

Investigating Aberrant Inflammatory Signalling in Asthma

Natalie Margret Niessen
MSc (Molecular Biosciences)



THE UNIVERSITY OF
NEWCASTLE
AUSTRALIA

School of Medicine and Public Health
Faculty of Health and Medicine
University of Newcastle, Australia

April 2021

A thesis submitted in fulfilment of the requirements for the degree
of Doctor of Philosophy in Medicine

STATEMENT OF ORIGINALITY

I hereby certify that the work embodied in the thesis is my own work, conducted under normal supervision. The thesis contains no material which has been accepted, or is being examined, for the award of any other degree or diploma in any university or other tertiary institution and, to the best of my knowledge and belief, contains no material previously published or written by another person, except where due reference has been made. I give consent to the final version of my thesis being made available worldwide when deposited in the University's Digital Repository, subject to the provisions of the Copyright Act 1968 and any approved embargo.

Natalie Niessen

THESIS BY PUBLICATION

I hereby certify that this thesis is in the form of a series of papers. I have included as part of the thesis a written declaration from each co-author, endorsed in writing by the Faculty Assistant Dean (Research Training), attesting to my contribution to any jointly authored papers.

Natalie Niessen

ACKNOWLEDGEMENTS

Today my PhD journey comes to an end. The last four years were both challenging and fulfilling. I have learned so much and grew a lot. I will forever be grateful for the entire experience and the outstanding support of my supervisors Dr Michael Fricker, Prof Peter Gibson, Prof Jodie Simpson and Dr Katie Baines. Your dedication to research is inspiring and each one of you is a great role model.

First of all, I would like to thank my primary supervisor Mike for giving me this incredible opportunity and for your guidance throughout my candidature. You were always approachable, answered my questions promptly and competently, and gave me the best advises on optimising laboratory methods. You found the right balance between supporting me and challenging me to become a confident, self-dependent researcher.

Many thanks to Mike, Peter, Jodie and Katie for your mentorship, your constructive feedback on my writing, the insightful discussions and for providing samples of clinical trials for my research. I am grateful to have been working along a professional team of renown clinicians and researchers.

This thesis comprises a series of papers and I owe my gratitude to my supervisors and co-authors. Without you, these publications would not have been possible, and I am proud of what we have achieved. Thank you, Dr Hayley Scott, for sharing your samples with me and co-authoring two of my papers. Special thanks to the entire AMAZES team for entrusting me with your precious samples and for your feedback on the manuscript. Thank you, Dr Daniel Barker for helping me with statistics related questions.

Further, I would like to thank the clinical and the laboratory processing team for coordinating study visits, collecting participant's clinical data, sample collection and processing. Special thanks to Dr Lakshitha Gunawardhana for keeping me updated on incoming samples and their processing status. Thank you Cat Delahunty and Lakshitha for your help with setting up my OpenSpecimen and RedCap accounts, your technical support and for easing my introduction into the clinical world.

Many thanks to Nicole Cole for your training and technical support in flow cytometry. During my first weeks I have had no clue what I was doing. Today I feel confident in setting-up an experiment, analysing samples and cell sorting. Thanks for answering my phone calls even outside working hours and taking the time to help me remotely with technical issues.

I would like to acknowledge the National Health and Medical Research Council, and the NHMRC Centre of Excellence in Severe Asthma for providing the financial support throughout my PhD candidature.

Special thanks go to my lunch group (and the morning tea crew earlier in my candidature). Mel, Cami, Teresa, Ev, Shaun, Virinchi, and all the other lovely people who have come and gone over the past years, thank you for the relaxing breaks in between. You have been my rock on hectic and stressful days. Valuable friendships arose and I wouldn't want to miss anyone of you. Mike (and Phil and Magnus!), even though you are back in Canada, you were part of this journey, and I can't wait to celebrate our graduations and your wedding in the Great White North, once the pandemic is over.

I don't want to miss out on thanking every one of the respiratory research team who I didn't name specifically. I appreciated working in a respectful and supportive environment and every one of you contributed to this experience.

Thanks to my family and friends on the other side of the world for their unconditional love and their support in everything I do. You always believed in me and cheered me up when I didn't. Mama, without you, living and working in Australia would have just remained a dream. Thanks so much for supporting me even though it meant for you to let me go. In your honour I chose this date for my thesis submission (5 days *before* the due date). I wish you a happy 60th birthday.

