts, accumulate in high numbers within fibrosis lesions, promoting progressive proliferation of lung myofibroblasts (Chong *et al.*, 2019). Upon the establishment of fibrosis, both resident and infiltrating immune cells, including macrophages and lymphocytes, adjust the ongoing immune reactions (Figure 2.5) (Desai *et al.*, 2018). Although the mechanisms of pulmonary fibrosis induction are not yet well understood, numerous studies have shown that many factors are inextricably linked to fibrosis, such as inflammation (Savin *et al.*, 2022), infection (Huang and Tang, 2021), autoimmune disorder (Diesler and Cottin, 2022), and tumour (Tzouvelekis *et al.*, 2019).

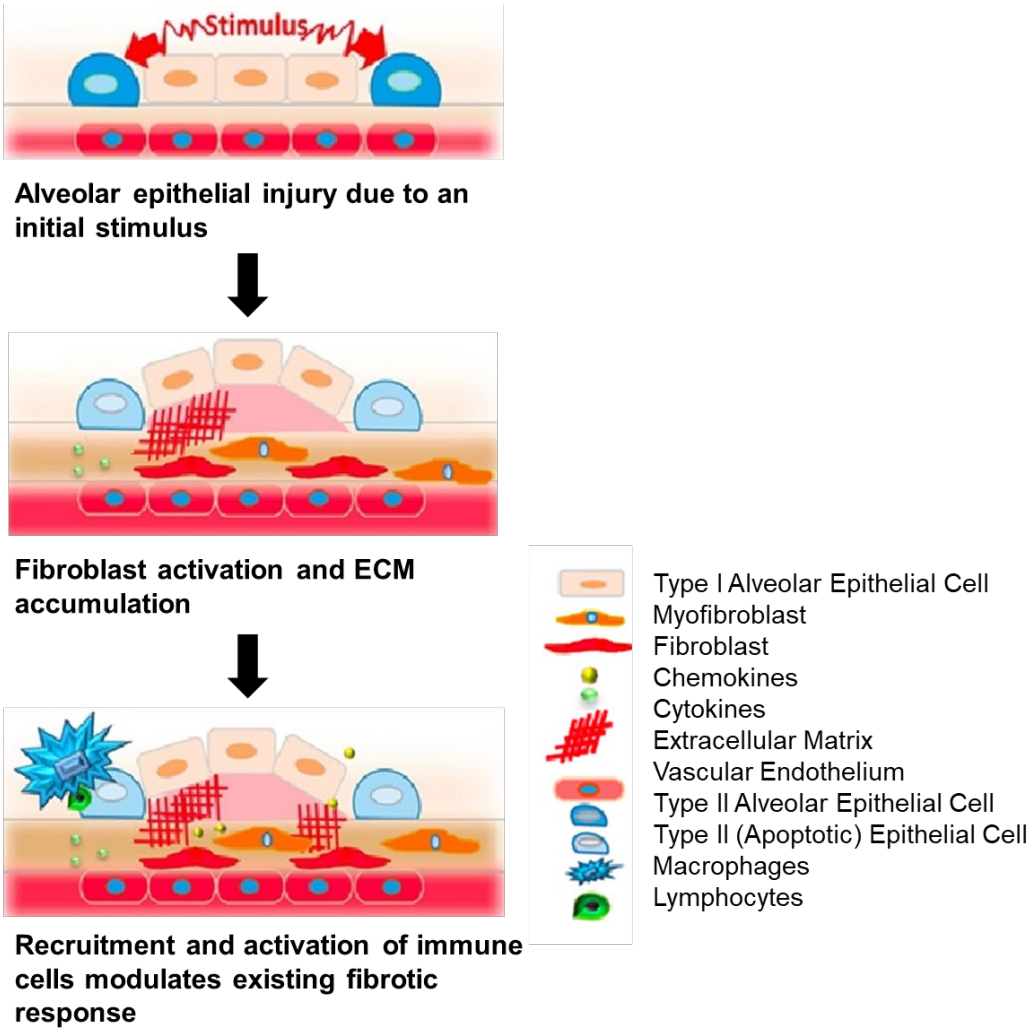

Figure 2.5 Pathogenesis of idiopathic pulmonary fibrosis (IPF). An initial stimulus affecting the lung epithelium leads to the activation of fibroblasts and the accumulation of extracellular matrix (ECM). This process results in the recruitment and activation of various immune cells, including different populations of macrophages and lymphocytes. Image adapted from (Desai et al., 2018).