Last but not least, I would like to thank all the volunteers who invested their time and efforts to participate in these studies.

PUBLICATIONS INCLUDED IN THIS THESIS

Niessen, N., Baines, K., Simpson, J., Scott, H., Qin, L., Gibson, P., Fricker, M. (2020). Neutrophilic asthma features increased airway classical monocytes. *Clinical and Experimental Allergy*, 51(2), 305-317. DOI: 10.1111/cea.13811

Niessen, N., Gibson, P., Simpson, J., Scott, H., Baines, K., Fricker, M. (2021). Airway monocyte modulation relates to TNF dysregulation in neutrophilic asthma. *Submitted to European Respiratory Journal – Open Research*

Niessen, N., Gibson, P., Baines, K., Barker, D., Yang, I., Upham, J., Reynolds, P., Hodge, S., James, A., Jenkins, C., Peters, M., Marks, G., Baraket, M., Simpson, J., Fricker, M. (2021). Sputum TNF markers are increased in neutrophilic and severe asthma and are reduced by azithromycin treatment. *Allergy*, online ahead of print. DOI: 10.1111/all.14768

OTHER RESEARCH ARTICLES & CONFERENCE ABSTRACTS RELATED TO THIS THESIS

Research articles

Fricker, M., Qin, L., **Niessen, N.**, Baines, K., McDonald, V., Scott, H., Simpson, J., Gibson, P. (2020). Relationship of sputum mast cells with clinical and inflammatory characteristics of asthma. *Clinical and Experimental Allergy*, 50(6), 696–707. DOI: 10.1111/cea.13609.

Conferences*** Presenting author**

Fricker, M.*, Qin L., **Niessen, N.**, Baines K., Scott H., Simpson J., Gibson, P. (2020). Relationship of sputum mast cells with clinical and inflammatory characteristics of asthma. *Respirology*, TO 117. DOI: 10.1111/resp.13777. *Oral presentation at the conference of the Thoracic Society of Australia and New Zealand (Online).*

Fricker M.*, **Niessen, N.**, Baines, K., Simpson J., Scott, H., Gibson, P. (2020). Neutrophilic asthma features increased airway classical monocytes. *European Respiratory Journal*, 56: 1109. DOI: 10.1183/13993003.congress-2020.1109. *E-poster presentation at the conference of the European Respiratory Society (Online).*

Fricker M.*, Qin, L., **Niessen, N.**, Baines, K., Scott, H., Simpson, J., Gibson P. (2020). Sputum mast cells associate with clinical and inflammatory features of asthma. *European Respiratory Journal*, 56: 1110. DOI: 10.1183/13993003.congress-2020.1110. *E-poster presentation at the conference of the European Respiratory Society (Online).*

Niessen, N.*, Simpson, J., Baines, K., Gibson, P., Fricker, M. (2019). Differential Tumor Necrosis Factor Ligand and Receptor Expression on Monocyte Subsets in Blood and Sputum. *American Journal of Respiratory and Critical Care Medicine*, 199:A3612. DOI: 10.1164/ajrccm-conference.2019.199.1_Meeting Abstracts. A3812. *Poster presentation at the conference of the American Thoracic Society in Dallas, TX, USA.*

Niessen, N.*, Simpson, J., Baines, K., Gibson, P., Fricker, M. (2019). Differential TNF alpha, TNFR1 and TNFR2 expression on blood- and sputum-derived immune cells in asthma. *Respirology*, TO 101. DOI: 10.1111/resp.13491. *Oral presentation at the conference of the Thoracic Society of Australia and New Zealand in Gold Coast, QLD, Australia.*

LIST OF ABBREVIATIONS

ACQ	Asthma control questionnaire
ACTRN	Australian clinical trials registration number
ADAM	A disintegrin and metalloproteinase
AHR	Airway hyperresponsiveness
AMAZES	Asthma and Macrolides: The Azithromycin Efficacy and Safety study
ANOVA	Analysis of variance
APC	Antigen-presenting cell
ATP	Adenosine triphosphate
ATS	American Thoracic Society
AZM	Azithromycin
BAL(F)	Bronchoalveolar lavage (fluid)
BD	Bronchodilator
BDP	Beclomethasone dipropionate
β₂-AR	Beta-2 adrenergic receptor
BMI	Body mass index
cAMP	Cyclic adenosine monophosphate
CBD	CREB-binding protein
CD	Cluster of differentiation
CM	Classical monocytes
COPD	Chronic obstructive pulmonary disease
CV	Coefficient of variability
DAMP	Danger-associated molecular pattern
DC	Dendritic cells
DTT	Dithiothreitol

EA	Eosinophilic asthma
EDTA	Ethylenediaminetetraacetic acid
ELISA	Enzyme-linked immunosorbent assay
Eos	Eosinophils
ERS	European Respiratory Society
FACS	Fluorescence-activated cell sorting
FCS	Fetal calf serum
FEV₁	Forced expiratory volume in one second
FMO	Fluorescence minus one
FSC	Forward scatter
FVC	Forced vital capacity
GINA	Global Initiative for Asthma
GM-CSF	Granulocyte-macrophage colony-stimulating factor
GR	Glucocorticoid receptor
GRE	GR response element
HAT	Histone acetyltransferase
HDAC	Histone deacetylase
HLA-DR	Human leukocyte antigen-DR
HMRI	Hunter Medical Research Institute
HREC	Human Research Ethics Committee
ICS	Inhaled corticosteroids
ICU	Intensive care unit
IFN-γ	Interferon gamma
IgE	Immunoglobulin E
IKK	I κ B-Kinase

IL	Interleukin
ILC	Innate lymphoid cell
IM	Intermediate monocytes
IQR	Interquartile range
LABA	Long-acting beta-agonist
LPS	Lipopolysaccharide
LTRA	Leukotriene receptor agonist
LUBAC	Linear ubiquitin chain assembly complex
Macs	Macrophages
MAPK	Mitogen-activated protein kinase
MCL	Myosin light chains
MFI	Median fluorescence intensity
MGA	Mixed granulocytic asthma
MHC	Major histocompatibility complex
MLCK	Myosin light chains kinase
MMP	Matrix metalloproteinase-9
Monos	Monocytes
mTNF(R)	Membrane-bound tumor necrosis factor (receptor)
NA	Neutrophilic asthma
NCM	Non-classical monocytes
NEMO	NF- κ B essential modulator
NETs	Neutrophilic extracellular traps
NF-κB	Nuclear factor kappa-light-chain-enhancer of activated B cells
NHMRC	National Health and Medical Research Council

NLRP3	Nucleotide-binding oligomerisation domain, Leucine rich Repeat and Pysin domain containing Proteins
OCS	Oral corticosteroids
PAMP	Pathogen-associated pattern
PBS	Phosphate-buffered saline
PCAF	p300/CBP-activating factor
PD	Provocative dose
PGA	Paucigranulocytic asthma
PKA	Protein kinase A
PBMCs	Peripheral blood monocytes
pred	Predicted
PRR	Pathogen recognition receptor
RCT	Randomized controlled trial
RIPK	Receptor-interacting serine/threonine-protein kinase
RT	Room temperature
RV	Rhinovirus
SABA	Short-acting beta-agonists
SD	Standard deviation
SSC	Side scatter
sTNF(R)	Soluble tumor necrosis factor (receptor)
Tab	TGF β -activated kinase 1 and MAP3K7-binding protein
TACE	TNF alpha converting enzyme
TAK	TGF β -activated kinase
TCC	Total cell count
Th	T helper cell

TLR	Toll-like receptor
TNF	Tumor necrosis factor
TNFR	Tumor necrosis factor receptor
TRADD	TNFR1-associated death domain
TRAF	TNFR-associated factor
Treg	Regulatory T cells
TSLP	Thymic stromal lymphopoietin