Genetic variations have been associated with the morbidity of pulmonary fibrosis. Studies of familial pulmonary fibrosis (FPF), which means that two or more family members have the disease, account for 5-20% of IPF cases (Moss *et al.*, 2022). The two most common mutated genes in IPF patients are Mucin-5B (MUC5B) and desmoplakin gene (DSP) (Moss *et al.*, 2022). MUC5B r35705950 allele-carrying rate increased threefold in IPF patients (Mota *et al.*, 2022). DSP genes are associated with intercellular adhesion, leading to structural changes in the alveoli, alveolar damage,

dysregulation of epithelial cell repair, and correlated with the expression of the ECM-associated genes MMP7 and MMP9 (Hao *et al.*, 2020).

Environmental exposures can also contribute to the development of pulmonary fibrosis. Pulmonary fibrosis usually occurs in these ILDs such as cigarette smoking-induced chronic obstructive pulmonary disease (COPD), antigenic substances induced asthma, dust dust-induced pneumosilicosis. Cigarette smoking causes epithelial and endothelial cell damage, active transforming growth factor (TGF)-β, more DNA methylation and miRNA dysregulation were also observed (Pardo and Selman, 2021).

Ageing is associated with susceptibility to pulmonary fibrosis, the hallmarks of ageing involved in the development of IPF (Meiners *et al.*, 2015). The incidence of lung fibrosis increases progressively with age, and the risk of developing ILD is seven times higher after the age of 70 than 40 (Choi *et al.*, 2018). Almost all features associated with senescence have occurred in IPF patients, such as genomic instability, telomere shortening, epigenetic changes, proteostasis, nutrient sensing, mitochondrial dysfunction, cellular senescence, stem-cell exhaustion, and alterations in intracellular communications (Cho and Stout-Delgado, 2020).

There is also a gender preference for pulmonary fibrosis, IPF is more prevalent and occurs in men, accounting for 70% of all cases (Jo *et al.*, 2017; Sese *et al.*, 2021) . Estrogen receptor (ER)α was found to be highly expressed in male fibrotic lungs in both IPF patients and fibrotic mouse models, thus ER activity was enhanced and fibrotic pathways were upregulated (Elliot *et al.*, 2019).

In recent years, the link between viral and microbial infections and the development of pulmonary fibrosis has garnered increasing attention (Figure 2.6), especially in the wake of the severe acute respiratory syndrome coronavirus 2

(SARS-CoV-2) pandemic, commonly referred to as COVID-19, which emerged in 2019. A significant number of individuals who recovered from COVID-19 were later diagnosed with pulmonary fibrosis. A survey revealed that out of 227 patients with COVID-19 infection, 60 patients were subsequently diagnosed with pulmonary fibrosis (Li *et al.*, 2022a). Furthermore, evidence has accumulated to suggest that infections with a range of pathogens, including human T cell leukaemia virus (HTLV), human immunodeficiency virus (HIV), cytomegalovirus (CMV), Epstein-Barr virus (EBV), murine gamma herpesvirus 68 (MHV-68), influenza viruses, avian influenza viruses, Middle East respiratory syndrome (MERS) coronavirus, and severe acute respiratory syndrome (SARS) coronaviruses, can contribute to the pathogenesis of pulmonary fibrosis in varying degrees (Huang and Tang, 2021). Patients with IPF exhibit higher bacterial loads in bronchoalveolar lavage fluid (BALF), which is positively correlated with their risk of mortality. The *Bacteroides* and *Prevotella* are significantly increased in the process of pulmonary fibrosis (Yang *et al.*, 2019a).