LIST OF TABLES

Table 1.1: Add-on therapy for moderate-to-severe asthma	16
Table 1.2: The three human monocyte subsets and their proposed properties	22
Table 2.1: Antibody cocktail composition to stain immune cells in blood and sputum.....	49
Table 3.1: Demographics and clinical characteristics of the study population	70
Table 3.2: Interrelation between flow cytometry quantified sputum immune cells	72
Table 3.S1: Sputum and blood cell counts of the study population.....	90
Table 3.S2: Demographics and clinical characteristics of asthma airway inflammatory phenotype subgroups.....	91
Table 3.S3: Sputum and blood cell counts of asthma airway inflammatory phenotype subgroups....	92
Table 3.S4: Demographics and clinical characteristics of the second independent sputum flow cytometry study population	93
Table 3.S5: Demographics and clinical characteristics of asthma severity subgroups.....	94
Table 3.S6: Sputum and blood cell counts of asthma severity subgroups	95
Table 3.S7: Correlation between monocytes/macrophages and clinical characteristics.....	96
Table 4.1: Demographics and clinical characteristics of the study population	120
Table 4.2: Spearman correlation matrix between soluble TNF marker levels in sputum supernatant and membrane-bound counterparts on sputum immune cells	123
Table 4.3: Spearman correlation matrix of the difference between membrane-bound TNF marker levels between blood and sputum with soluble variants in sputum supernatant	124
Table 4.4: Correlation between absolute numbers of sputum immune cells and soluble variants in sputum supernatant	126
Table 5.1: Clinical characteristics of the investigated AMAZES TNF sub-study cohort at baseline and comparison of the two treatment groups	151

Table 5.S1: Demographics and clinical characteristics of the AMAZES TNF sub-study cohort compared to the overall AMAZES RCT cohort at baseline	166
Table 5.S2: Differential sputum cell counts of the AMAZES TNF sub-study cohort compared with the overall AMAZES RCT cohort at baseline.....	167
Table 5.S3: Baseline clinical characteristics and sputum cell count of the investigated AMAZES TNF sub-study cohort by inflammatory phenotypes	168
Table 5.S4: Spearman correlation matrix between TNF markers at baseline and selected sputum immune cells proportions	170
Table 5.S5: Spearman correlation matrix between TNF markers at baseline and spirometry, age, asthma control, and BMI	171
Table 5.S6: Baseline marker levels of the investigated AMAZES TNF sub-study cohort and comparison of the two treatment groups	172
Table 5.S7: Differential changes in TNF marker levels between baseline and end of treatment with azithromycin (AZM) vs placebo.....	173
Table 5.S8: Differential changes in TNF marker levels between baseline and end of treatment with azithromycin (AZM) vs placebo by airway inflammatory phenotype (eosinophilic = EA and MGA, non-eosinophilic = NA and PGA)	174

LIST OF FIGURES

Figure 1.1: Pathological features of asthma	6
Figure 1.2: Pathophysiology of eosinophilic and neutrophilic asthma.....	7
Figure 1.3: Personalized management for adults and adolescents as recommended by the Global Initiative for Asthma to control symptoms and minimize future risks	10
Figure 1.4: Mechanism of action of β_2 -adrenergic receptor agonists	12
Figure 1.5: Mechanism of action of corticosteroids	13
Figure 1.6: Lineage tree of immune cells.....	20
Figure 1.7: Two distinct types of pulmonary macrophages	24
Figure 1.8: TNFR1-induced signalling	34
Figure 1.9: TNFR2-induced signalling	36
Figure 2.1: Antibody titration	51
Figure 2.2: Surface expression of mTNF, mTNFR1 and mTNFR2 relative to the isotype control.....	52
Figure 2.3: Gating strategy for blood cells in participants with asthma and non-asthma controls	54
Figure 2.4: Gating strategy for sputum cells in participants with asthma and non-asthma controls ..	55
Figure 3.1: Comparison of proportions and absolute numbers of sputum macrophages and monocytes quantified by flow cytometry in subjects with asthma vs non-asthma controls.....	72
Figure 3.2: Comparison of proportions and absolute numbers of sputum macrophages, monocytes and neutrophils quantified by flow cytometry between asthma inflammatory phenotypes	74
Figure 3.3: Comparison of classical monocytes in subjects with asthma vs non-asthma controls and between asthma inflammatory phenotypes in sputum	75
Figure 3.4: CD206 ^{-/+} monocyte subsets in sputum.....	77
Figure 3.5: CM in severe vs non-severe asthma.....	80
Figure 3.S1: Gating strategy for sputum cells and overlay of identified cell populations in a final dot plot.....	97
Figure 3.S2: Fluorescence minus one (FMO) controls and fully stained sputum samples	98

Figure 3.S3: Gating strategy for blood cells and overlay of identified cell populations in a final dot plot	99
Figure 3.S4: Correlation between the proportion of cells determined by flow cytometry vs differential sputum cell count and images of isolated immune cells via fluorescence-activated cell sorting	100
Figure 3.S5: Validation of key results in an independent cohort	101
Figure 3.S6: Comparison of CD14/CD16-based monocyte subsets in asthma vs non-asthma controls and between asthma airway inflammatory phenotypes in sputum	102
Figure 3.S7: Comparison of CD14/CD16-based monocyte subsets in asthma vs non-asthma controls and between asthma inflammatory phenotypes in blood	103
Figure 3.S8: Comparison of CD14/CD16 monocyte subset occurrence between CD206 ⁻ vs CD206 ⁺ monocyte populations in sputum of participants with asthma.....	104
Figure 3.S9: Blood monocyte subset composition in severe asthma vs non-severe asthma.....	105
Figure 3.S10: Sputum monocyte subset composition in severe asthma vs non-severe asthma	106
Figure 3.S11: Comparison of CD206 ⁻ vs CD206 ⁺ monocyte subsets in severe vs non-severe asthma.....	107
Figure 4.1: Comparison of soluble TNF- α , TNFR1 and TNFR2 in sputum supernatant (a-c) and plasma (d-f) between non-asthma controls (black, n=8), non-neutrophilic asthma (green, n _{plasma} =35, n _{sputum} =36) and neutrophilic asthma (red, n=9)	119
Figure 4.2: Surface expression of mTNF, mTNFR1 and mTNFR2 on immune cells in the circulation and the airways.....	122
Figure 4.3: Surface expression (median fluorescence intensity) of mTNF, mTNFR1 and mTNFR2 on sputum immune cells in neutrophilic asthma, non-neutrophilic asthma and non-asthma controls .	125
Figure 4.S1: Gating strategy for sputum cells and overlay of identified cell populations in a final dot plot.....	134
Figure 4.S2: Gating strategy for blood cells and overlay of identified cell populations in a final dot plot	135

Figure 4.S3. Gating of classical (CM), intermediate (IM) and non-classical monocytes (NCM) in blood (left) and sputum (right)	136
Figure 4.S4: Comparison of a) mTNF- α , b) mTNFR1 and c) TNFR2 expression on blood (red) and sputum (green) immune cells in non-asthma controls (n = 8)	137
Figure 4.S5. Differential TNF marker expression on monocyte subsets	138
Figure 5.1: Baseline sputum (A-C) and serum (D-F) TNFR1, TNFR2 and TNF by airway inflammatory phenotypes	152
Figure 5.2: Baseline sputum (A-C) and serum (D-F) TNFR1, TNFR2 and TNF in non-severe vs severe asthma	153
Figure 5.3: Baseline sputum (A-C) and serum (D-F) TNFR1, TNFR2 and TNF in non-frequent vs frequent exacerbators	154
Figure 5.4: Changes in TNF marker levels between baseline and end of treatment with azithromycin (green) and placebo (red)	156
Figure 5.5: Changes in TNF marker levels between baseline and end of treatment with azithromycin (green) and placebo (red) in eosinophilic (EA) vs non-eosinophilic asthma (non- EA).....	157
Figure 5.S1: Standard curves of TNFR1 standards prepared in assay diluent and assay diluent with DTT	175
Figure 5.S2: Dot plots of the change between baseline and post-treatment of each subject across treatment subgroup.....	176
Figure 5.S3: Dot plots of the change between baseline and post-treatment of each subject with eosinophilic (EA) vs non-eosinophilic asthma (non-EA) across treatment subgroup.....	177
Figure 5.S4: Changes in TNF marker levels between baseline and end of treatment with azithromycin (green) and placebo (red) in neutrophilic (NA) vs non-neutrophilic asthma (non-NA).....	178
Figure 5.S5: Changes in TNF marker levels between baseline and end of treatment with azithromycin (green) and placebo (red) in participants with bacteria positive vs negative sputum	179