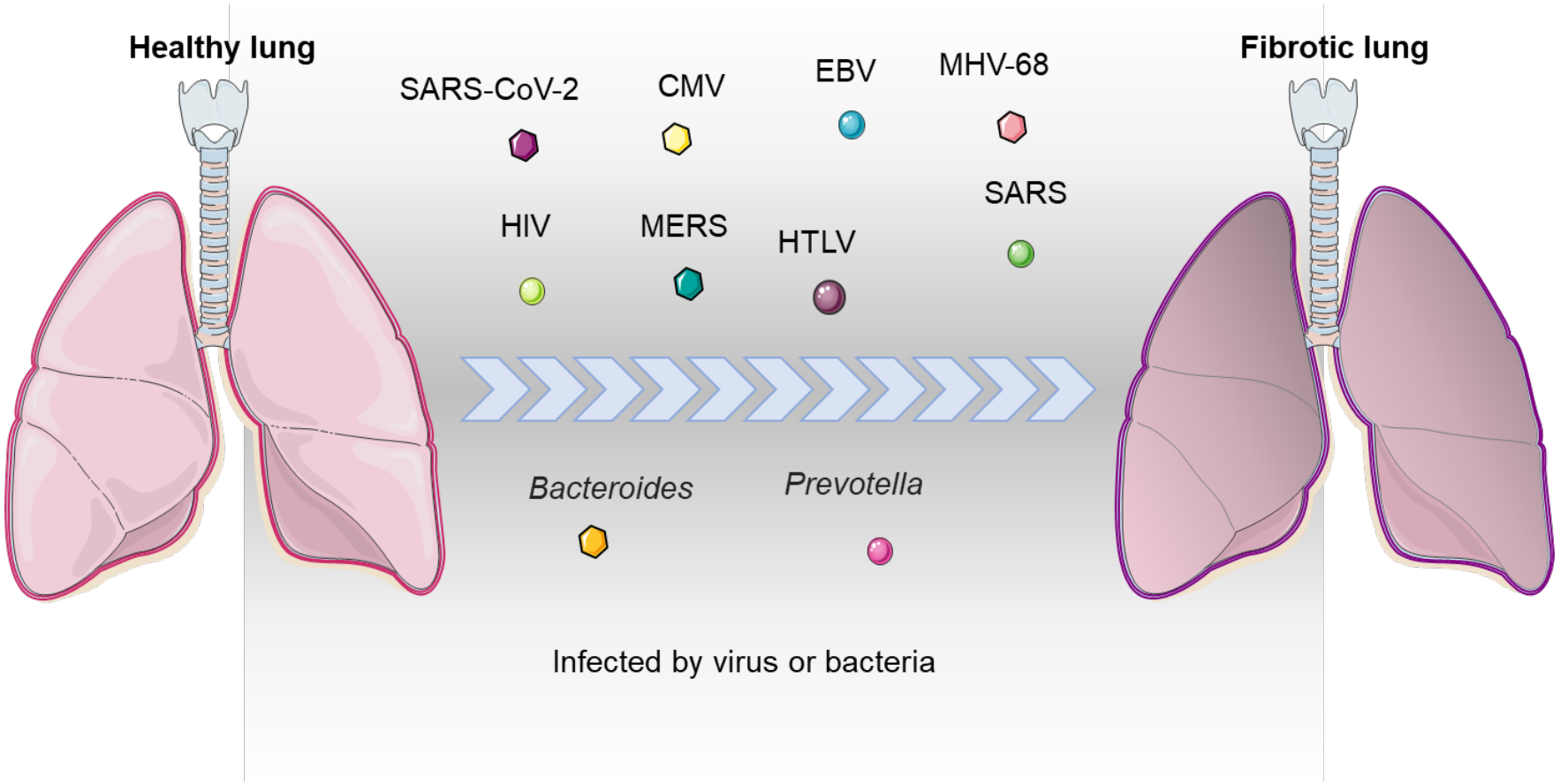

Figure 2.6 The viral and microbial infections involve in the development of pulmonary fibrosis. HTLV: human T cell leukaemia virus; HIV: human immunodeficiency virus; CMV: cytomegalovirus; EBV: Epstein-Barr virus; MHV-68: murine gamma herpesvirus 68; MERS-CoV: Middle East respiratory syndrome coronavirus; SARS-CoV: severe acute respiratory syndrome coronavirus; SARS-CoV-2: severe acute respiratory syndrome coronavirus 2.