Figure 6.1: Developmental pathways from human blood monocytes to macrophages in the lung.. 185

Figure 6.2: Proposed model of the mechanisms involved in neutrophilic asthma 195

TABLE OF CONTENTS

STATEMENT OF ORIGINALITY	i
THESIS BY PUBLICATION	ii
ACKNOWLEDGEMENTS	iii
PUBLICATIONS INCLUDED IN THIS THESIS	v
OTHER RESEARCH ARTICLES & CONFERENCE ABSTRACTS RELATED TO THIS THESIS...	vi
LIST OF ABBREVIATIONS	viii
LIST OF TABLES	xiii
LIST OF FIGURES	xv
THESIS ABSTRACT	1
CHAPTER 1: General Introduction.....	3
1.1. Asthma	4
1.1.1. Diagnosis	4
1.1.2. Pathogenesis	5
1.1.3. Asthma airway inflammatory phenotypes.....	8
1.1.4. Severe asthma.....	9
1.1.5. Asthma medication	10
1.1.5.1. Reliever medications.....	11
1.1.5.2. Maintenance therapy.....	12
1.1.5.2.1. Inhaled corticosteroids	12
1.1.5.2.2. Systemic corticosteroids	15
1.1.5.3. Add-on therapies	15

1.1.5.3.1.	Monoclonal antibodies	17
1.1.5.3.2.	Proposed treatments for non-eosinophilic asthma	18
1.1.5.3.3.	Macrolides.....	18
1.1.5.4.	Economic burden and unmet needs in asthma therapy.....	19
1.2.	Immune cells in asthma	19
1.2.1.	Mononuclear phagocytes	21
1.2.1.1.	Monocytes	21
1.2.1.2.	Dendritic cells.....	23
1.2.1.3.	Macrophages.....	24
1.2.2.	Granulocytes	26
1.2.2.1.	Neutrophils	26
1.2.2.2.	Eosinophils	27
1.2.2.3.	Basophils	28
1.2.3.	Mast cells	28
1.2.4.	Lymphocytes	29
1.2.4.1.	T cells.....	29
1.2.4.2.	B cells	30
1.2.4.3.	Innate lymphoid cells	30
1.2.5.	Sampling of pulmonary immune cells.....	31
1.3.	The TNF signalling pathway	32
1.3.1.	TNF	32
1.3.2.	TNFR1 and TNFR2.....	32

1.3.3.	TNFR1-induced signalling pathway	33
1.3.4.	TNFR2-induced signalling pathway	34
1.3.5.	Dysregulated TNF signalling in asthma	36
1.4.	Study rationale	37
1.5.	Aims & Hypotheses	40
1.5.1.	Study 1 (Chapter 3)	40
1.5.2.	Study 2 (Chapter 4)	40
1.5.3.	Study 3 (Chapter 5)	41
CHAPTER 2: Materials & Methods		43
2.1.	Participants	44
2.2.	Collection of clinical data	45
2.2.1.	Questionnaires	45
2.2.2.	Spirometry	45
2.3.	Sample collection	45
2.3.1.	Collection of blood	45
2.3.2.	Sputum induction and processing	45
2.4.	Definition of asthma subtypes	46
2.4.1.	Asthma inflammatory phenotypes	46
2.4.2.	Severe asthma	47
2.4.3.	Frequent vs infrequent exacerbators	47
2.4.4.	Age of onset	47
2.5.	Flow cytometry	48

2.5.1.	Staining of blood and sputum cells	48
2.5.2.	Flow cytometry experiment set-up.....	50
2.5.2.1.	Compensation	50
2.5.2.2.	Antibody titration.....	50
2.5.2.3.	Isotype controls.....	51
2.5.2.4.	Gating strategy.....	52
2.6.	Enzyme-linked immunosorbent assay (ELISA)	56
2.6.1.	Reagent preparation	56
2.6.2.	Validation and optimization.....	56
2.6.3.	Protocol.....	56
2.6.4.	ELISA quality control	58
2.7.	TNF marker shedding	58
2.8.	Statistical analysis	58
CHAPTER 3: Neutrophilic asthma features increased airway classical monocytes		60
3.1.	ABSTRACT.....	63
3.1.1.	Background	63
3.1.2.	Objective	63
3.1.3.	Methods.....	63
3.1.4.	Results.....	63
3.1.5.	Conclusion and Clinical relevance	63
3.2.	INTRODUCTION.....	65
3.3.	METHODS.....	67

3.3.1.	Participants:	67
3.3.2.	Spirometry, sample collection and processing	67
3.3.3.	Flow cytometry of whole blood and sputum.....	68
3.3.4.	Statistical analysis	69
3.4.	RESULTS.....	69
3.4.1.	Patients characteristics	69
3.4.2.	Flow cytometry enables direct airway sampling	69
3.4.3.	Reduced sputum macrophages in asthma are associated with airway neutrophilia ...	71
3.4.4.	Macrophages but not monocytes are decreased in neutrophilic asthma.....	73
3.4.5.	Neutrophilic asthma features increased sputum classical monocytes.....	73
3.4.6.	Neutrophilic asthma is associated with increased proportion and number of sputum CD206 ⁻ monocytes	76
3.4.7.	CD206 ⁻ monocytes feature increased CM phenotype vs CD206 ⁺ monocytes	77
3.4.8.	Circulating but not airway monocytes are altered in severe asthma.....	78
3.4.9.	Sputum macrophage number is negatively associated with age & asthma duration ..	78
3.5.	DISCUSSION.....	81
3.6.	FOOTNOTES AND SUPPLEMENTAL MATERIAL.....	86
3.6.1.	Supplemental methods.....	86
3.6.1.1.	Study inclusion and exclusion criteria.....	86
3.6.1.2.	Sputum induction.....	86
3.6.1.3.	Flow cytometry of whole blood and sputum.....	86
3.6.1.4.	Blood cell flow cytometry gating	87

3.6.1.5.	Sputum flow cytometry gating	88
3.6.1.6.	Calculation of absolute cell numbers as cells/mL sputum.....	89
3.6.2.	Supplemental tables	90
3.6.3.	Supplemental figures	97
3.6.4.	Acknowledgements.....	108
3.6.5.	Conflict of interests.....	108
3.6.6.	Author contributions.....	109
CHAPTER 4: Airway monocyte modulation relates to TNF dysregulation in neutrophilic asthma.....		110
4.1.	ABSTRACT.....	113
4.1.1.	Background	113
4.1.2.	Methods.....	113
4.1.3.	Results.....	113
4.1.4.	Conclusion.....	113
4.2.	INTRODUCTION.....	114
4.3.	METHODS.....	117
4.3.1.	Participants	117
4.3.2.	Spirometry, sample collection and processing	117
4.3.3.	Flow cytometry of whole blood and sputum.....	117
4.3.4.	Gating of immune cells and quantification of membrane-bound TNF proteins.....	118
4.3.5.	Enzyme-linked immunosorbent assays.....	118
4.3.6.	Statistical analysis	118
4.4.	RESULTS.....	119

4.4.1.	Participant characteristics.....	119
4.4.2.	Neutrophilic asthma features increased soluble sputum receptors	119
4.4.3.	Surface expression of TNF markers varies across immune cells.....	121
4.4.4.	TNF marker surface expression differs between compartments	121
4.4.5.	Blood monocyte subsets feature distinct TNF marker expression patterns	122
4.4.6.	Surface marker expression on airway immune cells is not altered in NA	123
4.4.7.	Soluble sputum TNFR levels correlate with sputum monocyte numbers	126
4.5.	DISCUSSION.....	127
4.6.	FOOTNOTES AND SUPPLEMENTAL MATERIAL.....	132
4.6.1.	Supplemental methods.....	132
4.6.1.1.	Study inclusion and exclusion criteria.....	132
4.6.1.2.	Sputum induction.....	132
4.6.1.3.	Collection of whole blood	133
4.6.1.4.	Flow cytometry of sputum and whole blood.....	133
4.6.2.	Supplemental figures	134
4.6.3.	Acknowledgements.....	139
4.6.4.	Conflict of interests	139
4.6.5.	Author contributions.....	139
4.6.6.	Support statement.....	139
CHAPTER 5: Sputum TNF markers are increased in neutrophilic and severe asthma and are reduced by azithromycin treatment		141
5.1.	ABSTRACT.....	144