As previously discussed, pulmonary fibrosis can arise from a multitude of etiological factors. Pulmonary fibrosis, as a definite pattern of lung disease, is considered to be comparable to cancer in terms of its high lethality as well as its irreversibility (Gu *et al.*, 2022). In particular, as the world has experienced a four-year outbreak of the novel coronavirus epidemic. This outbreak has resulted in widespread pneumonia infections and has led to fibrotic lesions in the lungs of many individuals (Zhang *et al.*, 2021). The need to elucidate the molecular mechanisms underpinning pulmonary fibrosis and to identify efficacious therapeutic targets for its management has become increasingly urgent in the current era.

In the pathogenesis of pulmonary fibrosis, multiple signalling pathways play a crucial role. The activation of TGF-β leads to excessive production of ECM components (Tie *et al.*, 2022). The Smad proteins are key receptors of TGF-β. The TGF-β/Smad signalling pathway is pleiotropic and plays a key role in inflammation, wound healing, and fibrotic processes, and is also an important inducer of ECM deposition and EMT processes in tissue fibrosis (Salton *et al.*, 2019). The supernatants of fibroblasts from IPF activated the TGF-β pathway in normal human fibroblasts (Epstein Shochet *et al.*, 2020).The Wnt/β-catenin signalling plays a crucial role in many pathological processes in the lung. Lung biopsy results from patients with IPF indicate that the heightened activation of the canonical Wnt/β-catenin signalling pathway is associated with tissue repair and fibroblast activation (Froidure *et al.*, 2020). Studies also suggest that Wnt/β-catenin signalling is involved in the induction of EMT during the development of fibrosis (Froidure *et al.*, 2020). In the lung tissues of animals treated with bleomycin, the expression of VEGF is significantly increased (Derseh *et al.*, 2019), and the pathological fibrosis and collagen deposition in the

fibrotic lung tissues of mice can be alleviated by using VEGFR antagonists (Derseh *et al.*, 2019). VEGF-A has also been shown to stimulate the activity of PDGF receptors (PDGFR), thereby regulating the migration and proliferation of mesenchymal cells. Certain members of the FGF family exert profibrotic effects by promoting the mitogenic activity of fibroblasts (Liu *et al.*, 2021). FGF-9 and FGF-18 promote the migration of human lung fibroblasts and inhibit the differentiation of myofibroblasts (Joannes *et al.*, 2016). The expression of FGF-7 and FGF-10 in IPF lungs is significantly enhanced compared to healthy lungs (El Agha *et al.*, 2018). PDGF is a potent fibroblast mitogen and plays an important role in the proliferation of myofibroblasts (Yao *et al.*, 2022). In animal models of IPF, targeting PDGFR-β can ameliorate bleomycin-induced pulmonary fibrosis (Kishi *et al.*, 2018).

2.1.4 Therapy for pulmonary fibrosis

Idiopathic pulmonary fibrosis (IPF) is a chronic lung disease characterized by its insidious onset, unknown etiology, and histological or radiological presentation of usual interstitial pneumonia (UIP), with progressive dyspnea and decline in pulmonary function as its hallmarks (Thiessen *et al.*, 2019). The use of objective, effective, and comprehensive methods to diagnose and assess the condition is crucial for clinical treatment. Currently, the GAP (Gender-Age-Physiology index) index and the CPI (Composite Physiologic Index) are two commonly used composite indices to measure the severity of the disease. The GAP index operates as a stratification tool founded on four foundational variables: gender (G), age (A), and the pulmonary function parameters of forced vital capacity (FVC) and diffusing capacity of the lung for carbon monoxide (DLCO), collectively denoted as (P). Patients are segmented into three stages by the GAP index: Stage I (scores 0-3), Stage II (scores 4-5), and Stage III (scores 6-8), with the latter stage signifying an elevated mortality risk. The CPI is