5.1.1.	Background	144
5.1.2.	Methods.....	144
5.1.3.	Results.....	144
5.1.4.	Conclusions	144
5.1.5.	Graphical abstract.....	145
5.2.	INTRODUCTION.....	146
5.3.	METHODS.....	148
5.3.1.	Participants	148
5.3.2.	Spirometry, sample collection and processing	148
5.3.3.	Enzyme-linked immunosorbent assays.....	149
5.3.4.	Statistical analysis	149
5.4.	RESULTS.....	150
5.4.1.	Participants characteristics	150
5.4.2.	Sputum TNFR1 and TNFR2 are increased in neutrophilic asthma	150
5.4.3.	TNF receptors are increased in severe asthma and in participants with a history of frequent exacerbations.....	152
5.4.4.	Azithromycin reduces soluble TNFR2 levels in non-eosinophilic asthma.....	154
5.5.	DISCUSSION.....	158
5.6	FOOTNOTES AND SUPPLEMENTAL MATERIAL.....	163
5.6.1.	Supplemental methods.....	163
5.6.1.1.	Participants	163
5.6.1.2.	Spirometry, sample collection and processing	163

5.6.1.3.	Enzyme-linked immunosorbent assay (ELISA) validation	164
5.6.1.4.	ELISA quality control	165
5.6.2.	Supplemental tables	166
5.6.3.	Supplemental figures	175
5.6.4.	Acknowledgements.....	180
5.6.5.	Conflict of interests.....	180
5.6.6.	Author contributions.....	180
CHAPTER 6: General Discussion		181
6.1.	Summary of key findings.....	182
6.2.	Monocytes: important immune cells of the airways	183
6.3.	Increased systemic and airway soluble TNF receptors are associated with worse asthma outcomes	186
6.4.	The therapeutic effect of azithromycin	189
6.5.	Strengths & Limitations.....	191
6.6.	Future directions.....	193
6.7.	Final conclusion.....	194
REFERENCES		197
APPENDIX		230
Co-author statements and endorsement by the Assistant Dean (Research Training)		230

THESIS ABSTRACT

Asthma is a chronic obstructive airway disease that is estimated to affect 340 million people worldwide. The underlying inflammation of the airways is heterogeneous and different asthma inflammatory phenotypes have been identified, that are associated with varying responses to treatment. Approximately 15 % of those with asthma feature neutrophilic airway inflammation, defined by elevated sputum neutrophils, that is associated with corticosteroid resistance and more severe disease. As yet, suitable treatment for neutrophilic asthma is lacking and a better understanding of the pathophysiological changes underlying this inflammatory phenotype is necessary in order to identify novel therapeutic targets.

This thesis reveals novel aspects of neutrophilic asthma, namely, altered airway immune cell trafficking and dysregulation of the TNF signalling pathway. In **chapter 3** I demonstrate that neutrophilic asthma is associated with increased recruitment of monocytes to the airways, while airway macrophages appear to be reduced. Dysregulation of the monocyte/macrophage lineage could relate to an altered inflammatory response, as these two cell types may execute distinct functions in tissue homeostasis and inflammation. **Chapter 4** investigates the relative abundance of the inflammatory markers TNF, TNFR1 and TNFR2 in the circulation and the airways. I demonstrate that neutrophilic asthma is associated with increased soluble receptor levels in the airways, whereas membrane-bound TNF markers do not differ across asthma inflammatory phenotypes or in comparison to non-asthma controls. These alterations could relate to aberrant inflammatory signalling and/or impaired inflammatory resolution and thus contribute to airway inflammation in neutrophilic asthma. In **chapter 5**, I show that increased soluble TNF receptors in both circulation and the airways are associated with clinical features of asthma, such as reduced lung function, more frequent exacerbation and more severe asthma, suggesting that dysregulated TNF signalling contributes to worse asthma outcomes. I further demonstrate that long-term low-dose administration of azithromycin significantly reduces soluble TNF marker levels. My results imply that the mechanisms

of action of AZM could be a combination of both general and specific mechanisms and potentially involve anti-inflammatory and anti-bacterial properties of the macrolide.

My observations in neutrophilic asthma prompt new hypotheses that require further investigation and validation in mechanistic studies.